Alcohol: Mode of Actions and Clinical Perspectives

Guest Editors
Albert Sun

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Albert Sun, Columbia, Mo.
Ting-Kai Li, Indianapolis, Ind.
Shih-Jiun Yin, Taipei
Jang-Yen Wu, Lawrence, Kans.

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Biomedical Vignette

In the current issue:

CYP2E1-Derived Oxidative Stress

It is believed that reactive oxygen species produced by ethanol plays an important role in ethanol-induced hepatotoxicity [2, 3]. Mari et al. [20] reported that overexpression of cytochrome P450 2E1 (CYP2E1) in HepG2-E47 cells increases total levels of antioxidants such as glutathione, and activity, protein or mRNA levels for other antioxidants such as catalase and glutathione transferases. These findings may reflect an adaptive mechanism to remove CYP2E1-derived reactive oxygen species through up-regulation of these antioxidant genes.

Oxidative Stress and Ethanol

It is well known that production of excessive reactive oxygen species could lead to structural and functional changes in the body [10]. Among various reactive oxygen species, nitric oxide (NO) appears to play a central role in regulatory functions in the nervous, immune and cardiovascular systems [17]. Zima et al. [38] reported in alcoholics an increase in NO-stable metabolites-nitrites and nitrates, antiphospholipid antibodies or oxidized LDL and a decrease in some important enzymatic antioxidant systems, including superoxide dismutase and glutathione peroxidase. These results provide additional evidence to support the long-suspected notion that free radicals play an important role in the development of alcoholic liver damage.

Structure and Function of Alcohol Dehydrogenases

Mammalian alcohol dehydrogenase (ADH) constitutes a complex system with different forms and extensive multiplicity (ADH1–ADH6) that catalyzes the oxidation and reduction of a wide variety of alcohols and aldehydes [7, 8]. Höög et al. [14] reported the functions of ADH1–ADH4 and cautioned that rodents are poor model systems for human ethanol metabolism since the rodent ADH2 enzymes almost lack ethanol-oxidizing capacity in contrast to the human form. The authors further proposed that, in contrast to the cytochrome P450 system, the function of the entire ADH system could be seen as a general detoxifying system for alcohols and aldehydes without generating toxic radicals.

P300 ERP and Alcoholism

Increasing evidence supports a role for genetic factors in the susceptibility to alcohol dependence [12, 23]. However, the identification of specific genes that contribute to this predisposition has proven elusive. Hesselbrock et al. [11] reported the use of P300 wave amplitude as an alternative approach to diagnose phenotype for alcoholism susceptibility genes. The authors identify differences in P300 amplitude between alcoholics and non-alcoholics, between unaffected relatives of alcoholics and relatives of controls, as well as between unaffected offspring of alcoholic fathers and offspring of controls [11]. These results indicate that P300 can be used as an endophenotype for alcohol dependence.

Dopamine and Alcohol Relapse

Relapse prevention is a major concern in therapeutic intervention of alcohol-dependent patients. Dopaminergic transmission has been implicated in alcohol dependence and hence dopaminergic agents have been regarded as putative therapeutics for preventing relapse [24, 26]. Walter et al. [30] reported that flupenthixol, a D1, D2, and D3 antagonist, increases the relapse rate significantly. The authors further showed that flupenthixol has detrimental effects only in types I and III and has no effect in types II and IV based on the Lesch typology. These findings demonstrate the value of the Lesch typology in the analysis of outcome of therapeutic intervention of alcohol relapse.

NMDA Receptor and Pharmacological Intervention

The central neurotoxic effects of alcoholism seem to be related to glutamate-induced excitotoxic damage and up-regulation of NMDA receptor [9]. Acamprosate is thought to have inhibitory activity on NMDA receptors [37]. It can also affect taurin and calcium channels. The preclinical study with the European Acamprosate Trial project has shown excellent methodological approach for relapse prevention and therapy [16]. There is also evidence that this compound can increase the sobriety rates and has only few and mild side effects.

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Mode of Action of Acamprosate and Neurotoxicity

Acamprosate (AC), N-acetyl-homotaurine, has recently been introduced for treating alcohol craving and reducing relapses in weaned alcoholics [27, 32]. However, its mechanism remains elusive. Wu et al. [35] proposed that AC might exert its action through the taurine, rather than the glutamatergic or GABAergic system. Whereas AC strongly inhibits the binding of taurine to taurine receptors, it has little effect on glutamate or $GABA_A$ receptors. In addition, AC is neurotoxic, at least in neuronal cultures. The underlying mechanism of AC-induced neuronal injury appears to be its action in increasing the intracellular calcium level, $[Ca^{2+}]_i$. Both AC-induced neurotoxicity and elevation of $[Ca^{2+}]_i$ can be prevented by taurine suggesting that AC may exert its effect through its antagonistic interaction with taurine receptors.

DETC-MeSO and Neuroprotection

S-methyl N,N-diethylthiolcarbamate sulfoxide (DETC-MeSO), the active metabolite of disulfiram, has recently been shown to exert an antagonistic effect on brain glutamate receptors [21]. It is also possible that the clinical efficacy of disulfiram in the treatment of alcoholism is due, at least in part, to its effect on glutamate receptors. The effect of DETC-MeSO on glutamate receptors may also explain the synergistic effect of acamprosate in combination with disulfiram in the prevention of alcoholic relapse [33]. Ningaraj et al. [22] reported that DETC-MeSO has neuroprotective function against glutamate-induced excitotoxicity in cultured neurons. Furthermore, the authors showed that this neuroprotective function of DETC-MeSO is due to the reduction in glutamate-induced elevation of intracellular Ca^{2+}. These observations point to an exciting possibility that DETC-MeSO may be useful for treatment of alcoholism as well as neurodegenerative diseases.

Lipid Carrier Proteins

Liver fatty acid binding protein is a lipid carrier protein that binds cholesterol, fatty acids, fatty acyl-CoA, retinal-heme, hematin, lysophospholipid, bilirubin, prostaglandins and many other amphipathic ligands. On the other hand, sterol carrier protein-2 is an intracellular protein that binds cholesterol and is involved in the intracellular trafficking of cholesterol [1]. Wood et al. [34] demonstrated that ethanol alters the cholesterol transport mechanism, leading to the accumulation of cholesterol inside the cells. Since cholesterol accumulation inside the cell may have great impact on cell structure and function, the changes in cholesterol homeostasis due to chronic ethanol administration may lead to activation of Fas pathway and apoptosis [36].

Alcohol and Brain Serotonin

It is commonly found that children of women who were heavy drinkers during pregnancy exhibit mental retardation, hyperactivity, cognitive deficit and behavioral abnormalities [5, 28] . These psychological problems have long been suspected to be related to central nervous system dysfunction. Sari et al. [25] reported that alcohol reduces the normal formation and growth of serotonin (5-HT) neurons in the midbrain. Furthermore, the projection of 5-HT fibers, in density as well as in distribution, is reduced in the major trajectory bundle. These findings may provide an explanation for some of the syndromes associated with fetal alcohol syndrome.

Ethanol and Signaling Pathways in Astrocytes

Chronic and excessive consumption of alcohol in humans and animals causes cellular damages in many body organs, including neurons and glial cells in the central nervous system [15, 18]. Wang and Sun [31] reported that in astrocyte cultures, ethanol inhibits nitric oxide (NO) production as well as cytokine-induced release of secretory phospholipase A_2 ($sPLA_2$). Furthermore, the inhibitory effect of ethanol on NO production corresponds well with the decrease in iNOS protein and NOS enzyme activity but not with iNOS and $sPLA_2$ mRNA nor binding of NF-κB to DNA. These results provide evidence for a post-transcriptional mode of ethanol action on the cytokine induction pathway for NO production in astrocytes.

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Alcohol Intoxication and Brain Damage

Alcoholics may show deficits in brain function as a result of interaction between ethanol and dietary factors [4]. Crews et al. [6] used a binge ethanol treatment model, which causes physical dependence, gene induction and alcohol-induced brain damage to examine the effects of diets on brain damage. Their studies demonstrated that nutritional components and total caloric intake do not affect behavior during ethanol withdrawal and that a nutritional complete diet may increase ethanol-induced brain damage. The exact mechanisms of binge ethanol-induced neurotoxicity remain to be elucidated.

Metabolic Fate of [^{14}C]Ethanol in Endothelial Cell Phospholipids

It is known that ethanol interacts with biological membranes and membrane-associated signal transduction mechanisms [13, 29]. However, it is not clear whether and how much of this ethanol is distributed into cellular lipid. Magai and Shukla [19] reported that [^{14}C]ethanol is incorporated into various endothelial cell phospholipids including phosphatidylethanol, phosphatidylcholine, neutral lipids, sphingomyelin (Sph), phosphatidylinositol and platelet-activating factor (PAF). The authors also reported that ethanol exposure has an opposite effect on the incorporation of [^{3}H]acetate into Sph and PAF. These findings provide a fresh look at the role of ethanol on specific lipid mediators in the altered responses of the endothelium in alcoholism.

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Review

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Role of Glutamatergic and GABAergic Systems in Alcoholism

Kathleen M. Davis Jang-Yen Wu

Departments of Molecular Biosciences and Medical Chemistry, University of Kansas, Lawrence, Kans., USA

Key Words

L-Glutamate · GABA · Alcoholism · Glutamate receptors · GABA receptors · Decarboxylase

Abstract

The pharmacological effects of ethanol are complex and widespread without a well-defined target. Since glutamatergic and GABAergic innervation are both dense and diffuse and account for more than 80% of the neuronal circuitry in the human brain, alterations in glutamatergic and GABAergic function could affect the function of all neurotransmitter systems. Here, we review recent progress in glutamatergic and GABAergic systems with a special focus on their roles in alcohol dependence and alcohol withdrawal-induced seizures. In particular, NMDA-receptors appear to play a central role in alcohol dependence and alcohol-induced neurological disorders. Hence, NMDA receptor antagonists may have multiple functions in treating alcoholism and other addictions and they may become important therapeutics for numerous disorders including epilepsy, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's chorea, anxiety, neurotoxicity, ischemic stroke, and chronic pain. One of the new family of NMDA receptor antagonists, such as DETC-MESO, which regulate the redox site of NMDA receptors, may prove to be the drug of choice for treating alcoholism as well as many neurological diseases.

'Alcoholism is a disorder of great destructive power' [213]. It is one of the world's leading health problems, costing the United States alone an estimated $150 billion annually [156]. Prior to the 1970s, it was generally accepted that the central effects associated with alcohol abuse were the result of fluidization of neuronal membranes [189]. It has also been suggested that ethanol inflicts its damage by producing free radicals [129], and by interfering with the supply of oxygen and glucose to the brain [181]. More recently it was found that the major effect of ethanol is not lipid bilayer disruption [54, 56, 166] but modification of the proteins in the lipid membranes [166]. It is now apparent that ethanol can alter neurotransmission in the central nervous system (CNS) by modifying neurotransmitter enzymes and receptors, and it is generally accepted that alcohol abuse and dependence has a biochemical basis related to abnormal neurotransmitter function [158, 184]. Many neurotransmitter systems have been studied including γ-aminobutyric acid (GABA) [68]; glutamate (Glu) [210, 212]; dopamine [105, 161]; serotonin [33, 125, 194]; acetylcholine [7, 13, 137, 204]; and endogenous opioids [38, 74, 158]. Neurochemical systems which have received the most attention have been glutamate, GABA, serotonin, dopamine, and the opioid peptides.

GABA and Glu are the major inhibitory and excitatory neurotransmitters, respectively, and greater than 80% of the neurons in the brain utilize these neurotransmitters [54]. The neuroadaptive changes that occur in these sys-

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Dr. Jang-Yen Wu
Department of Molecular Biosciences
1043 Haworth Hall, University of Kansas
Lawrence, KS 66045-2106 (USA)
Tel. +1 785 864 4557, Fax +1 785 864 5374, E-Mail jywu@falcon.cc.ukans.edu

tems therefore have primary relevance to the neurochemical and behavioral effects associated with ethanol dependence. The glutamatergic and the GABAergic systems have been implicated as target sites of brain adaptation after chronic exposure to ethanol [3, 103, 131, 140, 159, 201, 205]. In this review, we focus on recent advances in Glu and GABA systems with special focus on their roles in alcohol dependence and alcohol-withdrawal-induced seizures.

Glutamatergic System

Glutamate receptors in the mammalian CNS have been divided into two major families, namely, ionotropic and metabotropic receptors [34, 142]. The ionotropic receptors exhibit the best evidence for the alcohol effects on the CNS [158]. Ionotropic receptors can be categorized as responding to N-methyl-*D*-aspartate (NMDA), kainate (KA), or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). NMDA receptors are sensitive to low concentrations of ethanol (5–50 m*M*) and respond quickly to the action of ethanol [117, 121, 122]. The acute action of ethanol has been reported to be more potent at the NMDA receptor than at other glutamate-activated ligand-gated channels, and does not appear to be secondary to other electrophysiological consequences of ethanol application [123]. Thus it seems that ethanol acts directly on NMDA receptors. KA-receptor-mediated currents are also significantly inhibited by ethanol concentrations of 50 m*M* or more [36]. Contrary to most studies, it has been reported that concentrations as low as 20 m*M* significantly and reversibly depressed KA excitatory postsynaptic currents and suggested that ethanol may have an even greater inhibitory effect on glutamatergic synaptic transmission mediated by KA receptors than NMDA receptors in rat hippocampal CA3 pyramidal neurons [221]. It has been shown that AMPA receptors are inhibited by pharmacological-relevant concentrations of ethanol, however these receptors, unlike NMDA receptors, do not undergo adaptation with chronic ethanol exposure (CEE) sufficient to induce physical dependence [63]. It has been hypothesized that NMDA receptors mediate the common adaptive processes, which are involved in the development and maintenance of drug and alcohol addiction. We will focus on the NMDA glutamate receptor since this receptor is most likely a pivotal ethanol target, which leads to changes in many other neurotransmitter systems.

The NMDA receptors are coupled to a voltage-sensitive ion channel and permeable to calcium and monovalent cations (Na^+; K^+). The binding of glutamate and glycine to the NMDA receptor results in the opening of the ion-permeable channels [82]. Compared to the glutamate response at other receptor subtypes, the response of the NMDA receptor is relatively slow and results in a large amount of Ca^{2+} entering the cell through the activated NMDA receptor [79, 93]. This influx of Ca^{2+} mediated by NMDA receptors is important for synaptic plasticity and memory [10, 11, 89, 219]. When the receptor is excessively stimulated, the influx of Ca^{2+} can lead to excitotoxicity [41, 123]. Thus the function of NMDA receptors has been implicated in both health and disease, such as the development of epileptiform seizures [27, 46, 132], neurotoxicity [41, 94, 123], alcohol craving [128, 210, 231], Parkinson's disease [73, 120, 215], Alzheimer's disease, psychiatric syndromes, alcoholism, ischemic stroke [73], amyotrophic lateral sclerosis, Huntington's chorea, anxiety [110, 136] and chronic pain [12, 51, 130, 186].

Native NMDA receptor-channel complexes are thought to be heteromeric pentamers. There are at least five genes (NR1, 2A, 2B, 2C, 2D) encoding various subunits that have been cloned. NR1 is thought to be the essential subunit since it forms a functional channel by itself [48, 115], and the NR2 subunits are believed to modulate the properties of the channel [48]. In addition to these subunits, other proteins, which bind different NMDA ligands, have been reported and may function as receptor-associated proteins or subunits [142]. The glutamate binding protein has been cloned [101].

In situ hybridization and immunocytochemical studies have shown that NMDA receptor subunits show a high degree of regional and individual variability with some regions showing a strong preference for a particular subunit [64, 104, 153]. The NR1 subunits are found in the majority of central neurons. There are two NR1 splice variants, NR1a and NR1b. The NR1a isoform is present in the majority of NMDA receptors and the NR1b variant is present at high levels in the cortex and dentate gyrus of the hippocampus [139]. NR2 subunits are large glycoproteins whose specific expression profiles in the brain are developmentally and regionally regulated [108]. The NR2A-D and 3A subunits show distinct expression patterns in the mammalian brain, suggesting that NMDA receptor composition is unique to each region of the brain [64, 104, 172]. Immunological studies show that NR2A is located throughout adult rat brain, NR2B primarily in the forebrain, NR2C primarily in the cerebellum and NR2D is found mainly in the thalamus, midbrain and brainstem.

In the rat brain, NR2A and NR2C increase during postnatal development, whereas NR2B and NR2D are abundant at birth and declined with age [108]. Splice variants of the NR2C subunit have also been reported [172].

The electrophysiological and pharmacological properties of the NMDA receptor complex are largely determined by the combination of the NR2 subunits. Peak channel open probability is twofold to fivefold higher for NMDA receptors composed of NR1A/NR2A than NR1A/NR2B subunits. Thus a change in relative expression levels of NR2A and NR2B can regulate the open/close kinetics of the NMDA ion channels [29]. Therefore, unique combinations of subunits can determine NMDA receptor function [22, 87, 169, 188], and selected antagonists and agonists would have varying effects on particular NMDA receptors. Selectivity to particular subunits can be seen with infenprodil, an NMDA receptor NR2B-specific antagonist [168]. Therefore, subunit composition may determine vulnerability to ethanol and may explain why there is a differential effect of ethanol on various regions of the brain [102]. It has been reported that the NR1/NR2A and NR1/NR2B combinations are preferentially sensitive to ethanol inhibition [224], although it has also been reported that ethanol sensitivity of the NMDA receptor in primary cultured cerebellar granule cells is not related to subunit composition [168]. It is likely though that CEE may alter the subunit composition of the NMDA receptor, resulting in changes in binding of some ligands and not others [82, 206]. In fact, chronic ethanol treatment of rat cortical neuronal cultures leads to progressive increases in NR1, NR2A, NR2B and AMPA subunits GluR2/3 and decreases in the NMDA receptor subunit NR2D [26]. It also upregulates NMDA and AMPA receptor subunits but not KA receptor subunit proteins [26]. The increases in subunit expression are probably an adaptive response to the inhibitory effects of ethanol and implicate both NMDA and AMPA receptors in the adaptation of the CNS to chronic ethanol [26].

Ethanol and NMDA Receptors

Disulfiram, which has been used as a therapeutic agent for treating alcoholic patients for the last 50 years, is believed to exert its effect by inhibiting acetaldehyde dehydrogenase. However, recently we have shown that the active metabolite of disulfiram, DETC-MeSO, not only inhibits acetaldehyde dehydrogenase but also partially blocks glutamate receptors [155]. Furthermore, DETC-MeSO is quite effective in preventing seizures induced by NMDA or hyperbaric conditions [155]. Additionally disulfiram has been shown to markedly enhance the anti-craving activity of acamprosate [14], suggesting that DETC-MeSO may also have anti-craving activity. In this issue, we have further demonstrated that DETC-MeSO specifically antagonizes NMDA receptors without significantly affecting either KA or AMPA receptors [160]. These findings suggest that NMDA may play a role in alcohol dependence and alcohol-withdrawal-induced seizures.

The above notion is compatible with earlier observations that acute effects of ethanol disrupt glutamatergic neurotransmission by inhibiting the response of the NMDA receptor [76]. It has been reported that ethanol, at concentrations corresponding to those achieved in the blood after moderate ethanol intake, reduces NMDA-active ion currents, inhibits NMDA-evoked electrophysiological responses, and decreases glutamate binding in the hippocampus and cortex, thus acting as an NMDA antagonist [99]. Patch-clamp electrophysiological studies in Xenopus oocytes have also shown direct ethanol-protein interaction for the NMDA receptor [225].

No specific site of action for ethanol on the NMDA receptor has been found. Ethanol has been reported to interact with an allosteric site that reduces agonist efficacy by modulating the kinetics of channel gating [225]. It may interact with a discrete hydrophobic pocket within the receptor protein [166], thus modifying intermolecular forces and bonds leading to a conformational change [114, 121, 124, 166], thereby affecting the kinetics of channel opening and closing [39]. A possible interaction of ethanol with the phencyclidine site has been reported [45], although other laboratories have reported that ethanol does neither appear to interfere with the action of phencyclicine or Mg^{2+} [150], nor directly interact with the agonist binding, either at the glutamate recognition site of the receptor, or at any other known multiple modulatory sites, such as the glycine or polyamine site [45, 170, 224]. In cerebellar granule cells though, ethanol has been reported to reduce the potency of glycine to act as a co-agonist at the NMDA receptors and this inhibitory effect can be overcome by high concentrations of glycine [206]. In addition, antagonists which act at the glycine site of the NMDA receptor have been reported to control ethanol withdrawal seizures [196], suggesting an interaction with the glycine site. It has also been suggested that ethanol sensitivity of the receptor may be affected by intracellular C-terminal receptor domains that regulate the calcium-dependent inactivation of the receptor [6]. Ethanol has also been reported to decrease extracellular glutamate in

the brain of awake animals, by lowering presynaptic glutamate release [24, 146, 228]. Thus ethanol can also affect glutamatergic neurotransmission presynaptically by altering extracellular glutamate levels.

Chronic ethanol use results in CNS changes, which compensate for the effects of ethanol in an attempt by the body to normalize neurotransmission. Inhibition of NMDA glutamate receptors by acute ethanol exposure leads to the hypothesis that prolonged ethanol exposure would be accompanied by an increase in NMDA receptors. NMDA receptor upregulation is considered a major neuroadaptive response to the chronic blockage by ethanol. It has been reported that chronic ethanol exposure leads to an increase in NMDA receptor-ion channel complexes in certain brain areas of rodents [77, 82]. Michaelis et al. [141] reported increased glutamate binding in brains of rats after chronic ethanol treatment. In rodents there is an increase in brain dizocilpine binding after CEE, but no change in glycine binding or in the binding of the NMDA receptor antagonist, CGS-19755 [78]. In animal models, it has been reported that chronic ethanol exposure leads to the upregulation of NMDA receptors [67, 70, 80, 85] and an increase in mRNA and protein levels for functional receptor subunits [30, 31, 55, 198]. CEE leads to an increase of the NMDA receptor number at the transcriptional and posttranscriptional level [224]. The upregulation of NMDA receptor complexes in the hippocampus return to control levels over a 24- to 48-hour abstinence period.

Most reports agree that CEE leads to an increase in NMDA receptor number, but not all do. Some studies indicate that robust increases in NMDA receptor binding do not occur with chronic ethanol treatment and suggest that NMDA receptor supersensitivity during the development of tolerance and dependence to ethanol may not simply be due to changes in the density of NMDA receptors, but may involve other mechanisms [25, 182]. In 1999, Freund and Anderson [58] reported that the density or affinity of NMDA receptors in the cingulate cortex, hippocampus, and the cerebellar vermis in human alcoholics does not change. They concluded that a long-lasting upregulation of the number or affinity of NMDA receptors is not a key feature of chronic alcoholics, but in 1996 they reported that chronic alcoholism moderately increases the density of the NMDA receptors in the frontal cortex [57]. From that study they concluded that an upregulation of NMDA receptors may represent a stage of alcohol-induced chronic neurotoxicity. These results indicate that there are regional differences concerning the modulation of NMDA receptors by ethanol. Additionally results from human studies differ from animal models of alcohol dependence. These differences may be related to the longevity of alcohol exposure in human alcoholics, as well as variability in the dependence/withdrawal state of the human subjects. Thus further studies in human postmortem brain tissue are warranted [145]. The apparent inconsistencies in the literature can often be attributed to the complexity of the neuronal circuitry, differences in methodology, including different animal models, neuronal cultures, difficulty in identifying particular brain regions, etc.

It has been shown that NMDA receptor antagonists promote the release of dopamine in the nucleus accumbens [202], and can revert the reduced dopamine levels during ethanol withdrawal [179]. This indicates that NMDA receptors mediate dopaminergic activity [54]. The decreased dopaminergic function which occurs during alcohol withdrawal has been suggested to be involved in alcohol craving [43, 105], and this decreased dopaminergic function may occur secondarily to the increase in NMDA receptor function during withdrawal. In addition 5-HT3 receptors are involved in alcohol seeking behavior including alcohol intoxication and addiction, and it has been reported that ethanol alters the function of the 5-HT3 receptor [125, 232]. Dopamine has been reported to activate the serotonin 5-HT3 receptor and ethanol potentiates that activation [126]. Thus the increase in NMDA function upon ethanol withdrawal may lead to a decrease in both the dopaminergic and serotonergic systems. This may be an underlying mechanism for craving in the ethanol-dependent individual.

Acute ethanol inhibits NMDA receptor function, thus changing the intracellular calcium levels, which in turn affects several cell-signaling cascades including phosphorylation. Phosphorylation regulates the activity of various ion channels, G proteins, and enzymes as well as transcription and translation with diverse and potentially profound consequences for neuron function [71, 86]. Thus prolonged exposure to ethanol leads to many neuronal adaptations through changes in signaling cascades in the cell, the primary trigger being the inhibition of NMDA glutamate receptors. Hence, it is conceivable that the NMDA glutamate receptor is the key target responsible for many of the ethanol-induced alterations in the CNS, including changes in gene expression, which could underlie the development of tolerance and dependence.

Ethanol and Phosphorylation

Phosphorylation has been reported to alter the activity of both the GABAA receptor ion channels, and the NMDA subtype of glutamate receptors [72, 109, 173, 220], however this is a controversial issue [127, 192, 193]. Abnormalities in protein phosphorylation have been implicated in neurodegenerative diseases [218]. Protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and calcineurin are all regulated by Ca^{2+} levels, and have been shown to be altered in a variety of cell systems by acute and chronic ethanol exposure. Ca^{2+} influx through the NMDA receptor has been shown to activate PKC suggesting that the effect of ethanol on Ca^{2+}-dependent kinases and phosphatases may be modulated through NMDA receptors. It is possible that alterations in the phosphorylation cascades during CEE are the critical molecular events associated with the development of ethanol dependence [164].

The regulation of ligand-gated ion channels by phosphorylation is complex [203]. Ethanol has been reported to decrease the activity of PKC [100, 195], but chronic ethanol has been reported to increase the activity of PKC by increasing the expression of two PKC isozymes δ and ε [178]. It appears that the GABAA receptor is not affected by ethanol unless it contains the γ2L subunit, which can be phosphorylated by PKC [72, 184, 216, 217], but the data are complex. Both activation and inhibition of GABAA receptor currents as a result of PKA and PKC phosphorylation have been reported, while phosphorylation by protein tyrosine kinase (PTK) enhances function. PKC has also been reported to regulate the activity of NMDA receptors [123], some reports indicate activation by PKC [28, 92, 154], others indicate inhibition [37, 197]. This apparent contradiction may simply be a reflection of the NMDA receptor subunit composition. Grant et al. [66] reported that NMDA receptor subunits NR2A or NR2B in combination with NR1 are enhanced by PKC, whereas NR2C and NR2D in combination with NR1 are suppressed by PKC. Xiong et al. [227] reported NMDA receptor potentiation by PKC with the NR1a/NR2A subunits, but not with the NR1a/NR2B combination. Thus we must always keep in mind that seemingly contradictory data may actually have an underlying explanation. In addition to mediating NMDA receptor activity, PKC may also modulate the clustering of NMDA receptors [203]. CaMKII has also been reported to regulate NMDA receptor activity [134]. Ca^{2+} influx via the NMDA receptor leads to activation and autophosphorylation of CaMKII [200], which may then lead to the phosphorylation of NMDA receptors. The NMDA subunit NR2B but not NR2A or NR1 have been reported to be responsible for autophosphorylation-dependent targeting of CaMKII. Colocalization of CaMKII and NMDA receptors have been reported and a CaMKII/NMDA receptor complex has been isolated from brain extracts [200].

In addition to PKC and CaMKII, other protein kinases and phosphatases have been reported to regulate the activity of NMDA receptors. Yotiao, a scaffold protein that attaches type I protein phosphatase (PP1) and protein kinase A [PKA (cAMP-dependent)] holoenzyme to NMDA receptors, has been described. Anchored PP1 is active and limits channel activity. PKA activation can overcome constitutive PP1 activity and enhances NMDA receptor currents [222]. Additionally it has been noted that a PTK upregulates NMDA receptor function [230]. A PTK has been reported to regulate the function of the NR2B receptor subunit [95], and the NR2A subunit at high (sedative) ethanol concentrations [5]. PSD-95, a molecular scaffold protein which directly binds to and coclusters with NMDA receptors, may anchor PTKs to NR2A, thus promoting tyrosine phosphorylation of NR2A [207]. Thus although there is a general consensus that ethanol modulates both GABAA and NMDA receptor functions through protein phosphorylation, there is less agreement regarding the effect of protein phosphorylation on receptor functions [72, 217, 220], and which kinases and phosphatases are responsible for the phosphorylation state of the receptors.

GABAergic System

It is our belief that effects of ethanol on the dopaminergic and other neurotransmitter systems are secondary to the effects on both the glutamatergic and GABAergic systems, although this belief is not universally shared [18]. In addition, activation of NMDA glutamate receptors leads to a reduction in the activity of GABAA receptors [88]. Therefore inhibition of NMDA glutamate receptors, by ethanol exposure, should lead to an increase in the activity of the GABAA receptors, suggesting that NMDA receptors may be the primary target of ethanol. Chronic in vitro application of NMDA agonists and antagonists alters GABAA receptor function and mRNA expression. In vivo chronic blockage of NMDA receptor by MK-801 significantly increased hippocampal GABAA receptor α_4 and γ_2 subunit expression while decreasing α_2 and $\beta_{2/3}$ subunit expression. Thus glutamatergic activity mediated by NMDA may regulate the subunit expression of GA-

BAA receptors [133]. Therefore alterations in GABAergic function following chronic ethanol may be a compensatory response secondary to changes in glutamatergic neurotransmission [54]. Although this idea is reasonable, it must be noted that glutamatergic transmission can also be modulated by GABAergic transmission [46], apparently via presynaptic GABAB receptors regulating glutamate release [96]. The GABAB receptor agonist baclofen has been shown to decrease the intensity of ethanol withdrawal and to reduce ethanol intake in rats, and preliminary studies suggest that it also reduces ethanol intake and craving in human alcoholics [2, 35].

Like the glutamate receptors, GABA receptors have also been classified as either ionotropic and metabotropic receptors. To date there is only one well-characterized ionotropic GABA receptor: the GABAA receptor [162, 163]. It is a receptor-gated chloride channel that contains binding sites for GABA as well as a number of pharmacologically important drugs such as benzodiazepines, barbiturates, convulsants, certain steroids and ethanol [112, 113, 162]. The GABAA receptor is a pentameric complex composed of distinct polypeptides, which have been divided into five subunit classes on the basis of sequence homology [191]. At least 17 isoforms of the receptor subunits have been identified and cloned in mammalian brain and designated α_{1-6}, β_{1-4}, γ_{1-4}, δ and ρ_{1-2} [171]. In addition several isoforms exist in alternatively spliced forms [171]. The α-subunit appears to be responsible for GABA enhancement of benzodiazepine binding [112, 113]. Two alternatively spliced forms of the γ_2 subunit of GABAA receptor (γ_{2L} and γ_{2S}), which differ by an exon of eight amino acids, show different sensitivities to modulatory effects of ethanol of receptor activities. This γ_2-subunit variant may be important in the predisposition to alcoholism [32]. Recombinant studies have shown that variations in GABAA receptor pharmacology and function can result from altering its subunit isoform composition [111].

Most of the studies on effects of ethanol have focused on the GABAA receptor, but the metabotropic receptor has also been reported to be affected by ethanol [4]. There is at least one metabotropic GABA receptor: the GABAB receptor and its G-protein-coupled receptors [16, 49]. G-protein-coupled receptors usually influence neuron function through indirect effects on ion channels or neurotransmitter release. G-proteins may directly influence the activity of ion channels [75], or they may have indirect effects on ion channel activity through the activation of second messenger cascades (adenylyl cyclase, guanylyl cyclase, phospholipase C). Activation of GABAB receptors can lead to changes in calcium and potassium currents, as well as to inhibition of adenylyl cyclase activity [16, 49]. GTP binding proteins (G proteins) have a major role in cell signaling and are perturbed by ethanol in adenylyl cyclase-coupled signal transduction [214].

Ethanol shares several pharmacological actions with barbiturates and benzodiazepines, including anxiolytic and sedative activity. The similarities of the actions of these drugs were the basis for the initial theories of ethanol action on the brain leading to the suggestion that all three drug classes share some mechanism of action linked to the GABAA-receptor-coupled ion channel [54]. Alterations in the density or affinity of brain GABAA/benzodiazepine receptors following chronic ethanol treatment have yielded conflicting results, and the pharmacologically relevant dose range at which ethanol affects GABA receptors is still unresolved. [54]. Ethanol has been reported to potentiate GABAA receptor ion channels [3, 19, 201, 220], but other studies have reported no such effect [190]. This is probably due to subunit differences in various brain regions [69, 216], thus although ethanol increases GABAA-receptor-mediated inhibition, this does not occur in all brain regions, all cell types in the same region, nor at all GABAA receptor sites on the same neuron. The molecular basis for the selectivity of the action of ethanol on the GABAA receptor has been proposed to involve a combination of BZ subtype, a β_2 subunit and a splice variant of the γ_2 subunit, but substantial controversy on this issue currently remains. The enhancement of the GABAA receptor does not appear to be regulated by the binding of ethanol to the benzodiazepine, barbiturate or neurosteroid sites [127].

Acute ethanol enhances GABA neurotransmission, whereas chronic exposure may decrease receptor number or function thus leading to tolerance, dependence, and ethanol withdrawal syndrome [123]. There are still a number of controversies regarding the molecular mechanisms responsible for the effect of ethanol on GABAA-receptor-mediated anion flux [97]. Recombinant studies show that altering the subunit isoform composition modifies the pharmacology and function of the GABAA receptor [111]. Thus the decrease in ethanol action may involve changes in subunit expression. It has been suggested that chronic ethanol administration alters the expression of GABAA receptor subunit mRNAs differently [42, 140, 147, 152]. CEE alteration of GABAA receptor subunit expression may lead to hypofunction and reduction of receptor density, during withdrawal this would contribute to increased neural excitability [147, 151, 208, 209]. It is also possible that ethanol may affect posttranslational

modifications of GABAA receptors thus altering GABAA function [68].

Ethanol affects particular brain regions and does not have a global toxic effect on the brain [118]. Reduced levels of GABAA receptor in several cortical regions and the cerebellum of alcoholics have been reported [59, 60], although others report alcoholics show no difference in the density of GABAA/benzodiazepine receptor binding sites [61, 62, 97]. In addition, some groups report a greater density of GABA agonist sites (labeled with [^{3}H]muscimol) on the GABAA receptor in the superior frontal gyrus of alcoholics [47]. Thus differences in receptor density or affinity upon CEE are most likely dependent upon the brain region. Chronic ethanol administration can alter GABAA subunit expression which can alter GABAA receptor function and pharmacology [112, 113], although it does not result in downregulation of the GABAA α_1 subunit [135]. If alcoholics do indeed show a reduction in GABAA receptors, ethanol either has a toxic effect upon these receptors or the reduction in these receptors represents a vulnerability factor for developing alcoholism [1].

It has been theorized that alcoholics have a GABA deficit. To support this theory it has been reported that initially after detoxification alcoholics show normal plasma GABA levels, but after 3–4 weeks of detoxification a subset of alcoholics show a significant decrease in plasma GABA levels [167]. GABA is synthesized by a single enzymatic reaction catalyzed by *L*-glutamate decarboxylase (EC 4.1.1.15; GAD) [177]. It has been reported that GAD1, which is localized on chromosome 2 (GAD67), may be a genetic determinant for the development of physical dependence on ethanol. A suggestive link between GAD1 (GAD67) and alcohol withdrawal severity has been reported [21]. A genetic difference in GAD67 may thus predispose to alcoholism.

There are two well-characterized isoforms of GAD, which are encoded by two separate genes: GAD65 and GAD67. Comparison of the deduced amino acid sequences of these enzymes indicate that they are ~65% homologous [20]. The gene for human GAD65 has been localized to human chromosome 10 [20, 90] and the gene for GAD67 has been localized to chromosome 2 [17, 20]. The activity of GAD has been linked to the levels of Zn^{2+} [226], and pyridoxamine (vitamin B_6), both are necessary for the synthesis of the coenzyme pyridoxal-5′-phosphate of GAD [50]. It has been reported that the absorption of both Zn^{2+} and pyridoxamine is reduced upon CEE [143, 157], this may be one mechanism by which CEE affects the activity of GAD. Zinc has also been reported to modulate NMDA receptors, and a zinc deficiency has been shown to impair brain function [138, 165, 185].

The expression of GAD65 and GAD67 is differentially influenced by other neurotransmitter systems such as dopamine [106] and excitatory amino acids [107]. In addition, the level of GAD65 and GAD67 proteins are also regulated differently by GABA levels [175, 176]. There are also differences in the expression levels of the two isoforms of GAD in different brain regions [199].

It is generally believed that GAD67 is a soluble protein and is distributed more evenly throughout the cell, whereas GAD65 is concentrated in the nerve terminals in association with synaptic vesicles [52, 53, 83, 91]. Changes in neuronal function can regulate GABA synthesis by altering GAD [8, 65, 144]. Intracellular Ca^{2+} levels may influence the activity of GAD by modulating the phosphorylation state of GAD. It has been shown that GAD is regulated by reversible phosphorylation [8, 9, 83], and that the two major GAD isoforms GAD65 and GAD67 are also regulated by phosphorylation [40].

Additionally, an influx of Ca^{2+} may initiate a mechanism which leads to synaptic vesicle binding of GAD65 and subsequent activation through phosphorylation by a membrane-associated protein kinase [83, 84], this would lead to an increase in transmitter GABA synthesis. The increase in Ca^{2+} may also lead to the activation of GAD67, the soluble form of GAD [8, 9]. Thus, reduction in Ca^{2+} influx, caused by ethanol, could lower GABA synthesis and release, although this may be a mechanism by which GABA levels are lowered, the effect of ethanol upon GABA release is complex and controversial. From the above discussion it seems reasonable to propose that the effect of ethanol on the GABA system could be at the receptor level as well as at the GABA level through its effect upon GABA synthesis via GAD.

Tolerance, Dependence and Treatment

NMDA receptors may mediate the common adaptive processes that are involved in the development and maintenance of drug and alcohol addictions, thus a novel treatment approach is the modulation of glutamatergic neurotransmission with NMDA receptor antagonists. The adaptive changes in the brain caused by CEE creates tolerance and eventually leads to the dependence upon ethanol for normal neuronal function. During withdrawal, overstimulation of glutamate receptors may constitute a primary neurochemical mechanism for chronic ethanol-induced brain damage [44, 121, 122, 223]. Withdrawal of

chronic ethanol treatment results in increased glutamate concentrations in the brain [180], this response is presumably mediated by activation of adapted (upregulated) NMDA receptors. Overactivation of glutamate receptors may be a common pathway for the pathogenesis of many neurological diseases [119]. In fact, repeated ethanol withdrawals, rather than ethanol exposure itself, contribute to ethanol-induced degeneration of neurons. Thus ethanol-induced degeneration of neurons is not only related to the amount of ethanol consumed, but also to the patterns of drinking [174].

NMDA antagonists may have multiple functions in treating addictions, including normalization of the neurological adaptations induced by chronic addiction, which should thus reduce withdrawal symptoms [15]. NMDA receptor antagonists can prevent the calcium-dependent increases of glutamate levels induced by activation of NMDA receptors in vivo [23, 24, 148, 229], and have been reported to reduce ethanol withdrawal symptoms [67, 116, 150, 180]. In addition NMDA receptor agonists have been reported to worsen the withdrawal syndrome [150, 187].

The development of tolerance to many drugs of abuse may be blocked with antagonists of the NMDA receptor, making them useful as anti-craving drugs. Other research groups have reported that the NMDA receptor may be pivotal to the pathophysiology of common psychiatric syndromes including alcoholism. [73, 211]. Thus the development of pharmacological strategies aiming at the modulation of NMDA receptor-mediated neurotransmission in the treatment of alcoholism is becoming increasingly important. Treatment with NMDA receptor antagonists may not only attenuate withdrawal seizures, but may also prevent withdrawal-induced neuronal damage [82].

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Review

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Intragastric Ethanol Infusion Model for Cellular and Molecular Studies of Alcoholic Liver Disease

Samuel W. French

Department of Pathology, Harbor-UCLA Medical Center, Torrance, Calif., USA

Key Words

Hypoxia · Cytokine · Oxidative stress · Apoptosis · CYP2E1 · Oxidized proteins · Neoantigens · Proteasome · NFκB

Abstract

The intragastric alcohol infusion rat model (IAIRM) of alcoholic liver disease (ALD) has been utilized in various laboratories to study various aspects of ALD pathogenesis including oxidative stress, cytokine upregulation, hypoxic damage, apoptosis, ubiquitin-proteasome pathway and CYP2E1 induction. The basic value of the model is that it produces pathologic changes which resemble ALD including microvesicular and macrovesicular fat, megamitochondria, apoptosis, central lobular and pericellular fibrosis, portal fibrosis, bridging fibrosis, central necrosis, and mixed inflammatory infiltrate including PMNs and lymphocytes. The model is valuable because the diet and ethanol intake are totally under the control of the investigator. A steady state can be maintained with high or low blood alcohol levels for long periods. The cycling of the blood alcohol levels, when a constant infusion rate of alcohol is maintained, simulates binge drinking. Using this model the importance of dietary fat, especially the degree of saturation of the fatty acids on the induction of liver pathology, has been documented. The role of endotoxin, the Kupffer cell, TNFα, and NADPH oxidase have been demonstrated. The importance of 2E1 in oxidative stress induction has been shown using inhibitors of the isozyme. The importance of dietary iron in the pathogenesis of cirrhosis has been documented. Acetaldehyde has been shown to play a role in preventing liver pathology by preventing NFκB activation. Using the model, to maintain high blood alcohol levels is found to be necessary to demonstrate proteasomal peptidase inhibition. Ubiquitin synthesis is also inhibited at high blood alcohol levels in the IAIRM model. Oxidized proteins accumulate in the liver at high blood alcohol levels. Neoantigens derived from protein adducts formed with products of oxidation induce autoimmune mechanisms of liver injury. Thus, in many ways the model has revolutionized our understanding of the pathogenesis of ALD.

Introduction

The intragastric alcohol infusion rat model (IAIRM) was originally designed to overcome the natural aversion that rats have to oral feeding of ethanol and to insure that the diet was optimized to nutritionally support normal growth. The diet was defined making it possible to omit or supplement any ingredient. The technique had already

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Samuel W. French, MD
Department of Pathology, Harbor-UCLA Medical Center
1000 W. Carson Street
Torrance, CA 90509 (USA)
Tel. +1 310 222 2643, Fax +1 310 222 5333, E-Mail French@HUMC.edu

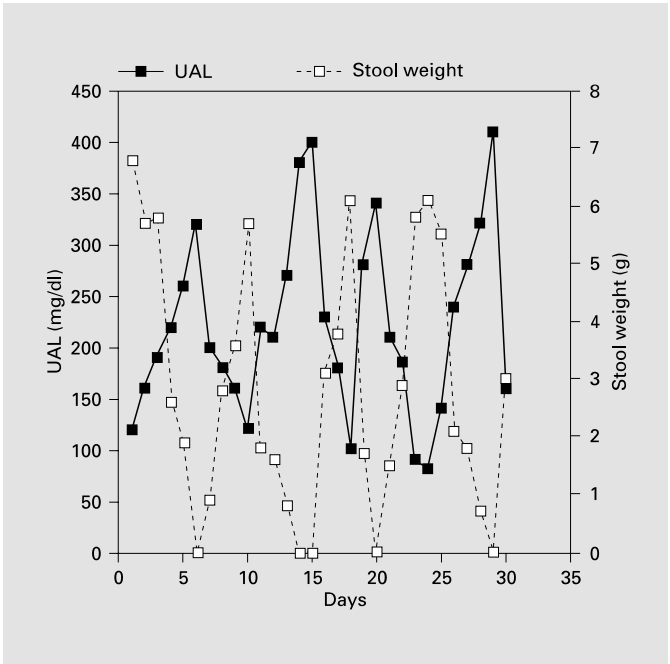

Fig. 1. Cycling of stool weights correlate inversely with the UAL during the UAL cycle.

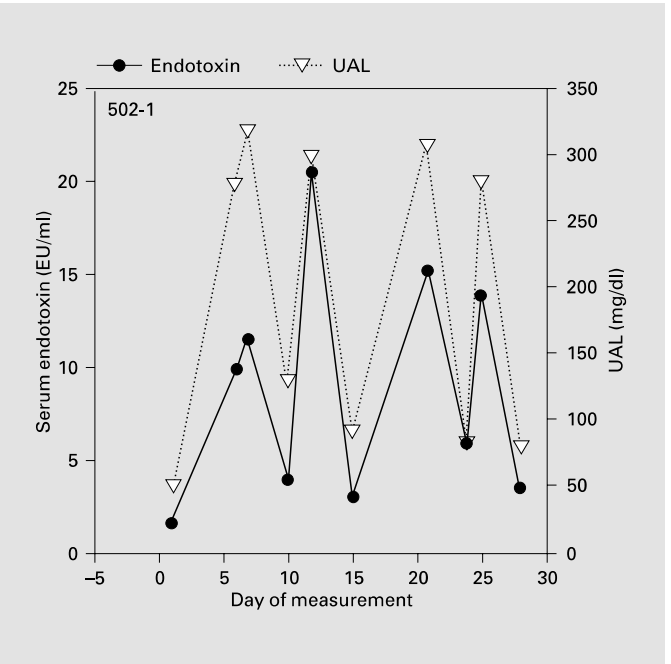

Fig. 2. Cycling of the blood endotoxin levels correlates positively with the UAL cycle.

been used to administer experimental drugs on a strict dosage schedule. We first applied the technique in a rat model of alcohol withdrawal. Rats became ethanol dependent in a week of ethanol feeding by IAIRM [45]. Pair-fed controls were fed isocaloric dextrose in parallel with the ethanol-fed rats. This made it possible, for the first time, to perform experiments where the control and ethanol-fed rats were in exactly the same state of nutrition.

Prior studies using rats fed ad libitum had the unfortunate problem where the pair-fed control rat would eat the diet quickly when given the daily portion, whereas, the ethanol-fed rat would eat the diet and ethanol very slowly over the entire 24-hour feeding period. Consequently, the state of nutrition was different at the time when the rats were assayed. The IAIRM-fed rats, however, have to be monitored daily for blood or urinary ethanol levels (BAL and UAL) because the UAL cycles over a 6- to 12-day span if the 24-hour ethanol dose/kg is given continually 24 h/day [54]. The ethanol levels cycle but so do a large number of other variables such as body temperature and O_2 consumption rate [26]. For instance, the stool weight (fig. 1) and the blood levels of endotoxin (fig. 2) decrease and increase, respectively, when the UAL peaks. To compensate for this, the daily dose of ethanol must be increased or decreased based on the BAL or UAL [19]. Therefore, ethanol must be delivered by a separate syringe and not the syringe that delivers the diet. The calories derived from the diet remain constant based on the rats' weight while the calories from ethanol vary depending on the UAL and the BAL. This regimen is required in order to optimize the progression of the liver pathology to fibrosis [16].

Four research laboratories have employed the IAIRM rodent experimental model of alcoholic liver disease (ALD) as recently reviewed at a conference in Bordeaux, France, and have published the proceedings [20]. The data produced by these four laboratories and their collaborators is the basis of this review. The laboratories are headed by Dr. Tsukamoto, Thurman, Nanji and French. The focus of their research is schematically shown in figure 3.

Role of Dietary Fat in the Pathogenesis of ALD

Initially, a low fat diet (5% of total dietary calories) was used in the IAIRM. This produced a fatty liver and apoptosis but no fibrosis [53]. However, when the fat content was increased to 25% of total calories, fibrosis developed [58]. When the source of dietary fat was changed from

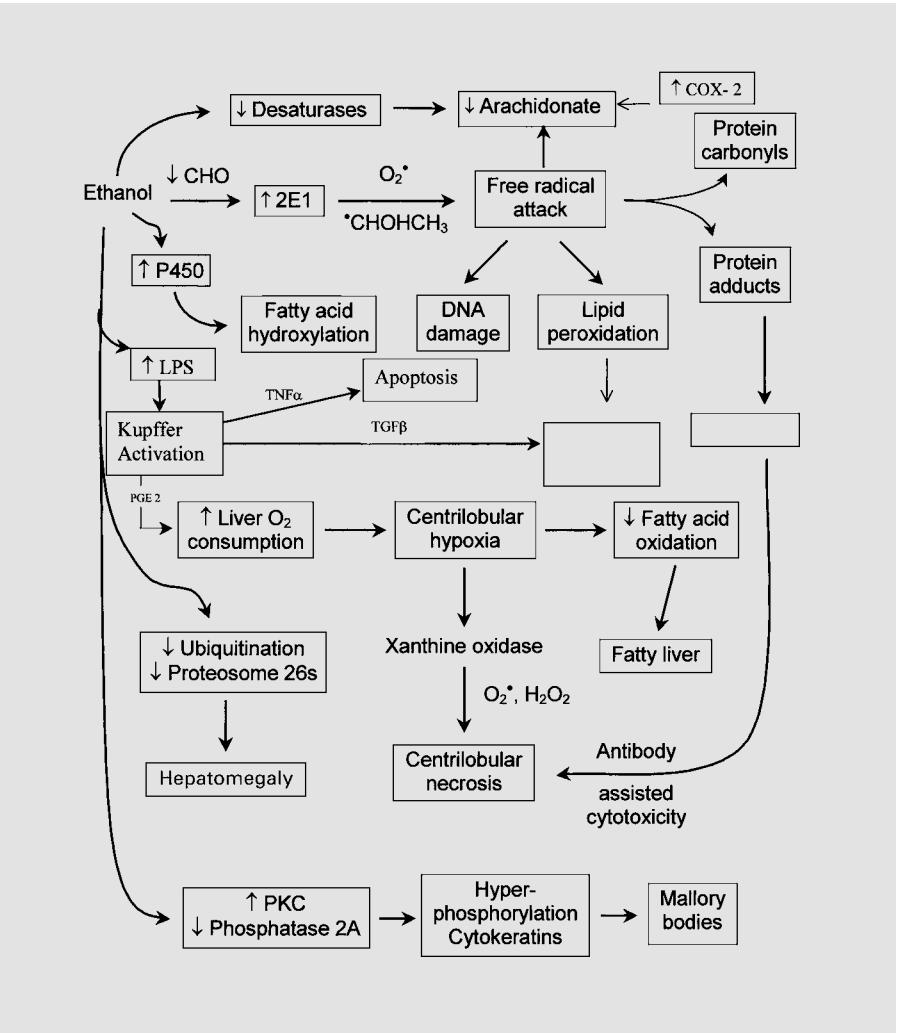

Fig. 3. Scheme of the multiple variables which may play a role in the pathogenesis of experimental ALD. This scheme is a modification of a similar scheme published previously [4].

corn oil to beef fat (tallow) which is deficient in the essential unsaturated fatty acid linoleate, all of the pathologic changes in the liver were prevented even when the diet was supplemented with ethanol for 6 months [40]. When linoleate was added to the tallow diet, this restored the ability of the diet to produce liver fatty change and fibrosis [35]. Currently, medium chain triglycerides are used instead of tallow to prevent ALD when IAIRM is studied. Subsequently, fish oil in the diet was found to exaggerate the severity of the pathology of liver using IAIRM [34]. Even the pair-fed control developed fibrosis in the absence of ethanol. This supports the concept that ethanol is not toxic to the liver unless the diet is rich in polyunsaturated fatty acids. The unsaturated fatty acids are necessary for ethanol metabolism to generate free radicals to attack lipids to form lipid peroxides which are damaging to the liver. Recently arachidonic acid in the ethanol-induced liver cell damage was shown to generate free radical changes in vivo and in vitro [5, 43] and stimulated collagen expression in vitro [44]. The high fat diet in IAIRM by itself partially activates Kupffer cells to release TGFβ in vitro to stimulate stellate cell synthesis of collagen [18]. Fibrosis in vivo is not increased until 2 months of feeding alcohol [17], and stellate cell activation in vivo is not demonstrable until 4–5 months of ethanol feeding. Even then, the scarring is focal [51]. Cirrhosis occurred only when the diet was enriched with carbonyl iron supplements [56] possibly by sensitizing Kupffer cells to endotoxin as indicated by NFκB activation [57]. Thus, a diet high in unsaturated fat and high in iron is important in the pathogenesis of IAIRM-induced ALD.

Role of Hypoxia in the Pathogenesis of ALD

Rats fed ethanol chronically by IAIRM developed a vulnerability to hypoxia which was induced by placing the rats in a chamber of 6% oxygen for 5 h [13]. Similarly, rats fed ethanol by IAIRM for 5 months in a chamber containing CO to induce chronic carboxyhemoglobin-induced chronic hypoxia develop evidence of increased liver damage compared with pair-fed controls kept in the same chamber [37]. These experiments using IAIRM established the vulnerability of the ethanol-fed rat to hypoxic injury. To establish a hypoxic condition in the liver, the levels of ATP, ADP, AMP and adenosine were measured using freeze-clamped liver tissue and HPLC [29, 30]. The level of ATP decreased and ADP, AMP and adenosine increased in the ethanol-fed rat livers tested monthly for 6 months. The question then was: Was the decrease in ATP levels due to hypoxia of the liver or was it due to mitochondrial injury caused by ethanol/acetaldehyde toxicity? To answer this question the rats were made acutely hypoxic or hyperoxic by breathing 5% or 100% O_2 for 3 min before measuring ATP levels. The response of the ethanol-fed rat livers to acute hypoxia exaggerated the decrease in ATP levels and increase in AMP, ADP and adenosine compared to pair-fed controls. The reverse was true under hyperoxia. Hyperoxia increased the levels of ATP and decreased the levels of ADP and AMP in an exaggerated way in the ethanol-fed rats. Using the IAIRM-fed rats where high BAL was maintained at the time that the measurements were made, it was possible to establish that ethanol-induced ATP deficiency was due to hypoxia not due to mitochondrial injury.

To further establish liver hypoxia in IAIRM the P_i/ATP ratio in vivo in IAIRM was monitored monthly for 6 months [49] using ^{31}P NMR. The P_i/ATP ratio was increased by ethanol feeding and the degree of increase correlated with the severity of the pathology score suggesting that hypoxia was linked pathogenetically to the liver injury. IAIRM made it possible to continue diet and ethanol during the measurement of the in vivo P_i and ATP. These studies were repeated in a mouse model of chronic intragastric ethanol feeding (IAIMM). Mice require up to 25 g/kg/day to maintain high BAL. The mice were subjected to normoxia and hypoxia (5% O_2) for 2 min [15]. The P_i/ATP ratios were measured by ^{31}P NMR while the mice were being fed diet and ethanol. The mice, like the rats fed ethanol, showed an increased P_i/ATP ratio which worsened with acute hypoxia with slowed recovery when normoxia was reestablished. This was the first IAIMM using ethanol-fed mice intragastrically.

It remained to actually measure hypoxia by measuring the O_2 tension in the hepatic venous blood in IAIRM [55, 59]. There was a significantly lower O_2 tension in the hepatic venous blood which was documented by direct measurement of centrilobular hypoxia as a cause for reduced levels of ATP. This suggested that the centrilobular necrosis observed in IAIRM was due to hypoxia.

Role of Oxidative Stress in ALD Pathogenesis

Many products of oxidative stress have been demonstrated in the liver of the IAIRM rat fed ethanol beginning with diene conjugates [55] lipid peroxidation, malondialdehyde, 4 hydroxynonenal [14], hydroxyethyl (HE) radical [18] and adducts of malondialdehyde and HE [18]. HE adducts which form with CYP2E1 have been detected and these adducts form neoantigens to which antibodies form [2]. NFκB activation, an indicator of oxidative stress, has been reported in IAIRM [28]. NFκB activation may result from endotoxin derived from a 'leaky' intestine caused by ethanol or it may result from oxidative stress or both [46]. In either case NFκB activation leads to upregulation of cytokines, the most notable being TNFα. Most likely TNF is increased as the result of Kupffer cell activation [61]. The activation of Kupffer cells can be aborted by feeding lactobacillus [38], by gadolinium treatment [24] and by TNFα antibody [40]. Likewise, TNFα-R2 knockout mice failed to develop liver pathology when fed ethanol in the IAIMM [61]. Paradoxically elevation of acetaldehyde levels by giving ethanol-fed rats aldehyde dehydrogenase inhibitors also inhibited NFκB activation [27]. TNF knockout mice fed ethanol (IAIMM) also failed to develop NFκB activation [61]. These interventions which block NFκB activation or TNFα upregulation also ameliorated the liver pathology caused by ethanol feeding. This has led some to conclude that TNFα mediates at least some aspects of the pathology observed in early experimental ALD.

The same approach has been applied to the problem of oxidative stress in studies on CYP2E1-induction where CYP2E1 levels increase 5- to 10-fold in IAIRM [50]. CYP2E1 generates free radicals and oxidative stress during ethanol metabolism by the liver [1]. Lipid peroxidation and oxidation of proteins are increased by ethanol-feeding in IAIRM and this increase correlates positively with severity of the liver pathology [47]. To investigate the importance of this link between oxidative stress and pathology, several CYP2E1 inhibitors were fed using the IAIRM model. Diallyl sulfide, phenethyl isocyanate and

chlormethiazole have been studied for this purpose. All three inhibitors reduced CYP2E1 induction or inhibited the enzyme in vivo. The inhibitors reduced the liver pathology significantly as well as reduced the products of oxidative stress including adduct formation and antibody production [21, 31–33]. However, where a saturated fat diet prevented ethanol-induced liver pathology using IAIRM, the CYP2E1 was induced by ethanol indicating that CYP2E1 requires cofactors in order to cause liver injury [50]. A negative correlation was found with the use of the mouse model where CYP2E1 knockouts were fed ethanol (IAIMM). Here, liver pathology was not reduced in the CYP2E1 knockout mice [23]. However, both the wild-type and the knockout mice generated the same degree of free radical formation in the bile when ethanol was fed in this study. This would suggest that the antioxidants in the liver tissue were reduced in the knockout mice and/or there was an induction of other free radical producing P450 enzymes. In either case oxidative stress was at the same level in the wild-type and the knockout mice fed ethanol. Thus it can be concluded that oxidative stress is a major contributor to the liver pathology in both the mouse and rat IAIM model in the presence or absence of CYP2E1 and that CYP2E1 plays a major role in the wild-type rodents.

Role of Fatty Acid Metabolism in IAIRM Experimental ALD

Arachidonic acid is reduced in IAIRM by several mechanisms. First, fatty acid hydroxylation and epoxidation is accellerated in the liver in IAIRM [4]. Second, arachidonic acid is utilized in prostaglandin and thromboxane synthesis because ethanol activates phospholipase A and phospholipase C and induces COX-2 [42, 43]. As a consequence thromboxane increases, as does PGE_2 [39]. Thromboxane A_2 is vasoconstrictive and may reduce liver blood flow and contribute to liver hypoxia caused by high BAL. PGE_2 stimulates the liver cell metabolic rate and may contribute to hypoxia of the liver by increasing the O_2 gradient in the liver lobule. The role for endotoxin in this process has been shown in acute ethanol exposure [46].

Arachidonic acid is the polyunsaturated fatty acid target for lipid peroxidation caused by ethanol metabolism. The level of arachidonic acid is inversely proportional to the level of lipid peroxidation [15]. Peroxisome proliferator activator receptor is down-regulated by the reduction of arachidonic acid by ethanol in IAIRM [60]. In vitro studies have shown that arachidonic acid was an inducer of oxidative stress which upregulates collagen type 1 gene expression in stellate cells which required CYP2E1 activity and involved H_2O_2, which initiated increased expression of COX-2 levels and the production of PGE_2 [44]. In summary, the oxidative stress, imposed by CYP2E1 induction by ethanol, initiates PGE_2 synthesis from arachidonic acid due to CYP2E1 induction of COX-2. This may involve Kupffer cells, hepatocytes and stellate cell synthesis of collagen leading to liver hypoxia, fatty liver and fibrosis.

Role of Protein Oxidation and the Ubiquitin-Proteasome Pathway

Ethanol increases protein carbonyls in the liver in IAIRM [47]. At the same time ethanol inhibits the proteasome system of removal of cytoplasmic proteins such as oxidized proteins, proteins synthesized in excess and missense, and misfolded proteins [6, 9, 22, 48] including ethanol-induced CYP2E1 [4]. As a consequence, proteins, including oxidized proteins, accumulate in the liver cells and this accounts for the increase in the liver weight in rats fed ethanol [6]. The 26S proteasome is more severely inhibited by ethanol than the 20s proteasome [6]. Ubiquitin protein and mRNA is markedly reduced including ubiquitin-protein conjugates [22]. Ethanol-induced Mallory body formation in IAIMM may result from proteasomal inhibition where cytokeratins form an aggresome in liver cells [12, 62]. Immunohistochemical studies of livers in IAIMM and IAIRM showed the loss of proteasomes in the centrilobular hepatocytes [12]. This might explain why Mallory bodies develop in centrilobular hepatocytes in human central sclerosing hyaline necrosis as seen in human alcoholic hepatitis.

The Role of the UAL in the Pathogenesis of Experimental ALD

The UAL cycle was first reported using IAIRM [54]. BAL was measured daily while feeding ethanol at a constant dose. Subsequently BAL has been followed by measuring UAL because the BAL and UAL are equilibrated when ethanol is infused at a constant rate [3]. The UAL cycle occurs in both IAIRM and IAIMM [26, 61].

The mechanism of the UAL cycle has been studied and found to depend on an intact thyroid, pituitary and hypothalamus [26]. It is eliminated by feeding propylthiouracil

or by cutting the pituitary stalk (fig. 4) [26]. First, the body temperature falls when the UAL rises above 300 mg/dl. This triggers the hypothalamic cold-sensitive neurons to release thyroid-releasing hormone which travels down the stalk to the pituitary in the portal system. The pituitary releases TSH which stimulates the thyroid to release T_4 which increases the metabolic rate and O_2 consumption rate. The latter generates NAD^+ through the electron transport chain of the mitochondria. Since the supply of NAD^+ is rate limiting in the oxidation of ethanol by ADH, this increase in the generation of NAD^+ increases the rate of ethanol elimination and UAL falls to low levels ($\sim$100 mg/dl). At this point the body temperature has increased, the release of T_4 has diminished and the metabolic rate has slowed so the supply of NAD^+ is diminished and the rate of ADH elimination of ethanol has slowed so the UAL begins to increase again and the cycle repeats itself [26].

The reason why the UAL cycle may be important is that the magnitude of the cycle oscillations correlates with the severity of the liver pathology observed [41]. Also, many changes in gene expression, redox state and fatty liver severity occur during the cycle [10]. This may account for the size of standard error in data derived from the IAIRM experiments when the time in the cycle is not controlled for. This means that the data will be significantly different if the rats are sacrificed at the peak of the cycle as compared with the troughs of the cycle. For this reason animal sacrifice should be synchronized with the UALs.

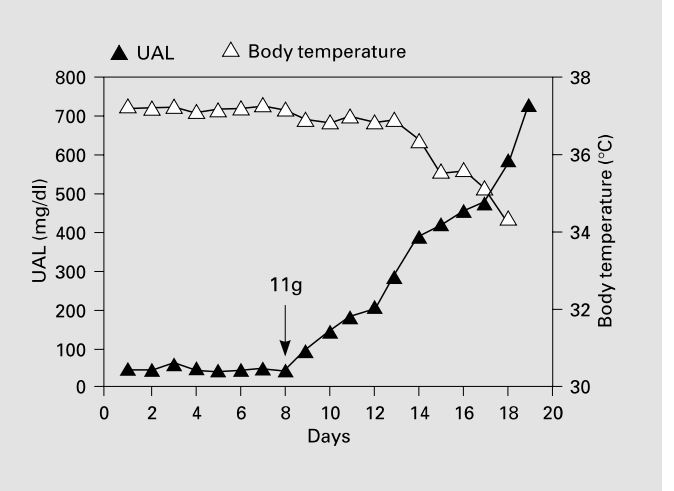

Fig. 4. When the pituitary stalk was cut, the rats could no longer cycle UAL, but instead overdose and die when the dose of ethanol is increased (arrow).

Role of Cytokines, Chemokines and Growth Factors in Liver Inflammation, Necrosis and Fibrosis

The liver pathology in IAIRM progresses from fatty liver, inflammation and necrosis to fibrosis over 6 months of ethanol feeding. The importance of these various changes in fibrogenesis is problematic. The degree of fatty change predicts the fibrosis that follows [41]. Centrilobular ischemic-like necrosis due to hypoxia is most likely to be the cause of fibrosis since focal fibrosis precedes stellate cell activation in the progressive time course [17, 51]. The inflammation and spotty necrosis appear to be an epiphenomenon. Nevertheless factors that stimulate the stellate cell to become activated to increase collagen in IAIRM may be important and have been summarized in a recent review [11]. Most of the data were derived from Kupffer cells and stellate cells isolated from livers from rats fed ethanol for 2–4 months [56, 57]. Endotoxin, carbonyl iron, nitric oxide, free radicals generated from Kupffer cells and leukocytes have been implicated [11, 25]. Cytokines such as TNFα, TGFβ, IL-1, -4, -8, -10 and -12; chemokines, MCP, MIP, CINC, ENA-78; and growth factors such as PDGF have been implicated [7, 36, 52, 57]. The data are difficult to sort out regarding the impact of the various factors involved but the most compelling mechanisms in fibrogenesis involve oxidative stress imposed by free radicals, H_2O_2 and products of lipid peroxidation [44].

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Review

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Application of DNA Microarrays to Study Human Alcoholism

Jo M. Lewohl[b] Peter R. Dodd[b] R. Dayne Mayfield[a] R. Adron Harris[a]

[a]Waggoner Center for Alcohol and Addiction Research, University of Texas at Austin, Tex., USA;
[b]Department of Biochemistry, University of Queensland, St Lucia, Australia

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Gene expression · Gene chip · Myelin · Frontal cortex

Abstract
An emerging idea is that long-term alcohol abuse results in changes in gene expression in the brain and that these changes are responsible at least partly for alcohol tolerance, dependence and neurotoxicity. The overall goal of our research is to identify genes which are differentially expressed in the brains of well-characterized human alcoholics as compared with non-alcoholics. This should identify as-yet-unknown alcohol-responsive genes, and may well confirm changes in the expression of genes which have been delineated in animal models of alcohol abuse. Cases were carefully selected and samples pooled on the basis of relevant criteria; differential expression was monitored by microarray hybridization. The inherent diversity of human alcoholics can be exploited to identify genes associated with specific pathological processes, as well as to assess the effects of concomitant disease, severity of brain damage, drinking behavior, and factors such as gender and smoking history. Initial results show selective changes in gene expression in alcoholics; of particular importance is a coordinated reduction in genes coding for myelin components.

While ethanol is one of the humanity's oldest drugs, knowledge of its mechanism of action is incomplete. The primary brain lesions and adaptive (and maladaptive) changes in neuronal function associated with chronic consumption have complex origins. Ethanol may damage the nervous system directly [29, 38], or via its oxidative metabolite acetaldehyde [3, 26], or through non-oxidative metabolites such as fatty acid ethyl esters [4, 9, 34] or phosphatidylethanol [20]. Malnutrition, vitamin deficiencies (especially of thiamin) and alcoholic liver disease complicate the cerebral effects of chronic alcoholism [63, 66]. These factors are all interrelated in the pathogenesis of ethanol-induced brain damage [5]. In addition to its intoxicant effects, long-term alcohol abuse produces consistent neurological and cognitive deficits in many alcoholic patients [62]. Associated with these deficits, and revealed in vivo by computerized tomography, is cerebral and cerebellar shrinkage [62]. Quantitative pathological studies confirm that there is a significant loss of brain tissue in a high proportion of cases [62], and that selective neuronal populations are damaged [24].

Neuroactive drugs such as ethanol influence neurotransmission to alter mood. Alcohol is also a drug of abuse, where long-term use can lead to addiction, the compulsion to take the drug and loss of control over intake [30, 31], or dependence, the need for continued drug exposure to avoid withdrawal [44]. Tolerance, where a reduced effect follows repeated exposure to a constant

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Dr. R.A. Harris
Molecular Biology Building 1.124
University of Texas A4800
Austin, TX 78712 (USA)
Tel. +1 512 232 2514, Fax +1 512 232 2525, E-Mail harris@mail.utexas.edu

dose, or an increased dose is needed to maintain the same effect, is also common [41, 44, 57].

The phenomena of tolerance and withdrawal have shaped hypotheses concerning the mechanism of drug dependence. The initial effect of a drug may be counteracted by homeostatic changes in systems that mediate the primary effect of the drug. With continued drug use these changes become more pronounced such that the brain is returned to near-normal function in the presence of the drug. However, when the drug is removed these neuroadaptations are unmasked, leading to the manifestation of the withdrawal syndrome. This has led to a hypothesis that long-lasting plastic changes in brain function underlie drug dependence [44]. Substantial evidence suggests that this plasticity is mediated, at least in part, by altered gene expression [42].

Alcoholism Causes Changes in Gene Expression

Under several experimental paradigms, sustained ethanol exposure results in changes in the expression of many genes, including some coding for neurotransmitter receptors, hormones and their receptors, signaling molecules, molecular chaperones, transcription factors, and cytokines [40]. Although most of these studies have been carried out using animal models or by exposing cells in culture to ethanol, it has recently been reported that chronic alcoholism in human cases results in changes in the expression of certain mitochondrial genes [14] and $GABA_A$ receptor subunit genes [35]. Further, a critical question is whether altered expression of the genes found in model studies also occurs in human alcoholism. It is likely that the alcohol-responsive genes identified so far provide a blurred glimpse rather than a full picture of the changes in gene expression that occur in human alcoholism.

It is not known whether alcohol has a direct effect on these alcohol-responsive genes, or an indirect effect involving many systems. For example, changes in transcription factor activation or in second messenger systems may initiate gene-expression cascades. The concentration of the second messenger cAMP is altered by acute and chronic ethanol exposure [18], and this, in turn, may alter the expression of cAMP-dependent genes. Activation or repression of alcohol responsive transcription factors is also likely to result in changes in the expression of many genes with the corresponding control elements [40]. Each of those possibilities would result in distinct patterns of gene expression. Such patterns are difficult to detect by traditional measurements of a few genes, but are well suited to array analysis. Changes in gene expression are likely to occur as an adaptive response to chronic alcoholism. Hence, identification of the genes which are differentially expressed between control and alcoholic cases will provide some insight into the pathogenic mechanisms of long-term alcohol abuse.

Neurological and Molecular Basis of Dependence

Drug addiction can have physical and psychological components. Loss of control over intake, compulsive drug-seeking, and use in the face of negative health and social consequences, which are hallmarks of the psychological component, are now considered to be the essence of addiction [53]. Further, the positive reinforcing properties of drugs of abuse, including alcohol, are considered to be a core cause of their addictive properties.

Defined Neuronal Pathways Are Associated with Dependency

Although each drug of abuse has some idiosyncratic mechanisms of action, virtually all of them have some effect, either directly or indirectly, on the mesocorticolimbic dopaminergic system [31]. This system is involved in stress responses and motivational state, and consists of dopaminergic neurons in the ventral tegmental area of the midbrain and various projection regions, notably the nucleus accumbens, olfactory tubercle, frontal cortex and amygdala. Animal studies suggest that ethanol reinforcement involves multiple neurotransmitter systems, including those for GABA, serotonin, opiates and glutamate, although the ultimate site of action appears be activation of the mesocorticolimbic dopaminergic system. For example, the GABAergic neurons in the substantia nigra pars reticulata and the amygdala have been implicated in controlling dopamine levels in the nucleus accumbens and ethanol intake, respectively [10, 39]; manipulation of serotonin levels alters ethanol intake [54]; opiate antagonists reduce ethanol self-administration [61]; and animals substitute glutamate antagonists for ethanol in drug discrimination studies [61]. Within the mesocorticolimbic dopaminergic system, the neuroanatomical entity termed the extended amygdala (which includes a portion of the nucleus accumbens) has been identified as a substrate for

acute drug reward. This region receives numerous afferents from the limbic system and projects to the hypothalamus and pallidum. Recently, adaptive changes in GABAergic function in the extended amygdala associated with ethanol dependence have been demonstrated [31].

Neuroadaptive models that rely on long-lasting changes at the molecular and cellular level have been developed to explain the establishment of compulsive drug use. Differences in these adaptive responses at the molecular level may account for individual differences in the susceptibility to ethanol and other addictive drugs. The models propose that adaptive changes that lead to the 'chronic drug state' (such as receptor adaptation) give rise to short-term withdrawal, but that other factors such as morphological or biochemical remodelling in sensitive cells are important as mediators of drug-related behaviors in the longer term [31]. In support of this concept are the observations of changes in second messengers and inducible transcription factors, and hence presumably in the expression of some 'downstream genes', in mesocorticolimbic structures in animals following repeated drug exposure [31]. These changes may underlie the neuroadaptive mechanisms in the short and long term. What is yet to be determined is the identity of the genes associated with the long-term adaptive change.

Adaptation of glutaminergic transmission represents a potential mediator of long-term drug effects, given its role in neural plasticity in general. Certainly, alterations in glutamate receptor subunit composition have been noted in the neurons of the ventral tegmental area after chronic opiate, cocaine or ethanol administration [15]. However, pharmacological data using receptor antagonists indicate that receptor adaptation, as an explanation of long-term changes, may be somewhat simplistic [43]. Recently, a novel mRNA coding for a putative neuroendocrine secretagogue that is transcriptionally regulated by chronic cocaine and amphetamine administration was identified [13]. The transcription factor ΔFosB, has also been shown to be induced in the nucleus accumbens by repeated exposure to drugs of abuse including cocaine [2, 45, 46, 51]. The induction of ΔFosB enhances cocaine sensitivity by altering the expression of down-stream genes such as GluR2 [28]. Similarly, PCR differential display has been used to reveal increased expression of mitochondria- and genomic-encoded genes in the brains of ethanol-treated rats and human alcoholics [7, 14]. Our experience illustrates an important point: *although changes in gene expression may be hypothesized, it is not possible to predict which particular genes will be involved from neurobiological or pharmacological considerations.*

In alcoholism, there are likely to be changes in gene expression that underlie the regional selectivity and variability of brain damage and cell death, as well as changes in gene expression that occur as an adaptive response to chronic alcohol ingestion. This project used microarray technology to assess the expression of known and as yet unidentified alcohol-responsive genes in the brains of human alcoholics. By careful selection and pooling of samples it should be possible to identify genes which underlie the pathogenic mechanism of alcoholic brain damage, cellular susceptibility, and protection, and the adaptive response to chronic alcohol abuse. The successful application of this technique to post-mortem human brain samples provides a useful tool for the generation of expression profiles of different brain regions and disease states, and is limited only by the availability of tissue samples.

Discrete Subsets of Neurons May Be Selectively Vulnerable

Quantitative stereometric analyses by Harper and Kril [23] have shown that the brain shrinkage associated with alcoholism is largely due to a reduction in volume of the cerebral white matter. However, studies of gray matter suggest that neurons in specific regions of the brain (cerebellar Purkinje cells and frontal lobe cortical neurons) are selectively damaged [24, 33, 50]; the alterations in frontal cortex are in line with known changes in cognitive function, and correlate with findings of a retraction of dendrites and a reduction in synaptic density due to ethanol toxicity. Hence, discrete subsets of neurons may be particularly susceptible to ethanol toxicity, as a consequence of possessing a specific receptor profile [12].

Alcoholics differ markedly in their neuropathological presentation, ranging from little or no damage on examination to severe frontal lobe and/or cerebellar atrophy. While neuronal and white matter loss are apparent in uncomplicated alcoholics, neuropathological studies have shown the severity of damage to be greater in alcoholics with concomitant cirrhosis of the liver, Wernicke's encephalopathy (WE) or Wernicke-Korsakoff syndrome (WKS) [23, 33]. Hence, liver damage and thiamin deficiency may have additive effects on alcohol neurotoxicity [5]. One possibility is that alcoholics with varying degrees of neuropathological abnormalities and concomitant pathologies represent stages on a dosage continuum with more severe damage correlating with greater lifetime alcohol consumption. Alternatively, alcoholics with liver

damage or thiamin deficiency may be more susceptible to alcohol neurotoxicity as a result of a genetic predisposition.

To determine the extent of brain damage due to alcohol abuse it is important to analyze changes in gene expression in alcoholics with varying magnitudes of pathological change. Thus, it is important to divide alcoholics on the basis of neuropathological and clinical data into the following groups: uncomplicated alcoholics, those with no concomitant disease and no neuropathological damage, cirrhotic alcoholics, alcoholics with WE and alcoholics with WE and cirrhosis. Ultimately, a comparison between these subgroups will enable us to delineate effects due to alcohol abuse and determine whether there is any correlation to extent of neuropathological damage.

Gene Expression Can Be Monitored in Many Different Ways

The techniques currently available for the analysis of gene expression fall into two categories: those that evaluate a small number of genes for which the sequence is known; and those that attempt to analyze all genes that are expressed in a given cell or tissue. The former, which include Northern hybridization, S1 ribonuclease protection assays and various RT/PCR strategies, can be adapted to provide at least semiquantitative information. Of the latter, competitive microarray hybridization, where large numbers of genes can be analyzed simultaneously to determine the differential expression of each gene between two samples, is rapidly becoming the technique of choice (see www.incytegenomics.com).

Microarrays or gene chips, now available from many companies, are silicone or glass slides on which partial or complete cDNA sequences are immobilized. They allow rapid and detailed analysis of thousands of transcripts simultaneously, and provide a direct readout as to the relative expression of the genes between samples. Because of the large number of genes that can be analyzed in each experiment, it is possible to generate gene expression profiles that can be used to track changes in cellular pathways, as opposed to changes in individual genes. Analysis is limited to those genes that are represented on the gene chips although, as the technology advances, so will the number of known genes and expressed sequence tags that can be assessed.

Microarrays are highly accurate for estimating differential expression between samples applied to the same array, i.e. when using a two-channel labelling system. The technique used at Incyte Genomics uses two fluorescent labels – Cy3-dUTP for one sample and Cy5-dUTP for the other sample. These labels are incorporated with high efficiency in the reverse transcription reaction resulting in a high yield of photo-stable cDNA targets. The two samples can be simultaneously applied to a single microarray as their excitation and emission spectra are widely separated. The application of two samples to the same array controls for a number of variables in the array manufacturing and data extraction process. While there are certain to be some differences in labelling between any two samples, this variability is expected to be fairly small.

Fluorescent cDNA probes representing all of the mRNAs expressed in each sample are generated using reverse transcription. After incubation, the microarray is rinsed to remove probes that did not find their counterpart. The relative expression of each element in each sample is determined by scanning the microarray. Each element of the microarray is scanned for the first fluorescent label. The intensity of the fluorescence at each array element is proportional to the expression level of that gene in the sample. The scanning operation is repeated for the second fluorescent label. The ratio of the two fluorescent intensities provides a highly accurate and quantitative measurement of the relative gene expression level in the two samples. If a microarray element is unlabeled, that element or gene is not expressed in either sample. If an element is labeled with one fluorescent probe, the gene is expressed in one sample but not the other. The appearance of both labels indicates that the gene was expressed in both samples.

The data are downloaded directly into GEMTools™, a software package available from Incyte Genomics which is used for storing, managing and analyzing microarray data. The GemTools software package has many capabilities for the analysis of the data including query-based search tools, grouping genes into related families, ranking genes on the basis of the balanced differential expression, as well as links to the sequence information and to Genbank.

Four preliminary experiments comparing control frontal cortex to alcoholic frontal cortex were carried out to determine the feasibility of using microarray hybridization to analyze gene expression in human post-mortem brain samples [36]. Two sets of control and alcoholic samples were selected for analysis. Each case group consisted of 5 alcoholics and 5 matched control cases. The first set of cases was identical to those used in PCR-differential display experiments by Fan et al. [14]. The alcoholics in this group represent a heterogeneous population consist-

ing of uncomplicated alcoholics, 1 alcoholic with cirrhosis and 1 alcoholic with concomitant WKS [36]. Case group 2 was selected for uncomplicated alcoholism and is more homogeneous.

Total RNA was extracted from the frontal cortex of each case using a modified guanidine isothiocyanate extraction procedure [8] that we have shown to produce good quality RNA from post-mortem human brain samples. In our hands, the yield of total RNA from a cortical region is in the range of 75–100 μg/g tissue. Each case contributed 30 μg of total RNA to the pool and polyA$^+$ RNA was extracted. Approximately 1 μg of polyA$^+$ RNA was extracted from 100 μg of the pooled total RNA using the Oligotex polyA$^+$ RNA extraction kit available from Qiagen. The polyA$^+$ RNA from each pool was shipped to Incyte Genomics for probe generation and microarray hybridization. The microarray hybridizations were carried out in duplicate on each case group, i.e. four microarray hybridizations were performed in total. Of the 7,075 genes analyzed in each experiment, approximately 2,400 elements were detected in all four hybridizations. To our knowledge this technique has not been applied to the human frontal cortex and information regarding gene expression patterns in human brain is only available for a small number of genes. Although our primary interest is to identify the genes which are differentially expressed between controls and alcoholics, much more information can be gleaned from the microarray data. It is possible to identify which genes are expressed in frontal cortex and over what range, and how the expression of a single gene or group of genes may relate to others.

The data were directly downloaded into GemTools for analysis. GemTools enables genes to be analyzed individually, or in functionally related groups. In addition, global trends can be identified by looking at the data set as a whole. One way of looking at the entire data set is to plot the Cy3 signal value against the corresponding Cy5 signal value for each element of the array. This gives an indication of the expression level for a each gene (e.g. from 100 to 60,000 relative fluorescence units) and the ratio between the signals. This ratio is a measure of the differential expression between samples with ratios of one indicating no difference between the samples. Overall, the Cy3:Cy5 graphs for these duplicate experiments are similar and the elements show a similar distribution pattern; very few of the genes have differential expression levels that are 2-fold or greater, and that the lower expressing genes are more variable than higher expressing genes.

Since the technology is relatively new we were interested in the reproducibility between the arrays. We conducted the arrays in duplicate for each case group so that assessments could be made with regard to overall reproducibility and reliability of the data. Although the two hybridizations for case group 1 were conducted 5 months apart, the results are very consistent. A total of 7,075 elements were analyzed; 2,322 of these were determined in both hybridizations. Data from each hybridization were expressed in terms of a ratio corresponding to the differential expression between the samples applied to the array. The differential expression ratio attained for each hybridization was compared and ranked according to the extent of the difference for each element. When compared in this way approximately 90% of the elements from hybridization 2 had ratios that were within 0.3 of the ratio attained in hybridization 1. Most remarkably, 22% of the genes had *identical* ratios in the two experiments. The duplicate hybridizations for case group 2 were also conducted several months apart. Of the 7,075 elements on each of the arrays, 5,237 were detected in both experiments. The data attained for case group 2 were also remarkably consistent with approximately 80% of the elements with ratios within 0.3 of their replicate and 18% of the elements had ratios that were identical between experiments. To identify which genes were differentially expressed in each group of alcoholics the data from each hybridization were analyzed individually. Elements with differential expression ratios of at least 1.4-fold were selected from each data set and compared between replicate experiments from the same case group. Genes which showed consistent changes, i.e. differential expression ratios of at least 1.4-fold in the same direction in both replicates, were selected. Fifty-four genes (2.3%) met this selection criteria for case group 1 and 88 genes (1.7%) met the selection criteria for case group 2. A comparison of the differential expression ratios for these elements was also made between case groups.

The most striking changes in expression are in genes coding for cytoskeletal and myelin proteins. Glial fibrillary acidic protein (GFAP), two keratin subtypes and three myelin mRNAs are decreased in alcoholics with respect to controls. These changes may indicate a loss of particular cell types or a change in cellular architecture or morphology. The latter is particularly interesting as changes in morphology may underlie changes in neurochemistry in the frontal cortex of alcoholics.

GFAP has been identified as an alcohol-responsive gene and its expression is altered in animal models of alcohol abuse [16, 19]. GFAP has been shown to be upregulated after acute ethanol treatment in animal models but decreased with chronic treatment. GFAP has also been

shown to be transcriptionally regulated by ethanol [65] so a decrease in GFAP expression does not necessarily imply a loss of astrocytes.

Demyelination and white matter loss has been extensively documented in neuroimaging [47, 49, 55, 59] and neuropathological studies [25, 32] of human alcoholics [reviewed in ref. 6, 27]. White matter loss and altered myelin biogenesis has also been documented in children with fetal alcohol syndrome [52, 60].

White matter loss is most severe in cirrhotic alcoholics and alcoholics with concomitant WE and WKS but can also be found in uncomplicated alcoholics [32] and is thought to account for the brain shrinkage seen in these cases [25]. Demyelination is also a characteristic of chronic WE lesions which can occur in many brain regions including the dorsal medial thalamus, locus ceruleus, periaqueductal gray, ocular motor nuclei and vestibular nuclei [reviewed in ref. 6]. White matter loss is reversible in abstinent alcoholics [48, 55] and is not correlated to alcohol dose [22, 32, 64]. Hence, white matter loss may represent a reversible change in myelination rather than an irreversible loss of axons or oligodendrocytes, and alcohol-induced changes in gene expression may play an important role in this process. The more severe white matter loss in alcoholics with WE and WKS indicates that thiamin deficiency may compound the effect of alcohol neurotoxicity on myelin expression.

The effect of alcohol on myelin gene expression may also shed some light on why alcoholics are more susceptible than non-alcoholics to two rare disorders of demyelination: the Marchiafava-Bignami syndrome and central pontine myelinolysis. The Marchiafava-Bignami syndrome is a disorder of demyelination of the corpus callosum and adjacent subcortical white matter. This disorder occurs predominantly in malnourished alcoholics lending weight to the idea that thiamin deficiency may compound white matter loss. In central pontine myelinolysis, myelinolytic lesions are most common in the pons but can also occur in the striatum, thalamus, cerebellum and cerebral white matter. Most sufferers are alcoholics but this condition can also occur in non-alcoholics and is associated with rapid correction of hyponatremia.

The expression of myelin genes is regulated by a number of signals including insulin-like growth factors [17, 21], fibroblast growth factor-2 [17], tumor necrosis factor [1] and thyroid hormones [37]. While the expression of these signalling molecules was not altered in these experiments, insulin-like growth factor I and II [56, 58] and tumor necrosis factor [67] have been shown to be altered in animal models of alcohol abuse. Whether alcohol affects myelin gene expression directly, or via one of these signalling pathways is an important area for further investigation.

The preliminary microarray experiments have identified a number of exciting changes in gene expression. While the fact that changes in myelin expression may reflect a loss of oligodendrocytes cannot be discounted, it is likely that changes in myelin gene expression precede changes in myelin protein expression resulting in demyelination and white matter loss in specific brain regions. Hence, these changes in myelin gene expression may be the cause rather than the result of white matter loss.

The data presented here highlight the power of using microarray analysis to identify changes in gene expression in complex disease processes. For example, our results suggest for the first time an extensive, but selective, reprogramming of gene expression in myelin. This may provide a molecular basis for the susceptibility of alcoholics to white matter loss and demyelinating diseases. Using this technique it is possible to confirm changes in known alcohol-responsive genes and to identify genes which have not been previously implicated in the disease, including genes of unknown function. These experiments represent only the first stage in identifying genes related to the pathogenesis of alcohol-induced brain damage.

Microarray hybridization is a relatively new technology and adequate assessment of the limitations and pitfalls is essential. cDNA microarrays are highly accurate for estimating differential expression between samples as they utilize a two channel labeling system. In a large scale study of microarray reproducibility conducted by Incyte Genomics using the UnigemV microarray, there was a high degree of correlation between expected and observed differential expression ratios (can be downloaded at http://gem.incyte.com/gem/GEM-reproducibility.pdf). The coefficient of variation over the entire array for these experiments was $\sim 15\%$. Statistical analyses of the data reveal that differential expression ratios of 1.8-fold are reliable using a single array. However, many of our expression ratios are in the 1.4- to 1.8-fold range, which is at the lower limit for reproducible detection. As such it is advisable to perform multiple arrays for each comparison.

Another factor which deserves consideration is the overall reproducibility of the microarray data. In their recent reproducibility study, Incyte Genomics also assessed the contribution of variables in the manufacturing, hybridization and detection processes to array variation. This study assessed the effects of five dependent variables; glass lot, GEM microarray lot, post-fabrication setup, hybridization setup, and microarray scan setup.

These manufacturing variables accounted for less than 15% of the overall variation, with GEM microarray lot and microarray scan setup accounting for approximately 5% of the variation each.

Robotic printing of elements on the array results in regular and precise arrangement of DNA probes, but it is also a source of error in array hybridizations. Data are digitally extracted from the array by overlaying a grid pattern which specifies the element locations. If the elements are even slightly off-set during the spotting process, the relative intensity of the element cannot be accurately determined during the scanning process.

In addition, individual elements may not be accurately determined due to a poor target density. The DNA arrayed as an element is generated by PCR and slight differences in the quality of the PCR between array lots may have a profound effect on the density of the DNA which is spotted onto the microarray. However, when two samples with different labels are applied to the same array it allows for element by element normalization. Hybridization efficiency may also differ between elements on the array. Factors which influence hybridization including base composition and sequence of the cDNA clones, temperature, pH and the purity of the applied RNA samples may all contribute to error. These factors are also minimized when using a two-channel system.

While the above-mentioned variables may not affect the 'intra-array' data, subtle differences between arrays may have a profound effect on the 'inter-array' reproducibility. An example of the effect of this type of variability is seen in the preliminary experiments. The four hybridizations presented in the Preliminary Data section were conducted over a period of approximately 10 months. During this period the GEM lot and scan setup were no doubt changed a number of times. The biggest difference between the four experiments was in the number of elements which were deemed to be 'within specifications'.

An element is out of specifications if it has a signal-to-noise ratio below 2.5, e.g. if the mRNA is a rare transcript or is not expressed in a particular cell or tissue type; if the element area is less than 40% of the expected area due to poor DNA density or an error in the spotting process; or if the element does not lie within the grid location, e.g. if it was offset during the spotting process. The number of elements which were within specifications for each of the hybridizations conducted to date were 2,649, 3,898, 6,401 and 5,611 for hybridizations 1, 2, 3 and 4, respectively. While the spotting and scanning processes appear to be improving (the number of elements within specifications in the two most recent hybridizations are double that of the first), the number of elements which can be analyzed in all four experiments is fairly small. Many of the interesting changes in gene expression identified in case group 2 could not be determined in case group 1, as they were among the elements that were deemed to be out of specifications in these arrays. This problem can be overcome by running all four hybridizations for each comparison with arrays from the same batch. This should substantially improve the replicability between data sets. Analysis of each set of array data will include assessments of reproducibility between replicate experiments using the same RNA samples and between case groups with the same diagnosis.

The final variable that contributes to error is biological variation. This variable is an inherent part of any experiment but is a particular problem in studies utilizing human samples. Human beings differ in many factors: environment, nutrition, genetics, etc, some of which can be controlled for by careful case selection. Cases will be selected and grouped into pools on the basis of clinical information. Efforts will be made to match the pools – controls and alcoholics – as closely as possible. Particular attention will also be paid to the neuropathological information which is available for each case.

One perceived limitation of human autopsy tissue is that it is 'end stage'. Thus, neurochemical changes responsible for tolerance and dependence may be confounded by other effects of the disease. To address this problem, we will assess brain damage using histochemical approaches and will study brain regions that differ in their vulnerability to alcohol toxicity. In addition, attention will be paid to the role of pathologies such as cirrhosis and WE in regulating gene expression. Another issue is 'state versus trait', i.e. do differences in gene expression seen between controls and alcoholics exist before the development of alcoholism and represent genetic predisposition? This is impossible to answer rigorously, but our studies of abstinent alcoholics will demonstrate which changes in gene expression are reversible. In addition, the proposition that many of the observed differences will be due to alcoholism, rather than precede it, is supported by several considerations. (1) A multitude of animal studies have shown that alcohol administration brings about clear-cut changes in gene expression [40]. (2) Human alcoholics show enhanced expression of $GABA_A$ α_1 receptor in pathologically relevant cortical regions [35]. Together, these data suggest that long-term alcohol exposure is likely to alter gene expression but do not preclude that differences in gene expression also precede, and even cause, alcoholism.

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Review

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Ethanol and Oxidative Mechanisms in the Brain

Albert Y. Sun[a] Grace Y. Sun[b]

Departments of [a]Pharmacology and [b]Biochemistry, University of Missouri, Columbia, Mo., USA

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Ethanol · Oxidative neuronal damage · Resveratrol · Polyphenols

Abstract
There is strong evidence showing that chronic and excessive ethanol consumption may enhance oxidative damage to neurons and result in cell death. Although not yet well understood, ethanol may enhance ROS production in brain through a number of pathways including increased generation of hydroxyethyl radicals, induction of CYP2E1, alteration of the cytokine signaling pathways for induction of iNOS and $sPLA_2$, and production of prostanoids through the PLA_2/COX pathways. Since many neurodegenerative diseases are also associated with oxidative and inflammatory mechanisms in the brain, it would be important to find out whether chronic and excessive ethanol consumption may exacerbate the progression of these diseases. There is evidence that the polyphenolic antioxidants, especially those extracted from grape skin and seed, may protect the brain from neuronal damage due to chronic ethanol administration. Among the polyphenols from grapes, resveratrol seems to have unique antioxidant properties. The possible use of this compound as a therapeutic agent to ameliorate neurodegenerative processes should be further explored.

Introduction

Chronic and excessive alcohol abuse is marked by a number of biochemical and physiological changes in the central nervous system (CNS). Some of these changes are pertaining to alteration of specific neurotransmitter systems [13] and intricate signaling pathways [34]. A recognized mechanism of ethanol action is its ability to enhance oxidative stress [3, 59, 84]. Due to the presence of high proportions of polyunsaturated fatty acids and low oxidant defense enzymes in the brain, this organ is particularly susceptible to oxidative stress, and free radicals are generated under normal as well as pathological conditions [29]. Besides chronic alcohol consumption, many neurodegenerative diseases are associated with increased production of free radicals in the brain, especially during aging [8, 31, 68]. Therefore, it is possible that oxidative changes exerted by chronic and excessive ethanol consumption may exacerbate the progression of other neurodegenerative disorders. The purpose of this review is to provide information relating ethanol effects on oxidative and inflammatory pathways in the CNS. Since Alzheimer disease (AD) pathology is also marked by an increase in oxidative and inflammatory pathways, possible interaction between ethanol effects and the progression of this neurodegenerative disorder will be discussed.

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Prof. Albert Y. Sun, PhD
Pharmacology Department
University of Missouri
Columbia, MO 65212 (USA)
Tel. +1 573 882 1565, Fax +1 573 884 4558, E-Mail suna@health.missouri

Ethanol and Oxidative Stress in the Brain

Excessive ethanol ingestion has been shown to cause oxidative damage in a number of body organs including the brain [59], heart and liver, and the bile of ethanol-treated deer mice [43, 44, 73, 74]. Using the spin trapping technique, our laboratory as well as others have detected hydroxyethyl free radical formation in biological systems following ethanol administration [1, 3, 72, 73]. Formation of hydroxyethyl radicals can be observed in biological systems involving ethanol together with xanthine oxidase [2] and 6-hydroxydopamine [66]. Since hydroxyethyl free radicals are very reactive and have a longer half-life than hydroxyl radicals, it is not surprising that these radicals can cause more cellular damage than the hydroxy radicals.

Ethanol may enhance the production of ROS through a number of mechanisms. In particular, a number of studies have described metabolic conversion of ethanol through induction of cytochrome P450 2E1 (CYP2E1) [71, 100]. Although CYP2E1 is present in low levels in the CNS [30, 94], induction of this enzyme together with increased ROS production has been reported in rats after chronic ethanol administration [59].

Ethanol and the Cytokine/NO Pathway in Astrocytes

Activation of glial cells in the brain is frequently associated with an increase in oxidative and inflammatory mechanisms leading to neuronal damage. Astrocytes are the most abundant cell type in the CNS. Besides providing nutrient supplies to neurons, these cells are immune active and actively participate in host defense mechanisms [78]. Astrocytes are capable of responding to pro-inflammatory cytokines, which in turn can cause transcriptional activation of genes including the inducible nitric oxide synthase (iNOS) [25, 62]. A study by Li et al. [48] demonstrated different cytokine profiles for the induction of iNOS and secretory phospholipase A_2 ($sPLA_2$) in immortalized astrocytes (DITNC). Under normal conditions, NO in brain is produced mainly by the neuronal NOS (nNOS), a constitutive enzyme present mainly in neurons. NO generated under these conditions may serve as a neuromodulator and can regulate specific synaptic functions [16, 49]. However, under pathological conditions and in the presence of cytokines, large amounts of NO are generated from glial cells through induction of iNOS. NO can interact with superoxide radicals to form peroxynitrite ($ONOO^-$), a potent oxidant compound with cytotoxic effects. Peroxynitrite can induce oxidation of proteins, lipids and DNA resulting in alteration of important enzymes and proteins such as glutamine synthetase [41] and synaptophysin [56]. Therefore, excess production of NO in the brain may play an important role in exacerbating oxidative damage to cells.

There is evidence that chronic ethanol consumption may enhance oxidative stress in the brain through increasing NO production. This notion is supported by studies demonstrating the increase in NOS activity in rat brain cortex and cerebellum after alcohol administration [64, 97]. A study by Xia et al. [97] also showed that despite the increase in NOS activity in the cerebellum, nNOS mRNA levels were not changed. These results suggest that ethanol may exert its action on the protein or its co-factors.

Ethanol may exert its action on the cytokine signaling pathways and alter transcriptional factors responsible for induction of iNOS. However, these effects appear to depend on the type of cell under study. Ethanol exposure is shown to suppress lipopolysaccharide (LPS)-induced NO production in alveolar macrophages [27], human monocytes [52], and astrocytes [89]. On the other hand, ethanol can enhance NO production in aortic vascular smooth muscle cells [18], blood-brain barrier cells [63], hepatocytes [91], and embryonic cortical neurons [60]. Studies with primary and immortalized astrocytes have demonstrated the inhibitory effect of ethanol on cytokine induction of NO in these cells [88, 93]. However, while the study by Militante et al. [57] had attributed the effect of ethanol to suppressing the transcriptional activation of iNOS mRNA, a study by Wang and Sun [93] with immortalized astrocytes seems to favor a post-translational site of ethanol action on the iNOS protein instead. These studies well demonstrate that ethanol may alter cytokine induction pathways but the final outcome may depend on many factors.

Ethanol and PLA_2/COX Pathways

PLA_2 are present in diverse cell types, including those in the brain [58, 61]. Besides their role in the maintenance and repair of cell membrane phospholipids, these enzymes are also involved in the release of arachidonic acid, which can be further converted to a number of bioactive lipids, including prostaglandins and eicosanoids. PLA_2 in the brain have been implicated in cell injury and death and elevated PLA_2 activity has been shown in a number of neurodegenerative diseases, particularly in association with AD and stroke [15, 21, 83]. Recent studies have focused on the cytosolic type IV cPLA2 present constitu-

tively in most mammalian cells and the secretory type IIa sPLA2, which is involved in inflammatory processes [61]. Activation of a cytokine signaling pathway is associated with induction of $sPLA_2$ [48, 65, 90].

Cytosolic PLA_2 is ubiquitously present in most mammalian cells and is regulated by phosphorylation, Ca^{2+} and translocation mechanisms [46]. In astrocytes, agents such as phorbol myristate acetate, A23187, and ATP/UTP can stimulate the release of arachidonic acid (AA) from these cells [99]. It is interesting that stimulated AA release from astrocytes is dependent on protein kinase C but not mitogen-activated protein kinase [98]. A unique property for $cPLA_2$ is its intimate association with oxidative mechanisms in the cell. H_2O_2, an oxidant stressor produced endogenously, can stimulate $cPLA_2$ and AA release from astrocytes [98]. Although the mechanisms for the increase in AA release due to oxidative stress are not well understood, it is clear that increase in oxidative stress in astrocytes can exacerbate cell membrane damage through activation of phospholipases and generation of bioactive lipid mediators.

There is evidence that chronic ethanol consumption may lead to an increased PLA_2 activity in the brain and that $cPLA_2$ is the target for ethanol action [6, 7]. A study by Knapp and Crews [42] further demonstrated the increase in COX-2 immunoreactivity in rat brain upon acute and chronic ethanol administration as well as during withdrawal. A recent study in our laboratory also indicated increased expression of COX-2 mRNA in the rat dentate gyrus after chronic ethanol administration [95]. More interestingly, ethanol-induced increase in COX-2 mRNA could be protected upon supplementing the ethanol diet with grape polyphenols. Increased production of eicosanoids upon ethanol ingestion has been regarded as an important factor underlying some of the behavioral manifestations of alcoholism [19, 20, 26]. Furthermore, inhibition of prostaglandin synthesis by COX inhibitors can effectively reduce a broad range of ethanol activities. Therefore, understanding the mechanisms of ethanol action on these enzymes may provide new opportunities for development of new therapeutic strategies to combat the deleterious effects resulting from chronic ethanol consumption.

Ethanol and AD

AD affects 7–10% of individuals over 65 years of age, and 40% of individuals over 80 years of age [70]. There is strong evidence that oxidative and inflammatory mechanisms play an important role in the pathogenesis of AD [53, 54, 55, 77]. Consequently, increases in oxidative products, including nitrotyrosine, protein carbonyls, 4 hydroxynonenal and oxidized DNA have been reported in AD brain [53]. The increase in oxidative products in AD brain further indicate the loss of calcium homeostasis in cells, which in turn result in the increase in phospholipid degradative enzymes, e.g. PLA_2 [38, 83]. Several studies also demonstrated the increase in COX-2 in AD brain [67]. In fact, the use of non-steroidal anti-inflammatory drugs has been shown to offer beneficial effects in delaying or retarding the progression of this disease [67].

One of the pathological landmarks for AD is the progressive deposition of amyloid plaques enriched in amyloid β peptide (Aβ). These peptides are comprised of 39–42 amino acids and are derived from enzymic cleavage of the amyloid precursor protein. There is evidence that Aβ, especially in their aggregated form, can directly or indirectly increase free radicals and oxidative damage to cells [4, 10–12, 54, 81, 82, 102]. The cytotoxic effects of Aβ can be attributed to their ability to disrupt ion homeostasis [28, 51, 101]. Special interest has been placed on copper, which can be released during synaptic activation and attains high levels in the synaptic cleft [39]. Increased ROS production from Aβ has been shown to result in protein oxidation and lipid peroxidation, and these effects can compromise cell membrane functions [102]. The ability of antioxidants and oxidant defense enzymes to alleviate Aβ-mediated cytotoxicity further supports the notion that AD pathology is associated with increased oxidative damage [9].

There is evidence that chronic ethanol consumption may contribute to the progression of AD pathology [24, 39]. In fact, high alcohol consumption is considered a risk for late-onset AD [23]. A study by Freund and Ballinger [24] indicated a special decrease in muscarinic and benzodiazepine receptors in alcoholic AD patients. However, plaque counts have not been performed in these patients. On the other hand, Hebert et al. [33] observed that neuronal cell loss is only found in heavy drinkers, and that mild and moderate consumption of alcohol has no implication on the incidence of AD. Thus, ethanol effects on AD pathology may depend on the level of alcohol intake and the type of beverage consumed.

It remains to be determined whether the effects of chronic ethanol on AD may be due to the action of ethanol on Aβ aggregation and its ability to bind other molecules, particularly apolipoprotein E isoforms [69]. Since Aβ can independently stimulate iNOS signaling pathways in astrocytes and microglial cells [35, 36], ethanol may

alter this signaling cascade and induction of iNOS as well as $sPLA_2$ in AD brain. Since excessive release of Cu^{2+} or Fe^{3+} in brain may also affect the ability of Aβ to produce ROS. Ethanol may also exert its action on this pathway and exaggerate production of H_2O_2 or superoxide anions. Indeed, preliminary studies with primary neuron culture have provided data illustrating the ability of ethanol to exacerbate Aβ toxicity and neuron cell death mechanisms [85].

Antioxidants Alleviating Ethanol-Mediated Cellular Damage in the Brain

Many polyphenolic compounds in plants, including those found in vegetables, fruits, wine and tea, exhibit biological properties that are beneficial to human health [76]. The strikingly low incidences of coronary heart diseases in France as compared with other Western countries with comparable dietary intake has been regarded as the 'French Paradox' [75]. Compounds such as resveratrol, quercetin and catachin are enriched in grape skin and seeds and the ability of these compounds to inhibit platelet aggregation and protect low-density lipoproteins from oxidation has been well demonstrated [22, 96]. These compounds can also inhibit cytokine-induced NO production in astrocytes [47, 92]. A study by Ledig et al. [45] demonstrated the ability of polyphenols extracted from grape skin and seeds to protect the brain against oxidative insults due to chronic ethanol administration. Lin et al. [50] further demonstrated that isoflavonoid compounds extracted from *Pueraria lobata* could effectively suppress alcohol consumption in the rats. Therefore, besides grape extract, it is reasonable that other bioflavonoids may ameliorate neuronal damage due to chronic ethanol consumption. Recently, our laboratory initiated studies to examine whether supplementation of polyphenolic compounds from grape skin and seeds might alter ethanol-induced changes associated with neurodegenerative processes. In these studies, rats were administered a Lieber-DeCarli diet with or without ethanol and/or grape polyphenols for 2 months [96]. Results from these studies clearly demonstrated that supplementation of grape polyphenols to the ethanol diet could protect the decrease in synaptosomal Na,K-ATPase and dopamine uptake activity due to chronic ethanol administration [86]. Ethanol feeding also caused changes in membrane lipids in the liver, due mainly to metabolism of ethanol to acetate. Interestingly, although grape polyphenol supplementation could alleviate morphological changes in hepatic tissue, this feeding paradigm did not alter the lipid changes due to ethanol metabolism [87]. These results further suggest that antioxidant properties of these polyphenolic compounds are most effective when tissue organs are insulted by oxidative stress.

Among the polyphenols in grapes, most focus has been placed on resveratrol [22]. Resveratrol is a phytoalexin and is originally identified as the active ingredient of an Oriental herbal medicine used for treatment of a wide variety of diseases including dermatitis, gonorrhea, fever, hyperlipidemia, arteriosclerosis and inflammation [5]. This compound is thought to be the major ingredient in grapes and red wine with protective effect against coronary heart disease [75, 80]. Due to the amphipathic property of this molecule, it is capable of scavenging both lipid hydroperoxyl free radicals as well as the hydroxyl and superoxide anion radicals [37, 40]. Studies by Chanvitayapongs et al. [14] and Draczynska-Lusiak et al. [17] demonstrated the ability of resveratrol to protect neurons against oxidative stress [86]. Taken together, these studies well demonstrated the antioxidant properties of resveratrol and the possibility of this compound as a therapeutic agent for ameliorating the progression of neurodegenerative diseases.

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Review

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Phosphorylation Cascades Control the Actions of Ethanol on Cell cAMP Signalling

Boris Tabakoff Eric Nelson Masami Yoshimura Kaisa Hellevuo
Paula L. Hoffman

Department of Pharmacology, University of Colorado Health Sciences Center, Denver, Colo., USA

Key Words
Alcohol · Adenylyl cyclase · cAMP · Protein kinase A · Protein kinase C

Abstract
Our studies indicate that, in the presence of particular isoforms of adenylyl cyclase (i.e., type 7 AC), moderately intoxicating concentrations of ethanol will significantly potentiate transmitter-mediated activation of the cAMP signaling cascade. Activation of this signaling cascade may have important implications for the mechanisms by which ethanol produces intoxication, and/or for the mechanisms of neuroadaptation leading to tolerance to, and physical dependence on, ethanol. We initiated a series of studies to investigate the phosphorylation of AC7 by PKC, the role of this phosphorylation in modulating the sensitivity of AC7 to activation by Gsα, and the PKC isotype(s) involved in the phosphorylation of AC7. The T7 epitope-tagged AC7 expressed in Sf9 and HEK293 cells was found to be phosphorylated in vitro by the catalytic subunit of PKC. Treatment of AC7-transfected HEK293 cells with phorbol dibutyrate (PDBu) or ethanol increased the phosphorylation of AC7 and its responsiveness to Gsα. In human erythroleukemia (HEL) cells, which endogeneously express AC7, ethanol and PDBu increased AC activity stimulated by PGE_1. The potentiation by both PDBu and ethanol was found to be sensitive to the PKC δ-selective inhibitor, rottlerin. The potentiation of AC activity by ethanol in HEL cells was also selectively attenuated by the RACK inhibitory peptide specific for PKC δ, and by expression of the dominant negative, catalytically inactive, form of PKC δ. These data demonstrate that AC7 can be phosphorylated by PKC, leading to an increase in functional activity, and ethanol can potentiate AC7 activity through a PKC δ-mediated phosphorylation of AC7.

The G-protein-mediated signal transduction pathways that use adenylyl cyclase (AC) as an effector are among the most common signal transduction pathways in both neuronal and non-neuronal cells. Neurotransmitters bind to Gs-coupled receptors and promote the dissociation of the heterotrimeric Gs protein and the exchange of GTP for GDP bound to the Gsα subunit. The Gsα subunit with GTP bound to it then activates the AC. The activated AC generates the second messenger cyclic adenosine 3′5′-monophosphate (cAMP), which in turn activates the cAMP-dependent protein kinase A (PKA). PKA is known to phosphorylate and regulate several target proteins in

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Boris Tabakoff, PhD
Department of Pharmacology, University of Colorado Health Sciences Center
4200 E 9th Ave, Box C236
Denver, CO 80262 (USA)
Tel. +1 303 315 3125, Fax +1 303 315 7097, E-Mail boris.tabakoff@uchsc.edu

different intracellular regions from the plasma membrane to the nucleus. Several studies have shown that ethanol *acutely* potentiates Gs-stimulated AC activity in various whole cell and membrane preparations [1, 50, 53], and it is not surprising that consequences of the stimulatory effects of ethanol on the Gs-AC signal transduction pathway can be observed in the plasma membrane as well as in the nucleus. In studies by Palmer's group, the cAMP-generating system has been shown to be a controlling element in the modulation of ethanol sensitivity of $GABA_A$ receptor-coupled ion channels [10, 26]. Activation of AC through β-adrenergic receptors potentiated Purkinje neuron responses to GABA, and sensitized GABA responses to the potentiating effect of ethanol [10, 26]. Since ethanol can increase β-adrenergic receptor-stimulated AC activity, this pathway suggests a mechanism by which ethanol can produce a 'a feedforward' sensitization of $GABA_A$ receptors on cerebellar Purkinje neurons to the action of ethanol. Acute exposure of rats to ethanol has also been demonstrated to activate AC signal transduction from the membrane to the nucleus. Increased cAMP production at the plasma membrane increased the phosphorylation of CREB in the nucleus of striatal and cerebellar tissues [60, 61]. In addition, Moore et al. [39] demonstrated in a recent behavioral study using *Drosophila* mutants that cAMP levels, and consequently PKA activity, determined the sensitivity of the flies to the intoxicating effect of ethanol.

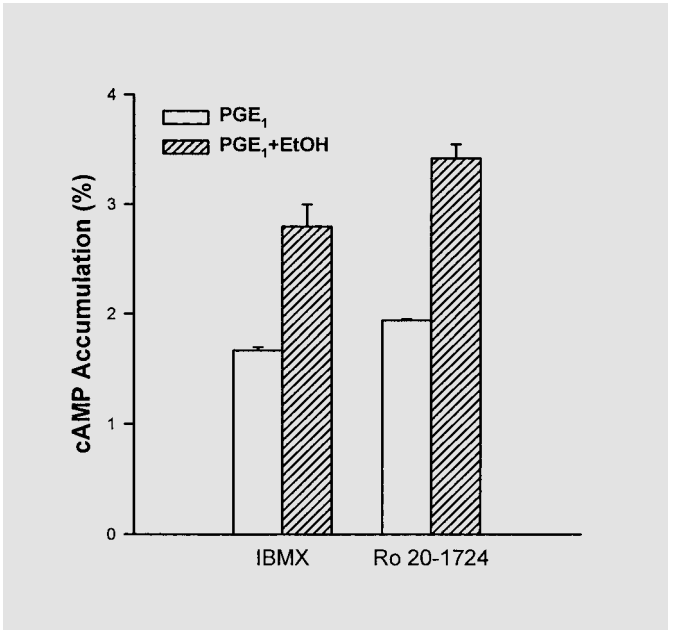
Adaptation of the AC system occurs after *chronic* exposure to ethanol, resulting in reduced responsiveness of AC to different stimuli, and leading to lowered cAMP production and PKA activity. These mechanisms have been suggested to play a role in behavioral tolerance to ethanol [9, 53].

Observations that the actions of ethanol on AC activity are evident primarily under conditions where Gs (the stimulatory G protein) is also activated have led to the hypothesis that ethanol acts to promote the rate of activation of Gs protein and/or to sensitize AC to the action of the Gs protein [19]. On the other hand, ethanol has also been shown to inhibit nucleoside transporters which internalize extracellular adenosine [8], and it has been proposed that the ethanol-induced increases in levels of extracellular adenosine lead to activation of AC through the interaction of adenosine with Gs-coupled A_2 adenosine receptors [8].

One of the difficulties in studying the mechanism of action of ethanol on AC activity in native tissues is the fact that ACs are a gene family. Currently, nine different genes that code for ACs (AC1–AC9) have been identified, and some of these genes also produce multiple splice variants [24, 51, 52]. Most cell types, including neurons, probably express two or more AC isoforms [16, 45]. In fact, practically all AC isoforms are expressed in brain, and each isoform has a unique but overlapping expression pattern, as demonstrated by in situ hybridization studies [38]. Since there is significant functional diversity in the family of ACs [24, 51, 52], it is important to realize that the complement of the ACs in a given cell type, as well as the ACs' subcellular localization and association with regulators and modulators, will probably determine how ethanol will influence AC signal transduction pathways both acutely and chronically.

All currently known AC isoforms can be activated through Gs-coupled receptors, although the conditions, magnitude and mechanisms of such activation vary from isoform to isoform [15, 24, 51, 52]. Similarly, ethanol's ability to potentiate Gs-stimulated AC activity varies among isoforms, with AC7 being the most sensitive to ethanol of all tested isoforms [64]. For instance, 50 m*M* ethanol produced a 56% increase in PGE_1-stimulated cAMP production in HEK293 cells transfected with type 7 AC [65], while activation of cAMP production in HEK293 cells transfected with other AC family members was, at best, one half to one third of this value. We wished to determine the characteristics of AC7 that result in the enhanced sensitivity to the potentiating effect of ethanol. Since in our prior work and the work of others, 3-isobutyl-1-methylxanthine (IBMX) has been used routinely as an inhibitor of phosphodiesterase to protect newly formed cAMP, and since IBMX can also act as a competitive antagonist at A_2 adenosine receptors, it became initially important for us to examine whether the actions of IBMX at the adenosine receptors were contributing somehow to our witnessed results regarding ethanol potentiation of AC7 activity.

To initiate the examination of the role of adenosine and adenosine A_2 receptors in the actions of ethanol on AC, we examined the effect of ethanol on AC activity in HEK cells transfected with AC7 in the presence of Ro 20-1724, a phosphodiesterase inhibitor lacking activity at the adenosine A_2 receptor. Figure 1 demonstrates that ethanol had equivalent effects in the presence of Ro 20-1724 or IBMX. To further examine the possible role of adenosine in the actions of ethanol, we examined the ability of adenosine per se to increase the accumulation of cAMP in HEK293 cells transfected with AC7. Adenosine (10 μ*M*) produced a small increase in cAMP accumulation in the AC7-transfected HEK293 cells (0.048%), whereas PGE_1 (10 μ*M*), which we routinely use for our studies [65], pro-



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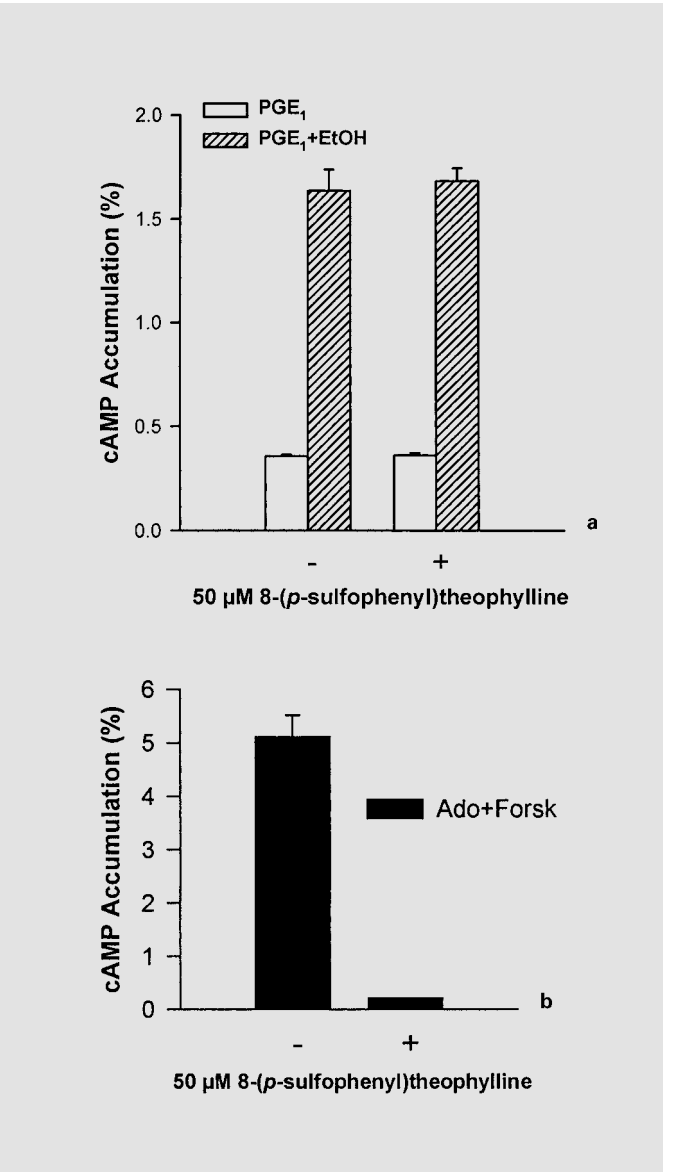

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Fig. 1. Effects of IBMX and Ro 20-1724 on ethanol-induced increases in cAMP production. cAMP accumulation was measured in HEK293 cells transfected with AC7. Cells were preincubated, prior to PGE_1 addition, for 10 min with either 500 µ*M* IBMX or 500 µM Ro 20-1724, and incubated for 5 min with 10 µ*M* PGE_1 in the presence or absence of 200 m*M* ethanol, as indicated. The effects of PGE_1 and PGE_1 + ethanol are reported as cAMP accumulation.

Fig. 2. Effect of an adenosine receptor agonist on cAMP accumulation. **a** HEK293 cells transfected with AC7 were incubated for 5 min with 10 µ*M* PGE_1 ± 200 m*M* ethanol in the presence or absence of 50 µ*M* 8-(*p*-sulfophenyl) theophylline, as indicated. cAMP accumulation was measured. The stimulation by ethanol was 358.8 ± 28.3% in the absence and 366.8 ± 17.2% in the presence of this adenosine antagonist. **b** The effect of the adenosine receptor antagonist on the actions of adenosine, per se, was examined by incubating the cells with 10 µ*M* adenosine (Ado) plus 10 µ*M* forskolin (Forsk) for 5 min.

duced a much higher increase (0.35%). In order to accentuate the effect of adenosine acting as an agonist at A_2 adenosine receptors, adenosine was added together with forskolin (fig. 2). A synergistic activation of AC activity was demonstrated when the Gs-coupled A_2 receptor agonist (adenosine) was added with forskolin to cells expressing AC7. The adenosine receptor antagonist [8-(*p*-sulfophenyl)theophylline] blocked the effect of adenosine on forskolin-activated AC activity (fig. 2b), but had no effect on PGE_1-stimulated AC activity or the potentiation of this activity by ethanol (fig. 2a). Because of its poor membrane permeability, 8-(*p*-sulfophenyl)theophylline would not be expected to inhibit intracellular events under our assay conditions.

As already mentioned, prior work [8] had suggested that ethanol acts to enhance extracellular adenosine levels, and it has been proposed that increases in AC activity in the presence of ethanol are wholly generated through the increased activation of A_2 adenosine receptors by the accumulated adenosine. Our current studies illustrate little or no role for adenosine in the actions of ethanol on PGE_1-mediated increases in cellular cAMP. A possible interpretation of the prior findings on the importance of endogenous adenosine in ethanol-induced actions on

cAMP signaling [8], is that activation of a Gs-coupled receptor is critical for evidencing ethanol-induced actions on AC activity. If an appropriate exogenous transmitter substance (e.g. PGE_1) is not added to a cellular assay system, ethanol would have no effect on cAMP levels unless an endogenous transmitter (such as adenosine) accumulated to levels sufficient to stimulate the cognate (adenosine A_2) Gs-coupled receptor.

We have additionally argued that the actions of ethanol on cAMP generation transcend the actions of ethanol on the Gs protein activation process and involve an ethanol effect on the Gs-AC interaction. Our contention was based on the already-stated fact [64] that certain members of the AC enzyme family were substantially responsive to the actions of ethanol while other members were not responsive. Since all members of the AC family of enzymes can, under specific conditions, be activated by Gsα [52, 54], the characteristics of a particular AC must contribute to the degree of ethanol potentiation of the catalytic activity of AC, irrespective of the actions of ethanol on the Gs protein.

An interesting feature of AC7 is that it is one of the AC isoforms that are sensitive to the effects of phorbol esters, through activation of protein kinase C (PKC) [17, 22, 59, 62]. Our studies showed that both the effects of ethanol and phorbol esters on AC7 are blocked by bisindolylmaleimide and staurosporine, a relatively selective PKC inhibitor and a Ser/Thr kinase inhibitor, respectively. These data suggested a role for PKC in the actions of ethanol on AC7. Further work in our laboratory [46] demonstrated that prolonged preincubation of cells (20 h) with a phorbol ester blocked the actions of ethanol on AC in human erythroleukemia (HEL) cells, which express AC7 as their primary endogenous AC isoform [16]. Although little published evidence exists for direct phosphorylation of AC7 by PKC, direct phosphorylation of the closely related AC2 by PKC α has been demonstrated [67]. Since both AC7 and AC2 have PKC consensus sites in functionally relevant regions, i.e. AC catalytic domains (C_{1a} and C_{2a}) and putative regulatory regions (C_{1b}) [24, 54, 56, 66], we hypothesized that AC7 activity can be regulated by PKC through direct phosphorylation, and that certain of these phosphorylation events may be involved in the actions of ethanol on AC7.

To determine whether or not AC7 could itself be a target (substrate) for PKC phosphorylation, a His- and T7-epitope tagged AC7 expression vector was constructed which would allow for efficient purification by immunoprecipitation. The tagged AC7 was overexpressed in Sf9 insect cells by infection with a recombinant baculovirus. The membranes from AC7- and control-infected Sf9 cells were solubilized, and the AC7 protein was immunoprecipitated using the monoclonal anti-T7 antibody. Western blot and autoradiographic evidence for in vitro phosphorylation of AC7 protein by PKC was obtained after incubation of the immunoprecipitated AC7 with rat brain PKC catalytic subunits in the presence of [γ-^{32}P]ATP. A phosphorylated band with the same mobility characteristics as AC7 was noted after polyacrylamide gel electrophoresis, while no phosphorylated band was noted in control assays which contained no AC7. Given these results, we also investigated whether ethanol could promote the phosphorylation of AC7 by PKC. AC7-transfected HEK293 cells were simultaneously incubated with PGE_1 and ethanol for 2–5 min, and the cell membranes were prepared under conditions which would preserve the phosphorylation state of AC7. We then performed a 'back' phosphorylation experiment by monitoring the extent of AC7 phosphorylation by PKC in vitro using the membranes of the ethanol-treated and control cells. The reduced phosphorylation of AC7 by PKC in vitro ('back' phosphorylation) indicated that ethanol had promoted phosphorylation of AC7 at PKC 'substrate' sites.

To show functional significance for the enhanced phosphorylation of AC7 seen with ethanol, AC7 activity was assayed in membranes that were prepared from ethanol-, PDBu-, or control vehicle-treated AC7-transfected HEK293 cells. When constitutively active Q227L-Gsα was added to the membranes from AC7-transfected HEK293 cells that had been pretreated with PDBu or vehicle immediately prior to membrane preparation, the activity of expressed AC7 in the presence of Q277-Gsα was significantly higher in membranes of PDBu-treated cell membranes than in vehicle-treated cell membranes. This indicated that the phosphorylation of AC7 by PKC may change the responsiveness of the enzyme to Gsα. As for PDBu, ethanol exposure of AC7-transfected cells also increased the responsiveness of the enzyme to added Q227L-Gsα, when tested in the cell-membrane-containing assay for AC activity.

There is a significant amount of diversity in the family of PKCs, which consists of at least 11 different isoforms [41]. The PKC family can be divided into three subfamilies: the conventional PKCs (cPKC: α, βI, βII and γ) are Ca^{2+} dependent and activated by phosphatidylserine and diacylglycerol (DAG) and its analogs, the phorbol esters; the novel PKCs (nPKC: δ, ε, η, θ and μ) are Ca^{2+} *in*dependent, but activated by phosphatidylserine and DAG and phorbol esters; the atypical PKCs (aPKC: λ and ζ) are not activated by Ca^{2+} or by DAG or phorbol esters [41]. An

important question is how the specificity of regulation (phosphorylation) of a target protein by a kinase arises. Signal transduction pathways, such as the receptor-G protein-AC pathway, appear to be organized into multi-element complexes by scaffold and anchoring proteins that ensure functional integrity, efficiency and spatio-temporal control of signaling [3, 57]. The specificity of PKC actions is thought to originate from the presence of PKC-isoform-specific anchoring proteins [36, 37] as well as the presence of PKC-isoform-specific substrate sequences [40] in the target proteins, such as AC7. It is probable that *both* the PKC-isoform-specific anchoring protein and the nature of the substrate sequence together determine the efficiency and level of phosphorylation of a protein and the functional consequences of the phosphorylation of a given protein such as an AC. Having shown that AC7 can be directly phosphorylated by PKC, and that ethanol can promote this phosphorylation, we next investigated the isoforms of PKC responsible for the effect of ethanol. For these experiments, we used HEL cells.

HEL cells express mRNA for AC6, AC7, and the ethanol-insensitive AC3 [64]. AC7 mRNA is by far the most abundant species of AC mRNA expressed in HEL cells [16]. Furthermore, using an AC2 family antibody, an immunoreactive band in the range of 110 kD was identified in the membrane fraction of HEL cells, which corresponded in size to an immunoreactive band of AC7-infected Sf9 cells and AC7-transfected HEK293 cells. Therefore, HEL cells seemed appropriate for further investigation of the role of PKC in ethanol potentiation of AC activity. Gö-6976 has been shown to selectively inhibit the conventional PKCs and PKC μ, while rottlerin selectively inhibits PKC δ and θ [13, 32, 58]. Pretreating HEL cells with increasing concentrations of Gö-6976 had little or no effect on the potentiation of PGE_1-stimulated AC activity by either PDBu or ethanol. However, when HEL cells were pretreated with rottlerin, the potentiation of AC activity by both ethanol and PDBu was inhibited in a concentration-dependent fashion. For example, with 2.5 μ*M* rottlerin in the assay, the potentiation by ethanol was reduced from 83 ± 10 to 28 ± 7%, and the potentiation by PDBu was reduced from 112 ± 11 to 59 ± 12%.

The role of a PKC anchoring protein is to target the appropriate PKC isoform in the vicinity of its substrate [36, 37]. So far only two PKC anchoring proteins, or receptors for activated C kinase (RACKs), have been cloned. RACK1 is selective for PKC β [36, 48, 49], and the second RACK is selective for PKC ε [7]. Interestingly, the PKC anchoring RACK proteins are structurally related to G protein β subunits [48] that stimulate the activity of AC2-family enzymes, including AC7 [55, 63]. Peptides containing specific sequences within the N-terminal V-1 regions which are unique to the various PKCs have been recently used to competitively inhibit the binding of PKCs to the RACK binding proteins. These peptides have been shown to be PKC-isotype-specific, and to have little cross-reactivity. When HEL cells were pretreated with RACK inhibitory peptides (kindly provided by Dr. Daria Mochly-Rosen, Stanford University, Stanford, Calif., USA) prior to stimulation by PGE_1, the potentiation of AC activity by ethanol was attenuated by about 50% (from 68 ± 16 to 36 ± 3%, $p < 0.05$, Student's t test) by the RACK inhibitory peptide specific for PKC δ (δV1-1). In contrast, the inhibitory peptide for PKC ε (εV1-2) or a control (scrambled sequence) peptide had no effect (ethanol potentiation was 62 ± 11%). The potentiation by PDBu was also significantly reduced by the δV1-1 peptide, whereas the PKC ε peptide and scrambled control peptide were without effect.

Overexpression of dominant negative (DN), catalytically inactive, forms of PKC has previously been shown to competitively inhibit the activity of the targeted PKC isoform, either by displacing the endogenous PKC from its substrate, and/or by competing with the endogenous PKC for co-factors and substrates. The DN construct for PKC δ has a lysine in position 376 mutated to a methionine within the ATP binding site, making it catalytically inactive [25b]. Both the wild-type and DN constructs of PKC δ have been incorporated into separate replication-deficient adenovirus (Ad5 DL312) vectors, kindly provided to us by Dr. Trevor Biden (Garvan Institute of Medical Research, Sydney, Australia). HEL cells were infected with the viral vectors carrying either the wild-type or DN form of PKC δ at two different adenovirus titers, 10×10^3 or 50×10^3 particles/cell. HEL cells infected with the DN-PKC δ adenovirus demonstrated a 174% increase in immunoreactive PKC δ protein, representing the expression of the DN mutant PKC δ in addition to the endogeneous expression of wild-type PKC δ. When HEL cells were assayed for AC activity 20 h after viral infection, the potentiation of PGE_1-stimulated AC activity by 100 m*M* ethanol was no longer statistically significant in cells infected with the DN-PKC δ adenovirus, at either virus titer. At the higher virus titre, the potentiation of ethanol was reduced to 16 ± 7%, as compared to 52 ± 18% in cells infected with the wild-type PKC δ adenovirus.

Our previous results [46] ruled out the involvement of the atypical PKCs in the effect of ethanol by demonstrating that the downregulation of the classical and novel PKCs by prolonged exposure to phorbol esters eliminated

ethanol potentiation of PGE_1-stimulated AC activity in HEL cells. Our current results also demonstrated that the PKC inhibitor, Gö-6976, which is selective for the classical PKCs, did not reduce the potentiation of AC activity produced by ethanol or PDBu. This lack of effect was evident at Gö-6976 concentrations that were 50- to 500-fold higher than the published Ki values for PKC α, β and μ [14, 32]. The sum of these data indicated the lack of involvement of the atypical and classical PKCs, as well as PKC μ, in the effects of ethanol or PDBu on AC activity.

On the other hand, our pharmacological and molecular biological studies suggested a role for a novel PKC (PKC δ) in ethanol and phorbol ester potentiation of agonist-stimulated AC7 activity. First, rottlerin has been shown to selectively inhibit PKC δ and θ function [58], and to selectively inhibit the PKC δ and θ isozymes (Ki = 3–6 μ*M*) with 10-fold greater potency than the α, β and γ isoforms and a 20- to 30-fold greater potency than the ε, η and ζ isoforms [2, 14]. Rottlerin has been used to implicate PKC δ in many cellular functions, such as the regulation of cell growth and differentiation, apoptosis, and tumor development [6, 29, 44, 47]. Our results demonstrated a reduced potentiation of AC activity by either PDBu or ethanol even in the presence of a concentration of rottlerin below its Ki for inhibition of PKC δ [13]. However, rottlerin inhibition alone could not distinguish between the involvement of PKC δ and θ, both of which are expressed in HEL cells. Rottlerin has also been shown to affect CaM kinase III and Trk receptor activity [42]. Thus, additional experiments were performed using a novel class of selective peptide PKC inhibitors.

A RACK inhibitory protein similar to the one we used was previously shown to prevent translocation of PKC δ upon cell stimulation [23]. A related peptide has also been used to demonstrate the involvement of PKC δ in mediating ethanol-induced upregulation of L-type Ca^{2+} channels [11]. Our results, demonstrating that the PKC δ RACK inhibitory peptide, but not the PKC ε peptide or a scrambled peptide, inhibit PDBu and ethanol-induced potentiation of AC activity, further support a role for PKC δ in the action of ethanol.

Finally, catalytically inactive DN forms of various PKC isotypes have also been successfully used as specific inhibitors of PKC actions, and a recent study used a DN PKC to delineate opposing effects by two different PKC isotypes, δ and α [20]. We can conclude from our studies using DN PKC δ that PKC δ is in large part responsible for the potentiation by ethanol of prostanoid receptor/Gs protein-coupled AC activity in HEL cells. The fact that some potentiation remained even in the presence of the RACK inhibitor peptide and the dominant-negative PKC δ isoform may, however, argue for an additional mechanism for the effects of ethanol on AC activity.

Only two prior studies have implicated the novel PKCs in regulating AC activity. Inhibition of AC6 activity by a novel PKC during adenosine A2a receptor desensitization in PC12 cells was implied by the lack of Ca^{2+} dependence of this process and the effects of overexpression of PKC δ or ε in the PC12 cells [25a]. The potentiation of PGE_1-stimulated AC2 activity by phorbol-12 myristate, 13 acetate in macrophages was also suggested to be due to the novel family of PKCs [28]. On the other hand, atypical PKCs were reported to mediate lysophosophatidic acid potentiation of PGE_1-stimulated AC2 activity in these same cells [27]. Neither study by Lin et al. [27, 28] showed evidence of direct phosphorylation of AC, and it is not clear which AC isoforms besides AC2 are present in macrophages. Given our work showing that AC7 can be directly phosphorylated by PKC, it is of interest that in addition to the putative PKC phosphorylation consensus sites in the catalytically relevant cytoplasmic loops of AC7, putative sites for which the novel PKCs show preference [40] are present within the Gsα binding domains. This is particularly intriguing since we have shown that phosphorylation of AC7 by PKC resulted in greater sensitivity of the enzyme to stimulation by Gsα. In early examinations of the actions of ethanol on AC activity, we demonstrated that ethanol increases the rate of activation of Gs [30]. If an increased availability of activated Gsα* occurs in the presence of phosphorylated AC7, our results would predict a significant activation of this AC isoform, which could explain why AC7 is the most sensitive to the actions of ethanol.

In addition to our demonstration of PKC δ involvement in ethanol modulation of AC activity, the novel family of PKCs has been implicated in various other acute and chronic effects of ethanol. Short-term ethanol exposure of NG108-15 cells can alter the subcellular localization and thus presumably the function of PKC δ and ε [12]. PKC ε has been found to be involved in ethanol-induced cardioprotection [5, 35], and the acute behavioral effects of ethanol mediated through $GABA_A$ receptors are dampened in PKC ε knockout mice [18]. Chronic ethanol exposure upregulated the density and function of L-type Ca^{2+} channels via a PKC-δ-dependent mechanism [4], while the upregulation of N-type Ca^{2+} channels by chronic ethanol treatment was recently found to be attributable to PKC ε [33]. Chronic ethanol treatment of PC12 cells also enhanced nerve growth factor-induced neurite outgrowth and activation of MAP kinases by a PKC ε-dependent mechanism [21].

The exact mechanism or mechanisms by which ethanol activates PKC δ to alter AC7 activity remains unknown. Although previous data have indicated that ethanol has little if any direct stimulatory effect on the catalytic activity of PKC in vitro [31, 34], ethanol could modulate the interaction between, and the co-localization of, AC7 and PKC δ. A pool of endogenously active DAG-sensitive PKCs residing at the cell membrane has been recently described in a number of cell types, including HEL cells [4]. Other PKCs were localized primarily in the cytosol. In murine erythroleukemia cells, PKC δ was found to be predominantly membrane associated, and in a constitutively active state [43]. Thus, the acute effects of ethanol on AC activity, which can occur in less than 1 min [46], could be mediated through a pool of already activated, membrane-bound PKC δ. Ethanol could promote a conformational change in AC which provides or enhances availability of a site(s) for PKC-mediated phosphorylation, or ethanol could promote the association of AC with PKC δ within a transducisome complex [57].

The conclusions from our experiments, overall, are that under basal conditions, PKC δ maintains AC7 in a modestly phosphorylated form to maintain its responsiveness to Gsα. In the presence of ethanol, the phosphorylation of AC7 by PKC δ is enhanced. The more phosphorylated form of this AC isoform becomes more sensitive to activated Gsα and, in this way, ethanol enhances receptor-mediated signaling through the AC system. The increased levels of cAMP during such a signaling process will produce a greater effect on PKA, and greater modification of downstream effectors dependent on cAMP signaling.

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CYP2E1-Dependent Toxicity and Up-Regulation of Antioxidant Genes

Montserrat Marí Defeng Wu Natalia Nieto Arthur I. Cederbaum

Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, N.Y., USA

Key Words

CYP2E1 · Glutathione · Antioxidants · Toxicity · HepG2 cells · Stellate cells · Collagen type I

Abstract

Induction of cytochrome P450 2E1 (CYP2E1) by ethanol appears to be one of the central pathways by which ethanol generates a state of oxidative stress. Glutathione (GSH) is critical in preserving the proper cellular redox balance and for its role as a cellular protectant. The goal of the present study was to characterize the GSH homeostasis in human hepatocarcinoma cells (HepG2-E47 cells) that overexpress CYP2E1. Toxicity in the E47 cells was markedly enhanced after GSH depletion by buthionine sulfoximine (BSO) treatment. The antioxidant trolox partially prevented the apoptosis and necrosis, while diallylsulfide, a CYP2E1 inhibitor, was fully protective. Damage to mitochondria appears to play a role in the CYP2E1- and BSO-dependent toxicity. CYP2E1-overexpressing cells showed increases in total GSH levels, GSH synthetic rate and in γ-glutamylcysteine synthetase (GCS) mRNA. This GCS increase was due to transcriptional activation of the GCS gene and could be blocked by certain antioxidants. Activity, protein and mRNA levels for other antioxidants such as catalase, α- and microsomal glutathione transferases were also increased in the E47 cells. Up-regulation of these antioxidant genes may reflect an adaptive mechanism to remove CYP2E1-derived oxidants. These oxidants are diffusable and were able to elevate collagen type I protein in a co-culture system consisting of the E47 cells + rat hepatic stellate cells. Such interactions between CYP2E1, mitochondria and altered GSH homeostasis, and elevation of collagen levels, may play a role in alcohol-induced liver injury.

Introduction

Cytochrome P450 2E1 (CYP2E1), the ethanol-inducible form, is of interest because of its ability to metabolize and activate many toxicologically important substrates including ethanol, carbon tetrachloride, acetaminophen and N-nitrosodimethylamine to more toxic products [15, 20, 35]. There is considerable interest in the role of oxidative stress and ethanol generation of reactive oxygen species (ROS) in the mechanism by which ethanol is hepatotoxic [3, 6, 11]. A major advance has been the development of the intragastric model of ethanol feeding in which prominent induction of CYP2E1 occurs along with significant alcohol liver injury [5, 26, 28]. In these models, large increases in lipid peroxidation have been observed, and

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Arthur I. Cederbaum
Department of Biochemistry and Molecular Biology, Box 1020
Mount Sinai School of Medicine
New York, NY 10029 (USA)
Tel. +1 212 241 7285, Fax +1 212 996 7214, E-Mail Arthur.Cederbaum@mssm.edu

the ethanol-induced liver pathology has been shown to correlate with CYP2E1 levels and elevated lipid peroxidation.

An approach that our laboratory has utilized to try to understand basic effects and actions of CYP2E1 is to establish a stable cell line that constitutively expresses human CYP2E1 [10]. To briefly summarize results published elsewhere [7, 34], ethanol was found to be cytotoxic to HepG2 cells which express CYP2E1 and not to the control cells lacking detectable CYP2E1. A polyunsaturated fatty acid, arachidonic acid, was also found to induce cytotoxicity and apoptosis in the CYP2E1-expressing HepG2 cells. Glutathione (GSH) appears to be essential in protecting HepG2 cells against CYP2E1-dependent cytotoxicity by these agents, since buthionine sulfoximine (BSO) treatment, which depletes cellular GSH, increased the cytotoxicity. The cytotoxicity and apoptosis found with ethanol and polyunsaturated fatty acids were prevented by several antioxidants, especially vitamin E and the vitamin E analog trolox. Induction of a state of oxidative stress appears to play a central role in the CYP2E1-dependent cytotoxicity.

Toxicity of ethanol or iron or polyunsaturated fatty acids was enhanced when cellular GSH levels were lowered by treatment with BSO. CYP2E1 is a loosely coupled P450 and can be reduced by NADPH even in the absence of substrate [2]; formation of ROS by microsomes isolated from the CYP2E1-expressing HepG2 cells was not altered by the presence of substrates for CYP2E1 [10]. In a new HepG2 cell model, established by plasmid transfection methods, with higher levels of CYP2E1 than the original lines, removal of GSH resulted in a loss of cell viability [8]. One goal of the current report was to further characterize this CYP2E1-dependent toxicity which occurs upon GSH removal, with respect to activation of caspases, production of apoptosis or necrosis and effects on mitochondrial membrane potential.

Adaptation to oxidant stimuli is critical for short- and long-term survival of cells exposed to oxidative stress. GSH is critical for preserving the proper cellular redox balance and protecting cells against oxidative stress [23]. While much of the focus on CYP2E1 has been from a toxicological point of view, the possibility that the hepatocyte attempts to respond to increased levels of CYP2E1 by upregulation of protective factors has not been examined. A second goal of this report was to evaluate whether CYP2E1 overexpression could mediate an effect on GSH homeostasis and modulate the levels of other antioxidant enzymes important for the removal of ROS.

Hepatic stellate cells (HSC) are central to the fibrotic response to liver injury. During fibrogenesis, HSC undergo activation, resulting in the production of extracellular matrix components, especially collagen type I [4, 14]. ROS have been viewed as potential mediators of HSC activation [1, 17]. Since CYP2E1 is an active producer of ROS, the possibility that CYP2E1 could eventually cause activation of collagen type I expression would be important to evaluate. Indeed, we developed HSC cell lines that overexpress human CYP2E1 and found a 3- to 4-fold increase in COL1A2 mRNA levels as compared to HSC transfected with empty vector [29, 30]. This increase was partially prevented by antioxidants. Since CYP2E1 is present largely in the hepatocyte, whereas HSC contain a low amount of CYP2E1, a possible interaction between CYP2E1, or CYP2E1-derived diffusable mediators, with HSC was evaluated by developing a co-culture model involving HepG2 cells with HSC.

Methods

In vitro Model and Cell Culture Conditions

Two previously established hepatoma HepG2 sublines [8] were used as a model in this study. E47 cells contain the human CYP2E1 cDNA inserted into the *Eco*RI restriction site of the pCI-neo expression vector (Promega, Madison, Wisc., USA) in the sense orientation. C34 cells contain the pCI-neo vector alone. The HepG2 transduced C34 and E47 clones were cultured in MEM, supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 m*M* glutamine in a humidified atmosphere in 5% CO_2 at 37°C. Cells were maintained in the presence of 0.5 mg/ml of geneticin. An immortal rat stellate cell line (T6-HSC cells) was used for experiments assaying collagen protein levels [19].

Biochemical Assays

Published methods, which are described in Marí and Cederbaum [25], were used to assay GSH levels, GSH synthetic rate using monochlorobimane, glutathione peroxidase, glutathione transferase, glutathione reductase, total superoxide dismutase activity and catalase. Activity of caspases was determined using the following caspase substrates: caspase 1, Z-YVAD-AFC; caspase 3, acetylated DEVD-AMC; caspase 8, Z-IEYD-AFC; caspase 9, acetylated LEHD-AFC.

Cellular Viability Assays

Cytotoxicity of various agents or treatments was determined by the MTT assay. Cell death by necrosis was determined using a trypan blue exclusion method. CYP2E1-mediated apoptosis was measured by a flow cytometry method after addition of propidium iodide to permeabilized cells. The mitochondrial membrane potential was analyzed from the accumulation of rhodamine 123, a membrane-permeable cationic fluorescent dye [12].

Northern and Western blots and nuclear run-on transcription assays were carried out by methods previously described [25, 29, 30].

Table 1. Effect of BSO treatment on viability of E47 cells

Treatment	Reaction			
	caspase 3 U/mg	apoptosis %	necrosis %	MMP %
None	25	5	6	100
BSO	295	40	30	42
BSO, trolox	ND	19	7	100
BSO, diallylsulfide	ND	9	8	70
BSO, pan caspase inhibitor	45	10	ND	70

ND = Not determined; MMP = mitochondrial membrane potential.

Reaction times were either 48 h (caspase 3) or 96 h (apoptosis, necrosis, MMP) after the addition of BSO.

Table 2. Antioxidant enzyme activities in C34 and E47 cells

	C34	E47
GSH, nmol/mg	36.2 ± 2.4	47.4 ± 3.1*
GCS-HS mRNA/GAPDH	1	2.0 ± 0.3*
GCS-LS mRNA/GAPDH	1	1.8 ± 0.4*
GSH synthesis rate, nmol/min/mg	0.235 ± 0.05	0.360 ± 0.05*
Glutathione reductase, mU/mg	60.15 ± 6.23	54.32 ± 7.2
Superoxide dismutase, U/mg	1.40 ± 0.08	1.37 ± 0.05
Glutathione peroxidase, mU/mg	16.40 ± 1.96	11.25 ± 2.34*
Catalase, U/mg	32.18 ± 2.75	60.48 ± 3.94*
Glutathione S-transferase, mU/mg	17.21 ± 2.56	36.94 ± 1.7*
Glutathione S-transferase microsomal fraction, mU/mg[1]	3.54 ± 0.76	7.45 ± 0.84*

Results are expressed as means ± SD, n = 6. * $p < 0.05$ when compared to C34.

[1] The microsomal fraction contains microsomal and cytosolic glutathione S-transferases.

Results

CYP2E1-Dependent Toxicity after BSO Treatment

BSO was added to the E47 and C34 cells, and caspase 1, 3, 8 and 9 activity was determined at various times after the BSO addition. Treatment with BSO did not increase the activities of caspases 1, 9 and 8 in the E47 or C34 cells. However, caspase 3 activity was increased 36 and 48 h after BSO treatment in the E47 cells without any increase in the C34 cells (table 1). The increase in caspase 3 activity in the E47 cells was about 7- and 10-fold 36 and 48 h after BSO treatment, respectively. A pan caspase inhibitor, added to the cells at the same time as BSO, prevented the increase in caspase 3 activity in the E47 cells (table 1). In view of the elevated caspase 3 activity in the E47 cells after BSO treatment, apoptosis was evaluated by a flow cytometry method. No significant apoptosis was observed in the C34 cells even 96 h after BSO treatment (data not shown). However, apoptosis was observed with the E47 cells (table 1). No significant apoptosis was observed in the absence of BSO, and the apoptosis produced in the presence of BSO was completely prevented by the pan caspase inhibitor (table 1).

Experiments were carried out to evaluate the role of oxidative stress, essentially at the level of lipid peroxidation on the CYP2E1 + BSO-mediated apoptosis. Trolox, a vitamin E analogue, was added to the cells with or without BSO treatment. To further validate a role for CYP2E1 in the BSO-dependent apoptosis, experiments were also carried out with diallylsulfide (DAS), an effective inhibitor of CYP2E1. Treating the E47 cells with BSO for 48, 72 or 96 h caused 3.7 ± 1.4, 12.8 ± 5 and 39.9 ± 13.1% of the cells to undergo apoptosis. Addition of DAS completely prevented this BSO + CYP2E1-dependent apoptosis, whereas trolox was partially protective (table 1, 96 h data).

Treatment of the E47 cells with BSO not only caused apoptosis, but also produced necrosis, as determined by trypan blue uptake. Treatment of the E47 cells for 48, 72 or 96 h with BSO resulted in 8.8 ± 1.3, 11.6 ± 1.1 and 30.9 ± 5.1% of cells taking up trypan blue, respectively (table 1). Less than 7% of the C34 cells took up trypan blue after 96 h of BSO treatment (data not shown). DAS and trolox largely prevented the CYP2E1 + BSO-dependent necrosis (table 1).

A decrease in the mitochondrial membrane potential may be an early event in some models of apoptosis. The E47 cells were incubated with rhodamine 123, a lipophilic cation whose uptake by mitochondria is proportional to the membrane potential [12], and fluorescence of this dye was determined by flow cytometry. Upon treatment with BSO for 48, 72 and 96 h, there was a decrease in the fluorescence from a control value of 100% to values of 62 ± 5, 67 ± 5 and 43 ± 7% respectively, in the E47 cells (table 1, 96 h data). This decrease in membrane potential was largely or partially (96 h, table 1) prevented by DAS, trolox and the pan caspase inhibitor.

Modulation of Antioxidant Enzymes and GSH Homeostasis by CYP2E1

E47 cells had a significant 30% increase in total GSH as compared to C34 cells (table 2). GSSG levels were very low and similar in both cell lines. To maintain the intra-

cellular GSH levels, GSSG is reduced back to GSH by glutathione reductase, or de novo synthesis of GSH by γ-glutamylcysteine synthetase (GCS) occurs. Organic hydroperoxides can be reduced either by glutathione peroxidase or glutathione S-transferases (GST). Superoxide is converted to H_2O_2 by superoxide dismutase, H_2O_2 can be removed either by reduction by GSH in the cytosol, as catalyzed by glutathione peroxidase, or by catalase in the peroxisomes. As shown in table 2, there was no difference in the activity of glutathione reductase or superoxide dismutase between C34 and E47 cells. There was a 30% decrease in glutathione peroxidase activity in CYP2E1-expressing cells. However, there was a 2-fold increase in the activities of total GST and catalase in E47 compared to C34 cells. An increase in GST activity in the microsomal fraction isolated from the E47 cells was also found and the percent increase in GST activity was similar in both cytosolic and microsomal fractions of CYP2E1-expressing cells.

The first step of GSH biosynthesis is rate limiting and catalyzed by GCS, which is composed of a heavy subunit (HS) and a light subunit (LS). Control cells (C34) expressed two transcripts of GCS-HS mRNA with sizes of 4.1 and 3.2 kb. CYP2E1-expressing cells (E47) showed a 2-fold increase in these two transcripts of GCS-HS mRNA (table 2). In other experiments, a 2-fold increase in GCS-LS mRNA was also found in the E47 cells, suggesting that there appears to be coordinate regulation of the mRNAs for both subunits of GCS in the E47 cells. To determine if the increase in GCS-HS mRNA is parallel with a higher enzyme activity, we determined the de novo capacity to synthesize GSH. Cytosolic fractions from CYP2E1-overexpressing cells showed a 50% increase in the rate of GSH synthesis (table 2).

An increase in GCS-HS mRNA may reflect either an increased transcription rate of the GCS-HS gene or a stabilization of the mRNA. The half-life of GCS-HS mRNA in C34 and E47 cells was assessed in the presence of actinomycin D (10 μg/ml). The half-life in C34 and E47 cells was similar (~4 h), indicating that posttranscriptional modification of GCS-HS mRNA stability cannot account for the increase in GCS-HS mRNA in CYP2E1-overexpressing cells. To determine whether there is increased transcription of the GCS-HS gene, nuclear run-on experiments were carried out. Indeed, the nuclear run-on revealed an increased capacity of E47 cells to transcribe the GCS-HS gene. The ratio of newly synthesized GCS-HS to GAPDH mRNA was elevated from a value of 1 in the C34 cells to a value of 1.7 in E47 cells. This increase in transcription under steady-state conditions is similar to the increase in mRNA levels as detected by Northern blotting.

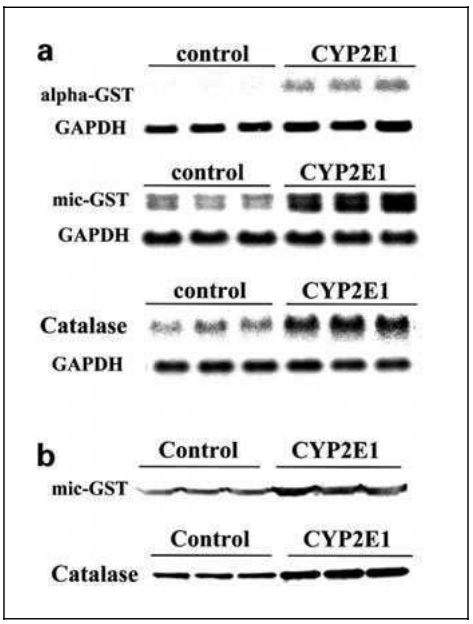

Fig. 1. Northern (**a**) and Western (**b**) blots for α-GST, microsomal (mic) GST and catalase mRNA and protein levels in control C34 cells and E47 CYP2E1-expressing cells (n = 3).

To characterize the nature of CYP2E1-induced transcriptional activation of GCS-HS mRNA, GSH, which may act as an antioxidant or feedback regulator of GSH synthesis, was added to the culture medium, and the effect on the GCS-HS mRNA was determined. Glutathione ethyl ester (GSH-EE) is efficiently transported into human cells and converted intracellularly to GSH and therefore was used as the source of added GSH. GSH-EE increased the content of GSH in C34 cells by 50% but produced only a 14% increase in the already elevated GSH levels in E47 cells; in fact, GSH-EE equalized the GSH content in the two cell lines (data not shown). GSH-EE had no effect on GCS-HS mRNA levels in C34 cells, but largely prevented the increase in this mRNA found in E47 cells. In other experiments, transfection with catalase decreased GCS-HS mRNA in E47 cells but not in C34 cells. The decreases produced by GSH-EE and by catalase suggest that H_2O_2 may be a critical ROS responsible for the increase in GCS mRNA found in the E47 cells.

With respect to the other antioxidant enzymes whose activity was elevated in the E47 cells (table 2), Northern and Western blots were performed to determine the nature of the induction. As shown in figure 1a, Northern blots for catalase display a single transcript of 2.7 kb.

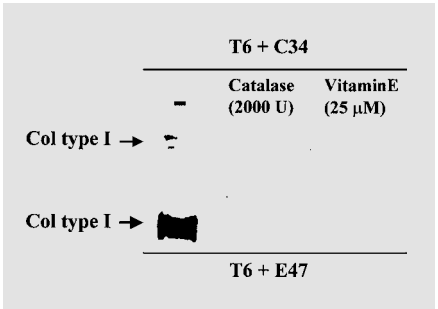

Fig. 2. Levels of collagen type I protein in co-culture of T6-HSC cells with either HepG2 C34 or E47 cells, in the absence or presence of the antioxidants catalase and vitamin E.

There was a 2-fold increase in catalase mRNA in CYP2E1-expressing E47 cells compared to control C34 cells. This increase in catalase mRNA correlates with an increased catalase protein content in the CYP2E1-expressing cells (fig. 1b). The fact that glutathione peroxidase activity was decreased whereas catalase was induced in the CYP2E1-expressing cells suggests the possibility that the catalase induction helps to remove the H_2O_2 derived from CYP2E1 expression.

Northern blots for the microsomal GST (fig. 1a) revealed the presence of two transcripts with sizes around 1 kb. There was a 2-fold increase ($p < 0.05$) in the microsomal GST mRNA in the E47 cells compared to the C34 control cells. The content of microsomal GST protein in E47 cells was 2- to 3-fold higher ($p < 0.05$) than in the C34 cells (fig. 1b), consistent with the increase in mRNA levels. The mRNA for the α-GST in control C34 cells is almost undetectable but was present in the CYP2E1-expressing cells (fig. 1a). Levels of GAPDH mRNA were similar between the two cell lines. Nuclear run-on assays revealed an increased capacity of E47 cells to transcribe both the α-GST and the microsomal GST gene (data not shown). The GST/GAPDH ratio of the newly synthesized mRNA was elevated from a value of 1 in the C34 cells to a value of 2 in the E47 cells for the α-GST, and from 1 to a value of 1.8 for the microsomal GST. This increase in transcription under steady-state conditions is similar to the increase found in microsomal GST mRNA levels as detected by Northern blot.

Increased Collagen Type I Protein Levels in Co-Cultures of E47 Cells with HSC

E47 or C34 cells were co-cultured with the T6-HSC and production of collagen type I protein was determined by Western blot analysis. The HepG2 cells and the HSC were separated from each other by an insert; therefore, the experimental protocol is designed to evaluate whether mediators (ROS, cytokines, growth factors) produced by HepG2 cells diffuse to the HSC and affect collagen type I protein levels. There was a time-dependent increase in collagen type I levels in T6-HSC when co-incubated with C34 cells, and this was further elevated when T6-HSC were co-incubated with E47 cells (data not shown). Whereas little collagen type I protein was detected in the incubation medium from the C34 + HSC co-culture, collagen type I protein was secreted into the incubation medium from the E47 + HSC co-culture (data not shown). These experiments suggest that E47 cells generate diffusable mediators which increase collagen type I protein levels in HSC.

To gain insight into the nature of these diffusable mediators, antioxidants were added to the medium of the co-cultures. Catalase and vitamin E markedly lowered the collagen type I protein in HSC with both co-cultures and completely blocked the increase produced by the co-culture with E47 cells (fig. 2). This suggests that the E47 cells are releasing ROS such as H_2O_2 and lipid peroxidation-derived products that contribute to the elevation in collagen type I protein. Enhanced collagen type I protein found with the E47 + HSC co-culture could reflect enhanced production of ROS and lipid peroxidation by the CYP2E1-expressing HepG2 cells.

Discussion

Induction of CYP2E1 by ethanol appears to be one of the central pathways by which ethanol generates a state of oxidative stress. GSH is the most abundant antioxidant in cells and plays a major role in the defense against oxidative stress-induced cell injury [23]. There is considerable interest in the effects of ethanol on GSH homeostasis and the role which GSH depletion plays in ethanol-induced liver injury. While acute ethanol treatment lowers hepatic GSH levels, largely as a consequence of inhibiting GSH synthesis [33], the effects of chronic ethanol treatment on GSH levels are less clear, with reports of decreased GSH levels [13, 24], unchanged levels [18, 27] or even increased levels [31]. Mitochondrial GSH levels are decreased after chronic ethanol treatment, and this decrease has been suggested to play a role in ethanol-induced liver injury [9, 13].

One goal of the current report was to continue to characterize the role of GSH in CYP2E1-dependent toxicity in HepG2 cell models that constitutively express human

CYP2E1. In a HepG2 cell model developed to express high levels of CYP2E1, removal of GSH resulted in a loss of cellular viability, suggesting that GSH was critical in protecting the cells against CYP2E1-dependent toxicity. Indeed, in the E47 cells expressing high levels of CYP2E1, GSH levels were 30% higher than the levels in control C34 cells due to a 2-fold increase in activity and expression of GCS, the rate-limiting enzyme of GSH synthesis [25]. We suggest that this up-regulation of GSH synthesis was an adaptive response to attenuate CYP2E1-dependent oxidative stress and toxicity. Removal of GSH from the E47 cells resulted in a time-dependent production of apoptosis as well as necrosis. It has been suggested that these two fundamental forms of death may share common upstream events [22], such as oxidative stress or impairment of mitochondrial function. Oxidants such as H_2O_2 may cause apoptosis at low concentrations and necrosis at high concentrations [16, 32]. Both the CYP2E1- and BSO-dependent apoptosis and necrosis could be partially prevented by trolox, suggesting that lipid-peroxidation-related events played a role in the developing toxicity. It is likely that CYP2E1-dependent production of ROS and catalysis of lipid peroxidation, coupled to the loss of the GSH antioxidant defense system upon treatment with BSO, is responsible for the apoptosis and necrosis.

Mitochondria are a main source for generating ROS and target for damage by ROS. Pro-oxidants can induce a mitochondrial permeability transition and disrupt the mitochondrial membrane potential [21, 36]. This potential decreased in the E47 cells treated with BSO and the decline shared similar characteristics with the developing apoptosis, e.g. prevention by DAS, trolox and the pan caspase inhibitor. The prevention by DAS and trolox suggests that CYP2E1-derived ROS play a role in the fall in mitochondrial membrane potential. While it is likely that several mechanisms contribute to alcohol-induced liver injury, the linkage between CYP2E1-dependent oxidative stress, mitochondrial injury and GSH homeostasis may contribute to the toxic action of ethanol on the liver.

The ability to maintain cellular functions under conditions of oxidative stress depends on the rapid induction of protective antioxidant systems. GSH synthesis, GCS-HS mRNA transcription and GSH levels were increased in CYP2E1-expressing HepG2 cells, along with other antioxidant enzymes such as catalase, α-GST and microsomal GST. It is likely that these increases reflect an adaptive response by the E47 cells to cope with CYP2E1-derived oxidative stress. Increases in the levels of these enzymes are due to increases in de novo synthesis as determined by nuclear run-on transcription assays. There appears to be some selectivity in E47 cells for increasing the activity of antioxidant enzymes important for GSH homeostasis, e.g. GST and GCS, as glutathione reductase activity is not altered, whereas glutathione peroxidase activity is decreased. The last is known to be sensitive to oxidative stress. The K_m value of catalase for hydrogen peroxide is much higher than that of GSH peroxidase. Hence, GSH peroxidase has been postulated to degrade low levels of H_2O_2 physiologically, while catalase might function when cellular levels of H_2O_2 are increased. In CYP2E1-expressing cells the two enzymes appear to compensate for each other in scavenging H_2O_2 since GSH peroxidase is decreased by 30% but catalase is increased 2-fold. GST can protect cells against oxidative stress and membrane lipid peroxidation by removing reactive metabolites as well as H_2O_2.

We evaluated whether CYP2E1 overexpression could result in the release of diffusable mediators that could have consequences on metabolic activities of adjacent cells. We utilized a co-culture model of E47 or C34 cells + stellate cells and assayed levels of collagen type I protein. Activation of HSC and increased production of collagen are key features in fibrogenesis, therefore it was important to assess whether CYP2E1 expression could ultimately result in increases in collagen type I protein. This proved to be the case as the collagen type I level was higher in co-cultures of E47 cells + HSC as compared to co-cultures of C34 cells + HSC. Moreover, antioxidants prevented this increase implicating CYP2E1-derived diffusable oxidants as participating in the overall mechanism resulting in the increase in collagen type I protein. Interactions between CYP2E1, mitochondria and altered GSH homeostasis, and elevation of collagen levels of HSC by CYP2E1-derived ROS, may act synergistically to play a role in alcohol-induced liver injury.

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Oxidative Stress, Metabolism of Ethanol and Alcohol-Related Diseases

Tomáš Zima[a] Lenka Fialová[b] Oto Mestek[d] Marta Janebová[b] Jiřina Crkovská[b] Ivan Malbohan[b] Stanislav Štípek[b] Ludmila Mikulíková[a] Petr Popov[c]

[a]Institute of Clinical Chemistry, [b]1st Institute of Medical Chemistry and Biochemistry and [c]Department for Alcohol and Addiction, First Faculty of Medicine, Charles University, and [d]Department of Analytical Chemistry, Institute of Chemical Technology, Prague, Czech Republic

Key Words

Oxidative stress · Antioxidants · Ethanol · Alcoholism · Trace elements · Antiphospholipid antibodies · Oxidized low-density lipoprotein · Nitric oxide · Autoantibodies · Tocopherol · Retinol

Abstract

Alcohol-induced oxidative stress is linked to the metabolism of ethanol. Three metabolic pathways of ethanol have been described in the human body so far. They involve the following enzymes: alcohol dehydrogenase, microsomal ethanol oxidation system (MEOS) and catalase. Each of these pathways could produce free radicals which affect the antioxidant system. Ethanol per se, hyperlactacidemia and elevated NADH increase xanthine oxidase activity, which results in the production of superoxide. Lipid peroxidation and superoxide production correlate with the amount of cytochrome P450 2E1. MEOS aggravates the oxidative stress directly as well as indirectly by impairing the defense systems. Hydroxyethyl radicals are probably involved in the alkylation of hepatic proteins. Nitric oxide (NO) is one of the key factors contributing to the vessel wall homeostasis, an important mediator of the vascular tone and neuronal transduction, and has cytotoxic effects. Stable metabolites – nitrites and nitrates – were increased in alcoholics (34.3 ± 2.6 vs. 22.7 ± 1.2 μmol/l, $p < 0.001$). High NO concentration could be discussed for its excitotoxicity and may be linked to cytotoxicity in neurons, glia and myelin. Formation of NO has been linked to an increased preference for and tolerance to alcohol in recent studies. Increased NO biosynthesis also via inducible NO synthase (NOS, chronic stimulation) may contribute to platelet and endothelial dysfunctions. Comparison of chronically ethanol-fed rats and controls demonstrates that exposure to ethanol causes a decrease in NADPH diaphorase activity (neuronal NOS) in neurons and fibers of the cerebellar cortex and superior colliculus (stratum griseum superficiale and intermedium) in rats. These changes in the highly organized structure contribute to the motor disturbances, which are associated with alcohol abuse. Antiphospholipid antibodies (APA) in alcoholic patients seem to reflect membrane lesions, impairment of immunological reactivity, liver disease progression, and they correlate significantly with the disease severity. The low-density lipoprotein (LDL) oxidation is supposed to be one of the most important pathogenic

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Tomáš Zima, MD, PhD
Institute of Clinical Chemistry, First Faculty of Medicine
Charles University, Karlovo nám. 32
CZ–121 11 Prague 2 (Czech Republic)
Tel./Fax +420 2 2223 2261, E-Mail zimatom@mbox.cesnet.cz

mechanisms of atherogenesis, and antibodies against oxidized LDL (oxLDL) are some kind of epiphenomenon of this process. We studied IgG oxLDL and four APA (anticardiolipin, antiphosphatidylserine, antiphosphatidylethanolamine and antiphosphatidylcholine antibodies). The IgG oxLDL (406.4 ± 52.5 vs. 499.9 ± 52.5 mU/ml) was not affected in alcoholic patients, but oxLDL was higher (71.6 ± 4.1 vs. 44.2 ± 2.7 μmol/l, $p < 0.001$). The prevalence of studied APA in alcoholics with mildly affected liver function was higher than in controls, but not significantly. On the contrary, changes of autoantibodies to IgG oxLDL revealed a wide range of IgG oxLDL titers in a healthy population. These parameters do not appear to be very promising for the evaluation of the risk of atherosclerosis. Free radicals increase the oxidative modification of LDL. This is one of the most important mechanisms, which increases cardiovascular risk in chronic alcoholic patients. Important enzymatic antioxidant systems – superoxide dismutase and glutathione peroxidase – are decreased in alcoholics. We did not find any changes of serum retinol and tocopherol concentrations in alcoholics, and blood and plasma selenium and copper levels were unchanged as well. Only the zinc concentration was decreased in plasma. It could be related to the impairment of the immune system in alcoholics. Measurement of these parameters in blood compartments does not seem to indicate a possible organ, e.g. liver deficiency.

Introduction

A role of free radicals in the development of alcoholic liver damage has been suspected since the early 1960s. Alcohol-induced oxidative stress is linked to the metabolism of ethanol. Each metabolic pathway [alcohol dehydrogenase, microsomal ethanol oxidation system (MEOS), catalase] produces specific metabolic and toxic disturbances. Ethanol per se, hyperlactacidemia and increased NADH induce xanthine oxidase activity with subsequent production of activated oxygen species. Increased generation of oxygen- and ethanol-derived free radicals occurs at the microsomal level, especially during the action of the ethanol-inducible cytochrome P450 2E1 (CYP2E1; MEOS). Lipid peroxidation and superoxide production correlate with the amount of CYP2E1. MEOS aggravates the oxidative stress directly as well as indirectly by impairing the defense system against it. Hydroxyethyl radicals appear to be involved in the alkylation of hepatic proteins. Glutathione depletion promotes lipid peroxidation as well.

Free radicals are highly reactive species characterized by one or more unpaired electrons in their outer orbital. Reactive oxygen species include oxygen radicals and substances closely related to oxygen radical reactions. Production of reactive oxygen species is a physiological process, but its increase and dysbalance between production of radicals and antioxidants could lead to oxidative stress with the affection of various biological functions and structural changes [30]. Biological effects of reactive oxygen species and other radicals are controlled by antioxidant mechanisms – enzymes and substrates. They are both lipophilic and hydrophilic and are closely related to each other. Retinol and tocopherol are the main representatives of lipophilic antioxidants.

Nitric oxide (NO) is a simple, free radical gas with important bioregulatory functions in the nervous, immune and cardiovascular systems. NO synthase (NOS, EC 1.14.13.39) is an enzyme, which generates NO from the terminal guanidino nitrogen of *L*-arginine during its conversion to *L*-citrulline. Three isoenzymes of NOS have been identified. They all require NADPH, tetrahydrobiopterin; flavin adenine dinucleotide and flavin mononucleotide as cofactors, and all contain heme [25]. Neuronal NOS (nNOS, type I) has an important function in neurotransmission (modulation of N-methyl-*D*-aspartate, NMDA, receptor). NO can originate from at least four different sources in the central nervous system: the endothelium of cerebral vessels, the immunostimulated microglia and astrocytes, noradrenergic noncholinergic nerves and the glutamate neurons [49].

Endothelial cells and several other cell lines, including phagocytes, synthesize and release NO. NO displays a broad spectrum of activities – smooth muscle relaxation, inhibition of platelet aggregation and adhesion, decrease in smooth muscle cell proliferation, cytotoxicity and modulation of neuronal transmission [4].

The inducible form of NOS (iNOS, type II) produces a much higher amount of NO and is an important mediator of the inflammatory reaction in the human body. Formation of NO by macrophages is a primary defense mechanism against pathogenic microorganisms and tumorous cells. Diffusion of NO into pathogenic cells leads to inactivation of some enzymes and to formation of reactive oxygen species [4, 86]. The activity of iNOS corresponds to increased gene expression, which is stimulated by lipopolysaccharides, bacterial endotoxins, interferon γ, α and β, tumor necrosis factor (TNF) α, TNF-β and interleukin

(IL) 1. Nuclear activating factor as well as free radicals are connected with the expression and release of NO [6, 44, 62].

NO is degraded to the stable metabolites nitrites and nitrates which could be used as parameters of NO production [4, 21].

The cell membrane consists of a phospholipid bilayer and proteins. Phospholipids can be modified by oxidative stress and free radicals. Antiphospholipid antibodies (APA) are a generic term describing antibodies that recognize various phospholipids – cardiolipin, phosphatidylcholine, phosphatidylethanolamine or phosphatidylserine. APA occur in patients with systemic lupus erythematosus, thrombosis, neoplastic disease, infections, advanced age and in women with repeated spontaneous abortions [5, 33].

Hepatocyte damage is a cardinal event in the course of alcoholic liver injury, and autoantibodies against native and modified phospholipids could play a role in this process. Antiphospholipid autoantibodies reflect the membrane lesion, but an eventual disorder of the immune system should also be taken into account. In general, formation of autoantibodies increases with age. It is still difficult to define the clinical importance of these antibodies. APA occur physiologically as 'cleaning mechanism' according to Grabar's autoimmune theory. Pathological production of these antibodies as well as other antibodies is a sign of the primary damage to the biological membrane, e.g. by lipoperoxidation or by impaired tolerance to organ-specific antigens.

Atherosclerosis is the most frequent cause of death in the civilized world. Low-density lipoprotein (LDL) particles transporting cholesterol can be modified by oxidation and glycation and so become harmful for the organism. Interaction of modified LDL with scavenger receptors on the surface of macrophages leads to the formation of foam cells, which represent the first phase of the atherosclerotic process [8, 66, 71, 82].

Oxidative modification of LDL is responsible for their antigen specificity and thus formation of autoantibodies. Introduction of their determination into clinical practice extends the possibilities of research of the immunological aspects in the development of atherosclerosis. These autoantibodies are not exclusively specific for atherosclerosis; nevertheless, their amount increases parallel with formation of oxidatively modified LDL (oxLDL) particles.

Oxidation of lipid particles is a complex process. In vivo, lipid peroxidation was observed above all in tissue macrophages, endothelial cells and smooth muscle cells [29, 83]. oxLDL have a wide range of biological effects, they are immunogenic, cytotoxic and chemotactic for monocytes and smooth muscle cells, and increase the adhesion of monocytes. They stimulate directly or by the means of smooth muscle cells the production of several mediators [plasminogen activator inhibitor 1, IL-1, IL-8, monocyte chemotactic protein 1 (MCP-1) or colony-stimulating factor (CSF)], they activate T lymphocytes, inhibit the formation of TNF and influence the production of NO [22–24].

Antioxidants, mainly α-tocopherol, β-carotene and ubiquinol Q_{10} [22–24, 42], protect LDL particles against oxidation. This leads to their consumption, if they are not effectively regenerated by antioxidants in the hydrophilic compartment, especially by ascorbic acid. Regular intake of vitamin E and other antioxidants can thus retard the oxidative modification of LDL [23, 48].

Vitamin A (retinol) is a fat-soluble vitamin from the group of carotenoids. It is rapidly oxidized in the presence of oxygen, transient metals and light [60]. Vitamin A has several functions in the organism – it is involved in the visual process, in growth and differentiation and belongs to important antioxidants. Carotenoids (especially β-carotene – provitamin A) protect against atherosclerosis (defense against lipid peroxidation of LDL and high-density lipoprotein (HDL) particles) and cancer [15].

Tocopherols are other fat-soluble vitamins. Absorption of vitamin E from the intestinal lumen is closely related to the digestion and absorption of lipids. In the circulation, tocopherol can be exchanged between all types of lipoproteins without passing through the liver [21]. α-Tocopherol is the most important chain-breaking antioxidant present in human membranes [30, 75]. It reacts with an organic peroxyl radical to form the corresponding organic hydroperoxide and the tocopheryl radical. The tocopheryl radical must be reduced back to tocopherol, and this requires an interaction with water-soluble reducing agents (ascorbate, glutathione, thioctic acid which reduces both tocopheryl radicals and ascorbate) [63]. Tocopherol protects mainly polyunsaturated fatty acids in cell membranes, VLDL and LDL. Vitamin E protects against atherosclerosis and cancerogenesis, and decreases mortality from cardiac ischemia. High concentrations of vitamin E may have prooxidative activity [16, 28, 30, 63, 75].

Alterations of blood and tissue concentrations of trace elements have been extensively investigated. Methodology represents the major limitation to valid studies on trace element levels in biological material [14, 32, 79]. Selenium, zinc and copper are elements, which play an important role in biological systems as components of proteins, enzymes and antioxidants.

Selenium (Se) is an essential trace element and it may play several roles in the human body. Se is a naturally occurring antioxidant and appears to preserve tissue elasticity by delaying oxidation of polyunsaturated fatty acids. Se is an essential component of glutathione peroxidase (EC 1.11.1.9), whose main role is to decompose safely mainly hydrogen peroxide and organic peroxides with the help of reduced glutathione. Se deficiency has been implicated as contributing factor to the development of cardiovascular disease (congestive cardiomyopathy), accelerated atherosclerosis, skeletal muscle myopathy, increased cancer risk, aging, cataract and deranged immune function [43, 46].

Zinc (Zn) has three well-defined physiological roles – catalytic, structural and regulatory. Zn is a structural component of an antioxidant enzyme, Cu,Zn-SOD, and plays also a role as a stabilizer of biological membranes and macromolecules. It is an important factor in transcriptional control of specific genes via zinc finger proteins associated with cellular proliferation and differentiation and intracellular signaling. Zn is essential for the development and proper function of the immune system [18, 19, 46].

Copper (Cu) is an important element, e.g. for the integrity of connective and bone tissues, blood vessels and production of neurotransmitters. Health-threatening deficiencies of Cu are fairly rare [46, 47].

The aim of our studies was to describe both formations of free radicals and antioxidant systems in alcoholics with incipient liver lesions.

Methods

Biochemical parameters were measured by standard assays on a biochemical analyzer Hitachi 717 (Boehringer Mannheim, Germany).

Nitrites/Nitrates and oxLDL

Serum was deproteinated by ultrafiltration (6,000 *g*, 180 min, 4°C) and frozen (–20°C). A method for simultaneous nitrite and nitrate estimation is based on the reduction of nitrate to nitrite with nitrate reductase. Nitrites/nitrates were measured in plasma spectrophotometrically (540 nm) by the modified Griess' method [20].

Blood was centrifuged freshly (10 min at 4°C) and serum was frozen at –70°C. OxLDL was measured by our modification of Ahotupa's UV method spectrophotometrically (234 nm) as concentration of conjugated dienes in the LDL fraction [88].

Tocopherol and Retinol

Blood was centrifuged (10 min at 4°C) and serum was stored at –18°C in the dark (retinol and tocopherols are sensitive to oxidation which is accelerated by light, heat, alkali or metal ions).

Tocopherols were extracted to hexane, evaporated and dissolved in ethanol. The concentration of tocopherol was estimated by HPLC with a UV spectrophotometric detector using a Sepharon SGX C18 column (4 × 250 mm, 7 µm particle size). The flow rate of the mobile phase (methanol) was 1.0 ml/min; detection was performed at 292 nm.

Retinol was extracted to butanol and ethyl acetate. The concentration of retinol was estimated by HPLC with a UV spectrophotometric detector using a Sepharon SGX C18 column (4 × 250 mm, 7 µm particle size). The flow rate of the mobile phase (methanol) was 1.0 ml/min; detection was performed at 325 nm [35].

Autoantibodies

We studied IgG oxLDL and 4 APA IgG and IgM: anticardiolipin antibodies (ACA), antiphosphatidylserine antibodies (APSA), antiphosphatidylethanolamine antibodies (APE) and total antiphosphatidylcholine antibodies (APCA). Serum was freshly frozen, and all samples were measured together.

IgG oxLDL was determined by a commercial ELISA kit (Biomedica).

APA were identified using an ELISA based on techniques described by Harris [31] with some modifications. The solution of purified phospholipids (Sigma Chemicals, St. Louis, Mo., USA) was placed in each well of the microtiter plate, dried and blocked with 10% adult bovine serum in phosphate-buffered saline (PBS). Serum samples were placed in duplicated wells in a 1:50 dilution in 10% adult bovine serum in PBS, and, after incubation, wells were washed and horseradish-peroxidase-conjugated goat antihuman total Ig or IgM or IgG (Sevac, CR) diluted 1:5,000 in 10% PBS was added. After washing coloration developed by addition of *ortho*-phenylenediamine with H_2O_2.

Antiphospholipid assays other than anticardiolipin are not standardized and no commercial standard positive sera are available for calibration. In our assay, positive and negative control as a standard was placed into each microtiter plate. We determined the cutoff values using sera from 30 blood donors. Sera with absorbances higher than or equal to 3 SD above the mean for that phospholipid antibody isotype were considered as positive [85].

Trace Elements

Heparinized blood was taken into polyethylene tubes washed with nitric acid solution and deionized water. Both plasma and erythrocytes were stored in the refrigerator. Analysis was performed within 24 h after taking the blood samples. Measurements were carried out using the method of inductively coupled plasma mass spectrometry (Elan 6000, Perkin Elmer, Norwalk, Conn., USA). This technique operates with extremely low detection limits, a high dynamic range and high accuracy. Analysis of isotopes ^{77}Se, ^{66}Zn and ^{65}Cu was performed [54, 55].

Study Groups

The examined group consisted of 35 alcoholic patients (heavy drinkers, mean age 42 ± 9 years) with slightly affected liver function at the beginning of their abuse treatment in the Department for Alcohol and Addiction. The alcoholics had no signs or symptoms of autoimmune diseases or any other diseases. They were not taking any substituent drugs including the studied elements and vitamins. All patients gave their informed consent prior to entering the study.

The control group consisted of 60 healthy blood donors (mean age 40 ± 8 years). They were not taking any supplementation of the stud-

ied elements and vitamins. All subjects gave their informed consent prior to entering the study.

Statistical Significance

The statistical significance was evaluated using the analysis of variance one-way test (ANOVA) and test of alternative distribution.

Results

We did not find any significant differences between the alcoholics and the control group concerning alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, bilirubin, albumin and amylase. The alcoholics had significantly higher γ-glutamyl transferase levels. Lipid parameters were altered in the alcoholics as follows: increased total cholesterol, triglycerides and LDL cholesterol. On the other hand, HDL cholesterol was diminished. The values of LDL cholesterol and HDL cholesterol in alcoholic patients are borderline in the normal range of these parameters (table 1). The alcoholics had only slightly affected liver functions without any sonographic signs of either fibrosis or liver cirrhosis. Alcoholic hepatitis was not found in the anamnesis of these alcoholics. We found a mild alteration of all lipid parameters and we could consider a diet common in our country.

Chronic alcoholics – heavy drinkers without serious liver pathology – have elevated nitrites/nitrates as degradation products of NO (34.3 ± 2.6 vs. 22.7 ± 1.2 μmol/l, $p < 0.001$). Another parameter of oxidative stress – oxLDL – was also significantly increased (71.6 ± 4.1 vs. 44.2 ± 2.7 μmol/l, $p < 0.001$). Both these parameters could be connected with endothelial dysfunction. Increased NO production could be discussed as a cause of excitotoxicity and cytotoxicity. Overproduction of NO is supposed to be via iNOS based on chronic inflammation and affection of the immune system.

Table 2 shows the prevalence of APA in alcoholic patients. All studied APA are more abundant in alcoholic patients than in the control group, but the difference is not statistically significant. One patient was positive for all types of APA and 2 patients were positive for 3 APA types.

The IgG oxLDL level (406.4 ± 52.5 vs. 499.9 ± 52.5 mU/ml) was not changed in alcoholic patients. The 95% confidence interval of our control group was between 395 and 605 mU/ml. We did not find any correlations between oxLDL and IgG oxLDL.

There were no changes of the serum levels of retinol and tocopherol (table 3).

Table 1. Biochemical parameters of alcoholics and controls

Parameter	Alcoholics (n = 35)	Controls (n = 60)
ALT, μkat/l	0.61 ± 0.40	0.52 ± 0.32
AST, μkat/l	0.52 ± 0.20	0.43 ± 0.26
ALP, μkat/l	1.29 ± 0.41	1.34 ± 0.52
Amylase, μkat/l	1.41 ± 0.45	1.26 ± 0.53
GMT, μkat/l	0.74 ± 0.95**	0.35 ± 0.30
Bilirubin, μmol/l	7.7 ± 4.0	8.1 ± 3.1
Albumin, g/l	46.7 ± 2.7*	48.3 ± 3.1
Triglycerides, mmol/l	1.99 ± 1.04**	1.47 ± 0.84
Cholesterol, mmol/l	5.63 ± 1.23*	5.0 ± 1.09
LDL-cholesterol, mmol/l	3.50 ± 1.15**	2.90 ± 0.95
HDL-cholesterol, mmol/l	1.16 ± 0.27*	1.30 ± 0.31

All results expressed as means ± SD. * $p < 0.05$, ** $p < 0.01$, ANOVA test. ALT = Alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; GMT = γ-glutamyl transferase.

Table 2. Prevalence of APA in alcoholics

APA	Alcoholics (n = 35)		Controls (n = 60)	
	%	n	%	n
Total ACA	17.1	6	8.3	5
IgG ACA	11.4	4	6.7	4
IgM ACA	8.6	3	3.3	2
Total APE	14.3	5	6.7	4
Total APCA	14.3	5	8.3	5
Total APSA	20.0	7	8.3	5

p = n.s., test of alternative distribution.

Table 3. Concentration of retinol and α-tocopherol in alcoholics

Parameter	Controls (n = 14)	Alcoholics (n = 14)
Retinol, mg/l	0.90 ± 0.23	0.50 ± 0.16
α-Tocopherol, mg/l	8.94 ± 3.57	11.26 ± 2.73

All results expressed as means ± SD. p = n.s., ANOVA test.

Table 4. Concentration of trace elements (μg/l) in alcoholics and controls

Element	Alcoholics (n = 25)	Controls (n = 42)
Cu plasma	955 ± 34	1,020 ± 54
Cu blood	801 ± 25	853 ± 22
Se plasma	84 ± 4	78 ± 3
Se blood	127 ± 6	106 ± 3
Zn plasma	800 ± 27*	924 ± 32
Zn blood	5,694 ± 149	5,521 ± 113

All results expressed as means ± SD. * $p < 0.05$, ANOVA test.

Copper and selenium were unchanged in both compartments – plasma and the whole blood (corpuscular particles). We have found only a mild decrease in zinc in the plasma compartment (table 4).

Our results show that heavy alcoholics, even without any signs of hepatic lesion, have increased production of free radicals via various mechanisms (elevation of nitrites/nitrates, oxLDL). Production of autoantibodies against phospholipids is increased as well, probably due to increased destruction of biological membranes, e.g. through lipoperoxidation etc. On the other hand, the antioxidant defense in the lipophilic compartment remains unaffected and trace elements, which are connected with the antioxidant defense, are not significantly changed.

Discussion

Nowadays, it is generally accepted that the oxidative modification of LDL plays an important role in the pathogenesis and progression of atherosclerosis. This modification is thought to involve peroxidation of polyunsaturated fatty acids and oxidative modification of the apoprotein B in LDL particles and depends, at least in part, on the fatty acid composition of LDL and its antioxidant content (mainly vitamin E).

A moderate alcohol intake may prevent atherosclerosis, whereas heavy drinking has the opposite effect as it promotes oxidation of LDL. The protective effect is linked to the increase in HDL cholesterol. There is evidence for a U-shape relationship between alcohol and coronary heart disease and the threshold at which the right side of the U begins to increase could be as few as 2 or as many as 6 drinks per day. So, chronic heavy drinking can abolish the cardioprotective benefits attributed to alcohol, and, moreover, it results in an increased risk of hemorrhagic and ischemic stroke as well as acute myocardial infarction and coronary death [57, 68]. The cardioprotective effect of alcohol is beverage specific – it is attributed rather to wine than to beer. Consumption of red wine or its major polyphenols may reduce the susceptibility to oxidation of LDL and may slow the progression of atherosclerosis [69]. Polyenylphosphatidylcholine markedly attenuates the ethanol-induced increase in LDL oxidation and so opposes one of the effects of alcohol – promotion of atherosclerosis [57]. Tocopherol, which has a protective effect against oxidative modification, was decreased in LDL particles in alcoholics. In general, ethanol has prooxidant effects, which may be responsible for the enhanced oxidizability of LDL [68].

oxLDL is probably also immunogenic. Thus, antibodies against oxLDL are some kind of epiphenomenon of the lipid peroxidation process and are detectable in humans. Anti-oxLDL autoantibodies were present in the plasma of the majority of patients with coronary atherosclerosis and they can also be detected before the onset of clinically relevant signs of atherosclerotic disease in patients considered to be at risk [50].

A high titer of IgG oxLDL autoantibodies was described in systemic lupus erythematosus, severe atherosclerosis and in preeclampsia. The increase in IgG oxLDL probably accompanies the immunological process of lipid peroxidation [38].

Our 95% confidence interval for IgG oxLDL is similar to the Vienna study describing the titer of IgG oxLDL in healthy working people (12,000). The peak of distribution was at 300 mU/ml, and two thirds of all samples were found within a range of 150–800 mU/ml. Young people have higher titers than elderly ones; however, a protective function of IgG oxLDL should be considered [38]. Production of autoantibodies depends on the 'quality' of the immune system.

LDL particles of alcoholic patients without serious liver disease contain oxidatively modified epitopes and acetaldehyde adducts in LDL. LDL of alcoholic patients has a lower vitamin E content, is chemically modified in vivo and exhibits altered biological functions. These changes in heavy alcoholics may make LDL more atherogenic and thereby may diminish the antiatherosclerotic effects of moderate alcohol consumption [45].

Wehr et al. [81] described higher IgG reactivity against both native and ethylated LDL in individuals with alcoholic liver disease than in alcoholics without liver injury. High IgG reactivity in alcoholics with liver disease was

observed against malondialdehyde-modified, methylated, acetylated and carbamylated LDL. A highly selective anti-ethylated-LDL IgG reactivity was observed in 11% of control subjects.

Vaarala et al. [78] reported cross-reactivity between APA and antibodies against oxLDL. With regard to the occurrence of IgG oxLDL also in healthy individuals and a very broad range of reference values (395–605 mU/ml), Zima et al. [87] do not consider this examination as a parameter suitable for the determination of the possible risk of atherosclerosis and oxidative stress. Paiker et al. [64] have the same opinion concerning patients with familiar hypercholesterolemia; in this case Ig oxLDL titers cannot be used as a predictive marker of the presence or severity of atherosclerosis either [64]. The decrease in Ig oxLDL titer was very slow and lasted for many months; that is why this parameter cannot be considered as suitable for the description of rapid changes during oxidative stress of the organism.

The cell membrane consists of a phospholipid bilayer and proteins. Phospholipids can be modified by oxidative stress and free radicals. APA have emerged as the subject of intense clinical and scientific interest in a wide spectrum of diseases over the last decade and the association with alcoholic intoxication deserves further investigations [74].

Alcoholic liver injury has been reported to be directed preferentially against the proteins of the cell membrane, sparing the phospholipids. However, APA against certain cell membrane phospholipids are known to be associated with a variety of diseases [17]. The prevalence of ACA was 57% in patients with alcoholic liver cirrhosis [27]; APA levels are also high in patients with alcoholic hepatitis without cirrhosis [9].

The antibody prevalence was 15% in alcoholic patients with normal liver function, 31% in alcoholic patients with abnormal liver function, 81% in patients with alcoholic hepatitis or cirrhosis and 0% in nonalcoholic controls. Twenty of 41 patients with alcoholic hepatitis or cirrhosis had antibodies against several cell membrane phospholipids (i.e. APE, APSA). Both IgA ($p < 0.01$) and IgM ($p < 0.008$) APE correlated significantly with the disease severity. APCA was not found, which is contrary to our positive findings [17]. APA seem to be frequently associated with chronic liver disease of various causes [10]. The prevalence of autoantibodies in patients with liver disease is higher than in patients with systemic diseases including systemic lupus erythematosus, where the prevalence of APA is 39% [3].

High prevalence of other autoantibodies – antinuclear antibodies (22%) and either anti-dsDNA (60%) or anti-ssDNA (60%) antibodies – was described in alcoholics [39]. These results suggest that autoimmune mechanisms may play an important role in the pathogenesis of alcoholic liver diseases in at least some patients.

In patients with no autoimmune liver diseases, ACA production is an epiphenomenon of the liver damage and is not associated with thrombotic complications [51].

APA are traditionally associated with arterial and venous thrombosis in patients with primary or secondary antiphospholipid syndrome. Recent studies, especially in patients with myocardial infarction, extend the concept of APA and suggest that they play a role in atherosclerosis as well. Antibodies against oxLDL may not interfere directly with blood coagulation but seem to have importance in the inflammation of the vessel wall in atherosclerosis and in vasculitis. These antibodies may contribute to the formation of atherosclerotic thrombosis by changing the balance of hemostasis toward a hypercoagulative state.

APA in alcoholic patients seem to reflect membrane lesions, impairment of immunological reactivity, liver disease progression and they correlate significantly with the disease severity [17, 33, 85].

According to the repeatedly demonstrated positive correlation of Ig oxLDL with APA and other antibodies it is possible to use Ig oxLDL as a marker for the description of the total production of autoantibodies in various diseases. The changes and correlations of Ig oxLDL, anti-β2-glycoprotein I IgG and APA support the immunological link between thrombotic and atherosclerotic processes in the human body.

Formation of NO in neuronal endings and endothelium belongs to the determinants of the blood pressure, which depends on constriction and dilatation of blood vessels. NO is an important relaxing factor of vascular smooth muscles; it causes vasodilatation and so influences blood flow and pressure. Physiological vasodilatation in healthy vessels is regulated by endothelial NOS (eNOS) and nNOS. In alcoholics, increased formation of NO can also be caused by chronic stimulation of the immune system with subsequent induction of iNOS. Increased production of NO and increased production of superoxide with vasoconstrictive effects can take part in cardiovascular instability and the evolution of hypertension in alcoholics. Moreover, atherosclerosis reduces NO-mediated relaxation of vessels. oxLDL decreases transcription of mRNA for eNOS in endothelial cultures. oxLDL binds to NO and inactivates it. Formation of oxidative products of NO, e.g. NO^{2-} or NO^{3-}, is increased in

atherosclerotic vessels and nitration of tyrosyl residues is increased as well. Decreased vasodilatation of endothelium depends either on increased NO formation followed by its inactivation or decreased formation of NO, which represents an important functional component in atherosclerotic vessel lesions [4, 6, 86]. Persson and Gustafsson [67] indicate that consumption of ethanol may influence the formation of endogenous NO both in the central nervous system and in other tissues.

The immune system is another important source of NO. Ethanol-induced free radical generation can also affect the synthesis of some cytokines (i.e. IL-2 and TNF-α), which stimulate NO synthesis. Monocytes produce a basal amount of nitrite, which could be stimulated more than 6-fold using endotoxin. This effect was blocked by the addition of an NO synthesis inhibitor, *L-n*-monomethyl-arginine. A striking difference was observed in monocytes obtained from alcoholics with and without evidence of alcoholic hepatitis. Whereas the latter behaved in a similar manner to the controls, the former had markedly increased basal levels. In the hepatitis group, there was also substantial inhibition of production by *L-n*-monomethyl-arginine. These results indicate that NO derived from monocytes may play a role in the pathogenesis of alcoholic liver disease, especially alcoholic hepatitis [34].

Cirrhotic patients showed an increase in cardiac output and a decrease in peripheral vascular resistance. These patients had higher levels of plasma endotoxin than those observed in the control group. N-ω-nitro-*L*-arginine-methyl-ester-inhibitable nitrite production from mononuclear lymphocyte cells was higher in patients than in the control group; the highest levels were found in nonalcoholic cirrhotic patients and the lowest levels in patients with noncirrhotic alcoholic liver disease. Immunocytochemistry studies revealed a positive immunoreactivity for the inducible isoform of NOS in lympho-mononuclear cells that was more evident in nonalcoholic than in alcoholic cirrhotic patients. iNOS mRNA expression was observed only in lymphomononuclear cells from nonalcoholic cirrhotic patients. These patients show a correlation between NO synthesis, endotoxin levels and hemodynamic parameters. These findings indicate that lymphomononuclear cell stimulation may play a role in elevated NO production in hepatic cirrhosis. Thus, this increased NO synthesis could be implicated in the pathogenesis of the hemodynamic disturbances frequently found in cirrhotic patients. This increase seems to be induced, at least in part, by activation of an inducible isoform of NOS [73].

In the gastrointestinal tract, NO takes part in muscular relaxation and motility. NO acts as 'nitrigenic' neurotransmitter – non-adrenergic non-cholinergic transmission in the myenteric plexus of the stomach and intestines. It takes part in the dilatation of the stomach and peristalsis. Alcohol abuse impairs the function of the intestinal barrier, which might enhance the translocation of bacterial toxins, thereby contributing to inflammatory processes and chronic endotoxemia (lipopolysaccharides) [65]. Modified LDL is a chemotactic factor for other monocytes and can up-regulate the expression of genes, which may help expand the inflammatory response in the endothelial lesion [72].

Chronic ethanol administration induces oxidative stress in the central nervous system, mainly increased lipid peroxidation of the cell membrane. This leads to increased membrane fluidity, disturbances of calcium homeostasis (increase in free intracellular calcium) and finally cell death [30, 59].

Treatment with an NOS inhibitor enhances the acute central depressant or anesthetic effects of alcohol and decreases some stimulatory effects of alcohol withdrawal after chronic alcohol treatment. Controversially, treatment with an NO donor inhibits the anesthetic effect of alcohol, blocks the effect of the NOS inhibitor on alcohol anesthesia and enhances the severity of some alcohol withdrawal signs. These results indicate that changes in NO synthesis mediate some aspects of alcohol intoxication and withdrawal and that the NO system represents an important therapeutic target for the development of agents to treat alcoholism and alcohol intoxication [1].

In an experimental study, Zima et al. [89] investigated the changes in neuronal NADPH diaphorase activity after prolonged exposure of rats to moderate doses of ethanol. The density of NADPH-diaphorase-positive neurons and positivity of the neuropil was clearly reduced in the cerebellar cortex and in the superficial layers of the superior colliculus of ethanol-treated rats. This could alter synaptic processes in the highly organized structures involving oculomotor and somatic motor coordination and is likely to contribute to the motor disturbances, which are associated with alcohol abuse [89].

NO may play a role in the development of tolerance to ethanol and this role may be similar to the role of NO in memory and learning, involving facilitation of transmission in certain NMDA synapses [36]. Chronic alcohol exposure may lead to excitotoxicity partially due to increased levels of NO. Excessive NO has been linked to cytotoxicity in neurons, glia and myelin [37]. On the other hand, Neiman and Benthin [58] did not find any increase

in NO metabolites in the cerebrospinal fluid of alcoholics.

Taken together, high NO concentration in alcoholic patients could be discussed for its excitotoxicity and also seems to be linked to cytotoxicity in neurons, glia and myelin. Formation of NO has been linked to increased preference for and tolerance to alcohol in recent studies. Production of NO could also play a role in the pathogenesis of liver disease. Increased NO biosynthesis may contribute to platelet and endothelial dysfunction.

Different results of vitamin A and E plasma levels were published for alcoholics. Some authors refer no significant changes of plasma retinol [2, 41, 56] and α-tocopherol [2, 56, 70]. Tanabe et al. [76] found higher retinol levels in drinkers than in nondrinkers. Both retinol and α-tocopherol levels were significantly higher in the group with smoking and drinking habits than in the group without them. The retinol level was positively dependent on the daily consumption of both cigarettes and alcohol, whereas tocopherol was dependent on the consumption of alcohol [77].

A significant decrease in concentration of α-tocopherol was found in all patients with cirrhosis or alcoholic skeletal muscle myopathy [80]. Heavy consumption of ethanol reduced the plasma level of α-tocopherol as well [12]. The serum concentration of α-tocopherol was 30% lower in the alcoholics [13]; in another study it was reduced by 37% as compared to controls [11]. Such results support a role of free-radical-mediated damage, which can develop diseases frequently observed in alcoholics [11, 80]. Reduced serum levels of α-tocopherol in alcoholics may be normalized by vitamin E supplementation [13]. With the only exception of supplemental vitamin E, and possibly vitamin C, being able to significantly lower lipid oxidative damage in both smokers and nonsmokers, the current evidence is insufficient to conclude that antioxidant vitamin supplementation materially reduces oxidative damage in humans [52].

Our study group of alcoholics had no signs of malnutrition and the serum concentrations of both lipophilic vitamins were unchanged either. However, this finding does not mean that there cannot be any changes including a decrease in the tissues or particles, e.g. LDL particles.

Methodology represents the major limitation of valid studies on trace element levels in biological material. The precise metal determination requires a very high level of skill, clean sampling, suitable methods of determination, internal and external quality control and a sufficient number of samples. Versieck and Cornelis [79] showed that the controversy concerning trace element concentration is mainly due to inadequate sampling and sample handling, air contamination, storage (leaking or absorption to the container), preservative reagents or defective analysis. Some degree of contamination was found in almost all steps [14]. Sampling and sample handling are the 'Achilles heel' of trace element research.

Many studies concerning trace element concentrations in serum or plasma have been published. Only some studies dealt with the trace element concentrations in erythrocytes or in the whole blood. These two compartments are very closely related and their balance is strongly regulated.

Health-threatening deficiencies in Zn and Cu are fairly rare. Some trace element deficiencies (Se, Cr, Mo) can initiate very serious complications and will require special caution. Other deficiencies (Cu, Zn) result in more slowly evolving clinical pictures, with lesser life-threatening potential, resulting in infections and prolonged wound healing [7]. Some authors reported different results dealing with trace elements. Plasma concentration of α-tocopherol, ascorbic acid and selenium were lower in alcoholics than in men who drank only low amounts of alcohol. α-Tocopherol and selenium concentrations remained unchanged after the withdrawal period [80].

In another study, the alcoholics group showed a significant decrease in plasma concentrations of β-carotene, zinc and selenium when compared to control subjects. When the patients were subdivided according to their liver histology, β-carotene showed a progressive decrease in plasma with increasing liver damage, whereas α-tocopherol was depleted only in patients with liver cirrhosis. Selenium and α-tocopherol levels were decreased in all patients with skeletal muscle myopathy, whereas patients with normal muscle biopsies showed adequate antioxidant status [40]. Heavy consumption of ethanol reduced the plasma levels of α-tocopherol and selenium [12].

Chronic alcoholism is associated with hypercortisolemia and a lower zinc serum level. It is hypothesized that low brain Zn, noted in chronic alcoholics, enhances NMDA excitotoxicity and the susceptibility to ethanol withdrawal seizures. Also, Zn deficiency can produce neuronal damage through increased free radical formation. Clinically, Zn replacement therapy may be a rational approach to the treatment of alcohol withdrawal seizures and alcohol-related brain dysfunction [53].

The serum zinc concentration reached normal values in alcoholics with or without liver cirrhosis by daily supplementation during 10 days to 2 months [84]. The serum zinc level was decreased in patients with chronic liver injury, and zinc deficiency seems to be related to hepatic

injury and not to alcohol intake [61]. Modest zinc deficits caused lymphopenia and reduced immune capacity among affected humans [26].

Decreased concentrations of serum zinc only described by us can be connected to the disturbed immune system of chronic alcoholics and maybe with cerebral dysfunction, too, as mentioned by Menzano and Carlen [53]. Zinc decrease is obvious yet in patients without liver disease.

Pro-oxidant effects of ethanol lead to an increased modification of LDL by oxidation, and this mechanism is one of the important mechanisms increasing the cardiovascular risk in chronic heavy alcoholics without liver disease. These changes in heavy alcoholics may diminish the antiatherosclerotic effects of moderate alcohol consumption. In patients with no autoimmune liver diseases, ACA production is an epiphenomenon of the liver damage and is not associated with thrombotic complications. Antibodies against oxLDL may not interfere directly with blood coagulation but seem to be important in the inflammation of the vessel wall in atherosclerosis and in vasculitis. Autoimmune mechanisms may play an important role in the pathogenesis of alcoholic liver diseases, and APA seem to reflect membrane lesions and impairment of the immunological reactivity. The changes and correlations of Ig oxLDL, anti-β2-glycoprotein I IgG and APA support the immunological link between thrombotic and atherosclerotic processes in the human body.

Increased NO synthesis can take part in the cardiovascular instability and evolution of hypertension in alcoholics and it may contribute to platelet and endothelial dysfunctions. Chronic inflammatory processes frequent in heavy alcoholics can stimulate production of NO via iNOS. Production of NO could also play a role in the pathogenesis of liver disease. High NO concentration in alcoholic patients could be discussed for its excitotoxicity and also seems to be linked to cytotoxicity to components of the nervous system.

Our study group of alcoholics had no signs of malnutrition and the serum concentrations of lipophilic vitamins, selenium and copper were unchanged too. A mild decrease in serum zinc levels can be discussed as a cause of affection of the immune system. However, this finding does not mean that there cannot be any changes including a decrease in the tissues or particles, e.g. LDL particles.

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Mammalian Alcohol Dehydrogenase – Functional and Structural Implications

Jan-Olov Höög Jesper J. Hedberg Patrik Strömberg Stefan Svensson

Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden

Key Words

Alcohol dehydrogenase · Enzyme kinetics · Protein expression

Abstract

Mammalian alcohol dehydrogenase (ADH) constitutes a complex system with different forms and extensive multiplicity (ADH1–ADH6) that catalyze the oxidation and reduction of a wide variety of alcohols and aldehydes. The ADH1 enzymes, the classical liver forms, are involved in several metabolic pathways beside the oxidation of ethanol, e.g. norepinephrine, dopamine, serotonin and bile acid metabolism. This class is also able to further oxidize aldehydes into the corresponding carboxylic acids, i.e. dismutation. ADH2, can be divided into two subgroups, one group consisting of the human enzyme together with a rabbit form and another consisting of the rodent forms. The rodent enzymes almost lack ethanol-oxidizing capacity in contrast to the human form, indicating that rodents are poor model systems for human ethanol metabolism. ADH3 (identical to glutathione-dependent formaldehyde dehydrogenase) is clearly the ancestral ADH form and S-hydroxymethylglutathione is the main physiological substrate, but the enzyme can still oxidize ethanol at high concentrations. ADH4 is solely extrahepatically expressed and is probably involved in first pass metabolism of ethanol beside its role in retinol metabolism. The higher classes, ADH5 and ADH6, have been poorly investigated and their substrate repertoire is unknown. The entire ADH system can be seen as a general detoxifying system for alcohols and aldehydes without generating toxic radicals in contrast to the cytochrome P450 system.

The mammalian alcohol dehydrogenase (ADH) system is divided into six classes, ADH1–ADH6, whereof five have been identified in man [4, 9]. These dimeric enzymes belong to the protein superfamily of medium-chain dehydrogenases/reductases [13] and are further divided into subgroups (ADH2), isoenzymes (ADH1 and ADH2) and allelic forms (ADH1). All ADH classes catalyze the reversible oxidation of alcohols to aldehydes/ketones using NAD^+/NADH as electron acceptor and donor, respectively, and have a broad but only partially overlapping substrate repertoire [5]. Apart from the formaldehyde scavenging of ADH3, functional roles of the other ADHs are not fully established. However, based on their catalytic activities they could play roles in the metabolism of steroids, retinoids, biogenic amines, lipid peroxidation products, ω-hydroxy fatty acids as well as xenobiotic alcohols and aldehydes.

The structure determinations for ADH1–ADH4 have provided a structural basis for the understanding of their different properties. All ADH subunits consist of one catalytic and one coenzyme-binding domain, and both

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Jan-Olov Höög
Department of Medical Biochemistry and Biophysics
Karolinska Institutet
S–17177 Stockholm (Sweden)
Tel. +46 8 728 7740, Fax +46 8 338 453, E-Mail jan-olov.hoog@mbb.ki.se

coenzyme and substrate bind in a cleft between the two domains [6, 15]. Low positional identities between the classes are especially observed in three segments, constituting parts of the substrate-binding pocket and subunit interaction areas [15]. This results in large differences in substrate pocket topology although the overall positional identity is high (~65%) and acceptance of residue exchanges at the substrate-binding site is likely to reflect the functional divergence of the classes.

ADH3, identical to glutathione-dependent formaldehyde dehydrogenase, is clearly the ancestral form of all mammalian ADHs and has been traced in all living species investigated [9, 10]. Further, this is the only ADH that has been ascribed a physiological substrate, S-hydroxymethylglutathione (HMGSH) and is identified as a functional formaldehyde scavenger [7]. ADH1, the major enzyme in the metabolism of ingested ethanol is the only human class where more than one isoenzymic form exists (α-, β- and γ-subunits, ADH1A, ADH1B and ADH1C in the new nomenclature system [4]). Mainly, rodents have been used to study the contributions of various ADHs in the metabolism of ethanol. The rodents do not show a setup of ADH1 isoenzymes, but the set of different classes is present (ADH1–ADH4 and ADH6). The ADH1 enzymes are involved in several metabolic pathways beside the oxidation of ethanol, e.g. norepinephrine, dopamine, serotonin and as recently shown bile acid metabolism [5, 12, 17]. This class is able to further oxidize aldehydes into the corresponding carboxylic acids, i.e. dismutation. The γγ-isoenzyme (ADH1C) shows the highest capacity for dismutation among the human ADHs and it seems to be a common theme that this form is involved in several specific pathways beside its high capacity for ethanol oxidation [12, 16].

Human ADH2 was isolated as a liver enzyme with a high K_m for ethanol [11] that was reinterpreted for the recombinantly isolated enzyme [18]. Reductions of the intermediate aldehydes in serotonin and norepinephrine catabolism are efficiently catalyzed [17] and it has further been shown that the human ADH2 is fairly efficient in retinoid metabolism [3]. For all these reactions, ADH1 and the extrahepatically expressed ADH4 have overlapping activities, and the ADH4 enzyme has been suggested to be the main bioactivator of retinoids [3]. ADH2 can be divided into two subgroups, one group consisting of the human enzyme together with a rabbit form and another consisting of the rodent forms. The ADH2 forms are found almost exclusively in the liver where the rodent forms almost lack ethanol dehydrogenase activity [18].

The higher classes, ADH5 and ADH6, have been poorly investigated, and nothing is known about their substrate repertoire. They are however liver expressed, and at least the human form, ADH5, shows an alternative splicing pattern.

In a further attempt to understand the interactions between different ADHs (classes and isoenzymes) as well as between different substrates, we here focus on the complex interplay between the participants in oxidoreductive cell defense.

Materials and Methods

Enzyme Preparations

Enzymes were recombinantly expressed in *Escherichia coli*, mainly using pET expression vectors for subcloning of mammalian ADH cDNAs. The recombinant proteins were purified to homogeneity essentially in a three-step procedure including ion exchange, affinity and gel permeation chromatography as described earlier [16, 18]. Protein concentrations were determined with the Bio-Rad protein assay with bovine serum albumin as standard and enzymatic activity was determined spectrophotometrically at pH 10 in glycine-NaOH buffer [16].

Kinetic Analysis

Ethanol and all-trans retinol oxidation were determined at pH 7.5 with a Hitachi U-3000 spectrophotometer, by monitoring the formation of NADH at 340 nm for ethanol oxidation and by the formation of retinal at 400 nm for retinol oxidation. Oxidation of HMGSH, spontaneously formed by formaldehyde and glutathione, was determined at pH 8.0 by monitoring the NADH formation. Reactions with serotonin metabolites were quenched by addition of perchloric acid, and metabolites were separated by HPLC and detected electrochemically [17].

Cloning and Expression

Human ADH5 and rat ADH6 were cloned with conventional techniques from liver cDNA libraries and the PCR technique with oligonucleotides designed after a published cDNA sequence [19]. Genomic DNA was prepared from blood samples and the ADH5 3′-end region was amplified with the PCR technique for sequence analysis. For *in vitro* translation the cDNA coding for the ADH6 was subcloned into transcription vector pTRIkan. This cDNA was further cloned into pEGFP adjacent to the coding sequence for green fluorescence protein for transfection into COS cells. The harvested cells were fixed and analyzed for protein expression.

Data Analysis

To fit lines to kinetic data points and to calculate kinetic parameters, a weighted non-linear-regression analysis program was used (Fig.P for Windows). DNA and deduced protein sequences were analyzed using the University of Wisconsin Genetics Computer Group Program and compared with EMBL data banks. For phylogenetic calculations, the programs Clustal W and Tree View were used.

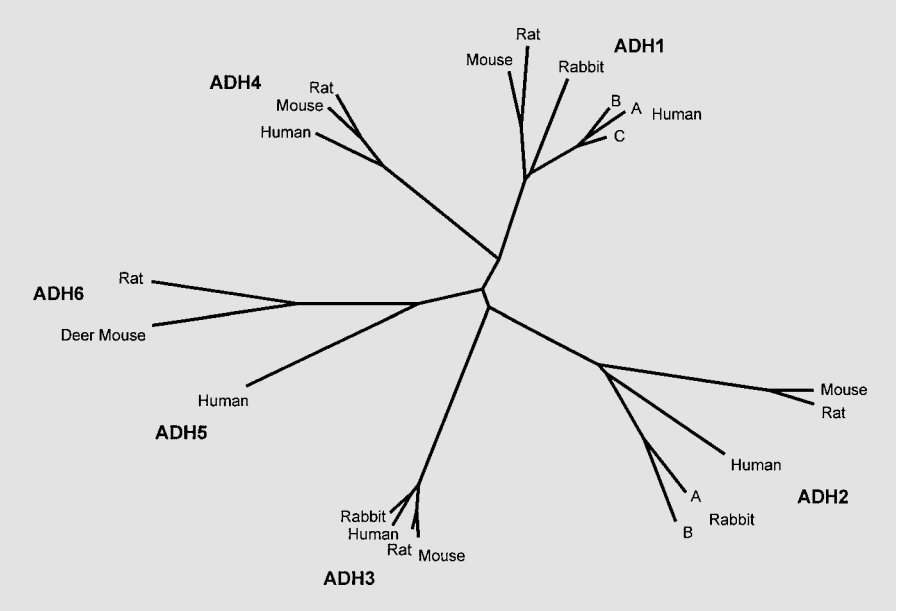


Fig. 1. Unrooted phylogenetic tree, relating the human, mouse, rabbit and rat ADH1–ADH6 enzymes. Isoenzymic forms are denoted A, B and C. Sequence data were from data banks, and line lengths are proportional to separation distances.

Results and Discussion

Mammalian ADH forms a setup of enzymes, isoenzymes and allelic variants with ADH3 as the ancestral form (fig. 1). All the different ADHs participate to some extent in the cell defense towards exogenous alcohols and aldehydes. ADH3 is the only enzyme, so far, within the system that has been ascribed a physiological substrate, HMGSH. All enzymes are capable of metabolizing ingested ethanol where the highly expressed ADH1 is established as the main ethanol-metabolizing enzyme. Several of the ADHs participate in specific metabolic pathways and metabolism of ethanol will therefore interact with these specific pathways (fig. 2). For example, ethanol oxidation affects human serotonin, bile acid and retinoid metabolism by increasing the relative formation of alcohol products, while decreasing the formation of carboxylic acid products [3, 12, 17]. These interactions are often underestimated and the contribution to the total ethanol metabolism of the higher classes is poorly understood. However, a direct effect of ethanol metabolism is a change in redox state within the cell, where the NADH/NAD^+ ratio has been shown to increase one order of magnitude in rat hepatocytes [2].

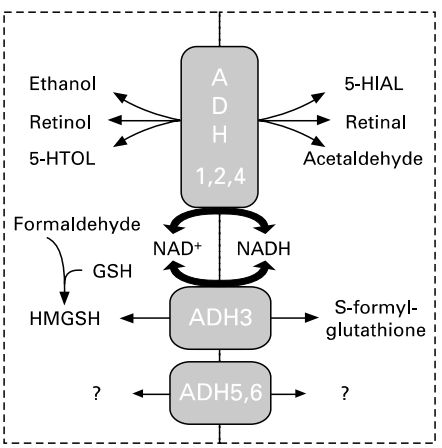


Fig. 2. Interaction of metabolic pathways where ADH is involved. 5-HTOL = 5-Hydroxytryptophol; 5-HIAL = 5-hydroxyindole-3-acetaldehyde; GSH = glutathione; HMGSH = S-hydroxymethylglutathione.

Ethanol Metabolism

The main function associated with mammalian ADH, ethanol oxidation, has been thoroughly described in the literature [5 and references therein]. All ADHs can participate in this metabolism; however, ADH3 is not able to saturate with ethanol and the rodent ADH2 enzymes show only traces of ethanol dehydrogenase activity. Furthermore, aldehyde oxidation catalyzed by ADH1 can be as efficient as the classical alcohol oxidation [16]. By sequential oxidation and reduction of an aldehyde the dismutation circumvents the slow step of coenzyme dissociation. Human ADH2 has a higher K_m value for ethanol

Table 1. Kinetic constants for human and rat ADHs

	EtOH		5-HTOL		All-trans-retinol		HMGSH	
	K_m m*M*	k_{cat}/K_m min^{-1} mM^{-1}	K_m m*M*	k_{cat}/K_m min^{-1} mM^{-1}	K_m m*M*	k_{cat}/K_m min^{-1} mM^{-1}	K_m m*M*	k_{cat}/K_m min^{-1} mM^{-1}
ADH1								
Human (AA)	4.2	6.7			0.056	92	–	NA
Human (BB)	0.05	29			0.045	20	–	NA
Human (CC)	0.52	100	0.22	350	0.29	19	–	NA
Rat	1.4	28			0.047	250	–	NA
ADH2								
Human	8	1.4	0.053	180	0.014	650	–	NA
Rat	NS	0.0006			0.0015	85	–	NA
ADH3								
Human	NS	0.045	–	NA	–	NA	0.004	50,000
Rat	ND	ND					0.00092	235,000
ADH4								
Human	28	65	1.3	80	0.031	190	–	NA
Rat	5,000	24					–	NA

All values are determined at pH 7.5, 25°C, except the values for HMGSH that are determined at pH 8.0.
Human ADH1: AA = αα-isoenzyme; BB = ββ-isoenzyme; CC = γγ-isoenzyme. Neither human ADH5 nor rat ADH6 have been isolated as active proteins. Therefore they are excluded from the table. Values are from the authors' laboratory or from Duester [3]. NA = No activity; NS = not possible to saturate.

than the ADH1 isoforms and therefore makes a significant contribution to ethanol metabolism only at high ethanol concentration [11]. Results from our determinations, however, show a fairly low K_m for ethanol, 8.0 m*M* at pH 7.5 (table 1) [18], as compared to literature values that are in the range from 34 to 120 m*M* [5, 11]. Not only acetate and 5-hydroxyindole acetate, but also chloride ions competitively inhibit ADH2 and this sensitivity for anions might explain the K_m anomaly. The contribution of the higher classes, ADH5 and ADH6, is unknown since they have not been isolated at the protein level. Both show amino acid residues at the substrate-binding pocket that suggests low ethanol dehydrogenase activity. In a large European study, two allelic ADH1 forms, ADH1B2 and ADH1C1, have been suggested to protect from alcoholism [1]. Notably, these two alleles are associated and are the most active ADH forms in ethanol metabolism.

Serotonin Metabolism

Ethanol intake significantly changes the cytosolic redox potential by increasing the NADH/NAD^+ ratio. This makes reductive ADH metabolism more favorable, which may explain the increased turnover of 5-hydroxyindole-3-acetaldehyde (5-HIAL) to 5-hydroxytryptophol (5-HTOL), observed after ethanol intake [17]. We have shown that 5-HIAL reduction is efficiently catalyzed by ADH1 (γγ-isoenzyme/ADH1C) and that 5-HIAL reduction is roughly 40-fold more efficient than 5-HTOL oxidation. The higher efficiency is due to higher k_{cat} for aldehyde reduction than for alcohol oxidation as observed for most alcohol/aldehyde pairs [17, 18]. In addition, a comparison of specificity constants shows that ethanol would at high concentrations competitively inhibit oxidation of 5-HTOL. ADH2 can partly contribute to this serotonin metabolism, where ADH3 lacks and ADH4 shows traces of this capability (table 1).

Retinol Metabolism

ADH is probably one of the main enzymes in the conversion of different isomers of retinol to the corresponding retinals. The extrahepatically distributed ADH4 has the highest specific activity for retinols (table 1). However, ADH1 and ADH2, both found in large amounts in the liver, can convert retinols into retinals. In rodents, the ADH2 cannot use all-trans-retinol as a substrate but 9-cis-retinol seems to be a substrate for rat ADH2 [14]. ADH4 involvement in cell differentiation has been established in several investigations, which is verified of the

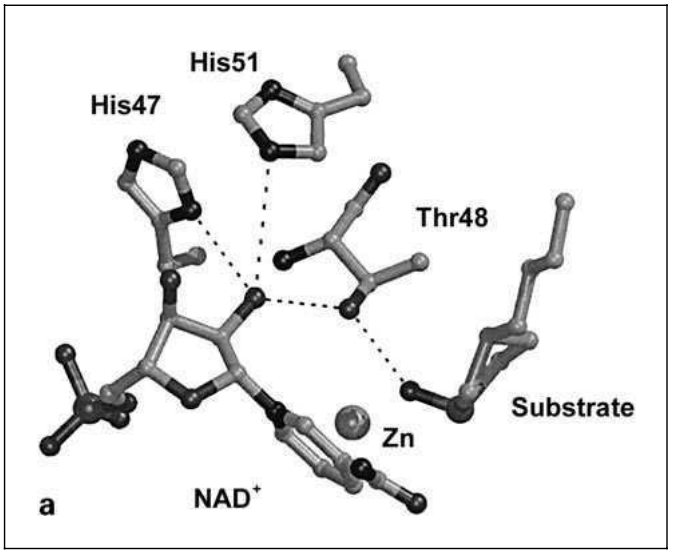
colocalization of ADH4 expression and conversion of retinols into retinoic acid [3].

Formaldehyde Metabolism

ADH3 is the only ADH that can participate in cell detoxification of formaldehydes. Formaldehyde spontaneously forms HMGSH with glutathione that is converted into S-formylglutathione by ADH3, an activity that can be traced in all living species that contain glutathione [10]. The mechanism for this reaction is identical to any alcohol oxidation by ADH and produces NADH, which thereby changes the redox state in the cell. Furthermore, recently it has been shown that ADH3 is able to reduce GSNO, a conjugation product between glutathione and NO, but the physiological relevance of this reaction is not known [8].

The above activities show the interference between different ADH substrates that competitively inhibit each other. Furthermore, ethanol oxidation will heavily change the NADH/NAD^+ ratio that directly disturbs other metabolic pathways (fig. 2). These examples of interactions can be one molecular explanation to fetal alcohol syndrome.

The higher classes, ADH5 and ADH6, showed unexpected gene arrangements. Notably, ADH5 was reported to be truncated at the C-terminus due to a deletion of the last exon [19]. We have shown, however, that the last exon is present in the ADH5 gene, that yields a shorter 'truncated' and a longer 'full-length' message, which can be explained by a complex transcription pattern. Rat ADH6, only 65% identical to ADH5, was isolated as a cDNA with an open reading frame that codes for 389 amino acid residues which yields an elongated N-terminus as compared to the other mammalian ADHs. Both these ADHs show residues at the active-site pocket that deviate from other mammalian ADHs. The coenzyme interacting residue at position 47 is Gly, in contrast to Arg and His in most mammalian forms. Thr at position 48 is identical with ADHs without steroid dehydrogenase activity; Lys51 corresponds to His in ADH1, which is proposed to interact in a charge relay system (fig. 3) [5]. Concomitantly, this amino acid residue setup suggests a low alcohol dehydrogenase activity. In vitro translation of the ADH6 mRNA yielded a protein product slightly larger than the product of ADH1 mRNA (43 kD as compared to 40 kD) and expression of ADH6 in COS cells, as judged from a fluorophore of ADH6 fused to green fluorescence protein, suggests a correctly folded protein. However, so far no active protein has been isolated when conventional substrates have been used.



Enzyme/Position	47	51
ADH1	Arg	His
Hu ADH2	His	Thr
Ra ADH2	Pro	Asn
ADH3	His	Lys
Hu ADH4	Arg	His
Ra ADH4	Gly	His
ADH5/ADH6	Gly	Lys

b

Fig. 3. Active site of mammalian ADH with His47 and His51. **a** A histidine at either position 47 or 51 has been shown to be a prerequisite for ethanol dehydrogenase activity to maintain the charge relay system. The active site zinc, NAD^+ and substrate are shown together with residues involved in the hydrogen-bonding network. **b** Amino acid residues at positions 47 and 51 in different mammalian ADHs.

For ADH2, the variability around the substrate-binding pocket is profound, with residue insertions and deletions as compared to the other classes of ADH. Several species variants of ADH2 have been described, and the rodent ones form a functionally distinct subgroup with interesting catalytic properties (fig. 1). Rodent ADH2s are by several orders of magnitude less efficient in alcohol oxidation as compared to other ADHs and are not able to saturate with ethanol (table 1). By replacing Pro47 with His, the ADH activity can be restored (fig. 3). In addition to alcohol/aldehyde oxidation/reduction, the ADH2 en-

zymes are capable of catalyzing the reduction of benzoquinones [18]. Notably, this activity is not affected by the Pro47His mutation. The structures provide new information on the generation of functional diversity between the ADH classes (fig. 1, 2) and give insights into the function of this particular ADH class. In a comparative study of human and rodent forms, the rodent ADH2 enzymes seem to be involved in reductive rather than in the oxidative catalysis.

The entire ADH system works as a general detoxification system that protects the cell from toxic alcohols and aldehydes. In many respects, the system can be compared to other detoxifying systems, e.g. the cytochrome P450 system, but without generation of cell-toxic radicals. However, the overall mechanism for the entire ADH system has as yet to be established.

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P_{300} Event-Related Potential Amplitude as an Endophenotype of Alcoholism –Evidence from the Collaborative Study on the Genetics of Alcoholism

Victor Hesselbrock Henri Begleiter Bernice Porjesz Sean O'Connor Lance Bauer

Department of Psychiatry, University of Connecticut School of Medicine, Farmington, Conn., USA

Key Words

Alcohol dependence · Event-related potentials · Endophenotype · Genetics · Electrophysiology

Abstract

There is substantial information supporting the role of genetic factors in the susceptibility for alcohol dependence. However, the identification of specific genes that contribute to this predisposition has proven elusive, although several theoretically relevant candidates, e.g. DRD2 or 5-HT_{1B}, have been considered. The difficulty in identifying specific genes may be related to the clinical heterogeneity of the disorder resulting in a poorly defined phenotype for genetic analysis. An alternative approach to the use of a diagnostic phenotype for identifying alcoholism susceptibility genes may lie in the examination of the neurobiological correlates of the disorder, the so-called endophenotypes. One possible endophenotype of alcohol dependence may be related to the P_{300} waveform of the event-related brain potential (ERP). Using data obtained from the Collaborative Study on the Genetics of Alcoholism (COGA), a multi-site family-based study, the utility of P_{300} amplitude as an endophentype was examined. Differences in P_{300} amplitude were found between alcoholics and nonalcoholics, between unaffected relatives of alcoholics and relatives of controls, as well as between unaffected offspring of alcoholic fathers and offspring of controls. A genetic analysis indicated that attributes of the P_3 ERP waveform are heritable, and a quantitative trait locus analysis found linkage to several chromosomal regions. These data provide significant support for P_{300} as an endophenotype for alcohol dependence.

An increasing number of studies have pointed to the importance of genetic factors in the vulnerability for developing alcohol problems, including alcohol dependence [28, 38]. Family pedigree studies [22, 23], twin studies [37] and adoption studies [9, 11] all provide supporting data implicating the role of genes in alcoholism susceptibility. It has been suggested that as much as 40–60% of the variance in liability for developing alcoholism may be due to genetic effects [24, 28]. However, the early promise of identifying specific genes that contribute to this vulnerability has not yet been fulfilled. Initial reports of finding the genetic bases of alcoholism [7] have not stood the test of replication [20]. This is not to say that progress in the search for susceptibility genes contributing to alcohol dependence has not been made. Several investi-

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Victor Hesselbrock, PhD
Department of Psychiatry, MC-2103, 263 Farmington Ave.
Farmington, CT 06030-2103 (USA)
Tel. +1 860 679 3913, Fax +1 860 679 4077
E-Mail hesselb@psychiatry.uchc.edu

gative teams using both animal models [14, 15] and studies of affected populations [17, 18, 32, 38] have identified chromosomal regions that show considerable promise for containing susceptibility genes. While some medical disorders appear to result from a single gene and follow Mendelian patterns of inheritance (cf. Huntington's disease), others do not. Hypertension, diabetes and many cancers, for example, do not show typical Mendelian inheritance patterns. Such traits or conditions are called 'complex', as they do not follow the classic Mendelian transmission patterns of recessive or dominant inheritance [31]. Consequently, the direct relationship between phenotype and genotype is disrupted, i.e. the same genotype may result in different phenotypes or different genotypes may result in the same phenotype. Such may be the case with alcohol dependence.

There has been considerable work to identify 'subtypes' of alcoholism and alcohol dependence. Unfortunately, many of the resulting findings appear to be unreliable due to small sample sizes and do not replicate across studies [25]. Further, the clinical features of the disorder frequently fluctuate over time, suggesting different diagnostic phenotypes within the same individual for different stages of the illness. To avoid the problems that may be inherent in using clinical diagnosis or clinical phenotypes for detecting susceptibility genes, Gottesman and Shields [21] have suggested the use of intermediate or endophenotypes. In their conceptualization, the manifestations of endophenotypes would be closely linked to gene expression and highly heritable. These characteristics thus allow for endophenotypes to be used to identify persons at genetic risk for the disorder even in the absence of symptoms. Gottesman and Shields specified six criteria that must be satisfied for a trait to be identified as an endophenotype. These criteria include: (1) the trait must be present in affected individuals, in both the well state and during the course of the illness; (2) the trait must be present in unaffected biological relatives; (3) the trait must be present in individuals known to be at high risk for developing the disorder; (4) the trait must be predictive of an increased likelihood for developing the disorder; (5) the trait must be heritable, and (6) the trait should have biological manifestations closely linked to gene expression.

Since many traits and symptoms are shared across different psychiatric disorders, an endophenotype may not be specific for a particular diagnosis. Thus, disorders sharing a common genetic diathesis may also share the same endophenotype. Further, the presence of an endophenotype could serve as an indicator of increased risk for a disorder or a set of disorders, and assist in the identification of susceptibility genes.

A biological trait that has received considerable attention by alcohol researchers and which appears to meet the criteria specified by Gottesman and Shields [21] is the amplitude of the P_{300} waveform, an evoked electroencephalographic (EEG) brain potential. The P_{300} waveform is identified as the largest positive peak voltage of the event-related potential (ERP) waveform occurring between 250 and 500 ms after presentation of a stimulus. This component is thought to index several aspects of cognitive functioning, including attention and the maintenance of working memory [34]. More recently, Begleiter and Porjesz [5] have also proposed that a low P_{300} amplitude may serve as an indicator of central nervous system disinhibition. However, the relationship between cortical disinhibition and disinhibitory behaviors (e.g. failure to obey rules, impulsivity, conduct problems) found in several psychiatric disorders, including alcohol dependence, remains to be tested.

This paper will examine the evidence supporting the use of P_{300} amplitude as an endophenotype for alcohol dependence. Each criterion specified by Gottesman and Shields [21] for the identification of an endophenotype will be addressed using data from the Collaborative Study on the Genetics of Alcoholism (COGA).

Methods

Data for this report were derived from the COGA, a 6-site family study of the genetics of alcohol dependence. To date, more than 1,800 families have been recruited, representing over 12,000 individuals. To be considered 'affected', probands were required to meet both DSM-III-R criteria for alcohol dependence [1] and the criteria of Feighner et al. [16] for definite alcoholism to be eligible for study. All probands were required to be in active treatment, with the majority being ascertained through inpatient treatment units. A multistage ascertainment procedure was used. Probands with no or only one additional first-degree relative affected with alcohol dependence were designated as stage I, while probands with at least two additional affected first-degree relatives were designated as stage II. Control families were recruited from the local communities of each site through a variety of recruiting schemes, and families were not excluded if a family member was found to have a history of alcohol dependence. All subjects provided a detailed psychiatric history via a structured interview, the Semistructured Assessment for the Genetics of Alcoholism. This is a polydiagnostic interview with good reliability [8] and validity [26]. A more detailed description of the study and assessment protocol can be found elsewhere [17, 38].

Only individuals from stage II families with a high risk for alcoholism provided a blood sample for genetic analysis and participated in an EEG/ERP assessment. For the EEG/ERP studies, each subject wore a fitted, 21-lead electrode cap following the 10/20 international

system. The reference electrode was placed on the tip of the nose, and a ground electrode was placed on the forehead. Three visual stimuli were presented: targets (probability = 0.125), nontargets (probability = 0.75) and novel infrequent nontargets (probability = 0.125). The experiment terminated automatically after a minimum of 25 target stimulus, 150 nontarget stimulus and 25 novel nontarget stimulus artifact-free trials had been acquired. Stimulus duration was 60 ms with an interstimulus interval of 1.6 s. Each stimulus subtended a visual arc of 2.5°. Trials with a response time greater than 1,000 ms were rejected. P_{300} amplitude was identified automatically and defined as the highest positive peak voltage within 275–575 ms after stimulus onset. P_{300} amplitude was measured at each lead as the difference in peak voltage relative to the prestimulus baseline. Data collection equipment and procedures are described in more detail elsewhere [12].

Table 1. P_{300} amplitudes (μV) and endophenotypic criteria: persons at high risk for alcoholism versus controls

		Family type	
		stage II	controls
Criterion 1	Alcoholics	15.9 ± 7.8	21.2 ± 6.8*
Criterion 2	Nonalcoholic relatives	17.5 ± 8.6	20.4 ± 8.4*
	Nonalcoholic offspring	23.6 ± 7.3	27.6 ± 6.8*

* $p < 0.01$.

Results

Each of the six criteria specified by Gottesman and Shields [21] that must be satisfied in order to consider P_{300} amplitude an endophenotype for alcohol dependence were examined.

(1) The trait must be present in affected individuals. This criterion has been directly addressed in the COGA in two separate studies. Porjesz et al. [36] compared the P_{300} amplitudes in response to a visual stimulus of alcoholics from COGA families with a high risk for alcoholism (designated as stage II) and of alcoholics identified in control families. The alcoholic subjects from stage II families were found to have significantly lower P_{300} amplitudes than the alcoholics from control families (table 1). Further, almost 25% of the stage II alcoholics had P_{300} amplitudes that were more than 2 standard deviations below the COGA adult sample average P_{300} voltage.

These data were re-examined in a slightly different way by Costa et al. [13], who compared P_{300} amplitudes in relation to a diagnosis of alcohol dependence and antisocial personality disorder. They also found reported reduced P_{300} amplitudes among persons with a lifetime diagnosis of alcohol dependence compared to nonalcoholics (9.5 vs. 11.4 μV; $p < 0.05$) in the anterior leads but not the posterior leads. However, Costa et al. also found that P_{300} amplitudes were reduced for younger (<30 years old) subjects with a diagnosis of antisocial personality disorder compared to those subjects without this diagnosis ($p < 0.01$), but only in the anterior leads.

(2) The trait must be present in unaffected biological relatives. To address this criterion, first-degree relatives of COGA probands were compared to individuals from families selected as community controls [36]. In this analysis, the unaffected family members of the alcoholic proband did have P_{300} amplitudes that were significantly lower than those of relatives of control probands ($p < 0.01$; table 1). Further, the unaffected stage II family members were almost 7 times more likely than control group family members to have a P_{300} amplitude 2 standard deviations lower than the entire sample mean.

(3) The trait must be present in individuals known to be at high risk for developing the disorder. This criterion was directly tested using information from the COGA by comparing 16- to 25-year-old unaffected offspring of an alcohol-dependent father with offspring of male probands from the control group (table 1). As predicted, the offspring 'at risk' for alcohol dependence of an alcoholic father had significantly lower P_{300} amplitude voltages to a visual stimulus than their age- and sex-matched controls [36].

(4) The trait must be predictive of an increased likelihood for developing the disorder. At this time, this criterion has not been tested in the COGA data set, as only cross-sectional baseline data are available. However, a 5-year follow-up study of the COGA sample is currently under way. The follow-up data on alcohol use and problems will allow us to determine the usefulness of P_{300} amplitude for predicting the occurrence of alcohol-related problems, including alcohol dependence, among the offspring of alcohol-dependent probands as well as control probands.

(5) The trait must be heritable. Using a sib pair analysis, the heritabilities of P_{300} amplitude voltages at the 19 leads assessed were determined from the first 103 families in the COGA data set. Using n = 758 pairs of siblings, the heritabilities (h^2) were found to range between 0.280 and 0.505, with most in the range of 0.35–0.45 [6].

Importantly, a quantitative trait analysis (QTL) has also been conducted on these data using the same sib pairs. The QTL analysis found a significant linkage for the P_{300} amplitude at the O2 electrode on chromosome 2

(LOD = 3.28; $p < 0.0299$)) and on chromosome 6 (LOD = 3.41; $p < 0.0219$) for the Cz electrode. LOD scores greater than 2.0 suggestive of linkage were also found for the T8 electrode on chromosomes 2, 5 and 13 [6]. A replication sample of an additional 157 COGA families containing 1,295 individuals is currently undergoing analyses. The preliminary findings suggest that the significant P_{300} QTL analyses reported by Begleiter et al. [6] will be replicated in the second portion of the COGA sample.

(6) The trait should have biological manifestations that can be directly linked to gene expression. Evidence supporting this criterion is found in a bivariate genetic analysis of the COGA data set that examined the correlation of P_{300} amplitude and a diagnosis of alcohol dependence to determine the extent of shared genetic influences [40]. The correlation of P_{300} amplitude and a formal diagnosis (DSM-III-R, DSM-IV, ICD-10) of alcoholism was negative at all leads. The central and temporal leads produced the highest genetic correlations, ranging from –0.61 to –0.71, $p < 0.01$. Trivial, nonsignificant genetic correlations were found at the occipital leads. When examined using a bivariate linkage analysis of P_{300} at Cz and a DSM-IV diagnosis of alcohol dependence, evidence for linkage was found for a region on chromosome 4 near the alcohol dehydrogenase gene cluster (LOD = 5.79) and for a region on the long arm of chromosome 6 (LOD = 3.49).

Discussion

This paper used data from the COGA to evaluate the potential utility of P_{300} amplitude as an endophenotype of alcohol dependence. Six separate criteria for an endophenotype, as specified by Gottesman and Shields [21], were examined. In each case, the COGA data supported the individual criterion, suggesting that P_{300} amplitude would be a useful endophenotype. It was found that alcoholics from stage II families with a high risk for alcoholism had lower P_{300} voltages compared to alcoholics from control families (criterion 1), and that the unaffected relatives of stage II probands had lower P_{300} voltages than relatives of control probands (criterion 2). Criterion 3 was also met as the unaffected offspring of alcoholics were found to have lower P_{300} amplitudes than offspring of controls. Together these data indicate that the P_{300} endophenotype is identifiable in persons with the trait (alcohol dependence), among the biological relatives of probands and among individuals at increased risk for developing the disorder. Criterion 4, regarding the value of P_{300} amplitude for predicting the later development of alcohol dependence, could not be tested in the current cross-sectional COGA data set. Criterion 5 and criterion 6 require that an endophenotype be heritable and directly tied to gene expression. In the COGA sample, P_{300} amplitude was found to be highly heritable among family members. Additional genetic analyses have also shown evidence of genetic linkage between P_{300} amplitude and certain regions of chromosomes 2 and 6. Importantly, bivariate linkage analyses using P_{300} amplitude and a diagnosis of alcohol dependence identified regions on chromosomes 4 and 6 that may contain susceptibility genes. For each of the criteria considered, the results described above based upon the COGA sample provide strong evidence in support of the use of P_{300} amplitude voltage as an endophenotype for alcohol dependence.

However, two other issues regarding P_{300} amplitude as an endophenotype deserve comment. First, a low P_{300} amplitude is not unique to persons with alcohol dependence or at risk for developing alcohol dependence. Reduced P_{300} amplitudes have been found in several other psychiatrically ill populations and among persons susceptible to these conditions. Low P_{300} voltages have been reported among samples with schizophrenia [39] and Alzheimer's disease [35]. Similarly, persons at risk for poor adult outcome, such as individuals with conduct disorder or antisocial personality disorder [2–4, 33] or other psychiatric disorders such as schizophrenia or bipolar illness [19] also display P_{300} amplitudes lower than control subjects.

Secondly, the relationship of a reduced P_{300} amplitude to behavior is not well understood. While Begleiter and Porjesz [5] identify a reduced P_{300} amplitude as an indicator of neuronal disinhibition, the relationship of P_{300} amplitude to behavioral manifestations is less clear. Although it is tempting to suggest that neuronal disinhibition is directly associated with behavioral disinhibition, direct evidence is lacking. As indicated above, Polich [34] has reported that several aspects of cognitive functioning, including attention and the maintenance of working memory, are positively correlated with P_{300} amplitude. Our laboratory [30] has reported similar results, including findings from a sample that contained individuals with antisocial personality disorder [29]. Using the sample from the latter study, the association of P_3 amplitude was also examined in relation to several different personality traits. No significant association was found between P_{300} amplitude and the Reward Dependence subscale of the Tri-dimensional Personality Questionnaire [10] with correlation coefficients ranging from 0.03 to –0.12 across the F8, Fz and Pz leads where P_{300} was recorded. Similarly,

the association between P_{300} and a childhood behavior rating scale indicating impulsivity [27] produced nonsignificant correlation coefficients which ranged from 0.06 to 0.17 [unpubl. data]. These preliminary findings suggest that P_{300} amplitude may be related to some cognitive skills, but not to more global indices of personality. Clearly further studies are required to examine the association between neural disinhibition and behavioral disinhibition to better understand the etiological importance of a reduced P_{300} amplitude for developing alcohol dependence. However, such a demonstrated association would not be mandatory for P_{300} amplitude to have value as an endophenotype for alcohol dependence in the search for susceptibility genes.

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Dopamine and Alcohol Relapse: D_1 and D_2 Antagonists Increase Relapse Rates in Animal Studies and in Clinical Trials

Henriette Walter[a] Katrin Ramskogler[a] Brigitte Semler[a]
Otto Michael Lesch[a,b] Werner Platz[c]

[a]Department of Psychiatry, University of Vienna, and [b]Anton Proksch Institute, Vienna, Austria; [c]Bonhoeffer Clinic, Berlin, Germany

Key Words
Alcohol · Relapse · Dopamine · Lesch typology · Flupenthixol

Abstract
A considerable number of animal studies on the effects of dopaminergic agents on alcohol intake behavior have been performed. Acute alcohol administration in rats induces dopamine release in the caudate nucleus and in the nucleus accumbens, an effect related among others to reinforcement. It has been repeatedly suggested that D_1 and D_2 receptor activation mediates reward. As alcohol consumption and dopaminergic transmission seem to have a close relationship, all kinds of dopaminergic agents may be regarded as putative therapeutics for preventing relapse. In a prospective European double-blind multicenter clinical trial, comparing the D_1, D_2, D_3 antagonist flupenthixol and placebo in 281 chronic alcohol-dependent patients (27.4% women), the application of the Lesch typology made an outcome differentiation possible. It could be shown in which patients flupenthixol administration was followed by a significantly higher relapse rate and in which patient groups no differences were found when compared to placebo.

Introduction

Relapse prevention is a major concern in clinical work with alcohol-dependent patients. In relation to the follow-up observation time, relapses occur in up to 90% (after 5 years approx. 80% and after 15 years approx. 90%), indicating that they are an inherent part of this disease. Depending on the individual illness course, relapses may represent quite different drinking patterns with different amounts of intake and duration. Therefore researchers have been attempting to establish course specifiers, symptom patterns or typologies with predictive values about future risks, drinking patterns and the further illness course [4, 11, 35, 50]. In principle, relapsing patients seek a positive reward or a relief from negative affective states. Dopamine (DA) seems to be involved in both. Animal studies revealed that acute ethanol intake induces a DA release in the caudate nucleus and in the nucleus accumbens (NAC) [14]. Dopaminergic transmission changes are generally related to reward. The use of microinjection techniques in animal studies permitted the identification of specific brain areas mediating the reinforcement. The ventral tegmental area (VTA) and the NAC represent key structures of the dopaminergic mesolimbic reward system [63, 75]. Morphine injections into the VTA, NAC, hypothalamus and septum elicited rewarding effects, which

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Prof. Henriette Walter, MD
University of Vienna, Department of Psychiatry
Währinger Gürtel 18–20, A–1090 Wien (Austria)
Tel. +43 1 40400 3528, Fax +43 1 40400 3560
E-Mail Henriette.Walter@akh-wien.ac.at

Table 1. Lesch types [35]

Lesch Type I
Main problem: alcohol metabolism, early and severe physical withdrawal (also withdrawal fits)
Withdrawal treatment (duration 7 days): benzodiazepines
Outpatient treatment: regular counseling
15 months relapse prevention, acamprosate, disulfiram or cyanamide
Relapse: Acamprosate to be continued, naltrexone and/or GHB for a few days
No neuroleptics
Lesch Type II
Main problem: anxiety, conflict solving with alcohol (disinhibition), relief drinking
Severe psychic withdrawal, mild physical withdrawal
Withdrawal treatment (duration up to 14 days): GHB
Motivation to psychotherapy
Outpatient treatment: psychotherapy
15 months relapse prevention: acamprosate
Relapse: acamprosate to be continued, naltrexone
No tranquilizers, no hypnotics
Lesch Type III
Main problem: seeking for social reward, seeking for relief of negative affective states
Severe psychic withdrawal, mild physical withdrawal (stable tension, suicidal tendencies, family history frequently positive for psychiatric diseases and alcoholism)
Typical is an episodic drinking pattern
Withdrawal treatment (duration up to 14 days): GHB
Diagnosis of the basic disturbance (depressed personality, affective disorder)
Psychotherapy and/or antidepressants, lithium/carbamazepine
15 months relapse prevention: acamprosate only additionally for strong craving
Relapse: GHB for up to 2 weeks
No neuroleptics
Lesch Type IV
Main problem: brain damage during brain development (before the age of 14)
Cognitive impairment, conduct disorders, mild physical and psychic withdrawal
Withdrawal treatment (duration up to 3 months): GHB
Outpatient therapy: arranging and organizing daytime and the social surroundings
Regular controls and outpatient treatment, self-help groups
15 months relapse prevention
Anticonvulsive drugs, nootropic drugs, biologically active light, regular care
Relapse: naltrexone (Sinclair 'extinction method') and/or GHB
No aversive medication or benzodiazepines

could be demonstrated in self-administration as well as in place conditioning studies [10, 74]. Thus, reward seems to be the primary function of this DA pathway. But, as practically all other signaling systems influence the mesolimbic dopaminergic system, seeking for relief might be an additional, secondary function. Besides adverse life events, negative mood states (5-HT via raphe) and sudden excitation or withdrawal-like signs (glutamate via amygdala and prefrontal cortex) are frequently reported symptoms leading to relapse. We know from 5-HT studies that agonists and antagonists (e.g. amperozide and ritanserin) can reduce alcohol intake. Also in the DA system we have conflicting results, as far as the effects of agonists and antagonists are concerned. Therefore it seems essential for testing any new medication to subgroup the attending patients according to their main reinforcement-seeking behavior (reward/relief). In a recent review three craving types, each related to different pathways (opioidergic/dopaminergic, serotonergic and GABA/glutamatergic neurocircuits), are differentiated [69]. Among the presently available typologies the Lesch typology comes closest to a clinical separation of these factors (types I and IV seek reward, II and III relief) and differentiates between four types according to their withdrawal symptomatology, their relapse pattern and their prognosis [34, 35] (table 1).

Animal Studies

The CNS DA contents are different in alcohol preferring (P) and non preferring (NP) rats in 2 regions: NAC and olfactory tubercle have 25–30% lower DA in P rats. Lower densities of DA fibers in the cingulum cortex and shell (medial part) of the NAC were found, whereby the VTA projections to the NAC are smaller and fire more frequently in bursts, which was interpreted as a reduced autoregulation and/or compensation [38, 39]. In one study, age-related changes in the nigrostriatal system, an up to 25% loss of total D_2 receptors in the rostral and caudal striatum and associated changes in motor function were found [65]. Chronic alcohol intake did not accentuate the age-related loss of D_2 receptors. They further found that the DA release in the NAC, which usually follows acute alcohol intake, is not attenuated by chronic intake. The authors interpret this finding that, if the effects of chronic alcoholism on DA release are selective for the NAC, the altered DA release could impact on the expression of the genes coding for D_2 receptors. On the other hand, the selective vulnerability of this tissue to eth-

anol might be caused by ethanol's impact on the regulation of transcription factors. Striatal D_2 receptors are situated on the large cholinergic interneurons and on efferent neurons that contain both GABA and enkephalin. One research group favors, besides the NAC, the central nucleus of the amygdala as an opioid/DA interaction site [31]. The authors put emphasis on the cytoarchitectural similarity between the shell of the NAC, the bed nucleus of the stria terminalis and the central nucleus of the amygdala, a fact from which the authors derive a possible functional entity, involved in eliciting reward.

The effects of chronic ethanol self-administration also depend on environmental variables (e.g. housing conditions). D_2 receptor binding density is downregulated after 5 weeks of chronic ethanol exposure in rats housed in groups (stress factor) but not in isolated rats [46].

Affinity Status

The high-affinity state of D_1, D_2 receptors is the agonist-receptor-G-protein complex. This form can be converted at 37 °C by the addition of guanine nucleotides and Na^+ to the low-affinity form [5, 51]. The activated G protein can react with the catalytic subunit of adenylate cyclase and activate D_1 receptors and inhibit some D_2 receptors. No alcohol-related differences in affinity or proportion of receptors in the high- or low-affinity form were found [36, 65]. Other researchers detected that D_2 receptors are predominantly in the high-affinity form, whereas the D_1 receptors are primarily in the low-affinity state [45]. Withdrawal of ethanol after a 7-day exposure reduces the efficacy of DA-stimulated adenylyl cyclase activity in the striatum of mice [64]. In vitro ethanol enhances, while chronic ethanol administration reduces, G-protein-activated adenylyl cyclase activity in the striatum [6, 29]. Chronic estrogen treatment seems to promote functional uncoupling of D_2 receptors and G proteins in rat pituitary glands [40]. During chronic intoxication of alcoholics, an increase in estradiol levels, which soon vanishes during abstinence, was found [26]. Thus, an estrogen-dependent postsynaptic signal transduction seems possible. The authors, as clinicians, are tempted to assume that the affinity status is influenced by many more endogenous and/or exogenous substances and might be one puzzle stone for clarification of tolerance (and consequently dosage increase) as well as for nonresponse to drugs (e.g. we know that adding estrogen is one of the clinical standard augmenting strategies for nonresponse in pharmacological depression treatment).

Animal Models of Relapse

Repeated episodes of intoxication in alcoholics can worsen the symptoms of dependence [7] and may produce kindling effects [66, 67].

An excessive drinking model in nondependent rats is the increase in ethanol consumption observed after a period of alcohol deprivation [28, 59]. Termed the 'alcohol deprivation effect', this phenomenon has been observed in rodents, monkeys and humans [59]. Rats, trained to lever press for ethanol in daily 30-min sessions, increase their consumption up to 200% of baseline after 5–14 days of deprivation [28]. One study could show that the alcohol deprivation effect could be blocked by chronic administration of acamprosate [27].

This animal model separates at best alcohol-induced relapse from cue-induced relapse. Environmental stimuli, conditioned to drinking, can trigger alcohol-like responses, withdrawal-like responses or a desire for the drug [43, 58]. In two animal studies, enhanced DA release in the NAC was found following the exposure to drug-associated cues [15, 18]. In rats, anticipation of ethanol consumption leads to a DA release, whereby this effect is more pronounced in P rats [71].

Further elucidation of the neurochemical/neurocircuitry basis for the alcohol deprivation effect and other aspects of relapse-related behavior may provide a next step to a better understanding of the biological basis of vulnerability to relapse and of the development of a different sensitivity to the effects of alcohol. We still have two basically opposite theories on the role of reward pathways in mediating relapse. One theory suggests that relapse is triggered by drug-imitating processes [47, 53, 54, 62, 73]. The other theory suggests that drug-opposite effects induce relapse by eliciting a hypofunction of reward pathways, leading to dysphoria and anxiety during withdrawal [30, 60]. The Lübeck Craving Scale meets both theories and identifies 4 main craving factors: euphoric mood, depressed mood, anger/tension and relaxation [68].

Human Relapse Studies

The evidence of underactivity in the withdrawal period is given in preclinical and clinical studies [12, 17, 24, 25, 49], which showed that subsequent relapsers have, already during withdrawal, a blunted response of growth hormone secretion in the apomorphine test, which the authors interpreted as a retarded adjustment of the DA

system. A recent study suggested iodobenzamide SPECT measurement during withdrawal as a predictive 'marker' for DA hypofunction and consequent relapse [23].

DA Agonists for Relapse Prevention

According to the above-mentioned results it would be logical to test DA agonists to substitute the functional deficit after withdrawal. Systemic priming injections of D_2-like, but not D_1-like, DA receptor agonists induce a heavy cocaine relapse in the reinstatement paradigm [52]. These findings suggest that D_2-like receptors are primarily involved in inducing drug-seeking. D_1-like receptor agonists fail to markedly induce cocaine-seeking behavior. D_1 receptors may have a permissive role in the priming effects mediated by D_2 receptors [53]. However, a pretreatment with D_1-like agonists completely abolishes the ability of priming injections of cocaine to induce relapse, whereas D_2-like agonist pretreatment potentiates priming with cocaine [53]. The authors of the above-mentioned studies suggest, as a possible explanation, that D_2-like receptors mediate drug seeking, while D_1-like receptors could mediate some aspects of drug reward related to gratification, drive reduction or satiety. Possibly their opposite effects on adenylyl cyclase and thereby on protein kinase underlie their opposing effects on relapse.

One study [48], using amphetamine, an indirect DA agonist, yielded differing results: Low intake was increased, while high intake was decreased. γ-Hydroxybutyric acid (GHB) has been shown to significantly increase brain DA [20, 21]. First clinical studies with direct (bromocriptine) and indirect (GHB) DA agonists have yielded contradictory results. A positive outcome, defined as a decrease in craving and improvement of social functioning, was reported in two studies [8, 9, 16]. A large multicenter trial with a parenteral application of bromocriptine showed that this substance did not reduce relapse rates [41]. GHB was effective in reducing relapses [2, 3, 13, 19]. In a randomized, double-blind, double-dummy study, performed in our center, 50 mg/kg GHB reduced craving during withdrawal effectively. In treating the withdrawal syndrome no difference between GHB and the comparison drug clomethiazole could be detected. As far as craving was concerned, the mean values were equal for both substances. Individual cases in the GHB group, all belonging to Lesch type III, reported also disgust at alcohol [42].

DA Antagonists for Relapse Prevention

While agonists may mimic alcohol effects, antagonists are expected to block the reward effects of alcohol. Stress-induced relapse could be partially attenuated by pretreatment with DA antagonists [56]. Stress is supposed to affect the NAC via the prefrontal cortex (via glutamate) and through release of corticotropin-releasing factor (CRF). It could be detected that intraventricular infusions of CRF cause an increase in DA release in the hypothalamus and prefrontal cortex (not in the NAC) [32, 61] and that those infusions mimicked the induction of stress-triggered heroin-seeking behavior, while similar infusions with a peptide-CRF antagonist partially reduced stress-induced relapse [55].

The D_1 antagonist SCH 23390 was tested by several researchers and found to abolish the reinforcing effect of morphine [33] and also the aversive effects of κ-receptor agonists and naloxone [57]. Furthermore, one study could indicate that the tonic activation of D_1-receptor-mediated pathways is antagonized with SCH 23390 followed by aversive states [1]. Two studies, one with low-dose fluphenazine which was injected into the NAC [44] and one with the D_2 antagonist raclopride [48], proved a reduction of alcohol intake. Another interesting drug, lisuride, is a partial D_2 antagonist with an unclear effect on D_1 receptors. In vitro full D_1 antagonistic effects of lisuride were proven [37], while in vivo studies yielded equivocal results [22, 70]. An unpublished placebo-controlled lisuride study showed an increase in relapses in the lisuride group [pers. commun., L. Schmidt].

Flupenthixol, a D_1, D_2, D_3 antagonist with less affinity to D_4 receptors, which also affects 5-HT_{2A} and 5-HT_{2C} and α_1-adrenergic receptors, is used in some countries as a relapse prevention drug. In a multicenter placebo-controlled, double-blind study with 281 alcohol-dependent patients (n = 142) versus placebo (n = 139), flupenthixol proved to increase the relapse rate significantly. The difference started after 30 days and lasted for the whole 6-month period. The mean time to first relapse was 48 (±39) days for flupenthixol patients and 48 (±40) days for placebo. The cumulative abstinence duration was 100 (±62) days in the flupenthixol group, significantly less than in the placebo group with 122 (±66) days of abstinence. 122 of these patients were diagnosed according to the Lesch typology before the study started. Flupenthixol had detrimental effects only in types I and III. In types II and IV, no difference between flupenthixol and placebo was found.

Therefore we conclude that a D_1 and D_2 antagonist leads to increased relapse rates in patients, who are char-

acterized by an illness course with severe physical symptoms at withdrawal (three-dimensional tremor, instability of functions of the autonomic nervous system, withdrawal fits), with frequent and heavy loss of control before treatment and with no psychiatric comorbidity (type I). Furthermore, this drug induced relapse in patients with a comorbidity of an affective disorder, depressed personality structure ('overachiever') and with major depression (with sleep disorders) and suicide attempts during sobriety, with a high stable tension at withdrawal (two-dimensional tremor, stable tensed, high blood pressure, anxiety, depressed, irritable mood) and with an episodic drinking pattern before treatment (type III). In these two types, the DA system seems to play a major role, whereby type I patients seek reward, while in type III patients relief is expected as the main alcohol effect. It might be challenging for basic research to investigate these symptom patterns and the role of different neurotransmitters. Most interesting seems also the question of how all these neurocircuits change after repeated episodes of ethanol intoxication in animals and humans and if there is an influence of repeated withdrawals on relapse patterns.

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The European Acamprosate Trials: Conclusions for Research and Therapy

O.M. Lesch[b] Anita Riegler[a] Karin Gutierrez[a] Ines Hertling[a] Katrin Ramskogler[a] Brigitte Semler[a] Ali Zoghlami[a] Norbert Benda[a] Henriette Walter[a]

[a]Department of Psychiatry, University of Vienna, and [b]Anton Proksch Institute, Vienna, Austria

Key Words

Alcohol · Relapse prevention · Lesch typology · Acamprosate

Abstract

In an excellent methodological approach, the European acamprosate study project showed that acamprosate increases sobriety times. In one randomized prospective study (n = 260) comparing acamprosate and placebo, with a 1-year treatment phase and 1-year follow-up phase, the authors found that acamprosate is effective only in Lesch type I and type II patients. To investigate the possible influence of diagnostic subgrouping, we applied the Lesch typology in a co-work with the main researchers of the UK study. The UK results concerning acamprosate's effects in the types do not mirror the Vienna results, but the numbers of type I and type II patients, retrospectively found as included in the UK centers, were too small for any conclusions. The distribution of the types points to the fact that too many type III and IV patients had been included to give acamprosate the chance to be effective. Following our typology and also these studies, we developed special treatment approaches. For relapse prevention studies, the cumulative abstinence duration together with the Lesch typology seems to be promising.

Introduction

The chronic process of alcohol dependence leads to changes in biological, psychological and social functioning. Treating alcohol-dependent patients we have to separate between withdrawal treatment, relapse prevention (time to first relapse, cumulative abstinence duration, CAD) and treatments to reduce the severity and duration of relapses. In all long-term treatment studies of alcohol dependence, it has been shown that only a small group of patients are totally sober in the long term. In an Austrian prospective long-term study of a catchment area with alcohol-dependent patients (DSM-III) we could show that 82% of all alcohol-dependent patients relapse in a period of 5 years after admission. During a further 12-year follow-up of these study patients, 91% of survivors relapsed [17]. Therefore improvements in relapse prevention and relapse treatment are most important. It was pointed out that high frequency of drinking, rapid onset of relapses and high level of related psychological symptoms may indicate a negative treatment prognosis. The importance of identifying these target patients as quickly as possible and of matching them with different types of treatment cannot be overestimated [12, 13, 45].

The chronicity of alcohol abuse in alcohol-dependent patients leads to adaptations in all cerebral functions. Therefore different pharmaceutical compounds are effec-

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Otto Michael Lesch
University of Vienna, Department of Psychiatry
Währinger Gürtel 18–20, A–1090 Wien (Austria)
Tel. +43 1 40400 3531, Fax +43 1 40400 3560
E-Mail Otto.M.Lesch@akh-wien.ac.at

tive in relapse prevention. Results of studies with these substances yielded differing and often contradictory results. Furthermore it is well known that negative results tend to be less frequently published. The European Acamprosate Trials offer the worlds largest database of a compound, investigating relapse prevention. Meanwhile all European studies are published. Therefore we used this database for conclusions on methodological considerations for the organization of further studies in relapse prevention and therapy.

Biological Basis for the Acamprosate Studies

The central neurotoxic effects of alcohol seem to be mainly due to ethanol, congener alcohols, aldehydes and brain catalase's ethanol metabolism. Their detrimental effects on the CNS seem to be closely associated with the major CNS excitatory neurotransmitter glutamate, which has been intensely investigated in the last years. Evidence suggesting that glutamate is involved in neuronal tissue damage, intoxication and dependence is given. Alcohol inhibits the function of glutamate receptors, particularly the N-methyl-*D*-aspartate (NMDA) receptors. Besides an increase in voltage-sensitive Ca^{2+} channels an up-regulation of NMDA receptors appears as the logic consequence contributing to an increased neuronal excitability. Chronic alcohol exposure may thus lead excitotoxic neuronal death. Furthermore, the increased NMDA receptor expression seems to contribute to the increased neuronal excitability, which can be observed during withdrawal and as suddenly occurring excitation and hyperactivity for some hours in the postwithdrawal period. We know nowadays that repeated withdrawal episodes increase the excitability of brain [11, 44]. The relationship between acute and chronic alcohol intake effects on the NMDA receptors and that of subunits still has to be investigated more profoundly. Nevertheless NMDA glutamate receptors may serve as targets of pharmacological interventions to alter the neuronal effects of alcohol.

It could be shown that acamprosate reduces the effects of iontophoretically applied excitatory amino acids agonists (e.g. *L*-glutamate, homocysteic acid) in neocortical neurons but does not alter the responses to applied γ-aminobutyric acid (GABA) [49]. A blunting of excitatory amino acids may reduce the hyperexcitability, being one of the cues for alcohol craving, elicited by increased excitatory amino acid receptors of an increased excitatory amino acid release [24, 25]. By such actions acamprosate may counteract neuronal hyperexcitability caused by chronic high alcohol intake. Acamprosate seems not to have any rewarding or reinforcing effects [49].

Acamprosate is thought to have inhibitory activity at NMDA receptors [31, 48]. Acamprosate decreases the glutamatergic excitation due to chronic alcohol intake, withdrawal and early abstinence [35, 43]. This effect may decrease the urge to drink. Alcohol preferring and alcohol nonpreferring rats were pretreated with acamprosate (400 mg/kg/day p.o.) during 4 weeks before microdialysis. Compared to untreated animals acamprosate increased the basal concentration of the extracellular accumbal glutamate, taurine and GABA with no effect on aspartate [6]. Summarizing this, acamprosate does not seem to have direct effects on GABA. Acamprosate affects taurine, which consequently has an impact on GABA. The substance affects glutamate, whereby time and alcohol seem to be influencing factors. Taurine also modulates glutamate. Acamprosate could therefore have an impact on glutamate via taurine. Acamprosate further modulates the calcium channels. Therefore acamprosate is now also defined as a partial co-agonist of the calcium channel [6].

These preclinical study results led to animal drinking experiments. Acamprosate produces a dose-dependent reduction in voluntary alcohol intake in animals with no effect on food and water consumption. There is no evidence of pharmacological interactions with ethanol or other compounds used in alcoholism treatment (anxiolytics, hypnotics, disulfiram) [3, 7, 10, 16, 34]. It has no other pharmaceutical effects than reducing alcohol intake (e.g. no sedation, no driving impairment, no excitation). In the Research Society on Alcoholism meeting in 1999, a report was presented that acamprosate could be combined with naltrexone without any drug combination problems [28]. The pharmacodynamics, kinetics and safety have already frequently been published [14, 29, 30].

The European Acamprosate Trials

Acamprosate was first marketed in France in 1989, followed by most European countries in the nineties of the last century. Studies in France showed optimal effects at 1.33 g/day and 2 g/day [26]. Controlled studies and this vast clinical experience with acamprosate confirmed the good tolerance of the drug [1]. The main side effects were diarrhea and pruritus. Acamprosate is eliminated by renal excretion and should therefore be cautiously applied in patients with renal dysfunctions.

In the 12 European studies, time to first relapse was measured, whereby relapse was defined as any intake of

alcohol (survival analysis, intention-to-treat analysis). Other efficacy criteria were CAD, a simple success/failure index for final outcome and alcohol intake at any visit. The efficacy of the drug was measured in all studies by GGT, ASAT, ALAT, MCV and in some studies also by CDT [21]. The studies in the different European countries were performed over different periods of time (treatment phase: 3–12 months; follow-up: up to 12 months). Methodological considerations of these studies had been extensively discussed within the Plinius Major Society and finally led to the development of the 'Guidelines on Evaluation of Treatment of Alcohol Dependence', published in 1994 and since then constantly up-dated by new research results [42] (updates in www.alcoweb).

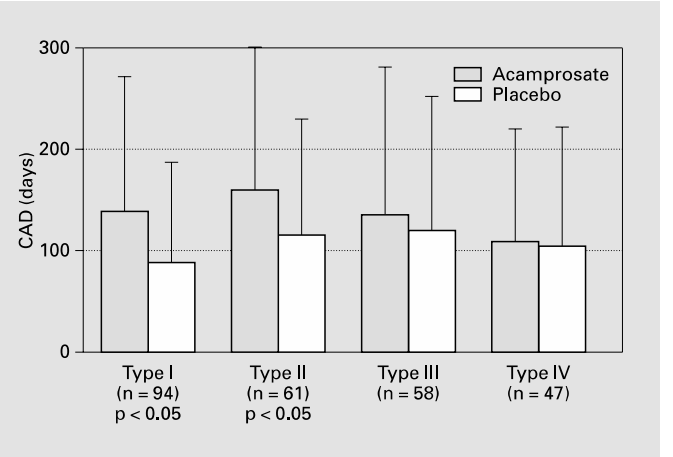

Fig. 1. Acamprosate in relapse prevention according to the Lesch typology: cumulative abstinence duration (CAD).

Results

Acamprosate was shown to be significantly superior to placebo in all but one of the 12 European clinical multicenter trials on more than 4,000 patients. In 11 studies, approximately twice as many patients remained abstinent during their treatment phase (3 months to 1 year) when given acamprosate. Side effects were reversible, rare and limited to diarrhea and mild dermatological problems [4, 5, 8, 15, 27, 28, 33, 37, 38, 41, 47]. Practical overviews have been given [14, 30]. All papers indicated the necessity for a better characterization of the patients, separating patients, who could benefit from acamprosate and who would not. In 1992, a paper reported that acamprosate only reduces the number of drinks in alcohol-dependent patients with no family history of alcoholism [9]. It has been reported recently that only the motivated patients profit from taking acamprosate [29]. In 1998, it was pointed out that concomitant administration of disulfiram improved the effectiveness of acamprosate [2].

The Austrian Acamprosate Trial

Designed as a multicenter double-blind, placebo-controlled trial, the Austrian study comprised 448 chronic alcohol-dependent patients and was divided into a 1-year medication period and a 1-year medication-free follow-up. The results showed that in the acamprosate group 18.3% did not relapse, compared to 7.1% nonrelapsers in the placebo group [47]. The need for a characterization of these 11.2% who benefit from acamprosate arises. In 1996, the authors published the results of one Austrian center, which took into consideration the patients' subgrouping according to the Lesch typology [18, 19, 22, 23, 25, 39]. This typology relies on the above-mentioned prospective long-term study. The diagnosis of the type-specific symptom patterns is validated with biological and physiological data and has already been available at that time in a computerized version, following the decision tree, published in 1999 [20]. In 260 alcohol-dependent patients (Vienna center, Anton Proksch Institute), the types were assessed (type I: 94 patients, type II: 64 patients, type III: 58 patients and type IV: 47 patients). In the 1-year treatment period, no type-related difference in the survival curve was detected, but the abstinent days (cumulative abstinence duration) were increased significantly in type I and II patients (fig. 1). There was no beneficial effect in types III and IV. We conclude from this that severe withdrawal syndromes (type I) and anxiety (type II) play a major role in the action of acamprosate. Severely cognitively impaired patients, with polyneuropathy and comorbidities, using alcohol as self-treatment for antidepressant action, and exhibiting an episodic drinking style, do not seem to profit from acamprosate.

As a consequence of these results we developed the hypothesis that one possibility of the UK negative result could be that the distribution of the types according to Lesch had been different from the Austrian trial [5]. Therefore we co-worked with the Edinburgh and London centers and retrospectively applied the Lesch typology. Taking into account the weakness of such as methodology (subgrouping after the trial had been completed, loosing patients etc.), we got quite interesting results. In the UK

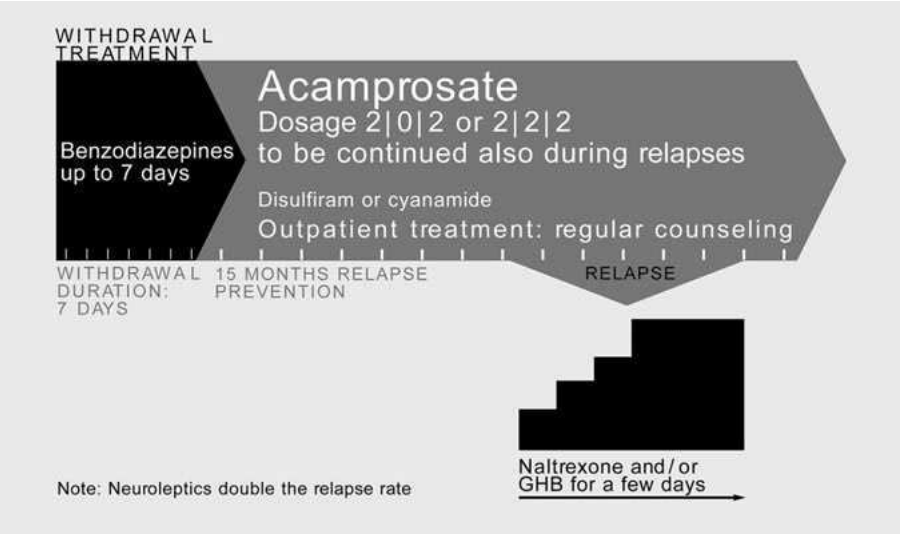

Fig. 2. Lesch type I (allergy model). GHB = γ-Hydroxybutyric acid.

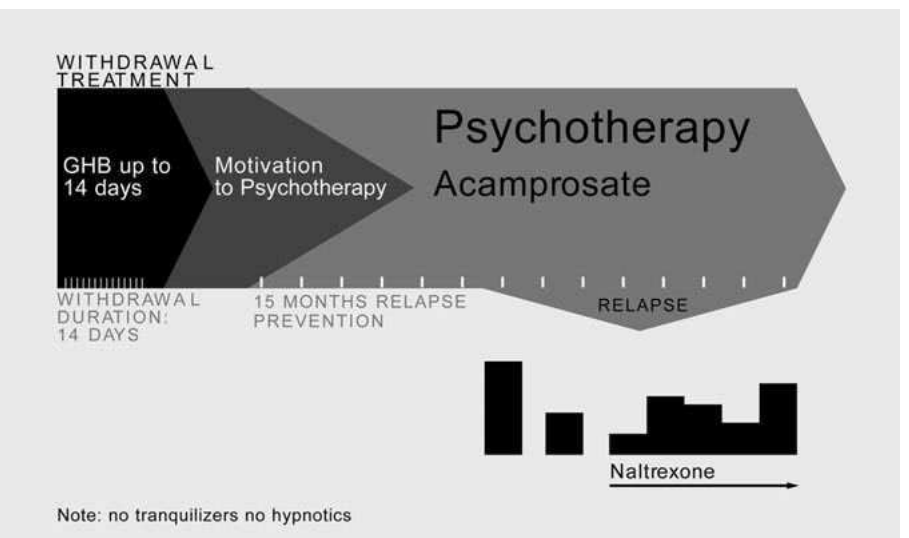

Fig. 3. Lesch type II (conflict model). GHB = γ-Hydroxybutyric acid.

study, 149 patients were included in Edinburgh and London. One of our coworkers, A.R., applied the Lesch typology in a personal interview. Two patients were cognitively too disabled to give an interview and 10 refused, 29 were untraceable and 32 had died. Therefore she could classify 76 alcohol-dependent patients according to the typology. Only 16 patients of types I and II had been in the acamprosate group, while 17 patients of type I and II had been on placebo. There was no difference between placebo and acamprosate in these patients, but the number of patients is far too small for interpretation. A comparison of the distribution of types between the Edinburgh/London samples and the Vienna sample showed that the Vienna sample had comprised more type I and II patients. The death rate in these two UK centers were extremely high (32 out of 149 patients), and the number of untraceable patients was also much higher (29 out of 149) than in the Vienna center. Our above-mentioned prospective long-term study showed that untraceable and deceased patients come mainly from types III and IV. This strengthens the hypothesis that in the UK study evidently more type III and IV patients had been included.

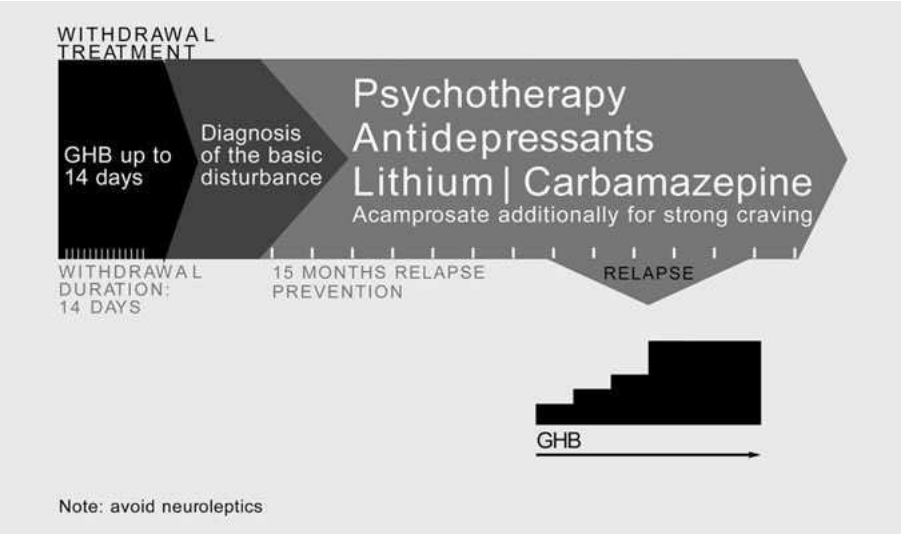

Fig. 4. Lesch type III (self-treatment model). GHB = γ-Hydroxybutyric acid.

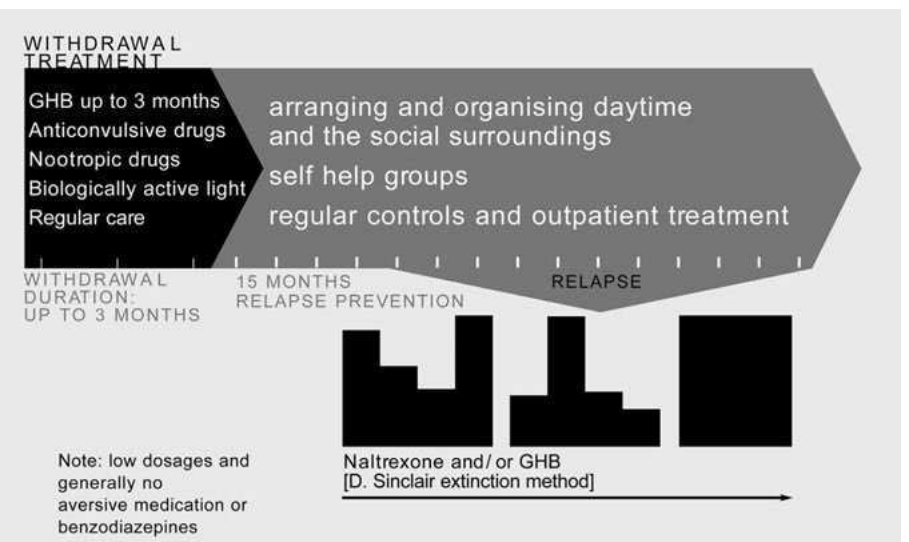

Fig. 5. Lesch type IV (adaptation model). GHB = γ-Hydroxybutyric acid.

Summary and Outlook for Research and Therapy

The international literature agrees on the assumption that drug therapy should be considered for all alcohol-dependent patients [40]. Early diagnosis of patient characteristics is necessary to treat withdrawal and to design the special treatment program for relapse prevention. In most alcohol-dependent patients relapses are common, therefore each development of a pharmaceutical agent reducing relapses should be welcomed [32].

The European Acamprosate Trial showed that acamprosate increases the sobriety rates and has only few and mild side effects. Using the Lesch typology it is possible to develop special research and treatment protocols. For future drug trials we recommend to stratify alcohol-dependent patients according to Lesch typology and to use as the main outcome criterion the cumulative abstinence duration (CAD). Time to first relapse does not seem to reflect the ups and downs of the long-term course of alcohol dependence [36].

We derive from these studies and from the present literature that for future research a good definition of patient subgroups (e.g. Lesch typology) is necessary. Following the Lesch typology we developed 4 different treatment programs (fig. 2–5).

Though only few substances are proven – according to their efficacy – to be as good as acamprosate, the authors think, that also other drugs, such as naltrexone and disulfiram, do have their place in the treatment concept of alcohol dependence. Motivated patients, with no severe cognitive impairments, profit from disulfiram, and naltrexone helps to decrease the severity and duration of relapses. Only acamprosate is able to prevent relapses in well-subgrouped alcohol-dependent patients. We have learned by now that if a drug is promoted for 'all' alcohol-dependent patients, we might loose compounds which are effective in subgroups (SSRIs, tianeptine, γ-hydroxybutyric acid etc.) [46].

Acknowledgement

The authors want to express their gratitude to Marsha Morgan and Jonathan Chick for their good cooperation and help in the UK typology assignment study.

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Neurotoxic Effect of Acamprosate, N-Acetyl-Homotaurine, in Cultured Neurons

Jang-Yen Wu[a] Hong Jin[a] John V. Schloss[b] Morris D. Faiman[c] Nagendra S. Ningaraj[c] Todd Foos[a] Weiqing Chen[a]

Departments of [a]Molecular Biosciences, [b]Medicinal Chemistry and [c]Pharmacology and Toxicology, University of Kansas, Lawrence, Kans., USA

Key Words

Acamprosate · N-acetyl-homotaurine · Taurine · Neurotoxicity · Alcoholism

Abstract

Acamprosate (AC), N-acetyl-homotaurine, has recently been introduced for treating alcohol craving and reducing relapses in weaned alcoholics. AC may exert its action through the taurine system rather than the glutamatergic or GABAergic system. This conclusion is based on the observations that AC strongly inhibits the binding of taurine to taurine receptors while it has little effect on the binding of glutamate to glutamate receptors or muscimol to $GABA_A$ receptors. In addition, AC was found to be neurotoxic, at least in neuronal cultures, triggering neuronal damage at 1 m*M*. The underlying mechanism of AC-induced neuronal injury appears to be due to its action in increasing the intracellular calcium level, $[Ca^{2+}]_i$. Both AC-induced neurotoxicity and elevation of $[Ca^{2+}]_i$ can be prevented by taurine suggesting that AC may exert its effect through its antagonistic interaction with taurine receptors.

Introduction

Addiction to alcohol produces numerous deleterious effects. Untreated alcohol abuse results in a variety of social, economic and medical consequences, all of which contribute to health care costs in the US in excess of 100 billion dollars annually [14]. Excessive alcohol use not only complicates treatment of general medical diseases, but can result in severe fatal outcomes. Hence, an effective therapeutic intervention of alcohol abuse is highly desirable.

Among various therapeutic agents that have been proposed for treating alcohol craving, naltrexone and acamprosate (AC) have received the most attention. Several preclinical studies have demonstrated that naltrexone, an opioid antagonist, reduced alcohol intake [15, 21, 22]. Unfortunately, a more recent follow-up study showed that the ability of naltrexone to facilitate abstinence compared to placebo diminishes over time [15].

Perhaps, the most promising therapeutic agent for reducing or preventing relapses in weaned alcoholics is AC, a homotaurine analog (N-acetyl-homotaurine). Numerous reports have shown that AC is effective in reducing alcohol craving and alcohol consumption and maintaining the abstinence [11, 17, 19, 20, 23]. In addition, it has also been shown to be effective in reducing excitatory amino acids (EAA)-induced neurotoxicity [1] and alcohol

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Jang-Yen Wu, PhD
Department of Molecular Biosciences, University of Kansas
Lawrence, KS 66045-2106 (USA)
Tel. +1 785 864 4557, Fax +1 785 864 5374
E-Mail jywu@falcon.cc.ukans.edu

withdrawal-induced seizures [3]. Although AC has already been used clinically in Europe [6, 17–20, 23] the mechanism(s) of action for its central effects remain elusive.

In this communication, evidence is presented to support the conclusion that AC has a neurotoxic effect, at least in the cultured neuronal system and it may exert its action through its antagonistic interaction with the taurine receptors.

Materials and Methods

Materials

Female Sprague-Dawley rats were obtained from Sasco (Wilmington, Mass.). Basal Medium Eagle (BME) and sodium bicarbonate were purchased from Life Technologies (Grand Island, N.Y.). Glutamine, poly-*L*-lysine (MW >300 kd), monosodium glutamate, β-NADH, sodium pyruvate, taurine and heat-inactivated fetal bovine serum were obtained from Sigma (St. Louis, Mo.). [^{3}H]-glutamate (Glu; 1 mCi/ml) and [^{3}H]-taurine (1 mCi/ml) were purchased from American Radiolabeled Chemicals (St. Louis, Mo.). [^{3}H]-muscimol (1 mCi/ml) was obtained from Dupont NEN Research Products (Boston, Mass.). Fluorescent dyes (Fura 2-AM and Calcium Green-AM) were purchased from Molecular Probes (Eugene, Oreg.).

Synthesis of AC

AC was synthesized by acetylation of homotaurine with acetic anhydride. AC thus obtained was characterized as N-acetyl-homotaurine primarily by NMR analysis.

Preparation of Neuronal Cultures

Primary neuronal cultures were prepared from fetal rat brains as previously described [9]. Procedures for all rats used in this study were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care Unit and Use Committee of the University of Kansas. The rats were housed in the animal care facility at the University of Kansas and bred weekly. Briefly, brains dissected from fetal rats were mechanically dissociated in BME (Gibco BRL) supplemented with 7.6 m*M* sodium bicarbonate, 26.8 m*M* glucose, 2 m*M* glutamine, and 20% heat-inactivated fetal bovine serum. This medium was referred to as GME. The dissociated cells were plated in either 24-well plates (1 ml/well), 35-mm tissue culture dishes or on 20-mm circular glass coverslip dishes (2 ml/dish), precoated with 5 mg/ml poly-*L*-lysine (MW >300 kd). After incubation in a humidified incubator (37°C, 5% CO_2) for 1–2 h, the incubation medium was then replaced with serum-free GME. Previously, it has been shown that neurons grown under similar conditions are morphologically and physiologically mature after 14 days in vitro (DIV) [9]. Furthermore, these cultures contain about 80–85% neurons as estimated by immunohistochemical staining using antibodies against neurofilament protein [9].

Treatment of Neuronal Cultures with Glutamate or AC

L-Glu-induced neurotoxicity was studied in neuronal cultures at 14 DIV as described previously [5]. Prior to Glu treatment, the original culture media were replaced with fresh serum-free GME and the cultures were equilibrated in the incubator for 2 h. The experiments were carried out at room temperature in an environmental hood. For Glu treatment, cultured neurons were stimulated with 0.25 m*M* Glu for 5 min. The stimulation was terminated by removal of the Glu-containing medium. Cultures were further washed twice and incubated with serum-free GME for 20–24 h to allow the process of neuronal injury to be completed. For AC treatment, the conditions were the same as those described above for the Glu treatment except Glu was replaced with various concentrations of AC. When AC was tested on Glu-induced neurotoxicity, cultured neurons were preincubated with AC for 10 min before Glu treatment and AC was present throughout the Glu treatment at concentrations indicated.

Determination of Neuronal Damage by Lactate Dehydrogenase Assay

Neuronal damage was determined based on the release of lactate dehydrogenase (LDH) due to neuronal injury [8]. The preparation of cell suspensions and the measurement of LDH were the same as described [5]. Change of absorbance at 340 nm upon addition of β-NADH was used for the determination of LDH activities. Release of LDH was expressed as the percentage of the amount of LDH activity in the medium to the total LDH activity. Total LDH activity is the sum of the LDH activity in the medium and the activity remaining in the cell. The percentage of LDH released was expressed as follows:

$$\text{LDH release (\%)} = \frac{\text{LDH media}}{\text{LDH media + LDH cells}} \times 100\%$$

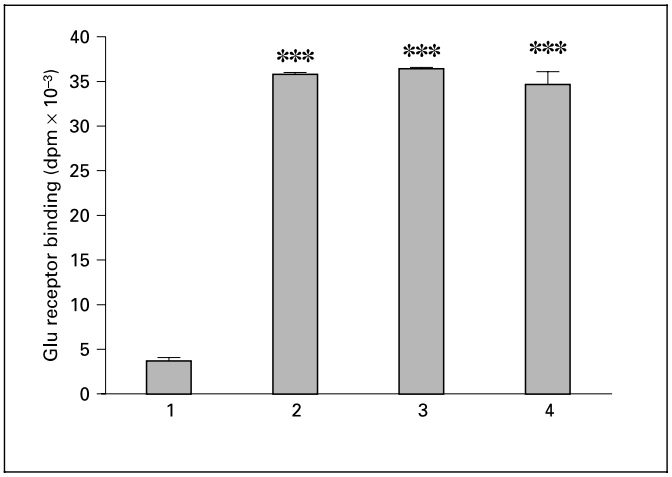
Glutamate Receptor Binding Assay

The preparation of synaptosomal membranes and the condition for Glu receptor binding assays were conducted according to Lee et al. [10] using [^{3}H]-Glu as the ligand. Briefly, male Swiss Webster mice were decapitated and the whole brain was removed and homogenized in a 10 m*M* Tris-HCl, pH 7.4 buffer containing 0.32 *M* sucrose and protease inhibitors (0.1 m*M* benzamidine HCl; 0.3 m*M* PMSF; 10 m*M* ACA; 0.1 m*M* EGTA). Synaptosomal fraction was obtained by differential centrifugation, followed by hypoosmotic lysis and high speed centrifugation to obtain the synaptosomal membranes (P_2 pellet).

Prior to the binding assay, the P_2 pellet was thawed and washed twice with 0.05% Triton X-100 in water and three more times with 50 m*M* Tris-HCl buffer, pH 7.6. For the total binding (TB), the reaction mixture contained about 100–150 mg protein of washed membranes, 15–20 n*M* [^{3}H]-Glu in 1 ml of 50 m*M* Tris-HCl buffer, pH 7.2. After 45 min incubation at room temperature, the reaction was terminated by brief centrifugation. The pellet was briefly washed twice before counting for radioactivity. For nonspecific binding (NB), the conditions were the same as those described above except that the membranes were preincubated with 1 m*M* unlabeled Glu for 10 min prior to the addition of [^{3}H]-Glu. In a typical assay, the ratio of TB to NB is about 5. The specific binding is the difference between TB and NB.

GABA Receptor Binding Assay

$GABA_A$ receptor binding assays were conducted as described [26]. [^{3}H]-muscimol was used as the ligand. The synaptosomal membranes used were prepared and washed as described above for the Glu receptor binding assay.



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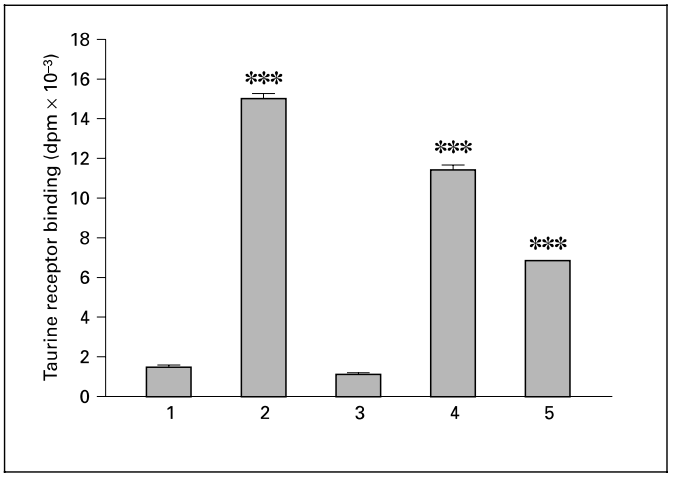

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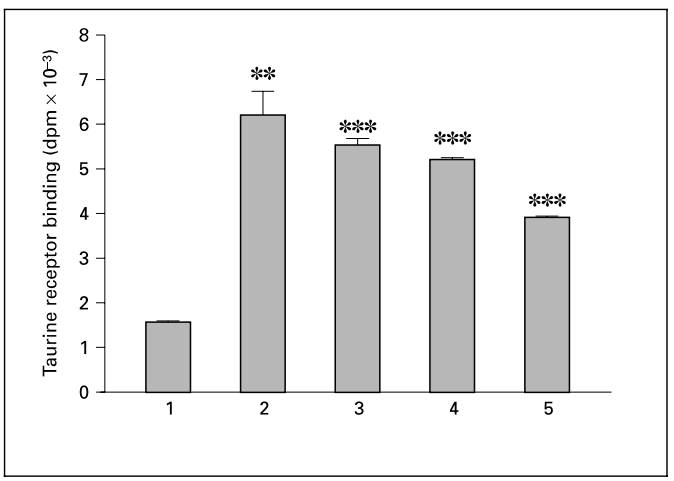

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Fig. 1. Effect of homotaurine and AC on Glu receptor binding. Glu receptor binding assays were conducted as described [10]. Glu receptor binding activity was expressed as [^{3}H]-Glu bound in dpm. 1 = NB; 2 = TB; 3 = binding in the presence of 10^{-4} *M* homotaurine; 4 = binding in the presence of 10^{-4} *M* AC. *** $p < 0.001$.

Fig. 2. Effect of homotaurine and AC on taurine receptor binding. The preparation of synaptosomal membranes for taurine receptor binding and the procedure for taurine receptor binding assays were carried out as described [25] using [^{3}H]-taurine as ligand. 1 = NB; 2 = TB; 3 = in the presence of 10^{-5} *M* homotaurine; 4 = in the presence of 10^{-6} *M* AC; 5 = in the presence of 10^{-4} *M* AC. *** $p < 0.001$.

Fig. 3. Effect of GTP on taurine receptor binding. The conditions used for taurine receptor binding were the same as described in figure 2. 1 = NB; 2 = TB; 3 = in the presence of 10^{-6} *M* GTP; 4 = in the presence of 10^{-5} *M* GTP; 5 = in the presence of 10^{-4} *M* GTP. ** $p < 0.01$; *** $p < 0.001$.

Taurine Receptor Binding Assay

Binding assays for taurine receptor were performed as described previously [25]. Briefly, the conditions described for [^{3}H]-Glu binding were followed with the following modifications. For taurine receptors, the washed synaptosomal membranes were further washed 4 times with 50 m*M* Tris-HCl, pH 7.6 containing 0.01% Triton X-100. The radioligand used was [^{3}H]-taurine (30 n*M*). The NB for the taurine receptors were obtained by incubation of washed synaptosomal membranes with 1 m*M* taurine prior to addition of [^{3}H]-taurine.

Measurement of Intracellular Free Calcium Concentration

Intracellular free calcium, $[Ca^{2+}]_i$, was measured with cultured neurons that were plated on 20-mm glass coverslips held by 35-mm culture dishes. After 14 DIV, the original media were replaced with 2 ml of EBSS media (Earl's buffered saline solution: 116.4 m*M* NaCl, 5.4 m*M* KCl, 0.8 m*M* $MgSO_4$, 1.0 m*M* NaH_2PO_4, 26.2 m*M* $NaHCO_3$, 1.8 m*M* $CaCl_2$) and cultures were equilibrated in the incubator for 1–2 h prior to loading of fluorescent dye. Calcium Green-AM was loaded at about 2–5 m*M* and the cultures were further incubated for about 20 min. The reaction was terminated by removal of excess dye followed by brief washes with EBSS.

$[Ca^{2+}]_i$ was measured using an MRC-1000 laser scanning confocal imaging system. Fluorescent emission signals following excitation at 488 nm were analyzed and pseudocolors corresponding to the intensity of fluorescence were added using the Confocal Assistance Program.

Results

Effect of Homotaurine and AC on Glutamate Receptor Binding Activity

Glutamate receptor binding activity was measured using [^{3}H]-Glu as the ligand. It was found that both homotaurine and its N-acetyl derivative, AC, have no effect on the binding of [^{3}H]-Glu to Glu receptor, even at a concentration of 100 m*M* (fig. 1). The ratio of TB to NB is about 8.

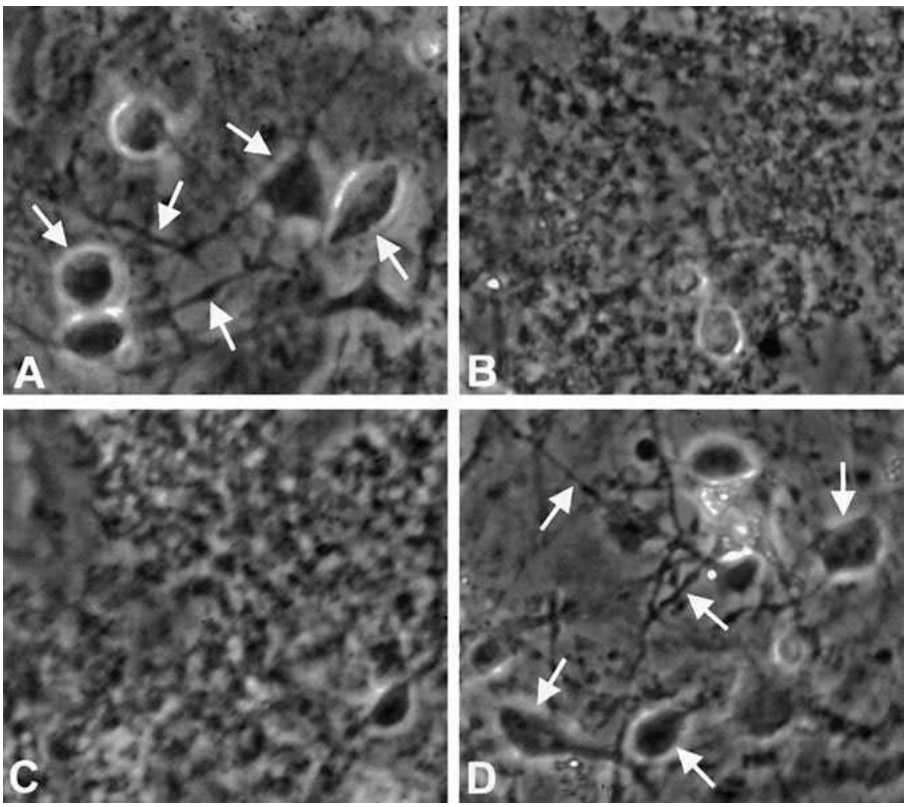


Fig. 4. Effect of AC on cultured neurons: morphological observation. Cultured neurons at 14 DIV were treated with either glutamate or AC as described in Materials and Methods. **A** Control group. **B** Treated with 0.25 m*M* Glu. **C** Treated with 1 m*M* AC. **D** Treated with 10 m*M* AC in the presence of 25 m*M* taurine. Arrows indicate healthy neuronal cell bodies and processes in the control group (**A**) and taurine-protected group (**D**).

Effect of Homotaurine and AC on Taurine Receptor Binding Activity

Taurine receptor binding activity was measured using [^{3}H]-taurine as the ligand. Homotaurine was found to be a very potent inhibitor inhibiting 100% of the binding of [^{3}H]-taurine to the taurine receptor at 10^{-5} *M*. Taurine receptor binding was also inhibited by AC to an extent of 30 and 70% at 10^{-6} and 10^{-4} *M*, respectively (fig. 2) suggesting that AC may exert its effect through its interaction with taurine receptors.

Specificity of Taurine Receptors

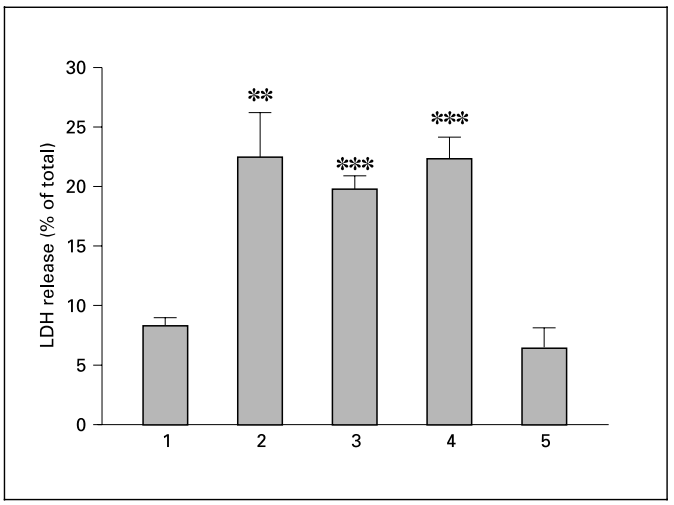
Taurine receptors were found to be highly specific for taurine since all of the 20 naturally occurring *L*-amino acids were found to have no effect on taurine receptor binding at 10^{-4} *M*. Furthermore, all the common agonists or antagonists of major amino acid neurotransmitter receptors such as glutamate, kainic acid, NMDA, quisqualic acid and kynurenic acid for the Glu receptor system, glycine and strychnine for the glycine receptor system, FNZP, GABA, bicuculline, picrotoxin, baclofen and phaclofen for the GABA receptor system all have little effect on taurine receptor binding at 10^{-4} *M*.

Effect of GTP on Taurine Receptor Binding

To test whether taurine may also bind to G-protein-coupled metabotropic receptors, taurine receptor binding was conducted in the presence of GTP. It is interesting that taurine receptor binding is inhibited by GTP in a dose-dependent manner inhibiting to an extent of 16, 25 and 54% at 10^{-6}, 10^{-5} and 10^{-4} *M*, respectively (fig. 3) suggesting that taurine may bind to both ionotropic and metabotropic taurine receptors.

Effect of AC on Cultured Neurons and on Glutamate-Induced Neuronal Damage

Culture neurons in the control group showed typical neuronal morphologies with numerous well-developed neuronal processes at 14 DIV (fig. 4A). In the treated groups, brief exposure of cultured neurons to either 0.25 m*M* Glu (fig. 4B) or 1 m*M* AC (fig. 4C) was sufficient to cause marked neuronal damage as indicated from morphological observations in which irregular cell bodies and fragmented processes were observed (fig. 4B, C). In the group treated with 10 m*M* AC in the presence of 25 m*M* taurine, the neuronal morphology appears to be well preserved (fig. 4D), suggesting that taurine may have a protective effects against AC-induced neuronal injury. In



addition to morphological examination, similar results were obtained from LDH assays. Glu at 0.25 m*M* and AC at 1 m*M* increased LDH release from the control level, 8% of total, to 23 and 20% of total, respectively (fig. 5). Furthermore, LDH release induced by 10 m*M* AC was reduced to even below the level of the control in the presence of 25 m*M* taurine (fig. 5). It is interesting that no fur-

Fig. 5. Effect of AC on cultured neurons in the presence or absence of taurine: LDH assay. Neuronal damage was determined by release of LDH from the damaged neurons. The amount of LDH released was expressed as described in Materials and Methods. 1 = Control group; 2 = treated with 0.25 m*M* glutamate; 3 = treated with 1 m*M* AC; 4 = treated with 0.25 m*M* Glu plus 1 m*M* AC; 5 = treated with 10 m*M* AC in the presence of 25 m*M* taurine. ** $p < 0.01$; *** $p < 0.001$.

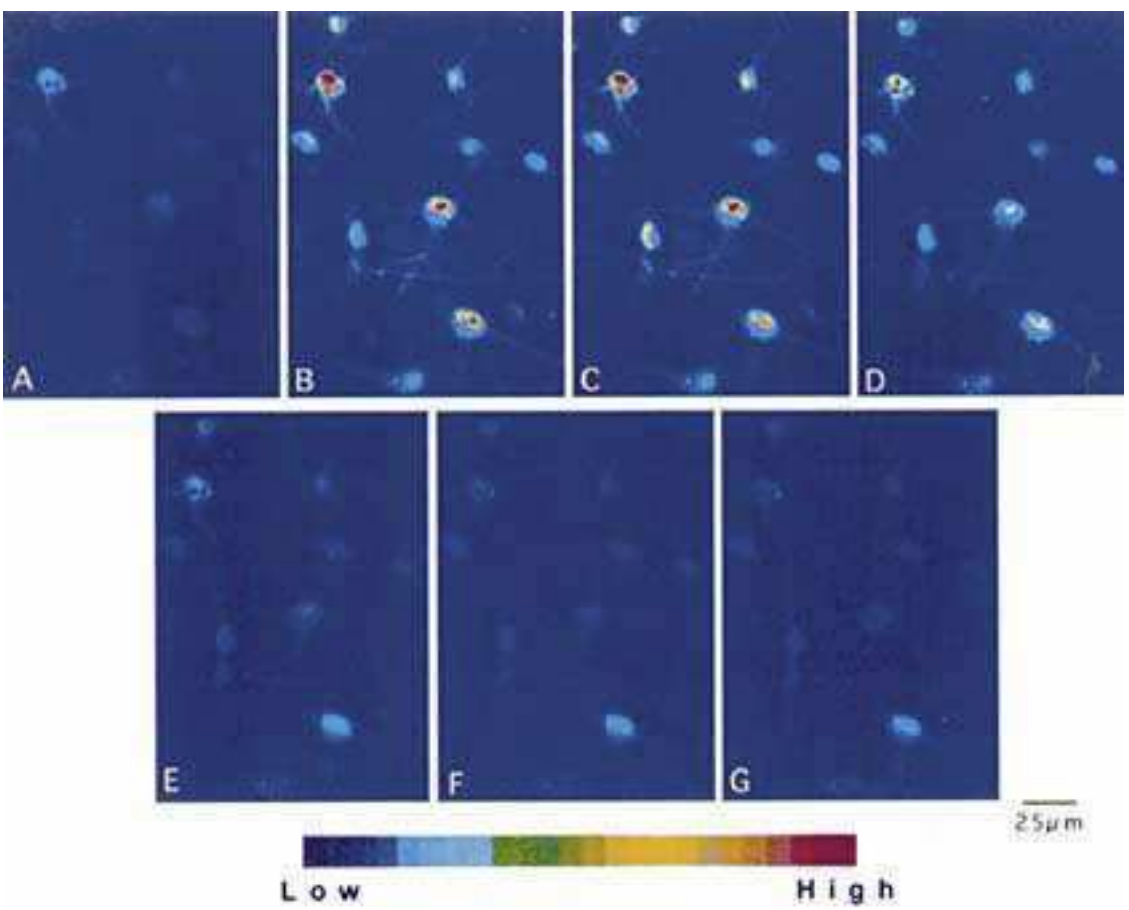


Fig. 6. Elevation of $[Ca^{2+}]_i$ by Li-AC and its inhibition by taurine. Cultured neurons (14 DIV) on coverslips were used for confocal image experiments. Before calcium dyes (Calcium Green-AM) were loaded, the original medium (GME) was removed and neurons were equilibrated in the EBSS medium for 2 h. After 20 min incubation with calcium dyes, neurons were treated with 1 m*M* Li-AC and the images were recorded. The intensity of the fluorescence in response to $[Ca^{2+}]_i$ level was monitored with a laser confocal microscope. **A–D** Intracellular Ca^{2+} level recorded at 0, 30, 60 and 120 s after the addition of 1 m*M* Li-AC. **E–G** The same cultured neurons as **A–D** were further treated with taurine and the $[Ca^{2+}]_i$ were recorded at 30, 60 and 120 s after the addition of 25 m*M* taurine. The color coding is included showing blue being the lowest and red the highest $[Ca^{2+}]_i$ concentration.

ther neuronal damage was observed by a combination of 1 m*M* AC and 0.25 m*M* Glu (fig. 5), indicating that either maximum neuronal damage has already been achieved under these conditions or both Glu and AC exert their neurotoxic effect on the same group of neurons.

Effect of AC on $[Ca^{2+}]_i$

To test the effect of AC on $[Ca^{2+}]_i$, two different salt forms of AC, namely, N-acetyl-homotaurine lithium salt (Li-AC) and N-acetyl-homotaurine calcium salt (Ca-AC), were used. Both Li-AC and Ca-AC were found to elevate $[Ca^{2+}]_i$ markedly although some neurons were more sensitive to AC than others (fig. 6B–D, 7G–I). Li-AC is more potent than Ca-AC in inducing an increase of $[Ca^{2+}]_i$ in neurons since 1 m*M* of Li-AC was sufficient to induce an elevation of $[Ca^{2+}]_i$ rapidly and markedly (fig. 6B–D) whereas Ca-AC at the same concentration appeared to have little effect on $[Ca^{2+}]_i$ in the same time intervals, namely 30–90 s (fig. 7D–F). However, at a higher concentration, e.g. 10 m*M*, Ca-AC could induce a rapid ele-

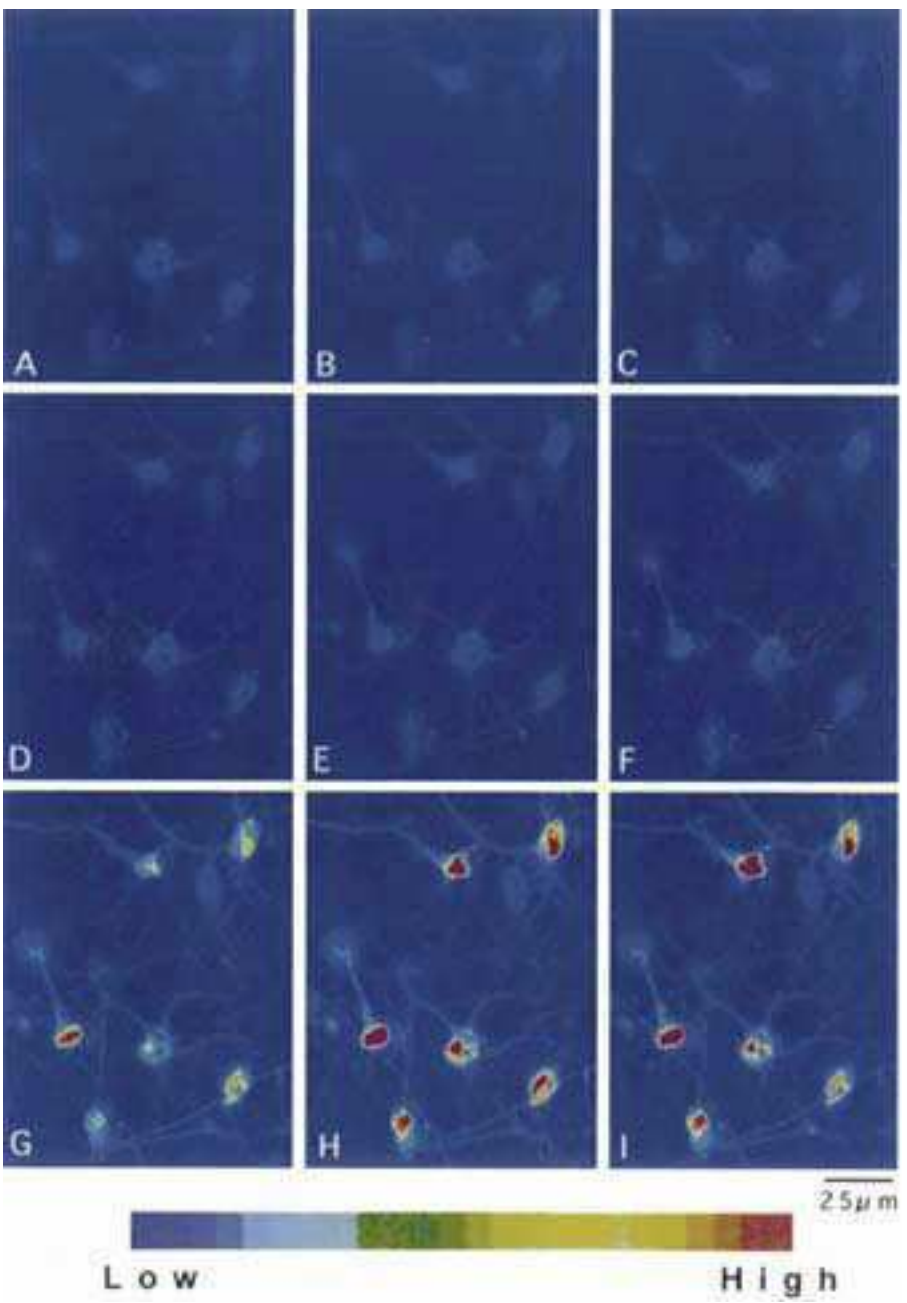


Fig. 7. Effect of Ca-AC on $[Ca^{2+}]_i$ level of cultured neurons. The conditions were the same as described in figure 6 except that Li-AC was replaced with calcium salt. **A–C** Intracellular Ca^{2+} level recorded at 0, 30 and 60 s after the addition of 0.1 m*M* AC. **D–F** Additional 1 m*M* AC was added and the images were recorded at 30-, 60- and 120-second intervals. **G–I** Further addition of 10 m*M* AC was made and the images were recorded at 30-, 60- and 120-second intervals. The color coding is included indicating blue being the lowest and red the highest $[Ca^{2+}]_i$ concentration.

vation of $[Ca^{2+}]$ at 30-, 60- and 120-second intervals (fig. 7G–I).

Effect of Taurine on AC-Induced Elevation of $[Ca^{2+}]_i$

Previously, we have shown that Glu-induced elevation of $[Ca^{2+}]_i$ was effectively reduced by taurine at 25 m*M* [24]. Interestingly, AC-induced elevation of $[Ca^{2+}]_i$ is also sensitive to taurine. Upon addition of taurine at 25 m*M*, the AC-induced increase of $[Ca^{2+}]_i$ was rapidly lowered to the control level within 30 s as indicated by a marked reduction in the intensity of Ca^{2+}-sensitive fluorescence (fig. 6E–G).

Discussion

Although extensive clinical studies have been conducted with AC, the molecular basis for its action remains speculative. It has been suggested that AC may exert its effects through its interaction with the GABA [2, 4, 13] or the EAA systems [12, 20, 27]. More recent data seem to suggest that the glutamatergic system, particularly the NMDA system, appears to play a major role in the action of AC. AC has been shown to interact with the NMDA receptor-mediated neurotransmission in various brain regions and to reduce Ca^{2+} flux through voltage-gated channels [20]. AC has also been shown to reduce postsynaptic efficacy of EAA neurotransmitters and to lower neuronal excitability in the neocortex of the rat [27]. However, AC was found to increase the evoked NMDA component of glutamatergic transmission in rat hippocampal CA1 neurons as well as the NMDA excitatory postsynaptic potentials in the nucleus accumbens, a structure believed to play a role in drug reinforcement [12].

The GABA theory for the action of AC is partially based on the fact that AC is structurally similar to GABA and may act as a GABA agonist. However, in this communication, we have shown that AC is a potent inhibitor for the taurine receptor. AC inhibited greater than 80% of $[^3H]$-taurine binding to the taurine receptor at 10^{-4} *M* concentration, whereas, at the same concentration, AC did not inhibit $[^3H]$-muscimol binding to the $GABA_A$ receptor, nor the binding of $[^3H]$-Glu to the Glu receptor. These findings may suggest that AC, which is a taurine analog, may exert its effect primarily through the taurine system instead of the GABA or the Glu system.

It is interesting that similar to Glu, AC is a potent neurotoxin inducing massive neuronal damage at 1 m*M*. The underlying mechanism of AC-induced neuronal damage appears to be due to its action in increasing $[Ca^{2+}]_i$, a mechanism which is also shared by Glu-induced excitotoxicity. Furthermore, AC-induced elevation of $[Ca^{2+}]_i$ is reduced to almost the control level by taurine. Previously, we have shown that taurine could protect Glu-induced neuronal damage through its action to prevent or to lower Glu-induced increase of $[Ca^{2+}]_i$ [24]. We have further shown that the mode of action of taurine in maintaining calcium homeostasis is through its inhibition of the reverse mode of Na^+/Ca^{2+} exchanger and its inhibition of the release of Ca^{2+} from the intracellular internal storage pools. The latter process is presumably due to the inhibition of phospholipase C activity by negative coupling of metabotropic taurine receptor to phospholipase C system. The decrease in phospholipase C activity will result in reduced formation of IP_3 and IP_3-gated release of Ca^{2+} from the internal Ca^{2+} storage pools. The inhibition of taurine receptor binding by GTP in a dose-dependent manner suggests the presence of metabotropic taurine receptor. This is analogous to the GABA system in which the binding of $[^3H]$-GABA to the metabotropic GABA receptors, namely, the $GABA_B$ receptors, is inhibited by GTP [7]. Naturally, further characterizations of taurine receptors are needed before one can fully understand their functions, especially regarding their role in alcohol craving and alcohol withdrawal-related disorders.

Acknowledgments

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S-Methyl-N,N-Diethylthiocarbamate Sulfoxide Elicits Neuroprotective Effect against N-Methyl-*D*-Aspartate Receptor-Mediated Neurotoxicity

Nagendra S. Ningaraj[a] Weiqing Chen[b] John V. Schloss[c]
Morris D. Faiman[a] Jang-Yen Wu[b]

Departments of [a]Pharmacology and Toxicology, [b]Molecular Biosciences and [c]Medicinal Chemistry, University of Kansas, Lawrence, Kans., USA

Key Words

S-methyl-N,N-diethylthiocarbamate sulfoxide · Disulfiram · N-methyl-*D*-aspartate receptor · Alcoholism · Neurotoxicity · Anticraving agent

Abstract

Glutamatergic neurotransmission, particularly of the NMDA receptor type, has been implicated in the excitotoxic response to several external and internal stimuli. In the present investigation, we report that S-methyl-N,N-diethylthiocarbamate sulfoxide (DETC-MeSO) selectively and specifically blocks the NMDA receptor subtype of the glutamate receptors, and attenuates glutamate-induced neurotoxicity in rat-cultured primary neurons. Other major ionotropic glutamate receptor subtypes, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and kainate, were insensitive to DETC-MeSO both in vitro and in vivo. Disulfiram, the parent compound of DETC-MeSO, also inhibits glutamate receptors partially in vivo; however, it fails to inhibit glutamate receptors in mice pretreated with N-butyl imidazole, a cytochrome P450 enzyme inhibitor, implicating the need for bioactivation of disulfiram to be an effective antagonist. We showed that glutamate-induced increase in $^{45}Ca^{2+}$ was attenuated in rat-cultured primary neurons following pretreatment with DETC-MeSO. The Ca^{2+} influx into primary neurons, studied by confocal microscopy of the fluorescent Ca^{2+} dye fura-2, demonstrated a complete attenuation of NMDA-induced Ca^{2+} influx. Similarly, DETC-MeSO attenuated NMDA-induced $^{45}Ca^{2+}$ uptake. Glutamate-induced $^{45}Ca^{2+}$ uptake and Ca^{2+} influx, however, were partially blocked by DETC-MeSO, and this is consistent with both in vitro and in vivo studies in which DETC-MeSO partially blocked mouse brain glutamate receptors. In addition, DETC-MeSO pretreatment effectively prevented seizures in mice induced either by NMDA, ammonium acetate, or ethanol-induced kindling seizures, all of which are believed to be mediated by NMDA receptors. These data demonstrate that DETC-MeSO produces the neuroprotective effect through antagonism of NMDA receptors in vivo.

Introduction

Alcohol abuse and dependence is a pathological behavior caused by long-lasting adaptations in the brain reinforcement systems [13]. The clinical management of alcoholism has been restricted to the use of agents aimed at GABA receptors [18], although the outcome is subopti-

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Jang-Yen Wu
Department of Molecular Biosciences, 1043 Haworth Hall
University of Kansas, Lawrence, KS 66045 (USA)
Tel. +1 785 864 4559, Fax +1 785 864 5374
E-Mail Jywu@kuhub.cc.ukans.edu

mal. Alcohol withdrawal symptoms manifested by seizures persist for days despite an aggressive treatment regimen with GABAergic agents. The lack of an effective treatment directed at the GABAergic system raises the possibility that other neurotransmitter systems are important for the pathophysiology of alcoholism. The present literature indicates that alcohol specifically and selectively affects the function of glutamate receptor-gated ion channels [34, 35], in particular N-methyl-*D*-aspartate (NMDA) receptor-gated ion channels [20, 33]. The NMDA receptors are both ligand- and voltage-dependent containing ion channels that are highly permeable to calcium ions. Long-term ethanol consumption elicits a temporary upregulation of the numbers of NMDA receptors in rodent and humans. Moreover, sudden withdrawal of ethanol results in withdrawal hyperexcitability (manifested as tremors and withdrawal seizures), possibly due to increased glutamate effects on NMDA receptors, which increases Ca^{2+} ion influx into the neurons [6, 16], and as expected, NMDA receptor activation has been directly implicated in the induction of alcohol withdrawal seizures [32]. However, the non-NMDA glutamate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate (KA) receptors, are less affected by pharmacologically relevant concentrations of alcohol [4].

Although, disulfiram (DSF) has been clinically used to treat alcoholism for more than 5 decades [5], in the last 5 years, studies have been published on anti-craving compounds as new pharmacological tools to treat alcoholism [30]. Acamprosate, the Ca^{2+} salt of N-acetyl-homotaurine, has been shown to reduce alcohol intake in rodents [14] and in humans [15]. The opioid receptor antagonist, naltrexone, also has been shown to reduce alcohol consumption [30]. Both acamprosate and naltrexone are thought to reduce the desire for alcohol or 'craving', while DSF is thought only to induce alcohol intolerance by inhibition of liver mitochondrial aldehyde dehydrogenase isoenzyme II [29]. Acamprosate may interact with NMDA or taurine receptor-mediated neurotransmission in various brain regions, and naltrexone may interfere with alcohol-induced reinforcement via the blockage of opioid receptors [30]. The physiology of alcohol is thought to be mediated, to a substantial extent, by its antagonistic effect on the glycine binding site of the NMDA receptors [34, 35]. Recently, it has been shown that S-methyl-N,N-diethyldithiocarbamate sulfoxide (DETC-MeSO), the active metabolite of DSF, has an antagonistic effect on brain glutamate receptors in mice [26]. It is conceivable that the clinical efficacy of DSF in the treatment of alcoholism is due, at least in part, to its effect on glutamate receptors. The effect of DETC-MeSO on glutamate receptors may also explain the synergistic effect of acamprosate in combination with DSF in a recently published clinical study [36]).

Studies from our laboratory showed that DSF must be bioactivated to form a metabolite believed to be responsible for $ADLH_2$ inhibition [8]. It was also shown that DSF elicits antialcohol effect in vivo only after cytochrome P450 enzymes mediated bioactivation to its active metabolite, DETC-MeSO [21]. We have shown that DETC-MeSO is a potent and selective carbamoylating agent for glutamate receptors [26], and glutathione [27]. We recently reported that DETC-MeSO partially blocks glutamate binding to synaptic membrane preparation from the brains of mice, and in addition DETC-MeSO prevents seizures in mice induced by glutamate analogs and hyperbaric oxygen [26]. Furthermore, we also reported that N-substituted S-carbamoylating agents block glutamate binding to glutamate receptors both in vitro and in vivo with varying efficacy [19].

The seizures, whether induced by hyperbaric oxygen, ethanol withdrawal or epilepsy, are complex processes involving several neuronal systems, including glutamatergic neurons. In the absence of a specific etiological understanding in any of the seizures or epileptic seizures, the therapeutic intervention must be directed at controlling symptoms, i.e. the attenuation of seizures [2]. Several new anticonvulsant drugs act by inhibition of glutamatergic excitation, specifically NMDA receptors. For example, NMDA receptor antagonists such as the noncompetitive antagonist D-CPPene [3-(2-carboxypiperazin-4-yl)propenyl-1-phosphonate] are shown to be effective anticonvulsants. Furthermore, the NMDA antagonists can block ethanol withdrawal seizures [6, 16, 24]. Most recently, the NR2B subunit containing NMDA receptors has been shown to be upregulated in mice after they were withdrawn from alcohol dependence, implicating NMDA receptors in alcoholism [28]. Moreover, MK-801 has been shown to attenuate kindling seizures caused by ethanol withdrawal in mice [25].

The present studies were performed to investigate whether or not a DSF-induced effect on glutamate receptors in vivo is caused by cytochrome P450-mediated bioactivation to DETC-MeSO. Our previous studies have shown that DETC-MeSO partially blocks glutamate receptors in vitro and in vivo. To investigate how DETC-MeSO only inhibits glutamate receptors partially in vitro and in vivo as reported by us earlier, we performed specificity studies on glutamate receptor subtypes. The anticonvulsive effect of DETC-MeSO on seizures induced by

NMDA, ammonia and alcohol withdrawal in mice was investigated. Furthermore, we studied the effect of DETC-MeSO on rat primary cultured neurons by monitoring the influx of $^{45}Ca^{2+}$ and intracellular Ca^{2+} concentration, primarily operated by NMDA-linked calcium channels.

Materials and Methods

Synthesis

DETC-MeSO was synthesized and the structure of DETC-MeSO verified by 1H NMR and FAB-MS/MS as described by Hart and Faiman [8]. All other chemicals including NMDA, KA and AMPA were either obtained from Sigma Chemical Co. (St. Louis, Mo.) or Aldrich (Milwaukee, Wisc.). The radioligands such as [^{3}H]-glutamate, [^{3}H]-CPP, [^{3}H]-KA and [^{3}H]-AMPA were purchased from Amersham Life Science (Buckinghamshire, UK).

Binding Studies

Synaptic membranes were isolated as described by Nagendra et al. [26] from whole brain homogenate of male Swiss Webster mice. The binding studies to determine the activity of glutamate receptor and glutamate receptor subtypes (NMDA, KA and AMPA) were carried out with [^{3}H]-glutamate, [^{3}H]-CPP, [^{3}H]-KA and [^{3}H]-AMPA, respectively. The equivalent of 100–150 μg protein of washed synaptic membrane was preincubated with DETC-MeSO, and in vitro and in vivo ligand binding assays were performed following the protocol described by us earlier [26].

Role of Cytochrome P450 Enzymes in the Effect of DSF and DETC-MeSO on Glutamate Receptor

To investigate the role of cytochrome P450 enzymes in the bioactivation of DSF to DETC-MeSO and its subsequent effect on glutamate binding to glutamate receptors, the mice (n = 5) were pretreated with 20 mg/kg i.p. N-butyl imidazole (NBI), a general cytochrome P450 enzyme inhibitor. Thirty minutes later, DSF (100 mg/kg i.p.) was administered to mice pretreated with NBI and 4 h later the brains were removed for isolation of synaptic membrane and determination of glutamate receptor activity. Other groups of mice were treated either with vehicle or DETC-MeSO (5.2 mg/kg i.p.), and 2 h later brains removed for synaptic membrane preparation and glutamate receptor binding study.

Animals and Seizure Studies

Male Swiss Webster and C57 BL mice (6–8 weeks old, 20–30 g) were used for the studies. Mice were maintained on a 12-hour light-dark cycle with access to chow and water ad libitum. The night before the experiment, only food was removed from the cages. All experiments that employed animals were conducted in strict compliance with the NIH guidelines and institutional regulations concerning animal experimentation. Unless otherwise specified, the ability of DETC-MeSO to prevent seizures was tested by intraperitoneal injection of 5.2 mg/kg DETC-MeSO 1–2 h prior to NMDA (125 mg/kg i.p.), ammonium acetate (7 mmol/kg i.p.) or chronic ethanol treatment and withdrawal. Seizures (except for ethanol-induced kindling seizures upon handling) were characterized by partial hind leg paralysis, labored breathing, stiff tail, followed by clonic-tonic seizures. However, tremor, handling-elicited convulsion and death in certain cases characterized handling-induced seizures in untreated C57BL mice after ethanol withdrawal.

Ethanol Administration in Mice

Male C57BL mice were given free access to a liquid diet containing 10% alcohol in 2% glucose solution for 10 days of alcohol conditioning. The control group had access only to regular water with 2% glucose. After discontinuation of ethanol, the mice exhibited the alcohol withdrawal syndrome characterized by tremor, handling-elicited seizures and death. Those mice that exhibited these symptoms were used for the kindling seizure studies.

Assessment of Kindling Seizures

Kindling seizures induced by lifting the mouse by its tail were measured according to the scale described by other investigators [24, 25]. A score of 0–4 was assessed: 0 = little or no reaction to handling by tail, 1 = a mild reaction, 2 = initial hyperactivity, clonic-tonic seizures within 5 s of handling by tail, 3 = spontaneous clonic seizures, and 4 = death as a result of seizures. The treatment group was administered DETC-MeSO (5.2 mg/kg i.p.) 2 h prior to inducing kindling seizures in mice.

Neuroprotection Studies

The cultured primary neurons isolated from rat fetal brains were used to investigate the neuroprotective effect of DETC-MeSO in vitro. The preparation of primary neuronal culture from rat embryos and the analysis of glutamate/NMDA-induced neurotoxicity were performed as described by us earlier [3]. In brief, the monolayer neuronal culture was exposed to *L*-glutamate (0.25 m*M*) without or 30 min pretreatment with DETC-MeSO (100 μ*M*). Twenty-four hours later, the LDH released was spectrophotometrically determined. Using light microscopy, the morphological study with rat primary neuronal culture was performed by incubating the cells with glutamate (0.25 m*M*) without or with DETC-MeSO (100 μ*M*) pretreatment.

$^{45}Ca^{2+}$ Uptake in Cultured Primary Neurons

The uptake of $^{45}Ca^{2+}$ in cultured neurons was determined following the procedure of Takuma et al. [31]. Briefly, the cultured neurons were washed twice with 1 ml EBSS medium and preincubated with 0.5 ml of EBSS at 37°C. The basal $^{45}Ca^{2+}$ uptake measurement was obtained by changing the medium to 0.5 ml of EBSS containing 37 kBq/ml $^{45}Ca^{2+}$. The neuronal culture was incubated for an additional 2 min, and the uptake was terminated by removal of the medium followed by three washes with ice-cold 4 m*M* EGTA in 0.9% saline. The cells were solubilized in 0.3 *M* NaOH and radioactivity measured. An aliquot was used to determine the protein assay. The culture without or with DETC-MeSO (100 μ*M*) pretreatment was incubated either with 100 μ*M* NMDA or glutamate for 5 min, and total $^{45}Ca^{2+}$ uptake measured at various time points for 10 min. The control $^{45}Ca^{2+}$ influx studies were performed with either glutamate or NMDA alone.

Intracellular $[Ca^{2+}]_i$ in Cultured Primary Neurons

The change in $[Ca^{2+}]_i$ was determined using specific fluorescent dye, fura-2 (Molecular Probes, Eugene, Oreg.) as described by Grynkiewicz et al. [7]. Briefly, after replacing the original medium (BME), the glass coverslip with monolayer neuronal culture was placed in a

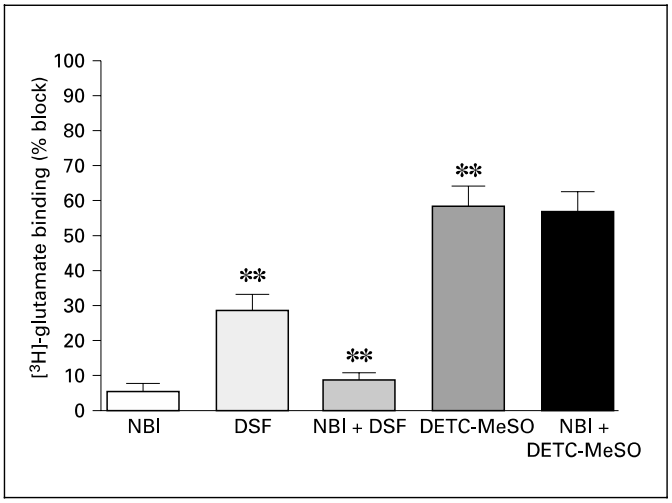


Fig. 1. Effect of DSF and DETC-MeSO on glutamate receptors in vivo in the absence or presence of NBI. In mice pretreated with NBI (20 mg/kg) 30 min prior to injection of DSF, the cytochrome P450-mediated formation of DETC-MeSO was blocked, thus preventing the DSF-induced glutamate receptor inhibition. However, NBI pretreatment did not have any effect on DETC-MeSO-induced glutamate receptor inhibition in vivo, suggesting that DETC-MeSO does not require cytochrome P450-mediated bioactivation. The values are % blockage of glutamate binding to glutamate receptor compared to the absolute control values which are means of n = 5 in triplicate. The percent inhibition, DSF vs. NBI, NBI + DSF vs. DSF only, and DETC-MeSO only vs. NBI have significant Bonferroni's ** $p < 0.001$ values determined by ANOVA.

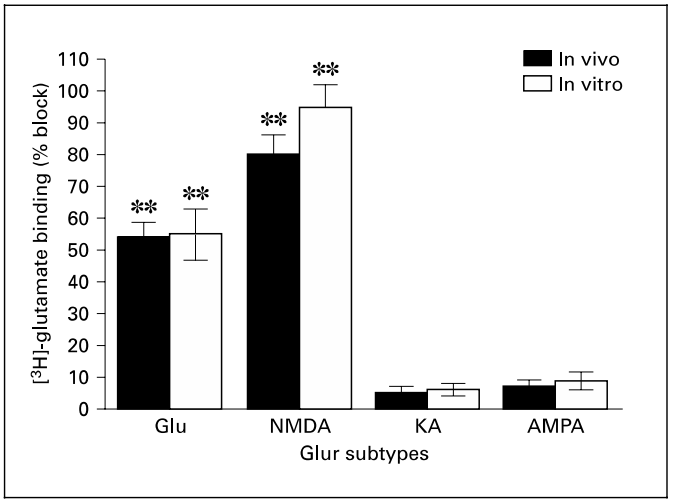


Fig. 2. Effect of DETC-MeSO on glutamate receptor (GluR) subtypes in vitro and in vivo. The synaptic membrane was incubated with DETC-MeSO (100 μM) for in vitro studies and mice were administered (5.2 mg/kg i.p.) and killed after 2 h for in vivo studies. The binding of specific radioligands, [^{3}H]-glutamate, [^{3}H]-CPP, [^{3}H]-KA and [^{3}H]-AMPA to glutamate, NMDA, KA and AMPA receptors, respectively, was determined with or without DETC-MeSO. For in vivo studies, the percent blockage values (mean ± SE of n = 5) are plotted. The in vitro data is the mean ± SE obtained from four samples performed in triplicate. The percent inhibition both in in vitro and in vivo studies is shown; untreated control vs. DETC-MeSO-treated mice have significant Bonferroni's ** $p < 0.001$ values determined by Student's t test.

perfusion chamber, washed twice at the rate of 1 ml/min with a loading buffer (EBSS). The cells were incubated with fura-2 for 30 min at 37 °C. The fluorescence as a result of the basal intake of the dye was recorded on a dual wavelength interfaced with a confocal microscope (SPEX, Edison, N.J.) setting at excitation and emission wavelengths of 346 and 386 nm, respectively.

Statistical Analysis

Evaluation of data for whole animal experiments with or without DETC-MeSO treatment was conducted with the program GraphPAD InStat from GraphPAD Software (San Diego, Calif.). Data were analyzed by either Student's t test or the unpaired two-tailed t test, ANOVA followed by a post hoc Bonferroni test, and data were considered significant at $p < 0.05$.

Results

Effect of DSF and DETC-MeSO on Brain Glutamate Receptors

Synaptic membranes were prepared from the brains of mice treated with either DSF (100 mg/kg i.p.) or DETC-MeSO (5.2 mg/kg i.p.) without or with NBI pretreatment, which effectively blocked cytochrome P450 enzymes required for DSF bioactivation to DETC-MeSO in vivo. The extent of [^{3}H]-glutamate binding to glutamate receptors was compared with similar preparations isolated from untreated mice, as illustrated in figure 1, which indicate that cytochrome P450 enzymes are required for the bioactivation of DSF to DETC-MeSO. In the presence of NBI, DSF failed to form DETC-MeSO and block glutamate receptors. NBI pretreatment, however, did not affect the antagonistic property of DETC-MeSO (fig. 1) on glutamate receptors. Furthermore, figure 2 showed the effect of DETC-MeSO on ionotropic glutamate receptor subtypes. When the radioligand binding studies were performed with synaptosomal membrane isolated from untreated (vehicle) and treated (DETC-MeSO; 5.2 mg/kg i.p.) mice brains, DETC-MeSO specifically blocked [^{3}H]-CPP binding to NMDA receptors. The nonspecific binding (radioligand bound in the presence of unlabeled glutamate, NMDA, KA and AMPA) averaged about 20–30% of total radioligand binding. It appears that DETC-MeSO at 100 μM (in vitro) and 5.2 mg/kg i.p. (in vivo) partially

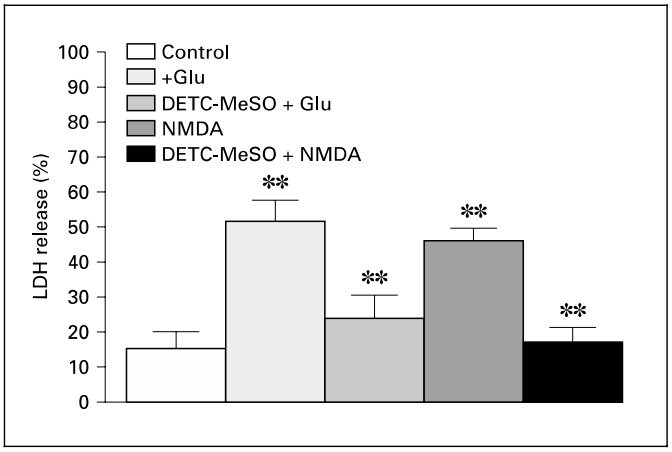


Fig. 3. Effect of DETC-MeSO on glutamate (Glu)/NMDA-induced neurotoxicity. The rat primary cultured neurons were pretreated with vehicle or DETC-MeSO (100 μ*M*) at 37 °C for 1 h prior to stimulation with either glutamate (0.25 m*M*) or NMDA (100 μ*M*), and incubated for another 30 min at 37 °C. The neurons were washed twice with the buffer and LDH released was measured after 24 h following the exposure to glutamate or NMDA by the spectrophotometric method in a multiplate reader. The pretreatment of neurons with DETC-MeSO attenuates glutamate-induced (about 50%), and NMDA-induced (greater than 70%) LDH release compared to untreated neurons. Each set is the mean ± SE of five experiments, each with its own control group. Values, Glu vs. control, DETC-MeSO + Glu vs. Glu only, NMDA vs. control, and DETC-MeSO + NMDA vs. NMDA are significantly different. Bonferroni's ** $p < 0.001$ values were determined by Student's t test.

Table 1. Effect of DETC-MeSO on seizures induced by convulsive agents in mice

Treatment	Vehicle-treated	DETC-MeSO-treated
NMDA (125 mg/kg i.p.)[a]	16 ± 3 (8/9)	>120 (0/9)
Ammonium acetate (7 mmol/kg i.p.)[a]	65 ± 10 (9/10)	>240 (2/10)
Ethanol (5% v/v for 10 days)[b]	3–4 (9/10)	0–1 (1/10)

DETC-MeSO (5.2 mg/kg i.p.) 1–2 h prior to inducing seizures in Swiss Webster mice (n = 9–10). The mean values for the time to the first clonic-tonic seizures are reported ± SE. The values in parentheses indicate the number of animals in that group (the number of animals to which value applies/total number of animals in the group). The comparison of the mean values between the untreated and DETC-MeSO-treated animals was performed by the two-tailed t test that gave $p < 0.001$. None of the mice in the DETC-MeSO-treated group (0/9) exhibited NMDA-induced seizures for the duration of the observed period (2 h). Only 2 out of 10 mice in the DETC-MeSO-treated group exhibited ammonium acetate-induced seizures for the duration of the observed period (4 h). However, a majority of the vehicle-treated mice exhibited seizure activity at the time shown above, but recovered completely after 180 min.

[a] Mean time (min) to clonic-tonic seizures is shown.

[b] Mice (C57BL) were administered DETC-MeSO (5.2 mg/kg i.p.) 1 h before initiating handling-induced seizures. Scale: 0 = little or no reaction to handling by tail; 1 = mild reaction; 2 = initial hyperactivity followed by clonic-tonic seizures within 5 s; 3 = spontaneous clonic seizures; 4 = death as a result of seizure.

blocks [^{3}H]-glutamate binding to glutamate receptor, and specifically and almost completely blocked [^{3}H]-CPP binding to NMDA receptors. However, the binding of [^{3}H]-AMPA and [^{3}H]-KA was unchanged indicating that both AMPA and KA receptors are insensitive to the drug (fig. 2). The results obtained for the group that received a single dose of DETC-MeSO and was sacrificed 2 h later were significantly (Bonferroni's $p < 0.01$ determined by ANOVA) different from the untreated group.

Antiseizure Effect of DETC-MeSO

Because DETC-MeSO blocks NMDA receptors, we investigated whether DETC-MeSO also prevents NMDA-mediated seizures. Two groups of Swiss Webster mice, one with DETC-MeSO pretreatment (5.2 mg/kg i.p., 2 h prior to induction of seizures) and the other without DETC-MeSO, were administered NMDA (125 mg/kg i.p.) or ammonium acetate (7 mmol/kg i.p.) to induce seizures. The mice pretreated with DETC-MeSO were effectively protected from both NMDA and ammonia-induced seizures (table 1). It has been shown that ethanol withdrawal seizures, including kindling seizures, are mediated by NMDA receptors. Therefore, we examined the effect of DETC-MeSO on handling-induced seizures in alcohol-conditioned mice. Similarly, pretreatment of ethanol-conditioned (10% v/v for 10 days) mice (C57BL) with DETC-MeSO (5.2 mg/kg i.p.) 2 h prior to the initiation of handling-induced kindling seizures dramatically prevented the intensity and severity of kindling seizures as compared to the untreated group (table 1). Nine out of 10 mice pretreated with DETC-MeSO did not exhibit kindling seizures upon handling (rated 0–1 on the arbitrary scale) as compared to the untreated group in which exactly the opposite effect was observed (9 out of 10 mice convulsed with a severity rating of 3–4), and 3 out of 10 mice died on account of clonic-tonic seizures.

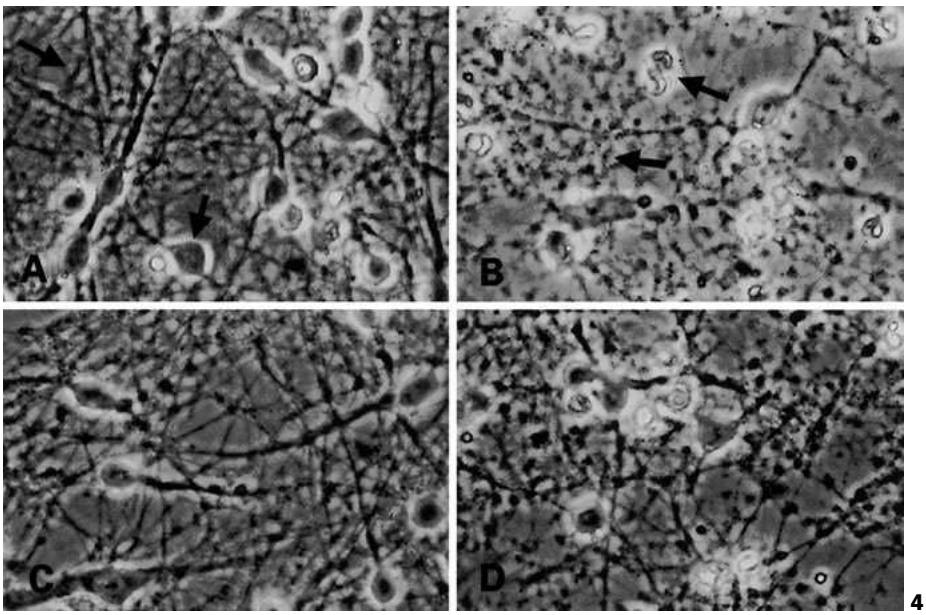


Fig. 4. Morphological analysis of rat primary neurons after stimulation with glutamate (0.25 m*M*) without or with pretreatment with DETC-MeSO. **A** Untreated neurons at 14 days show numerous cell bodies and extended processes (indicated by arrows). **B** After addition of glutamate, neurons show irregular cell morphology and damaged processes. **C** Neurons pretreated with DETC-MeSO alone show healthy morphology similar to untreated control, indicating that DETC-MeSO alone did not promote neuronal damage. **D** Neurons pretreated with DETC-MeSO prior to simulation with glutamate also show a healthy morphology similar to the control group.

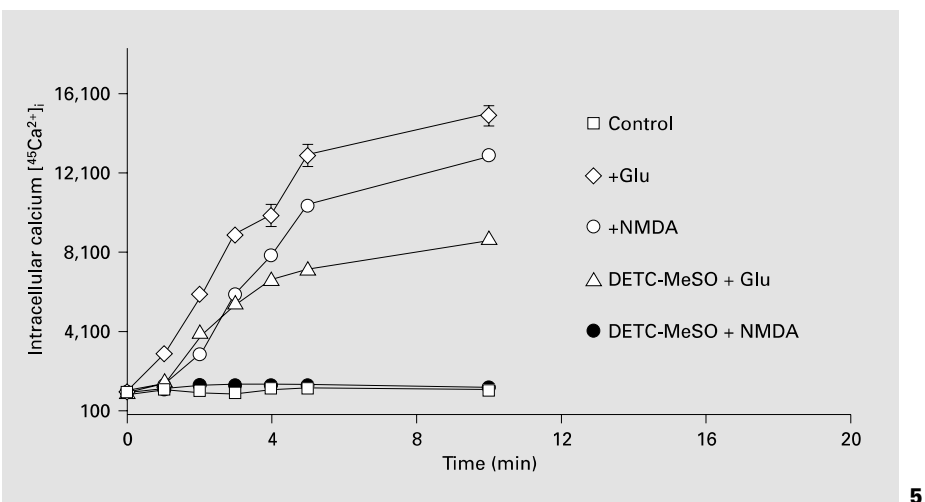


Fig. 5. Effect of DETC-MeSO on glutamate or NMDA-induced influx of $^{45}Ca^{2+}$ in rat primary cultured neurons. The cultured neurons were stimulated with either glutamate (0.25 m*M*) or NMDA (100 μ*M*) without or with DETC-MeSO pretreatment, to examine the Ca^{2+} channel-mediated $^{45}Ca^{2+}$ uptake. Both glutamate and NMDA elicited a significant ($p < 0.001$) increase in $^{45}Ca^{2+}$ uptake. Values (cpm) are mean ± SE of triplicate readings obtained separately in three experiments. The glutamate-induced $^{45}Ca^{2+}$ uptake was partially attenuated (about 50%), while NMDA-induced uptake was almost completely blocked by DETC-MeSO. This finding is consistent with the binding studies, in which DETC-MeSO partially blocks glutamate receptor, while completely blocking NMDA receptors.

DETC-MeSO Prevents Glutamate- and NMDA-Induced Neurotoxicity

The pretreatment of the monolayer primary neuronal cultures prepared from rat brain embryos with DETC-MeSO (100 μ*M*) protected the neurons from neurotoxicity induced by glutamate (0.25 m*M*) or NMDA (100 μ*M*) (fig. 3). The LDH release following the stimulation with glutamate and NMDA was attenuated by 50 and 70%, respectively, when the neurons were pretreated with DETC-MeSO (100 μ*M*). A partial neuroprotection against glutamate-induced neurotoxicity and a near complete protection against NMDA-induced neurotoxicity were observed (fig. 3). The amount of LDH leakage is indicative of the magnitude of the neurotoxicity. Furthermore, the morphological analysis of the DETC-MeSO (100 μ*M*) pretreated neurons after glutamate (0.25 m*M*) stimulation revealed that the neurons were partially protected compared to lack of protection in untreated neurons without DETC-MeSO pretreatment (fig. 4).

Effect of DETC-MeSO on Glutamate/NMDA-Induced $^{45}Ca^{2+}$ Uptake

A significant time dependent increase in $^{45}Ca^{2+}$ uptake was observed when 100 μ*M* glutamate or NMDA was added to the neurons. When the neurons, however, were pretreated with DETC-MeSO (100 μ*M*), the glutamate-induced increases in $^{45}Ca^{2+}$ uptake was partially blocked, while the NMDA-induced increase in $^{45}Ca^{2+}$ uptake was

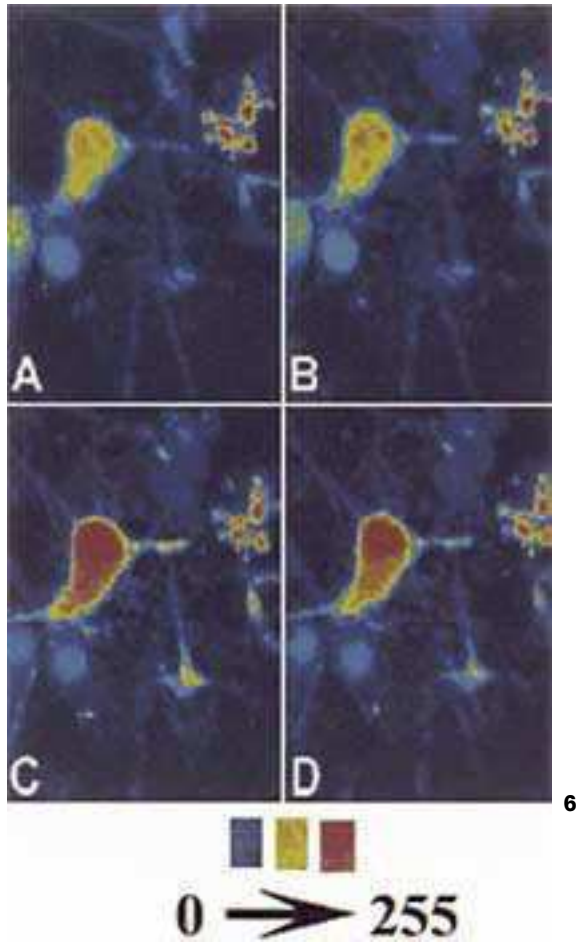
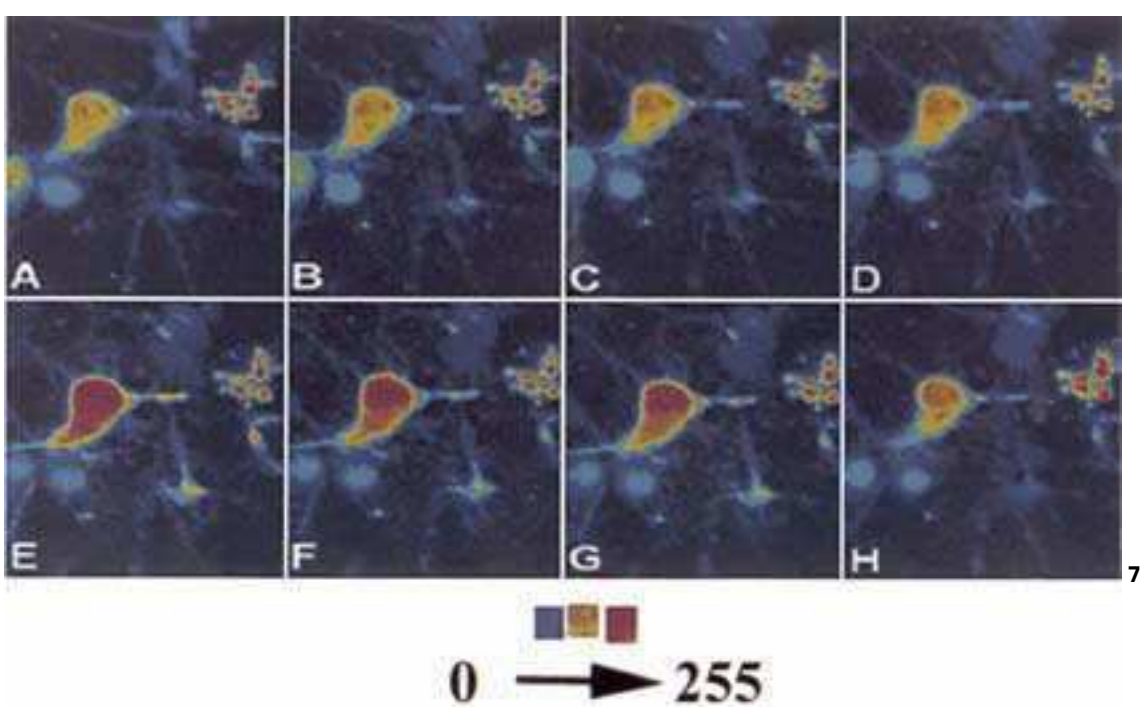


Fig. 6. Effect of DETC-MeSO on NMDA-induced elevation of intracellular calcium analyzed by confocal fluorometry. A single neuron of the rat cultured primary neurons showing the influx of calcium, as the dye fura-2 binds to free calcium. **A** Neuron in the presence of DETC-MeSO. **B** Neuron in the presence of DETC-MeSO showing appreciable attenuation of NMDA-induced Ca^{2+} influx. **C, D** Neuron in the presence of DETC-MeSO showing a low degree of attenuation of glutamate-induced Ca^{2+} influx. The intensity of the calcium-dye complex was compared on an arbitrary scale of 0–255.

Fig. 7. Confocal analysis of *L*-glutamate-induced calcium influx. A single neuron of the rat cultured primary neurons showing the influx of calcium, as the dye fura-2 binds to free calcium. **A** Basal $[Ca^{2+}]_i$ level of DETC-MeSO (10 μM) pretreated neurons. **B–D** Low level of $[Ca^{2+}]_i$ following NMDA addition. **E–H** Increased $[Ca^{2+}]_i$ level following *L*-glutamate addition. Notice that DETC-MeSO did not completely prevent the *L*-glutamate-induced calcium increase, but specifically prevented the NMDA-induced calcium increase. These results agree with the binding studies in which DETC-MeSO partially blocked glutamate receptors while completely blocking NMDA receptors. Thus calcium influx supposedly induced by AMPA and KA was not blocked by DETC-MeSO. The intensity of the calcium-dye complex was compared on an arbitrary scale of 0–255.



completely blocked (fig. 5). It appears that DETC-MeSO partially blocks glutamate receptors, while completely inhibiting NMDA receptors, which was consistent with the result obtained from ligand-binding studies.

DETC-MeSO on Calcium Influx in Primary Neurons

The influx of calcium was demonstrated in primary neurons after incubating the cells with fura-2 dye. The confocal microscopic observation of the cultured primary neurons indicated that DETC-MeSO completely blocked the influx of calcium induced by the addition of NMDA to the perfusion (fig. 6). DETC-MeSO, however, did not completely block glutamate-induced calcium influx (fig. 7), suggesting that it is a partial antagonist of glutamate receptor, and all the ionotropic receptor subtypes (NMDA, AMPA and KA) are not equally sensitive to DETC-MeSO.

Discussion

DSF has been currently used in the treatment of alcoholism, although the mechanism of its therapeutic action is still controversial, because some believe that DSF elicits an antialcohol effect by inducing an aversive response to ethanol intake, and others suggest that DSF has a CNS effect to reduce ethanol intake. Earlier, it was thought that DSF offers only aversive therapy and its anticraving components were never discussed. The anticraving component of DFS mediated by glutamate receptors was relatively unknown until we discovered its partial glutamate receptor antagonist property [26]. The alcohol dependence and craving involve several neural pathways and neurotransmitter systems. A lot of attention is focused on the realization that glutamatergic neurotransmission and NMDA receptors are critical components of the alcohol-CNS interaction [2]. One of the major problems with alcohol dependence and treatment is the alcohol withdrawal symptoms, manifested by seizures, once the alcohol is withdrawn. The current treatment protocol to manage these symptoms is the use of GABAergic agents, which are not clearly successful, and devoid of side effects. Another alternative treatment regimen proposed is the use of NMDA receptor antagonists. It has been shown that NMDA antagonists, CGP 39551 [18] and MK-801 [28], could attenuate ethanol withdrawal seizures. This protective effect of NMDA antagonists [12] on seizures induced by withdrawal of alcohol has been established for glycine (L-701 324) and polyamine (eliprodil) binding sites of the NMDA receptor. In the present study, we demonstrate that kindling seizures developed in C57BL mice as a result of the discontinuation of alcohol and handling can be effectively prevented by pretreatment with DETC-MeSO (table 1). This is consistent with other studies which showed that NMDA antagonist, MK-801, effectively prevented handling induced seizures in mice after withdrawal from chronic alcohol exposure [28]. The origin of alcohol dependence, development of tolerance and alcohol withdrawal syndrome, including kindling seizures after chronic alcohol conditioning, is believed to be mediated by NMDA receptors [13]. The above notion is supported by the following observations: firstly, the NMDA receptor density has been shown to be increased in animals following chronic alcohol exposure [13, 32], secondly, the subunit of NMDA receptors, NR2D, was implicated in seizure development in kindling epilepsy [1], and more recently, the NR2B subunit was reported to be upregulated in the initial development of alcohol dependence [28], and thirdly, the extensive animal literature and preliminary data suggest that NMDA receptor antagonists are excellent targets to treat withdrawal syndromes caused by alcohol withdrawal [2].

In this study we report that DETC-MeSO selectively and effectively blocks NMDA receptor subtype, while AMPA and KA receptors are insensitive to DETC-MeSO, both in vitro and in vivo (fig. 2). This is consistent with our previous observation that DETC-MeSO only partially blocks glutamate receptors [28]. Furthermore, DSF also blocks glutamate receptors partially, probably due to the bioactivation of DSF to the active metabolite, DETC-MeSO. We demonstrated in the present study that DSF bioactivation is essential for the in vivo inhibition of glutamate receptors, because the blockage of cytochrome P450 enzymes with NBI prevents DSF bioactivation preventing the glutamate receptor blockage (fig. 1). This, however, was not true with DETC-MeSO, because it may not require the bioactivation by cytochrome P450 enzymes to block glutamate receptors in vivo (fig. 1).

Earlier, we proposed that DETC-MeSO in vivo may carbamoylate glutamate receptor (NMDA subtype) via carbamoyl glutathione moiety (DETC-GS) to modify the redox modulatory site of the NMDA receptors [28]. In this context, Lipton et al. [17] showed that nitroglycerin (NO^+ donor) nitrosylate glutamate receptor partially blocks the receptor in vivo, and may represent the endogenous process of inhibiting the glutamate receptors. The neuroprotective effect of DETC-MeSO against NMDA and oxygen-induced seizures has been explained on the basis of this mechanism [28]. In the present study, DETC-MeSO prolonged the time to seizures, induced by NMDA

or ammonia, in mice compared to the untreated group. The pretreatment with DETC-MeSO prolonged NMDA-induced seizures well beyond (>120 min) compared to the time taken (about 16 min) by untreated mice to convulse. In case of ammonia-induced seizures, DETC-MeSO prolonged the seizure time by 3.5-fold compared to the untreated group (table 1). Furthermore, the pretreatment of mice with DETC-MeSO alleviated the typical symptoms of ammonia toxicity, including partial hind leg paralysis, labored breathing, a stiff tail and eventually clonic-tonic seizures. Although 9 out of 10 untreated mice seized in 65 min, they all recovered from ammonia toxicity (table 1). In the DETC-MeSO- treated group, 2 mice showed mild signs of ammonia-induced toxicity, but recovered well. Our observation is consistent with recently reported studies in which an NMDA-mediated seizure caused by ammonia was blocked by MK-801 [9, 23]. The DETC-MeSO was also effective in blocking ammonia-induced seizures mediated by NMDA receptors. Moreover, the mice pretreated with S-substituted sulfoxide analogs did not undergo ammonia-induced seizures, because these analogs are also partial antagonists of glutamate receptors in vivo, although they are not effective in vitro inhibitors (unpubl. data).

It is demonstrated that NMDA receptors are specifically blocked by DETC-MeSO, while AMPA and KA receptors are not sensitive to the drug (fig. 2). This result explains, in part, why DETC-MeSO produces a partial neuroprotection against glutamate neurotoxicity in cultured primary neurons (fig. 3). Glutamate-induced neurotoxicity is mediated by its ionotropic receptors including NMDA, AMPA and KA receptors. The excessive stimulation of NMDA receptors, in particular, triggers Ca^{2+} influx through the Ca^{2+}-gated channels as well as the mobilization of Ca^{2+} from the internal stores. In the present studies, DETC-MeSO was found to effectively block the Ca^{2+} influx induced by NMDA (fig. 6), but was partially effective in blocking the glutamate-induced Ca^{2+} influx (fig. 6). This observation is consistent with the binding study data showing that DETC-MeSO specifically blocks NMDA receptor, while partially blocking glutamate receptors. The failure of DETC-MeSO to completely block Ca^{2+} influx induced by glutamate (fig. 7) can be explained by the fact that AMPA and KA receptors were insensitive to the drug (fig. 2), and it appears that Ca^{2+} channels linked to AMPA and KA receptors are still operative. More precise real-time $[Ca^{2+}]_i$ studies, however, are required to demonstrate the effect of DETC-MeSO on various types of receptor-gated Ca^{2+} channels. Furthermore, pretreatment of primary neurons with DETC-MeSO attenuated the $^{45}Ca^{2+}$ uptake following the addition of either glutamate or NMDA (fig. 5). The ability of DETC-MeSO to block the uptake of $^{45}Ca^{2+}$ is an indication of the attenuation of Ca^{2+} fluxes through NMDA receptor-mediated calcium channels. Although we know that NMDA receptors also induce calcium efflux [22], the effect of DETC-MeSO on the calcium efflux is unknown at this time. Therefore, the protection against NMDA, ammonia and ethanol-induced seizures in mice, and the neuroprotective effect of DETC-MeSO against glutamate (partial)- and NMDA (complete)-induced neurotoxicity in cultured primary neurons can be explained on the basis of the blockade of NMDA-mediated Ca^{2+} influx. Undoubtedly, further studies are needed to determine the potential of DETC-MeSO as an effective therapeutic agent for treating alcohol craving, alcohol withdrawal seizures, as well as many excitatory-related neurological disorders.

Acknowledgments

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Lipid Carrier Proteins and Ethanol

W. Gibson Wood Nicolai A. Avdulov Svetlana V. Chochina Urule Igbavboa

Geriatric Research, Education and Clinical Center, VA Medical Center, and Department of Pharmacology, University of Minnesota School of Medicine, Minneapolis, Minn., USA

Key Words

Alcoholism · Apolipoproteins · Cardiovascular disease · Cholesterol · Ethanol · High-density lipoproteins · Lipids · Low-density lipoproteins · Reverse cholesterol transport

Abstract

Ethanol has a pronounced effect on lipid homeostasis. It is our overall hypothesis that certain lipid carrier proteins are targets of acute and chronic ethanol exposure and that perturbation of these proteins induces lipid dysfunction leading to cellular pathophysiology. These proteins include both intracellular proteins and lipoproteins. This paper examines recent data on the interaction of ethanol with these proteins. In addition, new data are presented on the stimulatory effects of ethanol on low-density-lipoprotein (LDL)-mediated cholesterol uptake into fibroblasts and direct perturbation of the LDL apolipoprotein, apolipoprotein B. A cell model is presented that outlines potential mechanisms thought to be involved in ethanol perturbation of cholesterol transport and distribution.

Lipid homeostasis is altered by chronic ethanol consumption. Ethanol-induced changes in lipid homeostasis have been reported in alcoholics, heavy drinkers and animal models of alcoholism [16, 23, 25, 29, 32]. Examples of ethanol-induced changes in lipid homeostasis are accumulation of triacylglycerols and other lipids in the liver, alterations in cholesterol transport, membrane cholesterol asymmetry, lipoprotein distribution and the polyphosphoinositide cascade. There is increasing evidence that certain lipid carrier or transport proteins including lipoproteins are targets of ethanol [2, 5, 13, 19, 22]. We have been studying the dynamic interaction of ethanol with some of the lipid carrier proteins, and those data are summarized in table 1. Liver fatty-acid-binding protein (L-FABP) is a lipid carrier protein that binds cholesterol, fatty acids, fatty acylcoenzyme A, retinol, heme, hematin, lysophospholipids, bilirubin, prostaglandins and other amphipathic ligands [20]. This protein is found in the liver and other organs. Expression of L-FABP was significantly increased in livers of mice maintained on an ethanol liquid diet for 8 weeks as compared with livers of pair-fed control mice [5]. There was a 43% increase in the amount of L-FABP in livers of the chronic ethanol-treated mice. An earlier study had also found that L-FABP was

Table 1. Lipid carrier proteins and ethanol

Protein	Ethanol dynamics			Ref.
	in vivo	sterol transport	lipid binding	
L-FABP	↑ Liver		No effect C, SA	5, 27
SCP-2	↑ Brain		↓ C, PC, SA	19, 3
Apo A-I			↓ PC, no effect C	4
ApoA-I-PC		↓ Efflux	↓ C	4
HDL		↓ Efflux	No effect C	4
BSA			↓ C, PC, PA	2

C = Cholesterol; PC = phosphatidylcholine; PA = parinaric acid; SA = stearic acid.

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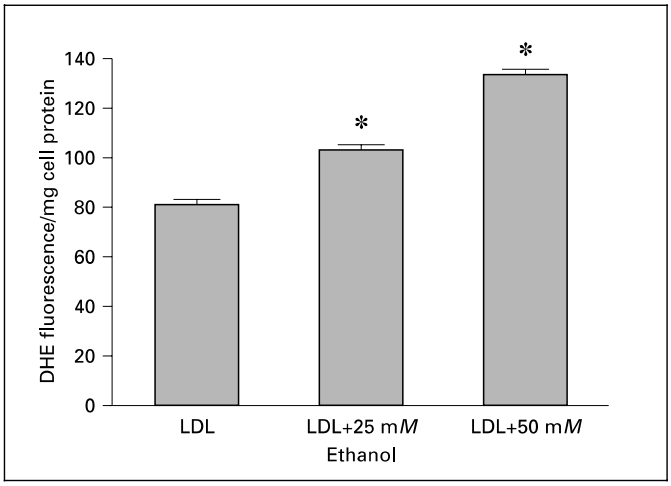
Dr. W. Gibson Wood
VA Medical Center, GRECC, 11G
Minneapolis, MN 55417 (USA)
Tel. +1 612 725 2051, Fax +1 612 725 2084
E-Mail Woodx002@tc.umn.edu

increased in livers of chronic ethanol-treated rats [21]. The increase in L-FABP expression in chronic ethanol-treated animals could be due to ethanol interfering with the binding capacity of L-FABP for its lipid ligands. This potential effect of ethanol on L-FABP was examined using fluorescent-dye-labeled cholesterol and stearic acid. We did not observe any effect of ethanol on cholesterol or stearic acid binding to L-FABP [27]. Chronic ethanol consumption could induce a posttranslational modification of L-FABP that could interfere with lipid binding. In our binding studies, we used rat liver recombinant L-FABP and not L-FABP from livers of chronic ethanol-treated animals. It was reported that the affinity for palmitate was greatly reduced in a delipidated cytosolic liver homogenate of chronic ethanol-treated rats [21]. In the same study, L-FABP amounts increased in the chronic ethanol-treated rats.

Sterol carrier protein 2 (SCP-2) is an intracellular protein that is found in peroxisomes and binds cholesterol, phospholipids and fatty acids [26]. This protein is found in the liver, brain and other organs. The only difference between the liver form of SCP-2 and brain SCP-2 is a single amino acid resulting in a conservative replacement of Ala^{55} for Val^{55} [19]. SCP-2 expression in liver was not affected by chronic ethanol consumption [5]. However, SCP-2 expression was increased in brain tissue of chronic ethanol-treated mice [19]. There was a 56% increase in SCP-2 in synaptosomes of chronic ethanol-treated mice as compared with the pair-fed control mice. In contrast to L-FABP, ethanol inhibited binding of lipids to SCP-2 with cholesterol binding being most affected [3]. The association constants (K_a) of the lipid ligand-SCP-2 complex were in the following order: NBD cholesterol > NBD phosphatidylcholine (PC) > NBD stearic acid. Ethanol, beginning at a concentration of 25 m*M*, significantly reduced the affinity of NBD cholesterol and NBD PC for SCP-2. Effects of ethanol on the K_a of NBD stearic acid were significant only at the highest concentration that was examined (200 m*M*).

The physiological function of SCP-2 has not been elucidated [6, 26]. SCP-2 may be involved in the intracellular trafficking of cholesterol [26]. SCP-2 decreases the half-life of sterol exchange between plasma membranes. SCP-2 is a peroxisomal protein, and transfer of cholesterol to the plasma membrane was found to be dependent on the amount of SCP-2 in peroxisomes [6]. Concomitant with ethanol-induced changes in SCP-2 expression in the brain was the finding that the transbilayer distribution of cholesterol was modified in synaptic plasma membranes of chronic ethanol-treated mice [31]. The amount of cholesterol in the exofacial leaflet was doubled in chronic ethanol-treated mice as compared with control mice. Total cholesterol amounts in synaptic plasma membranes did not differ between the ethanol and control groups. Mechanisms involved in regulating the transbilayer distribution of cholesterol are poorly understood. SCP-2 may be incorporated into the membrane and transports cholesterol from the cytofacial to the exofacial leaflet. Binding of SCP-2 to plasma membranes has been reported [33]. The increased expression of SCP-2 in brains of chronic ethanol-treated mice may be in response to ethanol interfering with binding of ligands to the protein. SCP-2, an intracellular protein, may then be taken up into the cytofacial leaflet that contains approximately 85% of the total synaptic plasma membrane cholesterol and this membrane-bound SCP-2 then translocates cholesterol to the exofacial leaflet. A recent report found that SCP-2 expression inhibited cholesterol efflux from L cell fibroblasts [1].

Cholesterol efflux from cells is an important function whereby cholesterol is removed from cells and transported to the liver. High-density lipoprotein (HDL), lipid-free and lipid-poor apolipoproteins act as acceptors for cholesterol efflux [7, 9, 18, 24, 35]. There has been substantial interest in the relationship between HDL levels and ethanol consumption. A generally accepted finding of both epidemiological and experimental studies is that HDL levels are increased in association with alcohol consumption [8, 11, 12, 14, 15, 23, 28]. Moreover, this lipoprotein and its subfractions are thought to play an important role in the reduced risk of coronary heart disease in moderate alcohol drinkers. What would appear to be an apparent contradiction is that HDL levels have been shown to be elevated in alcoholics but several studies report that the incidence of coronary heart disease is higher in alcoholics even when factors such as cigarette smoking and other factors are taken into consideration [11, 12, 14]. Recent data from our laboratory and another laboratory may contribute to an understanding of this apparent contradiction. We have recently reported that ethanol at concentrations commonly observed during periods of heavy drinking (25 and 50 m*M*) significantly inhibited cholesterol efflux from fibroblasts to HDL and to apolipoprotein A-I (apoA-I)-PC complexes [4]. While ethanol reduced cholesterol efflux to both HDL and apoA-I-PC, the mechanism of action was different. Ethanol inhibited incorporation of cholesterol into apoA-I-PC but did not affect incorporation of cholesterol into HDL. Cholesterol efflux mediated by HDL may result from direct contact of HDL with the cell exofacial leaflet of the plasma membrane, and ethanol may interfere with this contact. ApoA-



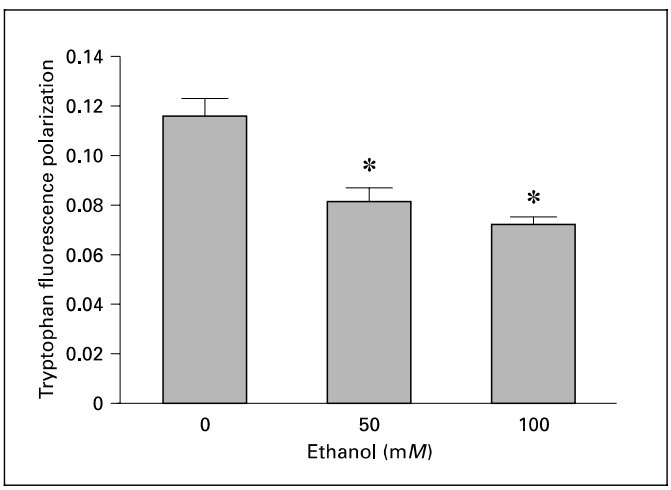


Fig. 1. Effects of ethanol on LDL-mediated cholesterol influx. The fluorescent cholesterol analogue dehydroergosterol (DHE) was incorporated into human plasma LDL. CREF fibroblasts were then incubated with LDL in the presence or absence of 25 and 50 m*M* ethanol for 5 h after which time cells were centrifuged and the fluorescence intensity of dehydroergosterol was measured. Data are means ± SEM of dehydroergosterol in fibroblasts (n = 4 experiments). $^{*}p \leq 0.005$ as compared to LDL control.

Fig. 2. Ethanol and tryptophan fluorescence polarization of apoB. Steady-state polarization was measured using tryptophan residues of apoB excited at 286 nm and emission at 335 nm in the presence and absence of 50 and 100 m*M* ethanol at 37 °C. Data are means ± SEM of polarization values for apoB tryptophan residues (n = 5 experiments). $^{*}p \leq 0.001$ as compared to 0 ethanol.

I-PC may incorporate cholesterol by the combined effects of membrane contact and aqueous diffusion. Lakshman's laboratory [22] has shown that HDL of alcoholic patients was less effective in removing cholesterol from mouse macrophages than HDL of control subjects. The uptake of HDL by HepG2 cells was significantly reduced in HDL samples of alcoholic patients. It should be noted that ethanol was not added in that study. A very important conclusion derived from the two studies is that the process whereby cells remove cholesterol is impaired by concentrations of ethanol routinely observed in heavy drinkers and by changes in HDL structure induced by chronic ethanol consumption.

Cholesterol influx mediated by low-density lipoprotein (LDL) is affected by ethanol and is opposite to effects observed for HDL on cholesterol efflux. It can be seen in figure 1 that ethanol significantly increased the uptake of LDL labeled with the fluorescent sterol dehydroergosterol into fibroblasts. There was approximately a 25% increase in fluorescence intensity of fibroblasts incubated with LDL and 25 m*M* ethanol, and 50 m*M* ethanol had even a larger effect on LDL-mediated sterol uptake (fig. 1). Potential mechanisms may include structural changes in apoB, the main apolipoprotein of LDL, providing a more energy-efficient conformation state or changes in the lipid environment associated with the LDL receptor or direct effects on the LDL receptor. We did observe that ethanol decreased fluorescence polarization of tryptophan residues of apoB (fig. 2). On the other hand, fluorescence polarization of tryptophan residues of apoA-I was increased by ethanol [4]. The restricted motion of apoA-I tryptophan residues induced by ethanol may interfere with lipid binding to the apolipoprotein.

Cholesterol transport into and out of cells is altered by ethanol concentrations that routinely occur in heavy drinkers (fig. 3). Ethanol stimulates the uptake of cholesterol into cells and inhibits cholesterol efflux from cells. Several potential mechanisms (LDL receptor binding and expression, LDL protein and lipid domains, selective uptake by coated pits) may explain this increase. Once cholesterol is internalized, it is delivered to lysosomes where the apolipoprotein component of LDL is degraded to amino acids, cholesterol esters are hydrolyzed by acid lipase and cholesterol is transported to different structures. Ethanol could modify the intracellular distribution of cholesterol by affecting the transport to different structures. Ethanol may directly act on HDL or lipid-poor apoA-I and alter protein conformation. Ethanol may perturb the membrane lipid environment that could affect cholesterol efflux. Ethanol could act on acylcoenzyme A cholesterol acyltransferase resulting in changes in the ratio of esterified cholesterol to free cholesterol and such changes affecting reverse cholesterol transport. Ethanol could perturb the Golgi apparatus that has been proposed to be an important component in both transport of cholesterol to the plasma membrane as well as transport from

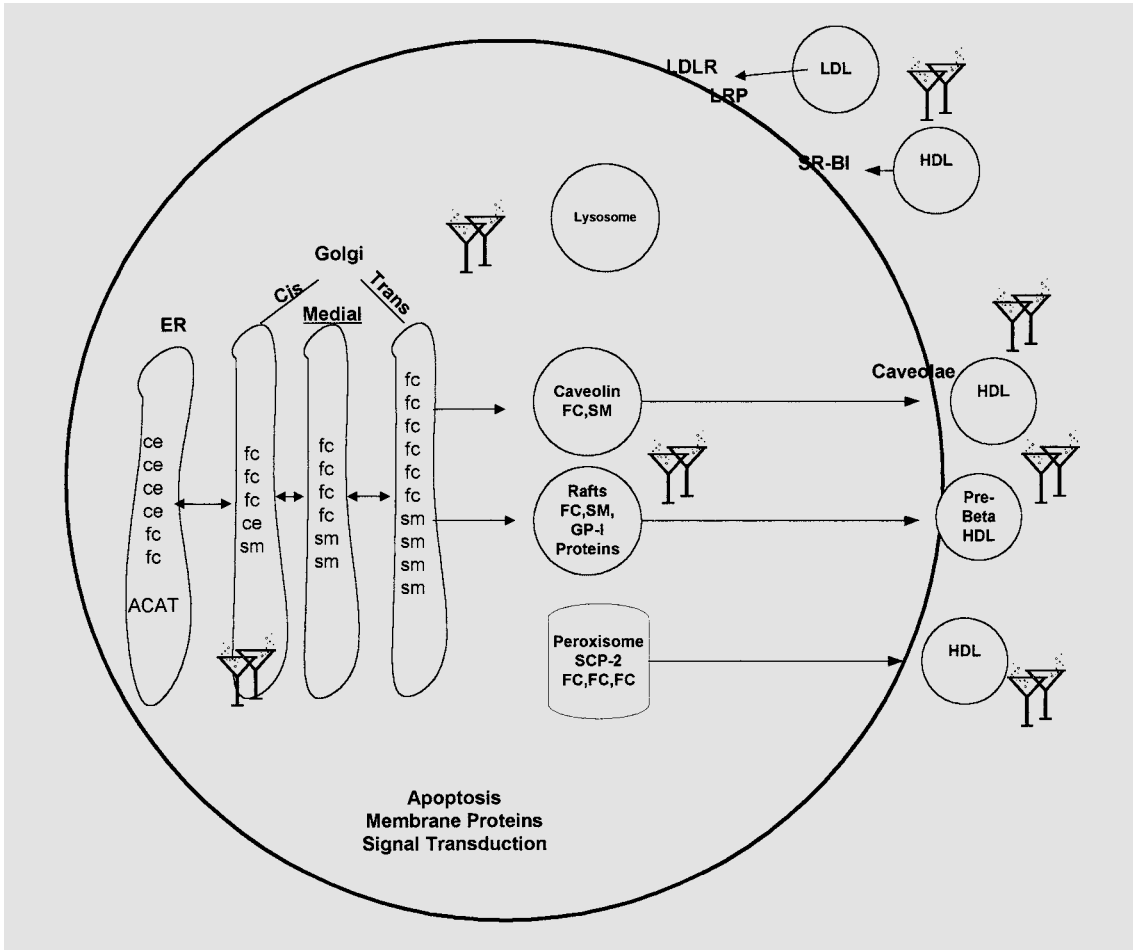


Fig. 3. Potential mechanisms of ethanol effects on cellular cholesterol transport and distribution. Ethanol increases LDL cholesterol uptake by directly altering apolipoprotein structure and possibly the receptor (LDLR). Internalized cholesterol may be processed through an endosome-lysosome pathway and transported to the endoplasmic reticulum (ER) and Golgi apparatus. Ethanol may increase the activity of acylcoenzyme A cholesterol acyltransferase (ACAT) resulting in an increase in esterified cholesterol that would not be available for transport. Transport of cholesterol to the plasma membrane may be inhibited by ethanol acting on caveolin, rafts or SCP-2. Additional mechanisms may include perturbation and structural changes of HDL, pre-β-HDL, caveolae and the scavenger receptor class B type 1 (SR-BI) receptor. The net result of ethanol-induced changes in cellular cholesterol homeostasis may include dysfunction of membrane proteins, the signal transduction pathway leading to apoptosis. ce = Cholesterol ester; fc, FC = free cholesterol; GP-I = glycosylphosphatidyl inositol; LRP = lipoprotein receptor-related protein; sm, SM = sphingomyelin.

the plasma membrane to the Golgi apparatus and other intracellular compartments [9, 17, 24]. Both monensin and brefeldin A inhibited cholesterol efflux to HDL from fibroblasts by acting on the trans-cisternae of the Golgi apparatus and cis- and medial Golgi cisternae, respectively [17]. Agents that disrupt the Golgi apparatus alter the intracellular cholesterol distribution to the plasma membrane and other structures [10]. Chronic ethanol consumption has been shown to alter cholesterol domains in brain plasma membranes, but there are little if any data on intracellular domains and ethanol in any cells [30, 31]. Marked changes in cholesterol distribution can occur in the absence of changes in the total amount of cellular cholesterol that could in turn affect cell function. Lipid transport involving caveolin, vesicles and SCP-2 to the plasma membrane could be altered by ethanol.

One conclusion is that cells of individuals who consume at least 5–6 drinks contain more cholesterol as compared to moderate drinkers. Therefore, cholesterol homeostasis of heavy drinkers is impacted by the acute and chronic effects of ethanol. It is well established that cholesterol plays an important role in cell structure and func-

tion. Membrane fluidity and activity of Na^+-K^+-ATPase are regulated by cholesterol. Apoptosis was induced by loading macrophages with cholesterol, and this lethal effect of cholesterol was thought to be due to activation of the Fas pathway [34]. Acute and chronic effects of ethanol on cellular cholesterol homeostasis could certainly contribute to pathophysiology occurring in alcoholics.

Acknowledgments

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Original Paper

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Alcohol Deters the Outgrowth of Serotonergic Neurons at Midgestation

Youssef Sari Teresa Powrozek Feng C. Zhou

Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, Ind., USA

Key Words

Fetal alcohol syndrome · Fetal alcohol effect · Ontogeny · Teratology · Neural tube defect

Abstract

We have previously demonstrated that treatment of pregnant C57BL mice from gestation days 8 to 14 with alcohol with 20% ethanol-derived calories (EDC) reduced the number of serotonin (5-HT) neurons and retarded their migration in the fetal brains. In the present study, we obtained similar results with the use of 25% EDC and extended our previous findings by demonstrating that besides the alteration of the number of 5-HT neurons, prenatal alcohol exposure also affects their projecting fibers in their early development. Pregnant C57BL mice were divided into an alcohol-exposed (ALC) group given 25% EDC (4.49%, v/v), a pair-fed group to the ethanol-fed group (PF) and a chow-fed group (Chow). The PF and Chow groups served as controls. Our results showed that in the ALC group, when compared with the control groups, prenatal alcohol exposure with 25% EDC reduced the number of 5-HT-immunoreactive neurons in both the median and dorsal raphe, and the amount of 5-HT-immunoreactive fibers in the medial forebrain bundle (MFB). The diameter of the 5-HT-immunoreactive MFB was also reduced as a result of treatment. No significant differences of the above parameters were found between the PF and Chow groups. The previous and present work confirmed that alcohol reduces the normal formation and growth of 5-HT neurons in the midbrain. Furthermore, the projection of 5-HT fibers, in density as well as in distribution, is reduced in the major trajectory bundle. This may affect the amount of 5-HT fibers available to the forebrain. In light of the importance of the 5-HT system in brain development, alcohol may affect the growth of the forebrain through its effect on 5-HT signaling.

In human fetuses, prenatal exposure to alcohol causes a spectrum of effects collectively termed fetal alcohol syndrome. It is commonly found that children of women who were heavy drinkers during pregnancy have mental retardation, hyperactivity, cognitive deficit and behavioral abnormalities [6, 41]. These psychological problems induced by prenatal alcohol exposure could be related to central nervous system dysfunction. Previous morphological studies in animals and humans showed that prenatal alcohol exposure reduces the volume of several areas in the brain [2–4, 21, 22, 29–31, 35, 36]. Our previous find-

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Dr. Feng C. Zhou
Department of Anatomy and Cell Biology
Indiana University School of Medicine, 635 Barnhill Drive
Indianapolis, IN 46202 (USA)
Tel. +1 317 274 7359, Fax +1 317 274 4934 or 278 2040, E-Mail imce100@iupui.edu

ings in C57BL mice showed that prenatal exposure to alcohol results in morphological defects in the fetal brain with incomplete neural tube fusion, which is closely related to the bilateral fusion of neural tissue and the enlargement of the ventricles [37, 48].

The neurotoxic effects of alcohol may also include disruption of several neurotransmitter systems such as the serotonergic [42, 50], dopaminergic [7, 9], noradrenergic [7], glutamatergic [15, 18], histaminic [33] and cholinergic systems [27, 38, 39]. In this present work, we focused on the serotonin (5-HT) system because earlier evidence indicates that 5-HT can act as a regulatory or trophic factor during neurogenesis for the development of 5-HT and other neurons [8, 13, 17, 24, 26, 45]. The actions of 5-HT in neurogenesis appear to be mediated by astroglial 5-HT_{1A} receptors and their stimulation results in the release of a serotonergic growth factor, S100β [1, 44]. 5-HT is implicated in brain development because of its ability to promote neuronal cell differentiation, migration and growth as well as synaptogenesis [5, 25]. In light of the importance of 5-HT as a signal for neurogenesis, the early 5-HT abnormalities associated with prenatal alcohol exposure could contribute to altering the central nervous system. In the present studies, we chose an earlier age than embryonic day 15 (E15) of the developing brain as the treatment endpoint for observation because that is when 5-HT neurons are known to be differentiating and projecting fibers.

Previous studies showed that prenatal alcohol exposure reduced the level of 5-HT and its metabolites in the offspring of rats [10] and mice [11]. Recent work in this laboratory showed that the same treatment also reduced the number of 5-HT neurons and retards their migration in the brain of E15 C57BL mice [49, 50]. Recently, it has been shown that alcohol exposure prior to conception and throughout pregnancy reduced the density of 5-HT-immunoreactive neurons in the dorsal raphe and B9 in postnatal day 5 rats [42]. It would, therefore, be of interest to examine if alcohol affects the development of the 5-HT system in a more defined stage (namely, E8–15) and if the neurons are already reduced in earlier development. Furthermore, it is important to investigate whether prenatal alcohol exposure would also affect the 5-HT projecting fibers in the early developing brain of C57BL mice, particularly in the major trajectory tract, the medial forebrain bundle (MFB).

Materials and Methods

Animals

Mice of the C57BL NHsd strain (Harlan, Indianapolis, Ind.) derived from C57BL/6J were used in these studies. They were housed in the Indiana University Laboratory Animal Research Center in a vivarium with a controlled climate room (temperature 22 °C, 30% humidity) with a 12:12 normal light-dark cycle.

Breeding and Treatment Procedure

Male mice were placed into female home cages in the afternoon (before the dark cycle). The day (next morning) when the sperm and plug were detected by vaginal smear was considered as E0. On E0, dams housed individually were fed with laboratory chow and water ad libitum.

On E8, dams were divided into three groups: (1) the alcohol group (ALC, n = 10) fed with chocolate sustacal (supplemented with vitamins and minerals) liquid diet with 25% (4.49%, v/v) ethanol-derived calories (EDC), (2) the pair-fed group (PF, pair-fed to the ethanol-fed group, n = 10) fed with a maltose-dextrin solution which was made isocaloric to the dose of ethanol used, and (3) the chow group (Chow, n = 10) fed with rat chow pellets and water. The PF and Chow groups served as controls.

Fetal Brain

All pregnant mice were anesthetized on gestation day 15 with deep anesthesia. The fetuses were removed, the fetal brains were dissected and fixed in 4% paraformaldehyde for 2 days. The fetal brains were further dissected from the base of olfactory bulb to the base of the metencephalon.

Immunohistochemistry

In order to keep the same conditions of immunostaining for both experimental and control groups, we embedded fetal brains of the ALC with those of the PF group or ALC with Chow brains in gelatin. All fetal brains were aligned at the same level in gelatin, and serial 50-μm coronal sections were then cut. The free-floating vibratome sections were prepared and incubated with 3% (v/v) H_2O_2 in 0.1 *M* phosphate-buffered saline (PBS, pH 7.4) for 10 min. Sections were washed three times and incubated overnight with 5-HT antibodies (from Incstar) diluted in PBS containing 0.3% Triton X-100 and 1.5% normal sheep serum (final dilution 1:250). Sternberger's peroxidase-antiperoxidase indirect enzyme method [40] was used for detection of 5-HT immunoreactivity. The second and third antibodies were added sequentially and followed by a 1-hour incubation after each addition. The peroxidase activity was revealed with 0.05% (w/v) 3′-3-diaminobenzidine tetrahydrochloride (Sigma) in 0.05 *M* Tris-HCl buffer (pH 7.6) supplemented with 0.003% H_2O_2.

Image Analysis for 5-HT-Immunoreactive Fiber Densities

The criteria for selecting a specific region of the brain for measurement of 5-HT-immunoreactive fiber densities were based on neuroanatomical landmarks within the coronal sections. We selected the areas of measurement following the *Atlas of the Developing Rat Brain* [32], with the assumption that the brain structures of E16 rats approximate those of E15 mice. The measurements of the density of 5-HT-immunoreactive fibers in the MFB were taken from the level of the habenular nucleus.

5-HT-immunoreactive fibers in the MFB were visualized and digitized using a SPOT camera mounted on a Leitz Orthoplan II

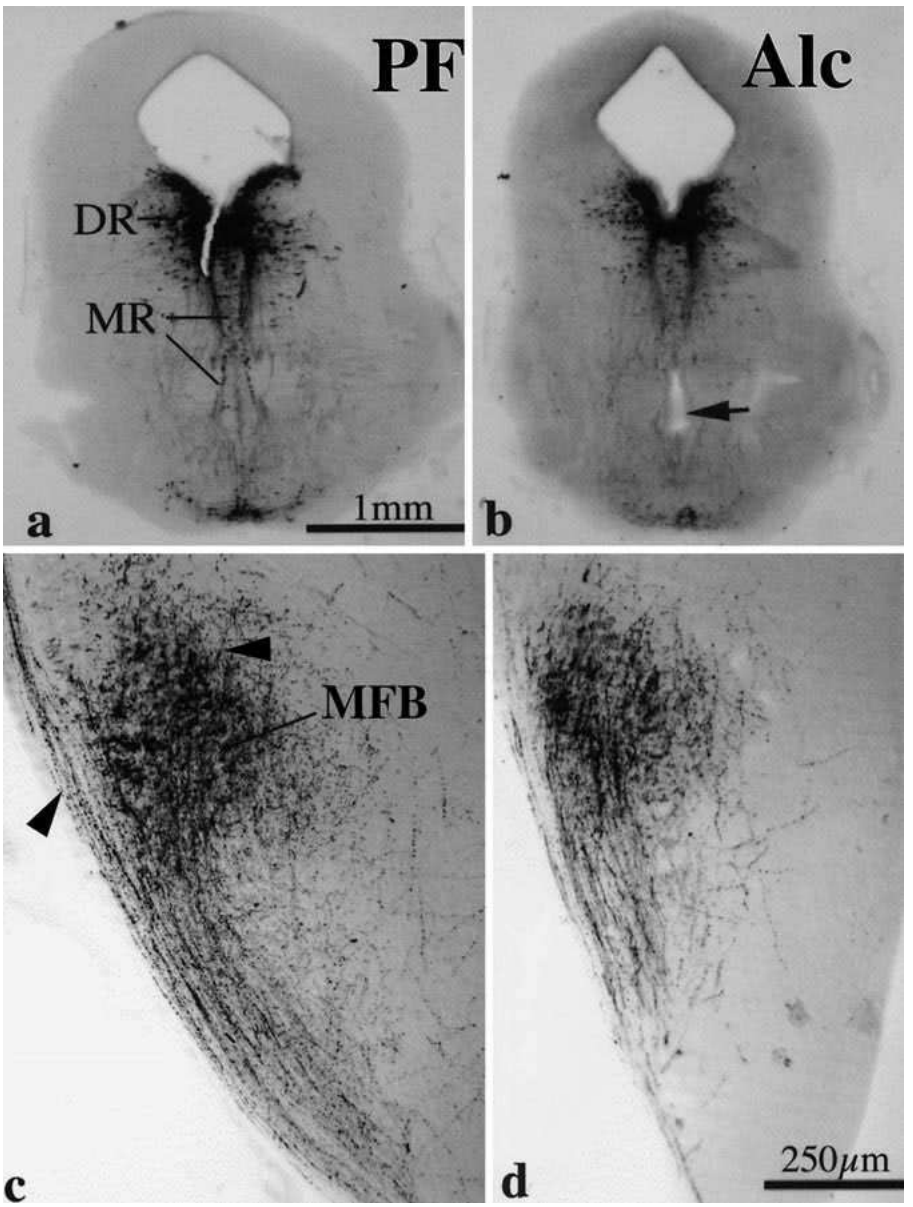


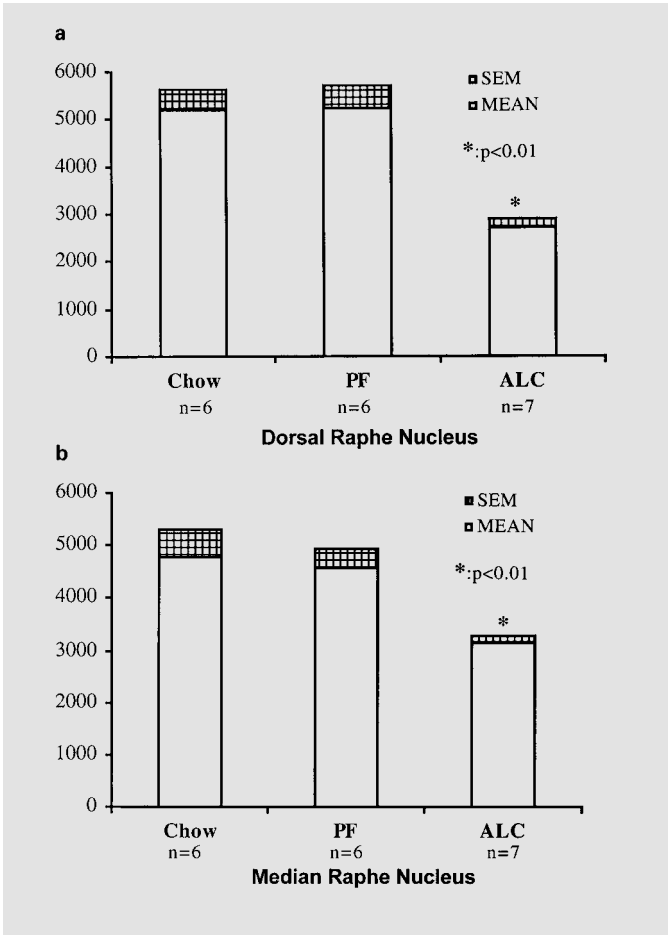
Fig. 1. The 5-HT-immunoreactive neurons in the rostral raphe nucleus (**a, b**) and 5-HT-immunoreactive fibers in the MFB (**c, d**) of the ALC and PF groups. The number of 5-HT-immunoreactive neurons is low in both dorsal (DR) and median raphe (MR) of the ALC group (**a**) as compared with those of the PF group (**b**) as shown in the sections of similar level in the brainstem. Marked opening (arrow) is frequently seen in the ALC brainstem. The great majority of the outgrowing 5-HT fibers from these cell bodies merged as a bundle within the MFB (arrowhead). The 5-HT-immunoreactive density is smaller in the ALC (**d**) as compared with that of the PF group (**c**).

microscope under a × 6.3 objective lens. The density of 5-HT-immunoreactive fibers was quantitated by box measurement of each of the selected brain structures using an image analysis system (NIH Image). The sum of the area optical density of 5-HT fibers obtained for each structure in the brain sections was divided by the sum of the box area of the sections, and the result was expressed as fiber density percent of background [47]. The density percent of 5-HT-immunoreactive fibers determined for the alcohol-exposed and control groups were compared using one-way analysis of variance. Student's t test was used for posthoc analysis between groups.

Results

5-HT Neurons in the Midbrain

The ascending 5-HT neurons were seen in the rostral raphe nucleus which extended throughout the pontine curvature. The dorsal (above the medial longitudinal fasciculari) and median raphe (midline and below the medial longitudinal fasciculari) are distinguishable at this stage. Fewer 5-HT-immunoreactive neurons were found in the dorsal and median raphe of the ALC as compared



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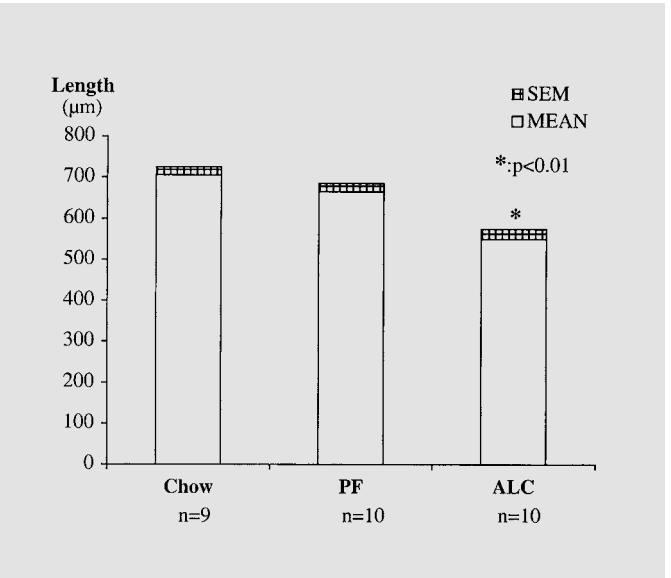

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Fig. 2. The number of 5-HT-immunoreactive neurons in the dorsal (**a**) and median raphe nucleus (**b**) of ALC mice is lower than those of the PF and Chow groups. No difference was found between PF and Chow.

Fig. 3. The length of the rostral raphe nucleus with rich ascending 5-HT neurons as outlined by 5-HT-immunoreactivity around the pontine flexure was found to be shorter in the ALC group as compared with those of the PF and Chow groups. No difference was found between PF and Chow.

with those of the PF ($p < 0.01$) or Chow group ($p < 0.01$; fig. 1, 2). No significant difference was observed between the two control groups.

Brainstem Length

The length of the rostral raphe nucleus (median and dorsal parts through the pontine curvature) of the ALC group was smaller as compared with that of the PF ($p < 0.01$) and Chow groups ($p < 0.01$). There was no significant difference between the two control groups (fig. 3).

Medial Forebrain Bundle

Densitometric analysis revealed that 5-HT-immunoreactive projecting fibers in the area of the MFB were significantly reduced by about 50% in the ALC group as compared with those in the PF ($p < 0.01$) or Chow group ($p < 0.01$; fig. 4). In addition, the diameter of 5-HT-immunoreactive fibers occupying the MFB is smaller in the ALC group as compared with that of the PF ($p < 0.01$) or Chow group ($p < 0.01$; fig. 5). The diameter of the 5-HT-immunoreactive MFB is not different between the PF and Chow groups.

Discussion

The present work shows the adverse effects of prenatal alcohol exposure on the development of 5-HT projecting fibers in mouse fetal brain. Substantial reductions in the density of 5-HT-immunoreactive fibers could be observed in certain areas of the brain as early as E15. We chose an earlier age of the developing brain, i.e. E8, as the starting point of alcohol treatment and E15 as the treatment endpoint because that is when 5-HT neurons are differentiating and projecting fibers. This is based on evidence which indicated that 5-HT and 5-HT transporter can be detected in neurons of the rostral raphe nucleus by E12–E13 and that cell differentiation occurs in these neu-

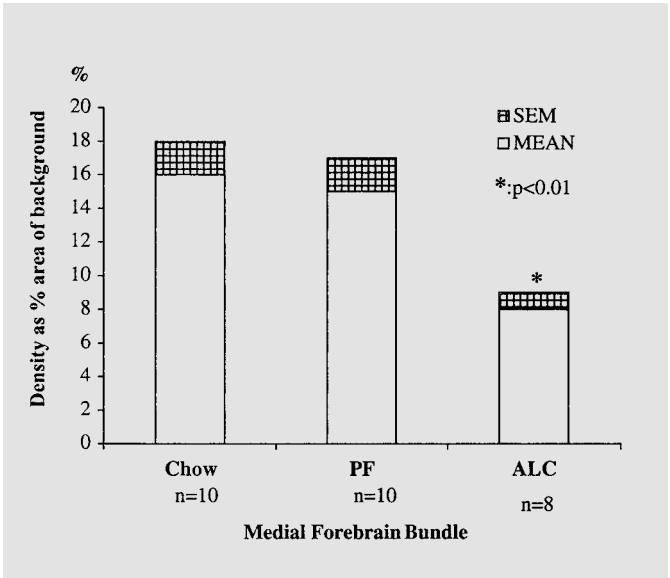

Fig. 4. The density of 5-HT fibers in the MFB is reduced in the ALC group as compared with those of the PF and Chow groups. No difference was found between PF and Chow.

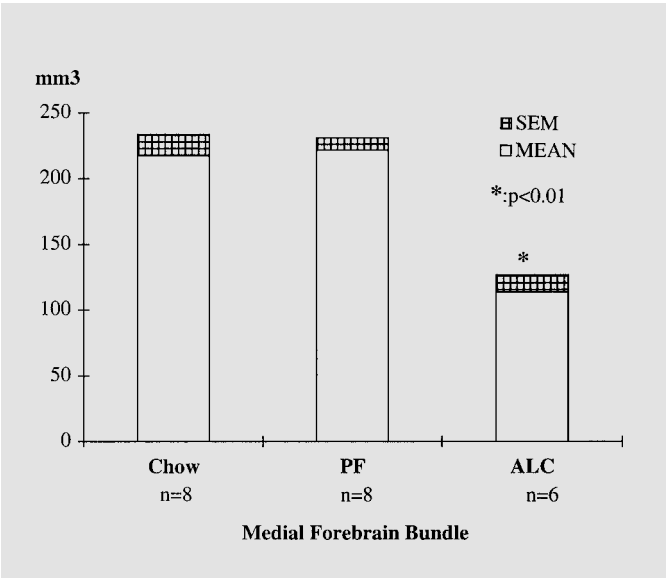

Fig. 5. The volume of the MFB as outlined with 5-HT fibers is smaller in the ALC group as compared with those of the PF and Chow groups.

rons on about E15–E16 in rats [26, 43, 46, 51], while in mice these events occur approximately 1 day earlier. We chose to examine the MFB because all long ascending 5-HT fibers course through here and it contains the highest density of 5-HT fibers on E15. Substantial reduction in this area at this stage implies a strong tendency for shortage of 5-HT projections in the forebrain.

It has previously been shown that in E15 C57BL/6J mice, prenatal alcohol exposure (20% EDC) reduced the number of 5-HT neurons and retarded their migration in the brain [49, 50]. We have now demonstrated that treatment with 25% EDC produced similar results. In addition, we found that alcohol at this dosage reduced the density of 5-HT-immunoreactive fibers in the MFB to half of the control value. Such changes presage a short fall of 5-HT projecting fibers in the forebrain. This expands on previous findings from studies in rats which demonstrated that 19- and 35-day-old offspring of dams with 6.6% (v/v) alcohol diet treatment 60 days prior to and during the entire pregnancy had a lower concentration of 5-HT and/or 5-hydroxyindoleacetic acid in the cortex, cerebellum and brainstem [10, 20]. The deficit in 5-HT persisted for 3–5 months in the brains of mice that had been exposed prenatally to alcohol [11, 12, 23]. In this present work, our densitometric analysis demonstrates clearly that prenatal alcohol exposure affects the development of 5-HT projecting fibers in the embryonic stage as early as E15.

Prenatal alcohol exposure induced a deficit in 5-HT projecting fibers, which could be related to the migration problems of these projections, as it has been described in our previous work that alcohol retards the migration of 5-HT neurons [49, 50]. The mechanism by which prenatal alcohol exposure induces a deficit in the 5-HT system is unknown. It could be explained by the fact that alcohol or its metabolite has a deleterious effect on the raphe neurons. Furthermore, prenatal alcohol exposure could result in a diminished production of certain essential neurotrophic factors that are synthesized by the astrocytes. Indeed, the damaging effect of alcohol on the proliferation and differentiation of astrocytes has been documented in studies conducted both in vitro and in vivo [14, 16, 19, 34, 50]. Such effects could lead to a decrease in the production of neurotrophic factors by these astrocytes, which are essential for the development of the 5-HT system. One of these factors, S100β, has been shown to play an important role in the developing serotonergic system [1, 28, 45] and to be present in lower levels in the brains of alcohol-exposed E15 C57BL mice [49, 50].

It is interesting to note that the length of the rostral raphe nucleus, where 5-HT neurons reside, and the MFB, where the majority of 5-HT ascending fibers course

through, are both reduced in correspondence with the lower density of 5-HT neurons or fibers in the ALC group as compared with those of the PF and Chow groups. This indicates that alcohol not only caused fewer 5-HT neurons and a lower density of 5-HT fibers, but also reduced the areas they occupied. A study is ongoing to investigate whether this phenomenon is widespread throughout the brain. The central 5-HT system is one of the earliest developed neuronal systems. Given the implication that serotonin plays an important role in brain development by acting on differentiation, migration and cell growth [5, 25], the early 5-HT abnormalities induced by prenatal alcohol exposure could have profound effects on the morphological defects observed in the fetal brain.

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Ethanol Inhibits Cytokine-Induced iNOS and $sPLA_2$ in Immortalized Astrocytes: Evidence for Posttranscriptional Site of Ethanol Action

Jing-Hung Wang Grace Y. Sun

Departments of Nutritional Sciences and Biochemistry, University of Missouri, Columbia, Mo., USA

Key Words

Ethanol · Astrocytes · Cytokines · iNOS · $sPLA_2$

Abstract

Chronic and excessive ethanol consumption is known to alter neuron and glial cell functions in the central nervous system (CNS). Astrocytes comprise the major cell type in the brain. These immune active cells are capable of responding to proinflammatory cytokines and endotoxins, which stimulate transcriptional pathways leading to induction of genes, including the inducible nitric oxide synthase (iNOS) and secretory phospholipase A_2 ($sPLA_2$). In this study, we investigate the effects of ethanol on cytokine-induced iNOS and $sPLA_2$ in immortalized astrocytes (DITNC). When DITNC cells were exposed to ethanol (0–200 m *M*) for 4 h prior to subsequent stimulation with cytokines for 16 h, NO production decreased with increasing ethanol concentrations starting from 50 m*M*. At ethanol concentrations higher than 100 m*M*, ethanol also inhibited cytokine-induced $sPLA_2$ release into the culture medium. The inhibitory effect of ethanol on NO production corresponds well with the decrease in iNOS protein and NOS enzyme activity, but not with iNOS and $sPLA_2$ mRNA nor binding of NF-κB to DNA. The inhibition of cytokine-induced NO production by ethanol was also dependent on the time of ethanol exposure to the cells, but addition of acetaldehyde up to 200 μM did not elicit any changes. Taken together, these results provide evidence for a posttranscriptional mode of ethanol action on the cytokine induction pathway for NO production in astrocytes.

Introduction

Chronic and excessive consumption of alcohol in humans and animals has been shown to cause cellular damages in many body organs, including neurons and glial cells in the central nervous system (CNS) [11, 18, 21]. Astrocytes are the most abundant cell type in the brain, and they are known to play important roles in providing nutrient supplies to neurons, neuronal guidance during development as well as regulation of ion homeostasis [30]. Recent studies have provided evidence that astrocytes may be an important target of ethanol action in the brain, especially during the early developmental stage [6]. A characteristic property of astrocytes is their ability to respond to proinflammatory cytokines, which lead to stimulation of transcription factors and induction of genes including the inducible nitric oxide synthase

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Dr. Grace Y. Sun
M121 Medical Sciences Building
Biochemistry Department, University of Missouri
Columbia, MO 65212 (USA)
Tel. +1 573 882 5377, Fax +1 573 884 4597, E-Mail sung@health.missouri.edu

(iNOS) [3, 24] and secretory phospholipase A_2 ($sPLA_2$) [29, 34]. Although mechanisms not yet clearly understood, many of the cytokine signaling pathways can be targets of ethanol action [9].

There is increasing evidence indicating sensitive effects of ethanol to suppress or enhance cytokine-induced NO production in immune cells. Ethanol was shown to suppress lipopolysaccharide-induced NO production in alveolar macrophages [4, 5], human monocytes [19] and astrocytes [20, 32], but enhanced NO production in aortic vascular smooth muscle cells [2], blood-brain barrier cells [25], hepatocytes [35] and embryonic cortical neurons [23]. Some studies attributed the inhibitory effects of ethanol to alteration of nuclear transcription factors and iNOS mRNA [4, 5, 19, 20].

Our recent studies have demonstrated the ability of immortalized astrocytes (DITNC) to respond to proinflammatory cytokines (TNF-α, IL-1β and IFN-γ) in the induction of iNOS and $sPLA_2$ [17, 34]. In this study, these cells were used to examine the effects of ethanol on the cytokine signaling pathways as well as iNOS protein and enzyme activity. Our results suggest a posttranscriptional site of ethanol action for this pathway.

Materials and Methods

Cell Culture

Immortalized astrocyte cells (DITNC) were purchased from ATCC (Rockville, Md., USA). Cells were cultured in 75 cm^2 flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum, 1% of penicillin/streptomycin (100 units/100 mg/ml) and 1% of fungizone (250 mg/ml) and were maintained at 37 °C in the incubator under a humidified atmosphere of 95% air and 5% CO_2. Cells were subcultured twice weekly, and confluent cells in 75-mm flasks were subcultured into 35-mm dishes and used for experiments after reaching 90% confluence.

Before the experiment, cells were washed with phosphate-buffered saline (PBS) twice and replenished with a serum-free medium containing DMEM and 0.5% bovine serum albumin (BSA). Ethanol (0–200 m*M*) was added to cells 1 h after change of medium, and the cultures were placed in a tray inside a Ziploc plastic bag. A culture dish containing 1 ml of ethanol at the same concentration as in the culture was also placed in the bag. For cytokine treatment, a mixture containing 50 units of TNF-α (recombinant murine, specific activity 1 U = 0.05 ng; R&D Systems, Minneapolis, Minn., USA), 50 units of IL-1β (recombinant murine, specific activity 1 U = 10 pg; R&D systems) and 25 units of IFN-γ (recombinant rat; Life Technologies, Gaithersburg, Md., USA) was added to the culture 4 h after ethanol exposure. The cytokine mixture was based on results of a previous study showing optimal induction of iNOS and $sPLA_2$ in these cells [17]. After treating cells with cytokines for 16 h, the culture media were removed for determination of NO release and $sPLA_2$ activity, and cells in the dishes were recovered for protein assay or RNA extraction.

NO Determination

NO produced by the cells was determined by assaying the levels of nitrite (NO_2^-) in the culture medium. For this reaction, 400 µl of culture supernatant was added to a mixture containing 200 µl of 7.5 m*M* of sulfanilamide, 200 µl of 0.75 *M* of HCl and 200 µl of 7.5 m*M* N-(1-naphthyl) ethylenediamine (Sigma Chemical Co.; St. Louis, Mo., USA). After reaction for 10 min at room temperature, the absorbance of the reaction mixture was read at 548 nm, and NO_2^- concentrations were calculated against a standard curve constructed with $NaNO_2$.

RT-PCR Assay of iNOS, $sPLA_2$ and β-Actin mRNA

Total RNA was isolated from cultured cells in 35-mm dishes using TRI^{ZOL} reagent (Life Technologies). The concentration of RNA was determined by reading the absorbance at 260 nm. An aliquot of 0.5 µg of total RNA was converted to cDNA and amplified using the access RT-PCR System (Promega, Madison, Wisc., USA). The iNOS primers (reverse: 5′-CCACAATAGTACAATACTTGG-3′ and forward: 5′-ACGAGGGTTCAGCGTGCTCCACG-3′) amplified a 395-bp fragment from the iNOS cDNA. $sPLA_2$ primers (reverse: 5′-TCTCAGGACCCTCTTAGGTACTA-3′; forward: 5′-TGACTCATGACTGTTGTTACAACC-3′) amplified a 493-bp fragment from rat $sPLA_2$ cDNA. β-Actin primers (reverse: 5′-GTGCCACCAGACAGCACTGTGTTG-3′ and forward: 5′-TGGAGAAGAGCTATGAGCTGCCTG-3′) amplified a 289-bp fragment from β-actin cDNA. RT-PCR conditions were: reverse transcription at 42 °C for 45 min, denaturating at 94 °C for 30 s, annealing at 60 °C for 45 s, and polymerization at 72 °C for iNOS, 68 °C for $sPLA_2$ and 45 s for β-actin. After 35 cycles (15 cycles for β-actin mRNA), a final 10-min incubation at polymerization temperature was carried out. After amplification, a 5-µl aliquot of the reaction mixture was applied to 1% agarose gel in TAE buffer containing 0.1 mg/ml ethidium bromide. The mRNA for the constitutive β-actin was used as the reference cellular transcript.

$sPLA_2$ Assay

Aliquots of [^{14}C]arachidonoyl-phosphatidylethanolamine (PE) (~8,000–10,000 dpm) together with 15 µl of unlabeled PE (10 µmol) were added to each tube, and organic solvent was removed under nitrogen. Then 10 µl of ethanol was added to the substrate together with 100 µl of incubation buffer containing 0.25 *M* sucrose, 1 m*M* EDTA, 1 m*M* EGTA, 6 m*M* $CaCl_2$, 1 mg/ml of BSA, and 50 m*M* Hepes (pH 7.4). The enzyme reaction was initiated by adding 50 µl of blank medium (0.5% BSA DMEM) and 50 µl of culture medium in each tube. Incubation was carried out at 37 °C for 30 min. The reaction was terminated by adding 2 ml of chloroform/methanol (2:1, vol/vol) and 0.3 ml of 1% acetic acid. After phase separation, the lower organic phase was dried, and lipids were spotted on an HPTLC plate coated with silica gel G (Whatman, Fairfield, N.J., USA). The free fatty acids were separated using a solvent system containing hexane-ethyl ether-acetic acid (85:15:2, by vol). After exposure of the HPTLC plates to iodine vapors, lipid bands corresponding to phospholipids (origin) and free fatty acids were removed and transferred to scintillation vials for measurement of radioactivity by a Beckman liquid scintillation spectrometer LS5800 (Beckman, Sunnyvale, Calif., USA).

Electrophoretic Mobility Shift Assay

For this study, cells were cultured in 60-mm dishes and after exposure to ethanol for 4 h, they were treated with cytokines for 1 h.

After cytokine treatment, media were removed, and cells were washed once with cold PBS. Cells were scraped and transferred to 15-ml cell culture tubes, centrifuged and washed in cold PBS. The cells were resuspended in 400 ml of cold buffer A (10 m*M* HEPES, pH 7.9, 10 m*M* KCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 1 m*M* DTT, 0.5 m*M* PMSF, 10 mg/ml leupeptin and 10 mg/ml aprotinin). After 15 min on ice, cells were lysed by the addition of NP40. Lysis was completed by vortexing vigorously for 10 s. The homogenate was centrifuged for 30 s in a microcentrifuge (12,000 *g*), and the nuclear pellet was resuspended in 50 µl of cold buffer C (20 m*M* HEPES, pH 7.9, 0.4 *M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* DTT, 1 m*M* PMSF, 10 mg/ml leupeptin and 10 mg/ml aprotinin). This suspension was agitated at 4°C for 15 min, followed by centrifugation in a microcentrifuge for 5 min at 4°C. The resulting supernatant was stored in small aliquots at –80°C . Protein concentration was determined using the BioRad Protein Assay kit (BioRad, Hercules, Calif., USA) with bovine serum albumin as standard.

Oligonucleotides specific for nuclear factor-κB (NF-κB; 5′-AGTTGAGGGGACTTTCCCAGGC-3′) and mutant probe (5′-AGTTGAGGCGACTTTCCCAGGC-3′) were obtained from Promega and Santa Cruz Biotech Inc (Calif., USA), respectively. For labeling the probes, 2 µl of oligonucleotides were incubated with [γ-^{32}P]ATP (New England Nuclear, Boston, Mass., USA), T4-kinase (Promega) and buffer (× 10) for 20 min at room temperature. Reaction was terminated by adding 1 µl of 0.5 *M* EDTA and 89 µl of TE buffer (pH 8). After labeling, the oligonucleotide probe was purified by eluting the reaction mixture through a G-25 column. The labeled probe was incubated with nuclear extract (2 µg protein) for 10 min. The samples were applied to SDS-polyacrylamide gel (7.5%). After electrophoresis, the gel was placed in fixing buffer and dried for 4 h. To visualize binding of NF-κB to DNA, the gel was exposed to X-ray film. In each experiment, a 100-fold nonlabeled oligonucleotide probe was used to displace the labeled probe, and mutant oligonucleotide probes were used as negative controls.

Western Blot Analysis

Cells were cultured in 60-mm dishes and treated with the same conditions as described previously. After 4 h of ethanol exposure and followed by 16 h of cytokine treatment, cells were scraped and homogenized in ice-cold lysis buffer containing 50 m*M* Tris-HCl (pH 8.0), 137 m*M* NaCl, 1% Triton X-100, 10% glycerol, 0.2 m*M* 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 mg/ml aprotinin, and 1 m*M* sodium orthovanadate. Cell debris was removed by centrifugation at 14,000 *g* for 30 min. Cell extracts (40 µg) were separated by electrophoresis using a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (0.45 µ*M*, Bio-Rad) using a Mini-Trans-Blot cell (Bio-Rad). After washing 3 times with TBS buffer, the membrane was incubated with both mouse anti-iNOS IgG2a antibody at 1:5,000 dilution (Transduction Laboratories, Lexington, Ky.) and mouse anti-β-actin antibody at 1:5,000 dilution (Sigma), and followed with peroxidase conjugated goat anti-mouse IgG (1:5,000 dilution). Immunoreactivity was visualized using the Lumi Glo enhanced chemiluminescence reagent (Kirkgard and Perry Laboratory, Gaithersburg, Md., USA).

Assay of NOS Activity

Cells were cultured in 60-mm dishes, and after appropriate treatments, media were removed and washed twice with cold PBS. Cells were scraped and homogenized in 1 ml of HEPES buffer (20 m*M* HEPES, 1 m*M* EDTA, 1 m*M* DTT and 1 m*M* PMSF, pH 7.2). Cell debris was removed by centrifugation at 14,000 *g* for 4°C 15 min. The resulting supernatant was stored in small aliquots at –20°C . Protein concentration was determined by the Bradford method using the Bio-Red reagent. NOS activity was measured by determining the conversion of [^{3}H]*L*-arginine to [^{3}H]*L*-citrulline. The reaction was incubated at 37°C for 30 min in a mixture containing 20 m*M* HEPES, 1 m*M* EDTA, 4.5 m*M* Ca^{2+}, 5 µ*M* arginine, 4 m*M* NADPH, 3.64 µg/ml calmodulin, 10 µ*M* BH_4, 6 m*M* FAD and 0.5 µCi [^{3}H]*L*-arginine (pH 7.2). Enzyme reaction was initiated by the adding of 100 µl (360 µg) cytosolic protein. Reaction was terminated by adding 800 µl ice-cold 50 m*M* HEPES with 2 m*M* EDTA, 2 m*M* EGTA (pH 5.5). Aliquots of 1 ml were passed through an ion-exchange column (Bio-Rad AG®50W, Na^{2+} form converted to H^+ form) for removal of [^{3}H]*L*-arginine, followed by 1 ml of HEPES buffer. Eluted radioactive [^{3}H] *L*-citrulline was quantified by a Beckman liquid scintillation spectrometer (LS5800).

Statistics

Statistical treatment was made using one-way ANOVA analysis followed by Bonferroni's multiple comparison test. Significant differences between treatment groups are defined as $p < 0.05$.

Results

Ethanol Inhibits Cytokine-Induced NO Production and sPLA$_2$ Release

Similar to results obtained previously [17], DITNC cells respond well to cytokines in the induction of NO and sPLA$_2$. When different concentrations of ethanol (0–200 m*M*) were added to the cells 4 h prior to cytokine stimulation, there was a decrease in NO production with increasing concentrations of ethanol (fig. 1a). Significant ($p < 0.001$) decrease in NO level could be observed upon exposing cells to 50 m*M* ethanol and the decrease reached approximately 50% at 100 m*M* ethanol. When the same culture media were taken for assay of sPLA$_2$ activity using [^{14}C]PE as substrate, a significant ($p < 0.05$) decrease in sPLA$_2$ activity was observed only at an ethanol concentration of 200 m*M* (fig. 1b).

After treatment of cells with ethanol and cytokines, cell morphology was examined using a phase-contrast inverted Nikon Diaphot 300 microscope (Nikon Inc., Melville, N.Y., USA). There was no obvious change in cell morphology with respect to ethanol or cytokine treatment in these cells. Furthermore, culture medium was used for lactate dehydrogenase assay to assess cell membrane integrity. No obvious release of the enzyme was observed upon exposure of cells to ethanol or cytokines (data not shown).

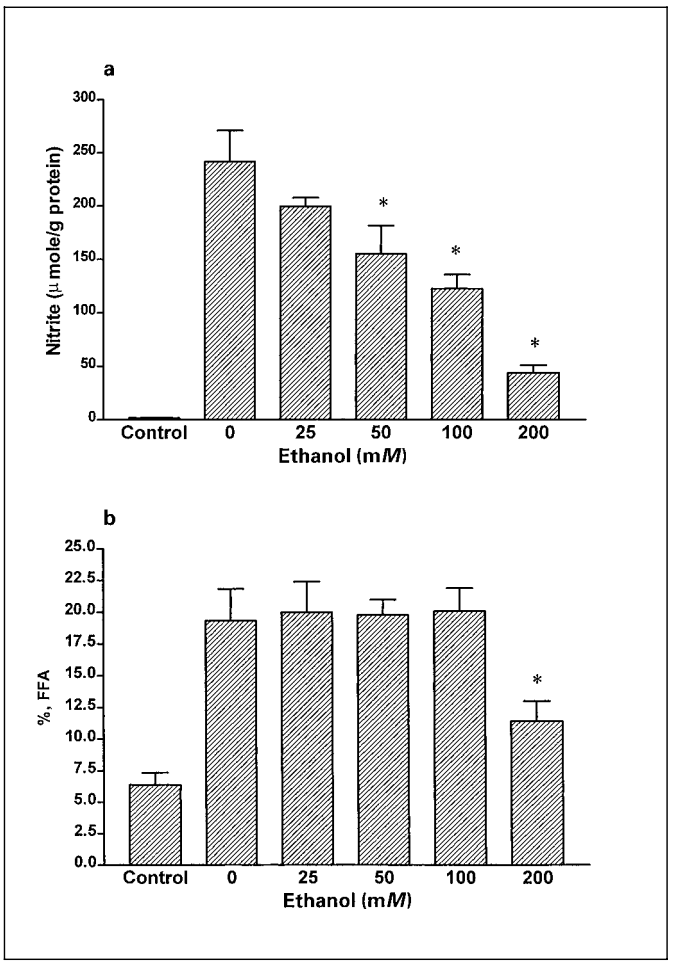


Fig. 1. Effects of ethanol on cytokine-induced NO production (**a**) and $sPLA_2$ release (**b**) in DITNC cells. Cells were cultured in 35-mm dishes as described in the text and were stimulated with a cytokine mixture containing TNF-α (50 units), IL-1β (50 units) and IFN-γ (25 units). Ethanol was added to the cells 4 h prior to addition of cytokines. After 16 h of cytokine treatment, culture media were removed for determination of nitrite concentration using the Greiss reagent and determination of $sPLA_2$ activity, using [^{14}C]arachidonoyl-PE as substrate. Results are means ± SD from 3 dishes from a typical experiment with similar results from repeated experiments. Control = no cytokine added. * $p < 0.05$, one-way ANOVA followed by Bonferroni's multiple comparison test indicates significant differences due to ethanol treatment.

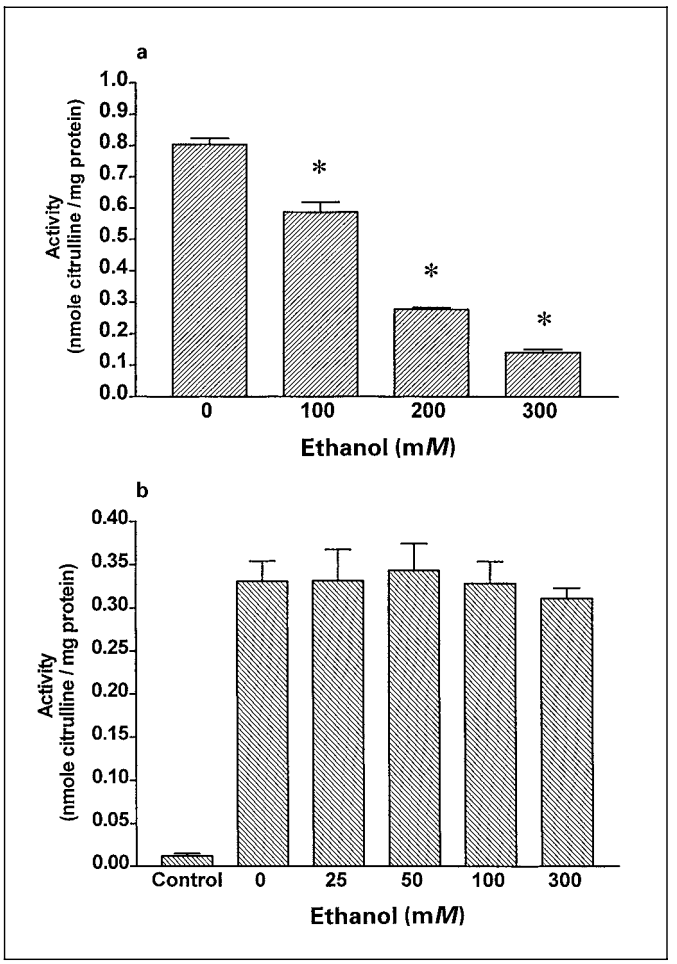
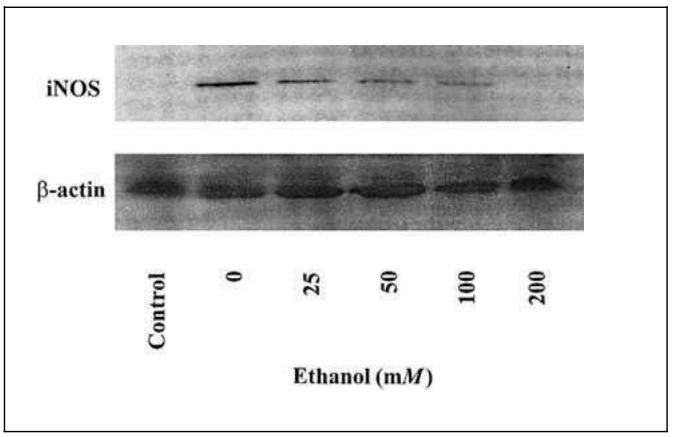


Fig. 2. Effects of ethanol on cytokine-induced NOS activity in DITNC cells. **a** Cells were cultured in 60-mm dishes as described in the text and were treated with ethanol and cytokines as described in figure 1. After treatments, cells were harvested and cell cytosol was used for assaying NOS as described in the text. **b** DITNC cells were treated with cytokines, and cell protein samples were prepared. Prior to the enzyme assay, different concentrations of ethanol were added into the reaction buffer. Results are means ± SD (n = 3). * $p < 0.05$, one-way ANOVA followed by Bonferroni's multiple comparison test indicates significant differences due to ethanol treatment.

Ethanol Treatment to DITNC Cells Inhibits NOS Catalytic Activity

In order to delineate the target site(s) of ethanol on the cytokine-induced intracellular signaling pathways, NOS activity in cell extract was assayed by incubation with [^{3}H]*L*-arginine together with necessary cofactors. As shown in figure 2a, exposure of DITNC cells to ethanol (100–300 m*M*) for 4 h prior to stimulation with cytokines for 16 h resulted in a dose-dependent decrease in NOS activity in the cells. On the other hand, addition of ethanol (0–200 m*M*) directly to the cell extract obtained after cytokine treatment did not alter NOS enzyme activity (fig. 2b).



Ethanol Treatment Inhibits Cytokine-Induced iNOS Protein

In order to determine the effect on iNOS protein, cells were exposed to ethanol and cytokines and then subjected to Western blot assay using iNOS polyclonal antibody. As shown in figure 3, cytokine treatment caused the increase

Fig. 3. Western blot analysis of iNOS protein. Cells were cultured in 60-mm dishes and were treated similarly as described in figure 1. After treatment, cells were removed from dishes and lysed in buffer. Cell extracts (40 μg protein) were subjected to electrophoresis and blot with antibodies against iNOS and β-actin as described in the text. Results represent a typical blot from repeated analysis.

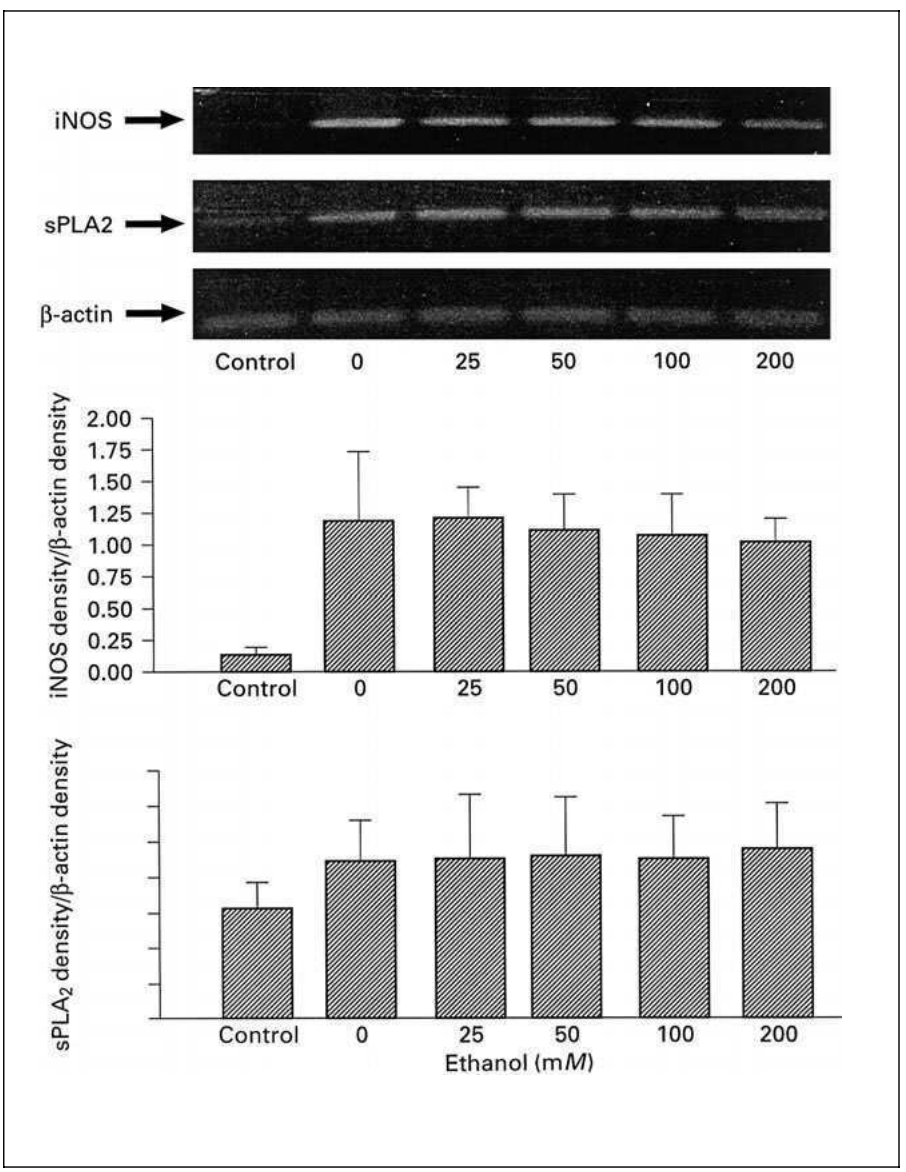


Fig. 4. Effects of ethanol on cytokine induction of iNOS and $sPLA_2$ mRNA in DITNC cells. Cells in 35-mm dishes were treated with ethanol and cytokines as described in figure 1. After treatment, total RNA was isolated from cells and 0.5 μg of RNA was used for RT-PCR with each primer set as described in the text. The relative density of iNOS and $sPLA_2$ of each sample was plotted against β-actin using an image analyzer. Results are means ± SD (n = 3) from one typical experiment.

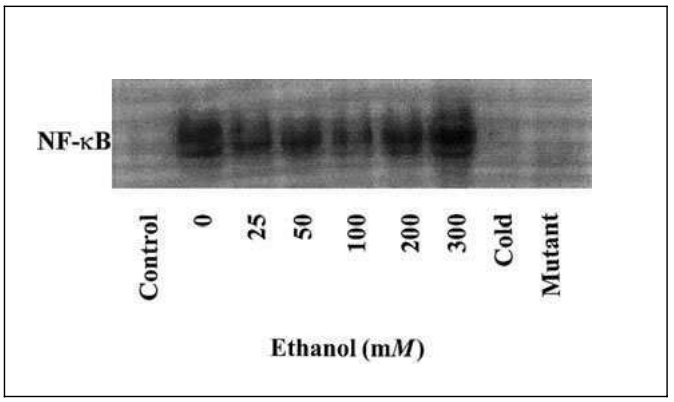


Fig. 5. Effects of ethanol on NF-κB/DNA binding using the electrophoretic mobility shift assay. Cells were cultured in 60-mm dishes and treated with ethanol and cytokines as described in figure 1. After adding cytokines for 1 h, cells were removed for isolation of nuclei as described in the text. Nuclear extract was used to incubate with oligonucleotide probes for NF-κB and followed by electrophoresis. Results represent autoradiograph from a typical experiment.

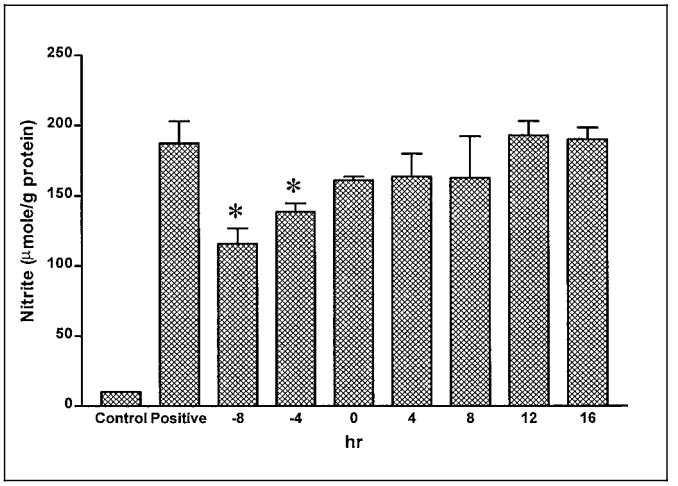


Fig. 6. Effects of different time of ethanol exposure on cytokine-induced NO release. Cells in 35-mm dishes were treated with ethanol (100 m*M*) for different time intervals prior to or after cytokine adding. After 16 h of cytokine treatment, cell culture media were taken for measurement of NO release as previously described. Results are means ± SD from 3 dishes. * $p < 0.05$, one-way ANOVA followed by Bonferroni's multiple comparison test indicates significant differences due to ethanol treatment.

in a protein band (130 kD) corresponding to the molecular weight of iNOS. By comparing with β-actin as a standard, it can be shown that iNOS protein levels in the cells decreased with increasing exposure to ethanol.

Ethanol Treatment Did Not Alter iNOS and sPLA$_2$ mRNA

In this study, DITNC cells were treated with ethanol and subsequently stimulated with cytokines. Samples were subsequently subjected to RT-PCR for assay of iNOS and sPLA$_2$ mRNA. Same samples were also used for RT-PCR of β-actin as a standard. Results in figure 4 show that although cytokines induced the increase in iNOS and sPLA$_2$ mRNAs, there are no apparent changes due to ethanol treatment.

Ethanol Did Not Alter Cytokine-Induced Increase in NF-κB Binding to DNA

In this experiment, cells were exposed to different concentrations of ethanol for 4 h followed by stimulation with cytokine for 1 h. Cells were recovered for isolation of nuclei and for measurement of NF-κB/DNA binding using the electrophoretic mobility shift assay (EMSA). As a positive control, NF-κB binding was competed by 100 × nonlabeled NF-κB probe. Results of EMSA show that unstimulated cells contain low levels of NF-κB in the nuclei, and that cytokine treatment greatly stimulated NF-κB/DNA binding activity (fig. 5). However, there is no apparent effect of ethanol on NF-κB/DNA binding.

Time Course of Ethanol Exposure on Cytokine-Induced NO Production

In order to further examine the time intervals for inhibitory action of ethanol on cytokine induction of NO, cells were exposed to ethanol (100 m*M*) for 8 and 4 h before and 4, 8, 12 and 16 hours after cytokine treatment. As shown in figure 6, significant inhibitory effects of ethanol could be observed when ethanol was added to the cells at 4 and 8 h prior to cytokine stimulation. Results further show that ethanol no longer can effectively inhibit cytokine-induced NO production when added to cells after addition of cytokine.

Acetaldehyde Did Not Inhibit Cytokine-Induced NO Production

Acetaldehyde is the major metabolic product of ethanol in the cell, and this compound can be toxic to cells when present at elevated levels. In this experiment, different levels of acetaldehyde (0–200 μ*M*) were added to cells for 4 h prior to addition of cytokines. As shown in figure 7, acetaldehyde up to 200 μ*M* did not alter cytokine-induced NO production in these cells.

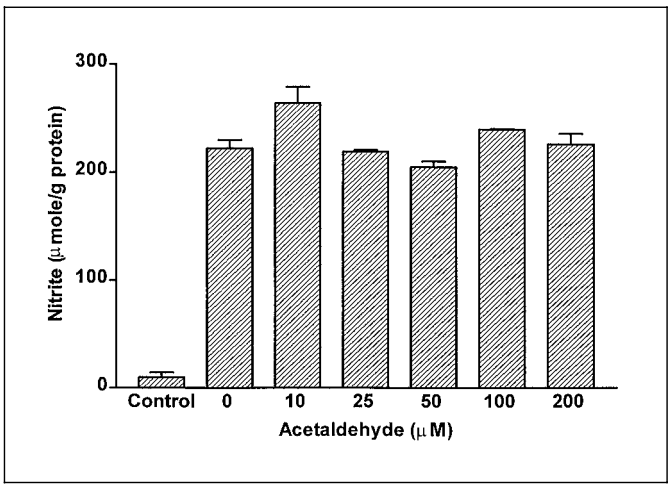


Fig. 7. Effect of acetaldehyde on cytokine induction of NO production. Cells were cultured in 35-mm dishes and acetaldehyde (0–200 μ*M*) was added to the cells 4 h prior to addition of cytokines. After 16 h of cytokine treatment, culture media were removed for determination of NO release. Results are means ± SD from 3 dishes.

Discussion

Due to the active and predictable response of immortalized astrocytes (DITNC) to cytokines, this type of cell culture can serve as an excellent model for assessing effects of compounds that may alter the cytokine signaling pathways in astrocytes [16]. In this study, we demonstrated sensitive effects of ethanol on cytokine-induced NO production in these cells. Under similar conditions, higher concentrations of ethanol (>100 m*M*) are needed to inhibit the induction of $sPLA_2$. Since induction of iNOS mRNA in these cells requires IFN-γ together with TNF-α or IL-1β, whereas, induction of $sPLA_2$ does not require IFN-γ [17], it is reasonable to attribute differences in ethanol sensitivity to the mechanisms for transcriptional activation of the iNOS and $sPLA_2$ mRNA. Indeed, the study by Xie et al. [36] also observed differences in ethanol sensitivity in suppressing cytokine induction of iNOS and TNF-α in rat lung.

Activation of the cytokine signaling pathways may lead to the increase in lipid peroxidation and generation of metabolic products such as acetaldehyde and/or reactive oxygen species [1]. Chronic ethanol-mediated oxidative damage has been demonstrated in hepatic as well as extrahepatic tissues [28]. Despite a lesser extent as compared to hepatic tissues, chronic ethanol consumption also increases reactive oxygen species formation and lipid peroxidation in rat brain, and part of these changes are attributed to the induction of the 2E1 form of cytochrome P450 (CYP2E1) [8, 22]. In cells in the hepatic system, it has been shown that ethanol as well as acetaldehyde can alter the cytokine signaling pathway and induction process [7, 12]. Although acetaldehyde was shown to increase intracellular calcium levels, DNA fragmentation and transglutaminase activity in astrocytes [10], results from the present study indicate no apparent effects of acetaldehyde on the cytokine induction of NO.

Previous studies by Militante et al. [20] and Syapin [32, 33] had obtained similar inhibitory effects of ethanol on NO production in C6 glioma cells and primary astrocytes. These studies also implicated the effect of ethanol on iNOS mRNA levels [20]. However, we are surprised that in our study, ethanol neither altered iNOS mRNA nor NF-κB binding to DNA in DITNC cells. Differences in ethanol effect may be due to a number of factors, e.g. the cell types, the stimuli used for induction of NO, and the time duration of ethanol treatment. The study by Militante et al. [20] had used C6 glioma cells stimulated with phorbol 12-myristate 13-acetate and lipopolysaccharide. In our studies, ethanol was added to cells for 4 h prior to treating with cytokines. Thus, depending on the conditions of the induction process, cytokine-induced iNOS expression can be regulated at multiple levels, including transcriptional, posttranscriptional, translational and post-translational steps as well as modifications of cofactors for the enzyme [26]. Our results, however, reveal a posttranscriptional site for ethanol action. Indeed, there is increasing evidence supporting the perturbation of different protein synthesis steps by ethanol. These steps include alteration of translation initiation factors [14, 15], post-translational modification of proteins [13], intracellular processing in the Golgi complex [27] and direct effect of ethanol on protein synthesis, transport and processing [31]. Taken together, immortalized astrocytes can be a useful cell model for further investigation of the molecular site(s) of ethanol action on synthesis and degradation of iNOS in astrocytes.

Acknowledgment

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Interaction of Nutrition and Binge Ethanol Treatment on Brain Damage and Withdrawal

Fulton T. Crews Christoper J. Braun Robin Ali Darin J. Knapp

Bowles Center for Alcohol Studies, School of Medicine, University of North Carolina at Chapel Hill, N.C., USA

Key Words

Ethanol, chronic · Diet · Withdrawal · Neurodegeneration · Silver stain · Cortex · Hippocampus · Nutrition

Abstract

To determine if nutrition plays a role in ethanol withdrawal and alcohol-induced brain damage, the effects of a 4-day ethanol binge treatment using ethanol in a nutritionally complete liquid diet compared to ethanol mixed with water were studied. The nutritionally complete diet group (ETOH-diet) received a complete diet of sugars, proteins and fats with vitamins and minerals with approximately 53% of calories from ethanol while the nutritionally deprived group (ETOH-H_2O) received 100% of calories from ethanol. No difference in withdrawal behavior was found between the ETOH-diet and ETOH-H_2O groups during the 72-hour period studied. In addition, no difference was seen for serum levels of magnesium and zinc taken at last dose or following 72 h of withdrawal. Serum alanine aminotransferase (ALT) and ammonia were increased in both groups with ETOH-diet showing a greater increase in ALT than ETOH-H_2O. Both groups showed damage in the olfactory bulb, perirhinal, agranular insular, piriform and lateral entorhinal cortical areas as well as hippocampal dentate gyrus and CA-3. Interestingly, the ETOH-diet group displayed more damage at last dose in the posterior dentate and CA-3 of hippocampus than did the ETOH-H_2O group. This study suggests that nutritional components and total caloric intake do not effect behavior during ethanol withdrawal and that a nutritionally complete diet may increase ethanol-induced brain damage.

Several human and animal studies indicate that chronic alcohol intoxication damages the brain [8, 17]. Human studies using computed tomography and magnetic resonance imaging of living human brain [8, 31] as well as postmortem studies [16] indicate that alcoholics have a reduction in brain size including enlargement of the cerebral ventricles and sulci. In addition to reduced brain mass, alcoholics also show deficits in brain function on a continuum of moderate deficits in the majority of long-term alcoholics ranging to much more severe deficits of Wernicke's disease and Wernicke's encephalopathy with Korsakoff's amnestic syndrome in the most severe cases [1, 27]. Wernicke's disease is most often found in alcoholics. It is related to thiamin deficiency resulting from a combination of inadequate dietary intake, reduced gastrointestinal absorption, decreased hepatic storage and impaired use of the vitamin [4, 37]. Although many alco-

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Fulton T. Crews, PhD
Center for Alcohol Studies, CB#7178, Thurston Bowles Bldg.
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-7178 (USA)
Tel. +1 919 966 5678, Fax +1 919 966 5679, E-Mail ftcrews@med.unc.edu

holics appear to have thiamin deficiency, only a subset develop Wernicke's disease, a finding that suggests additional factors may be involved. Wernicke's encephalopathy has characteristic brain lesions exhibiting symmetrical discoloration of the structures surrounding the third ventricle, aqueduct and forth ventricle with atrophy of the mammillary bodies in many cases, as well as neuropil loss, demyelination and neuronal loss in the medial thalamus [4, 37]. Thus, an interaction between ethanol and dietary factors can contribute to neuropathology often associated with chronic alcoholism.

Decreased brain mass in human studies occurs after years of alcohol abuse, and in the case of Wernicke's encephalopathy, years of poor nutrition. Recent studies in animals have found that as little as a 2–4 days of alcohol intoxication can lead to neuronal loss in several brain areas including entorhinal cortex and hippocampal dentate gyrus [6, 9]. These findings are consistent with recent human studies reporting damage to entorhinal cortex [18] and significant hippocampal shrinkage in alcoholics [15].

In this study, we use a binge ethanol treatment model of alcohol abuse previously shown to cause physical dependence, gene induction and alcohol-induced brain damage [6, 20, 22] to investigate the effects of nutrition on binge drinking-induced brain damage. This binge treatment protocol models the heavy alcohol consumption common among regular alcohol abusers and alcoholics. For example, Urso et al. [36] reported on alcohol blood levels in 76 representative subjects seen in the emergency room of a community hospital. While appearing sober, the group mean blood level was approximately 270 mg/dl, with levels as high as 540 mg/dl. In another study of individuals admitted, all talking and alert, to an alcohol detoxification unit, approximately 10% had blood alcohol levels over 500 mg/dl, with one individual alert at 894 mg/dl. In most cases, these measures were taken 4 h after the last drink; therefore, these values underestimate the peak levels [2]. An emergency room study of 640 patients found that approximately 4% had blood levels over 350 mg/dl [35]. In a recent study of a southwestern American Indian tribe, binge drinking – defined as more than 24 drinks daily for 3 or more days occurring at least 3 times in a lifetime – was very frequent, being reported by approximately 70% of men and 25% of women [28]. Thus, very high blood alcohol levels are common among alcoholics and can cause neurodegeneration. All of the 4-day binge drinking model studies performed to date showing neurodegenerative changes in animals used nutritionally complete diets. To determine the effects of diet on brain damage associated with binge drinking, we administered ethanol either added to a complete nutritional liquid diet or added to water. Our studies found damage in both groups with a trend toward more damage in the complete nutritional diet group.

Table 1. Average daily caloric, vitamin and mineral intake, and body weights of 4-day binge treatment with either ETOH-H_2O or ETOH-diet

Caloric sources	ETOH-diet	ETOH-H_2O
Composition, kcal/kg/day		
Dextrose	19.0	0.0
Lactoalbumin	23.0	0.0
Corn oil	11.2	0.0
Ethanol	59.6	58.2
Total	113.0	58.2
Dietary vitamins and minerals, mg/day		
$Ca^{2+}Cl^-$	501.0	0.0
Ca^{2+}pantothenate	0.7	0.0
$MgSO_4 \cdot 7\ H_2O$	75.0	0.0
$MgSO_4 \cdot H_2O$	4.5	0.0
$ZnCl^-$	0.8	0.0
Thiamin	1.0	0.0
Pyridoxine	1.0	0.0
Mean body weight, g		
Mean initial weight	305 ± 4.3	299 ± 4.5
Mean final weight	254 ± 3.3	242 ± 2.8*

Weight loss for the ETOH-H_2O group was greater than weight loss for the ETOH-diet group ($p < 0.053$). * $p < 0.05$.

Methods

Subjects and Treatments

Male Sprague-Dawley rats (n = 22, Charles River Laboratories, Raleigh, N.C.) were surgically implanted with intragastric cannulae at approximately 90 days of age. After 3 days of postsurgical recovery, all animals were exposed to 4 days of alcohol binge-type treatment. Animals were administered either 15% alcohol in water (ETOH-H_2O group) solution or 15% alcohol in nutritionally complete diet (ETOH-diet) solution [14] (table 1). Both groups received an average of 8.4 g/kg/day of ethanol (8.4 ± 0.24 for the ETOH-H_2O group and 8.4 ± 0.44 for the ETOH-diet group) administered over 4 days 4 times/day (12 a.m., 6 a.m., 12 p.m. and 6 p.m.). The first dose was a 5 g/kg priming dose. Each subsequent dose was determined based on the animal's body weight and behavioral score using a slightly modified version of the scale used by Majchrowicz [22]. A score of 0 indicated a state in which no signs of intoxication were observable, and a score of 5 indicated coma and loss of righting reflex [9, 21]. Mean behavioral states throughout the 4 days of treatment were 2.25 ± 0.25 for the ETOH-diet group and 2.25 ± 0.48 for the ETOH-H_2O group. Blood ethanol levels (BELs) were determined spectrophoto-

metrically with Sigma alcohol dehydrogenase kits (Sigma, St. Louis, Mo.). BELs taken immediately before and 1 h after the noon infusion on the 2nd and 4th day were 420 ± 30 and 530 ± 15 (mg/dl), respectively, for the ETOH-diet group and 450 ± 70 and 540 ± 20, respectively, for the ETOH-H_2O group. All animals periodically received 2–3 ml boluses of water between infusions of alcohol to maintain hydration. After the last infusion, animals were sacrificed within 1–2 h (T0 group) or after 72 h of withdrawal (T72 group).

Neurodegenerative Assay Using Amino Cupric Silver Stain

Animals were sacrificed following an 80 mg/kg overdose of pentobarbital (Abbott Labs, Chicago, Ill.). Animals were transcardially perfused [2 m*M* cacodylate (Sigma), 0.9% NaCl (Mallinkrodt-Baker, Paris, Ky.), 22 m*M* dextrose (Fisher Scientific, Fair Lawn, N.J.), 22 m*M* sucrose (Mallinkrodt-Baker), and 2 m*M* $CaCl_2$ (ICN Biomedical, Aurora, Ohio) at pH 7.4] at a flow rate of 27 ml/min for 4 min followed by fixation, 4% paraformaldehyde (Fisher Scientific), 90 m*M* sodium cacodylate and 115 m*M* sucrose, at a flow rate of 27 ml/min for 7 min. Skulls were postfixed for 24–48 h prior to brain removal. Brains were cut on an AO sliding microtome and amino cupric silver-stained to assay for neurodegeneration (Neuroscience Associates, Knoxville, Tenn.). Every eight 40-μm section (coronal plane) was stained for neurodegeneration using a similar technique to the amino cupric silver stain developed by Crews et al. [9] and de Olmos et al. [11].

Blood Chemistry Assays

At the time of sacrifice, 5 ml of blood were collected into a tube from the right atrium of the heart. Blood samples were centrifuged at 2,600 *g* for 6 min in a refrigerated Beckman centrifuge and the plasma was frozen at –20°C until assays were run. Calorimetric and atomic absorption assays were done to measure levels of Mg and Zn, respectively (LabCorp, Burlington, N.C.).

Tail vein blood samples were collected into heparin-containing tubes on day 3 of the 4-day binge alcohol treatment. Samples were centrifuged at 2,600 *g* for 5 min, and serum was frozen until assays were done. Alanine and aminotransferase levels (ALT) and ammonia were measured with activity assays. ALT, AST and ammonia levels were assayed using a Johnson & Johnson model 250 Analyzer. BELs were measured using an alcohol dehydrogenase assay (Sigma).

Intoxication and Withdrawal Behavior Scoring

A 6-point scale from 0–5 was used to measure alcohol intoxication (0 = minimal intoxication, 5 = maximal intoxication). The dose to be administered to each animal was determined based on this scale and the animal's body weight in grams. The 6-point scale that was used in our ethanol binge protocol is based closely on the scale published by Majchrowicz [22]. Animals with a behavioral score of 0 (no behavioral signs of intoxication) received the maximal dose of 5 g/kg of ethanol. Animals with a behavioral score of 1 (hypoactive, but no signs of ataxia) received 4 g/kg ethanol. Animals with a score of 2 (mildly ataxic and hypoactive) received 3 g/kg ethanol. Animals with a score of 3 (severe ataxia, absence of abdominal elevation and rigidity, delayed righting reflex) received 2 g/kg ethanol. Animals with a score of 4 (loss of righting reflex, but retaining eye blink reflex, pain response, and spontaneous movement when righted by the experimenter) received 1 g/kg ethanol. Animals with a score of 5 (complete loss of righting and eye blink reflexes, pain sensation and voluntary movement) received 0 g/kg ethanol.

In order to measure behavioral observations of ethanol withdrawal, we used a 5-point scale with 13 gradations based on the ethanol withdrawal scale published by Majchrowicz [22]. After the last dose of ethanol, all animals were observed for symptoms associated with ethanol withdrawal. A withdrawal score of 0 indicated that an animal was behaviorally neutral or was still sedated, an effect of elevated blood ethanol. A score of 1 indicated general hyperactivity. A score of 1.4 indicated tail tremors and rigidity. A score of 1.6 indicated tail spasticity and tail flicking. A score of 2.0 indicated general tremors of the hind limbs, pelvis and tail. A score of 2.4 indicated general spasticity and splayed limbs. A score of 2.6 indicated general tremors of the body. A score of 3.0 indicated head tremors. A score of 3.2 indicated induced running episodes where the animal would uncontrollably run around the cage. A score of 3.4 indicated that the animal experienced wet shakes and severe whole body tremors. A score of 3.6 indicated that the animal experienced chattering of the teeth and hyperactivity of the jaw muscles. A score of 3.8 indicated that an animal experienced spontaneous convulsions that sometimes lead to death. A score of 4 indicated death. This scale represents a progression of the symptoms of ethanol dependence and subsequent withdrawal which generally begins approximately 6 h after the last dose of a binge treatment and lasts until 24 h later in the most severe cases.

Digital Imaging Analysis of Amino Cupric Silver Staining

Digital image analysis was performed using a Zeiss Axiovert 100 microscope (Carl Zeiss, Thormwood, N.Y.), Dage Newvicon Red analog CCD camera (Dage Instruments, Michigan City, Ind.), and KS-400 ver. 2.0 digital image analysis software (Kontron Electronik, Eching bei München, Germany). Macro software was written to allow detection of argyrophilic regions and the area of these regions was measured in serial 40-μm brain sections.

Statistics

All groups were analyzed using ANOVA with Fisher's PLSD post hoc analysis to determine statistical significance ($p < 0.05$).

Results

Withdrawal Behavior, Serum Enzymes and Ions

To determine if nutrition would alter weight loss, physical dependence to ethanol following binge drinking, or serum levels of liver enzymes and minerals, blood was collected at the time of sacrifice and serum prepared. Rats in both groups were paired at the beginning of the study and received an average 8.4 g/kg/day of ethanol. Group mean weights for the ETOH-H_2O and ETOH-diet at the time of first treatment were 299 ± 4.5 and 305 ± 4.3 g, respectively. Although the ETOH-diet group received almost double the calories during treatment, both groups lost similar amounts of weight (table 1). Serum magnesium and zinc levels have been implicated in the symptoms of alcohol withdrawal [25] and similarly were not significantly changed at either T0 (just after the last dose of ethanol) or T72 (72 h after the last dose of ethanol) (table 2).

Table 2. Blood serum levels of Mg^{2+} and Zn^{2+} following 4 days' ETOH-H_2O, ETOH-diet, or normal chow diet control treatment

Treatment group	Mg^{2+}, mg/dl	Zn^{2+}, µg/dl
Chow control	1.6 ± 0.4	144 ± 15.8
ETOH-diet		
T0	2.1 ± 0.1	268 ± 66.4
T72	1.8 ± 0.1	148 ± 38.9
ETOH-H_2O		
T0	1.8 ± 0.1	121 ± 32.2
T72	1.9 ± 0.1	119 ± 28.9

Table 3. Blood serum levels of ALT and ammonia during 4-day binge treatment with ETOH-H_2O or ETOH-diet

Treatment group	ALT, U/l	Ammonia, µmol/l
Control diet	40 ± 10	227 ± 19
ETOH-diet	121 ± 18[a,b]	308 ± 64
ETOH-H_2O	64 ± 12	263 ± 32

Blood serum levels of ALT and ammonia were assayed from the serum of heparinized tail blood samples taken on day 3 of the 4-day binge treatment. ALT and ammonia values of untreated rats with ad libitum access to rat chow are approximately 50–60 U/l and 25 µmol/l, respectively (n = 9/group). [a] $p = 0.025$ vs. ETOH-H_2O; [b] $p < 0.01$ vs. control diet.

Since diet can alter ethanol metabolism, liver serum enzymes were also measured. Only the ETOH-diet group showed a significant increase in ALT levels being 3-fold greater than the control diet group (table 3). Interestingly, the ETOH-diet had significantly greater ALT levels than the ETOH-H_2O and the control diet groups. Thus, although the ETOH-diet group did not differ in ethanol-induced weight loss, ethanol withdrawal symptoms, or serum magnesium or zinc levels, this group did show increased serum ALT levels over those found in the ETOH-H_2O and control diet groups. Neither the ETOH-diet nor the ETOH-H_2O groups showed an increase in ammonia levels as compared to the control diet group (table 3).

The binge ethanol treatment produced a robust withdrawal syndrome indicative of physical dependence in both the ETOH-diet and the ETOH-H_2O group. Characteristic behaviors of ethanol withdrawal observed were splayed limbs, tremors and seizures. The peak of withdrawal was approximately 25 h after the last dose of ethanol. The time course of withdrawal lasted approximately 60 h, and the animals returned to a neutral state by 72 h after the last dose. No significant differences were found between the ETOH-diet and the ETOH-H_2O group regarding duration or severity of ethanol withdrawal.

Binge Ethanol-Induced Brain Damage

To investigate neurodegeneration due to the 4-day binge-type alcohol exposure, amino cupric silver staining was performed in all groups. We confirmed amino cupric silver staining using hematoxylin and eosin stains [26], and others have characterized this stain as a marker of neurodegenerating neurons [34]. Degenerating neurons were quantitated both by cell counting and computer-assisted measurement of argyrophilic area. These two methods complement each other, in that one is focused on neuronal cell bodies (counting), and the other includes degenerating neuropil as well as cell bodies by measuring the silver-stained area [9, 26]. Control diet animals showed essentially no silver staining – neuronal counts were zero and the argyrophilic area was low, but analyzed to provide values above zero for meaningful comparisons. Control animals have never exhibited argyrophilia in any of the brain regions that were measured [e.g. 9, 26]. Counts of argyrophilic cells are depicted in table 4 while corresponding pictures of damaged brain regions are depicted in figures 1–4. Both ETOH-treated groups showed damage in mesocorticolimbic areas, including the anterior and posterior dentate gyrus (fig. 1), lateral entorhinal (fig. 2), posterior perirhinal (fig. 3) and piriform (fig. 4)

Table 4. Cell counting of argyrophilic cell bodies in various brain regions of the rat brain 0 or 72 h (T0 or T72) following a 4-day binge ethanol treatment with ETOH-H_2O or ETOH-diet

Brain region	ETOH-diet		ETOH-H_2O	
	T0	T72	T0	T72
Posterior dentate	50 ± 20[a]	6 ± 3	13 ± 7	15 ± 5
Hippocampus CA3	46 ± 17[a]	6 ± 5	2 ± 0	6 ± 2
Posterior piriform	55 ± 17[b,c]	25 ± 8	14 ± 2	18 ± 7
Lateral entorhinal	41 ± 9	37 ± 14	61 ± 8	51 ± 5
Frontal dentate	11 ± 5	2 ± 1	0	8 ± 7
Posterior perirhinal	14 ± 3	12 ± 6	7 ± 1	12 ± 3
Agranular insular	6 ± 1	5 ± 2	4 ± 2	9 ± 2

[a] $p < 0.05$ vs. ETOH-H_2O T0 group; [b] $p < 0.02$ vs. ETOH-diet T72 group; [c] $p < 0.05$ vs. ETOH-H_2O T72 group (Fisher's PLSD post hoc analysis, n = 4/group).

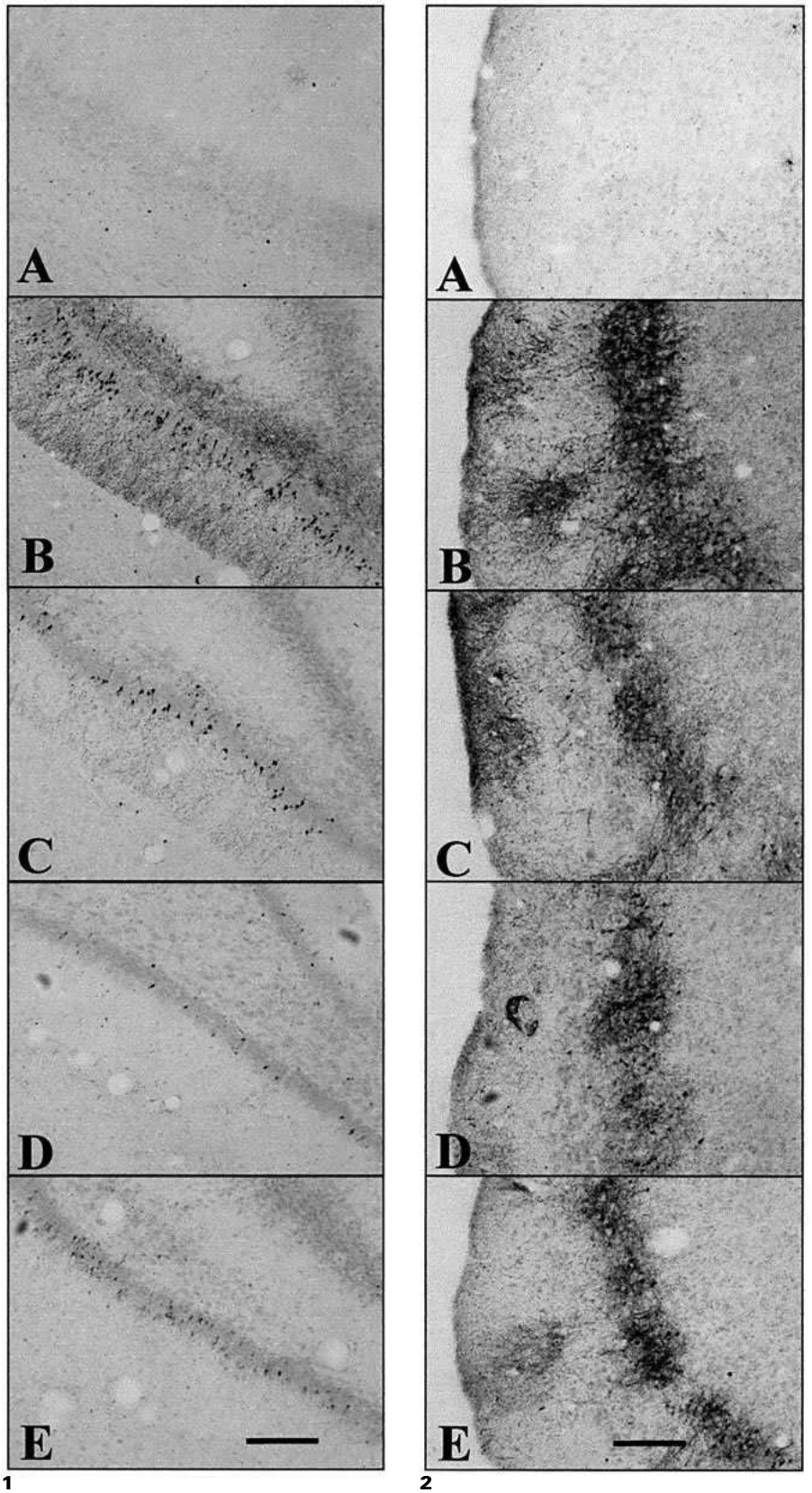
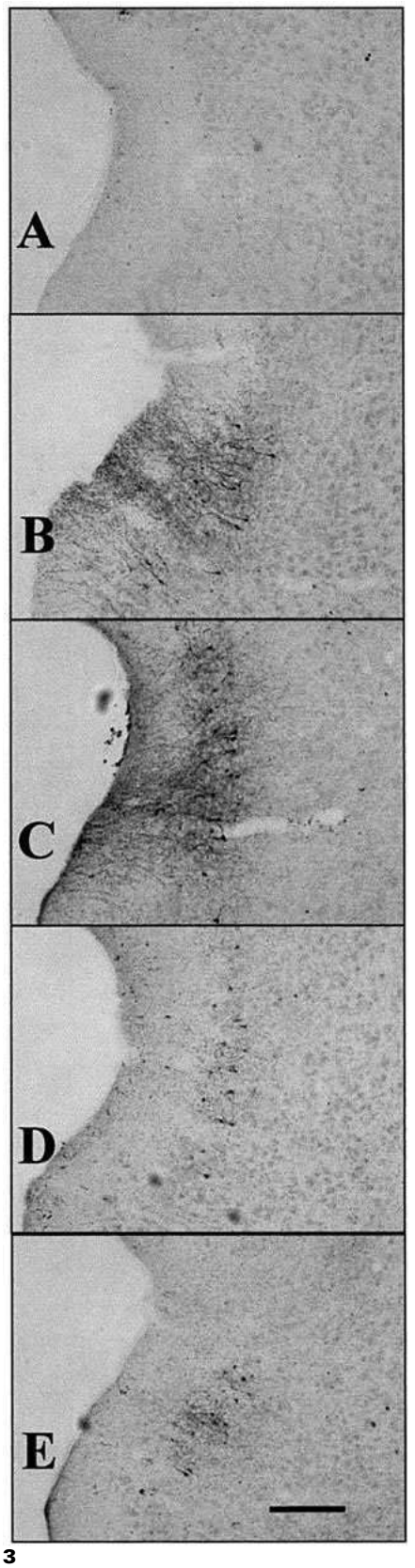


Fig. 1. Amino cupric silver-stained sections of the rat brain following 4 days of binge-type intragastric ethanol treatment alone or ethanol treatment in a nutritionally complete diet. Depicted are digital images of the posterior-ventral dentate gyrus of the hippocampus (bregma –5.0 mm). **A** Control diet. **B** ETOH-diet T0 (0 h after withdrawal of ethanol treatment). **C** ETOH-H_2O T0. **D** ETOH-diet T72. **E** ETOH-H_2O T72. Note the absence of staining in control tissue and the extensive staining in the granule cell layer and neuropil staining within the outer blade of the dentate gyrus and polymorphic layer especially in **B**. Bar represents 200 μm.

Fig. 2. Amino cupric silver-stained sections of the rat brain following 4 days of binge-type intragastric ethanol treatment alone or ethanol treatment in a nutritionally complete diet. Depicted are digital images of the entorhinal cortex (bregma –6.5 mm). **A** Control diet. **B** ETOH-diet T0 (0 h after withdrawal of ethanol treatment). **C** ETOH-H_2O T0. **D** ETOH-diet T72. **E** ETOH-H_2O T72. Note the absence of staining in control tissue and the extensive staining in cell bodies and processes of layers 2/3 with additional staining into layer 1 (neuropil) of ethanol-treated tissue. Bar represents 200 μm.



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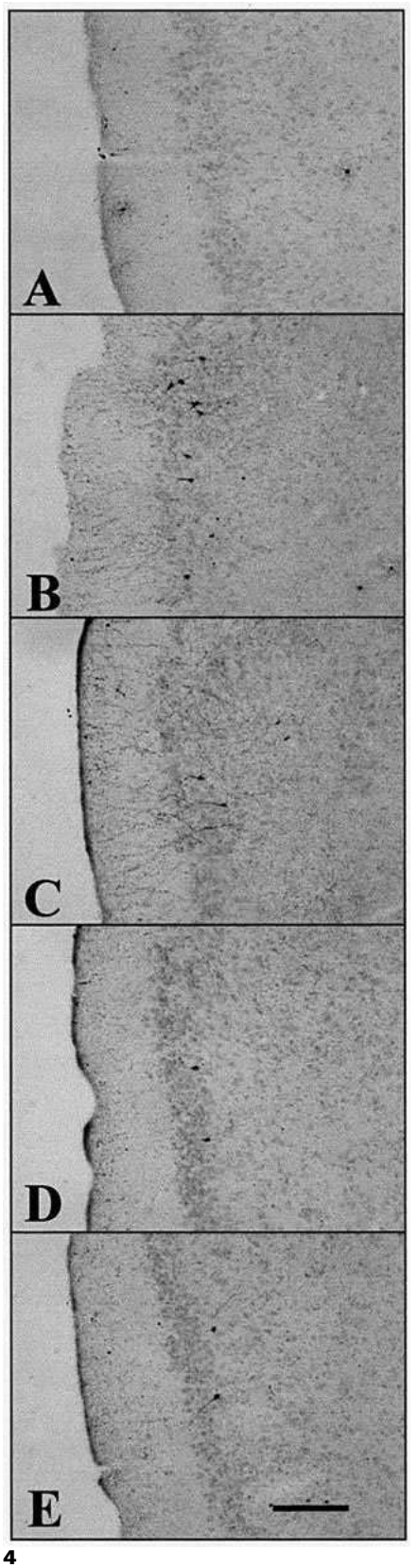

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Fig. 3. Amino cupric silver-stained sections of the rat brain following 4 days of binge-type intragastric ethanol treatment alone or ethanol treatment in a nutritionally complete diet. Depicted are digital images of the perirhinal cortex (bregma –3.3 mm). **A** Control diet. **B** ETOH-diet T0 (0 h after withdrawal of ethanol treatment). **C** ETOH-H_2O T0. **D** ETOH-diet T72. **E** ETOH-H_2O T72. Note the absence of staining in control tissue and the extensive staining of cell bodies and processes of cortical layers 2/3 with additional staining into layer 1 (neuropil) of ethanol-treated tissue. Bar represents 200 μm.

Fig. 4. Amino cupric silver-stained sections of the rat brain following 4 days of binge-type intragastric ethanol treatment alone or ethanol treatment in a nutritionally complete diet. Depicted are digital images of the posterior piriform cortex (bregma –3.3 mm). **A** Control diet. **B** ETOH-diet T0 (0 h after withdrawal of ethanol treatment). **C** ETOH-H_2O T0. **D** ETOH-diet T72. **E** ETOH-H_2O T72. Note the absence of staining in control tissue and the extensive staining of cell bodies and processes of layers 2/3 with additional staining into layer 1 (neuropil) of ethanol-treated tissue. Bar represents 200 μm.

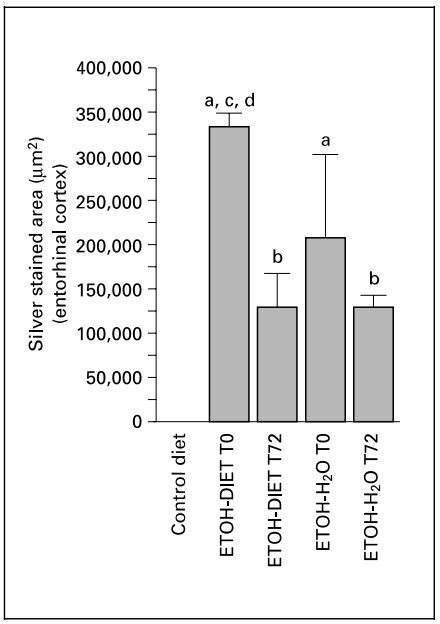

Fig. 5. Binge ethanol-induced brain damage as assessed with computer-assisted image density analysis. Silver-stained areas are summed across four 40-μm slides (one slide every 320 μm) through the entorhinal cortex. Silver-stained area was measured using digital imaging following 4 days' binge ethanol in ETOH-diet, ETOH-H_2O or nutritionally complete calorie-matched control diet. [a] $p < 0.01$ vs. control (Sheffé's post hoc); [b] $p < 0.05$ vs. control; [c] $p < 0.005$ vs. ETOH-diet T72 and ETOH-H_2O T72; [d] $p < 0.055$ vs. ETOH-H_2O T0. ANOVA, Fisher's PLSD post hoc.

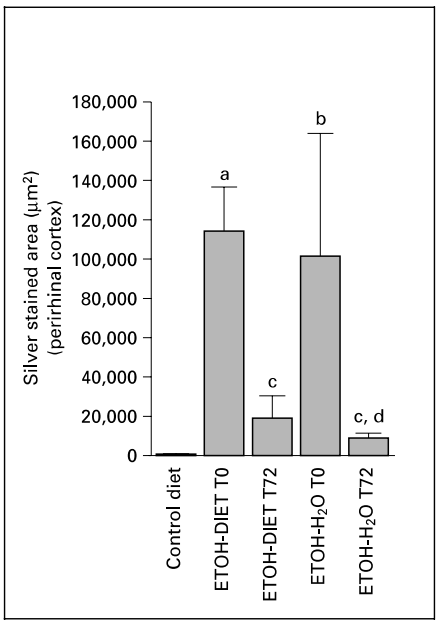

Fig. 6. Binge ethanol-induced brain damage as assessed with computer-assisted image density analysis. Silver-stained areas are summed across four 40-μm slides (one slide every 320 μm) through the perirhinal cortex. Silver-stained area was measured using digital imaging following 4 days' binge ethanol in ETOH-diet, ETOH-H_2O or nutritionally complete calorie-matched control diet. [a] $p < 0.01$ vs. control; [b] $p < 0.02$ vs. control; [c] $p < 0.03$ vs. ETOH-diet T0; [d] $p < 0.04$ vs. ETOH-H_2O T0. ANOVA, Fisher's PLSD post hoc.

cortices, CA3 of the hippocampus and agranular insular cortex. Both ETOH-treated groups also showed the greatest damage at T0 (just after the last ethanol dose) with reduced damage at T72 (72 h after the last ethanol dose when the withdrawal syndrome was complete). Computer-assisted quantification of image densities of cell body and neuronal processes revealed significantly greater damage in the lateral entorhinal and posterior perirhinal cortices of ETOH-diet-treated T0 animals relative to the remaining groups (fig. 2B and 3B quantified in fig. 5 and 6). Cell counting for these two regions revealed significant agyrophilic cell numbers in both the lateral entorhinal and posterior perirhinal cortices that were not significantly different between ETOH-treated groups; however, in posterior dentate and hippocampal CA3 ETOH-diet-treated T0 animals had significantly more agyrophilic cell bodies than ETOH-H_2O-treated animals (table 4). Thus, ETOH-treated groups showed significant brain damage with the ETOH-diet-treated T0 animals showing the greatest damage.

Discussion

Several clinical reports have found a correlation between low serum magnesium levels and the appearance of withdrawal signs and symptoms in chronic alcoholics with the more severe withdrawal states associated with more severe decrements in serum magnesium levels [13, 24, 32]. Both magnesium and zinc are known to reduce NMDA-glutamate receptor activation, and several studies have suggested that hyperactivity of the NMDA-glutamate receptor is involved in the ethanol withdrawal syndrome [10]. Mendelson et al. [23] administered ethanol equivalent to approximately one quart of bourbon per day for 21 days to human volunteers and found serum magnesium levels to drop at 24 h after cessation of drinking and to remain significantly decreased for 72 h, returning to control levels by the 5th withdrawal day. These levels returned to control levels without supplements. Although our animals exhibit significant signs of ethanol withdrawal including seizures, we found no changes in serum magnesium at T0 or T72 after cessation of ethanol, and no

differences in ethanol withdrawal syndrome between ETOH-diet and ETOH-H_2O-treated animals. We found that rats show significant ethanol withdrawal syndrome after binge ethanol treatment similar to previous studies [5, 9, 22]. However, there were no changes in serum magnesium or zinc at the time points we measured, a finding that suggests that the ethanol withdrawal syndrome does not require pronounced changes in serum levels of these ions.

Binge ethanol treatments induced damage to the piriform, perirhinal, entorhinal cortexes and hippocampus in both ETOH-diet- and ETOH-H_2O-treated animals. There are extensive associational connections linking olfactory, perirhinal, piriform and entorhinal cortical areas which project to the hippocampus [30]. These brain regions likely play a role in olfactory sensory processing and memory tasks. Chronic alcoholism causes both cognitive deficiencies and olfactory deficits [12, 19]. Further, olfactory impairment in alcoholics has been correlated with brain damage as assessed by increased MRI-measured cerebral spinal fluid volume, indicating decreased brain mass [29]. These studies suggest that the binge ethanol treatment-induced brain damage found in animal models has, at least in part, components of deficits found in human alcoholics.

The mechanisms of binge ethanol-induced brain damage remain obscure. The connections between olfactory, perirhinal, piriform, entorhinal and dentate gyrus suggest that glutamate-aspartate-containing pyramidal cells connecting to each brain region might be related to binge ethanol neurotoxicity. Chronic ethanol has been shown to increase neuronal sensitivity to NMDA receptor excitation and NMDA receptor-mediated excitotoxicity [3]. However, efforts to block binge ethanol-induced neurotoxicity have not been consistent with this hypothesis [6, 7]. NMDA antagonists such as MK801 produce damage somewhat similar to that caused by binge ethanol treatment [7] and do not appear to reduce binge ethanol-induced damage when given at doses below their neurotoxic threshold. Furthermore, in a single daily ethanol dose protocol that produces brain damage, MK801 did not have a neuroprotective action [6]. Similarly, nimodipine, a calcium channel antagonist, and DNQX, a glutamate AMPA receptor antagonist, did not have neuroprotective properties in binge ethanol-induced damage [7]. It has been proposed that brain edema and status epileptic seizures that involve these brain regions may mediate binge ethanol-induced neurotoxicity, and furosimide has been found to reduce ethanol neurotoxicity with once-daily treatment and in vitro ethanol-induced neurotoxicity [6]. However, we did not find a protective effect of furosemide in our 4-day binge model, although we confirmed amino cupric silver staining damage using hematoxylin and eosin stains [26]. Greater damage was found in ETOH-diet posterior dentate cell counts than in ETOH-H_2O. In addition, we saw a greater elevation of serum ALT. Alcohol is known to cause liver toxicity, and hepatic encephalopathy is known to cause brain damage. Thus, it is possible that nutrients in combination with ethanol actually increase liver toxicity in a manner that accentuates brain damage. However, the differences between ETOH-diet and ETOH-H_2O groups were small relative to controls. The exact mechanisms of binge ethanol-induced neurotoxicity remain to be elucidated but clearly seem to be related to the interconnecting pathways from the frontal cortical olfactory areas to the mesocorticolimbic association and memory-consolidating areas of brain.

We found our greatest damage just after the last dose of ethanol, with less damage at 72 h after the last dose. This finding is consistent with that of previous studies in which we found no remaining detectable silver-stained damage at 168 h after the last dose [9]. Since the time required for degeneration of cell bodies and dendrites to become visualized by silver degeneration stain is about 36–48 h after neuronal insults [34], the insult caused by the 4-day binge ethanol treatment is likely to be increasing during days 2 and 3 of dosing. Collins et al. [5] found binge ethanol treatment-induced brain damage 8 and 36 h after the last ethanol dose with the 36-hour time point showing a trend towards reduced silver stain damage. Posterior perirhinal cortex and entorhinal cortex argyrophilic area of T0 ETOH-diet rats showed significantly greater damage than in T72 ETOH-diet-treated animals with a similar trend in the ETOH-H_2O-treated rats. Although the cupric silver stain of the Olmos that we used was developed specifically to identify degeneration of neurons, axons and other neuronal processes, neuronal debris is likely cleared in the days following neuronal death such that the silver stain is no longer present [34]. Taken together, these findings suggest that binge ethanol-induced brain damage occurs during ethanol treatment, before the peak onset of withdrawal symptoms that occur between 12 and 24 h after the last dose.

In summary, binge ethanol treatment leads to physical dependence upon ethanol in both ETOH-diet- and ETOH-H_2O-treated groups which is followed by a pronounced but similar ethanol withdrawal syndrome. No changes in serum magnesium or zinc are associated with the withdrawal syndrome. The 4-day binge ethanol treatment results in brain damage in perirhinal, piriform, entorhinal cortexes and hippocampus in ETOH-diet groups consistent with previous findings [5, 9, 33] and is

similar to damage found in ETOH-H_2O treatment with two exceptions: ETOH-diet groups had slightly greater damage in hippocampus and increased serum ALT levels. These findings suggest that a nutritionally complete diet may not protect the brain from binge ethanol-induced brain damage, and in fact may exacerbate damage under certain conditions.

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Metabolic Fate of [^{14}C]-Ethanol into Endothelial Cell Phospholipids Including Platelet-Activating Factor, Sphingomyelin and Phosphatidylethanol

Roberta M. Magai Shivendra D. Shukla

Department of Pharmacology, University of Missouri School of Medicine, Columbia, Mo., USA

Key Words

Phospholipids · Ethanol · Platelet-activating factor · Sphingomyelin · Phosphatidylethanol · Endothelial cells

Abstract

The metabolic fate of ethanol into the phospholipid pool of calf pulmonary artery endothelial cells was studied. [^{14}C]-ethanol was incorporated into various endothelial cell phospholipids including phosphatidylethanol (PEth), which may represent a substantial fraction in microdomains of membrane phospholipids. The incorporation into phospholipids was reduced in the presence of pyrazole and cyanamide, inhibitors of ethanol metabolism. Wortmannin, the phosphatidylinositol 3-kinase inhibitor, increased [^{14}C]-PEth formation. [^{3}H]-acetate was also incorporated into endothelial cell phospholipids but in a different pattern. Distribution of [^{3}H]-acetate and [^{14}C]-ethanol into the fatty acyl moiety versus the glycerophosphoryl backbone of the phospholipids was also different. Stimulation of the endothelial cells with ATP increased [^{3}H]-acetate incorporation into platelet-activating factor (PAF) and ethanol decreased it. Ethanol exposure increased ATP-stimulated [^{3}H]-acetate incorporation into sphingomyelin. However, ATP had no effect on the incorporation of [^{14}C]-ethanol into any phospholipids. The results suggest that the two precursors contribute to a separate acetate pool and that the sphingomyelin cycle may be sensitized in ethanol-treated cells. Thus, metabolic conversions of ethanol into lipids and the effect of ethanol on specific lipid mediators, e.g PAF, PEth and sphingomyelin, may be critical determinants in the altered responses of the endothelium in alcoholism.

Early research suggested that ethanol strongly interacts with biological membranes by partitioning into the lipid bilayer [19]. More recently, ethanol has been shown to interact with membrane-associated signal transduction mechanisms that rely on the reaction of phospholipases with their phospholipid substrates in the membrane [9, 20]. However, it is not known whether and how much of this ethanol is distributed into the cellular lipid. The main pathway for ethanol metabolism involves hepatic alcohol dehydrogenase (ADH), an enzyme that catalyzes the formation of acetaldehyde. Acetaldehyde is converted to acetate through the activity of another enzyme, acetaldehyde dehydrogenase. Acetate is in turn converted into acetylcoenzyme A (CoA), which can enter a number of path-

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Dr. S.D. Shukla
Department of Pharmacology
School of Medicine, 1 Hospital Drive
University of Missouri-Columbia, Columbia, MO 65212 (USA)
Tel. +1 573 882 2740, Fax +1 573 884 4558, E-Mail shuklasd@health.missouri.edu

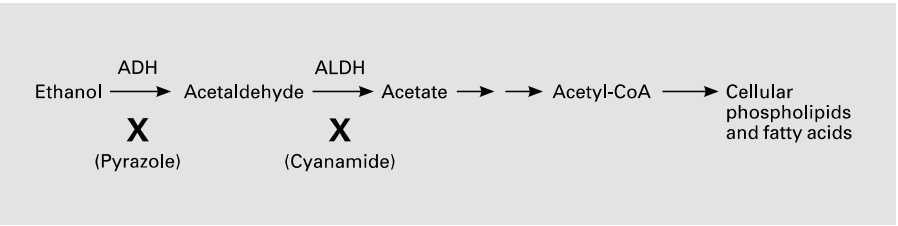


Fig. 1. Metabolism of ethanol. Ethanol is metabolized primarily in the liver, first to acetaldehyde by ADH, and then to acetate by aldehyde dehydrogenase (ALDH). Acetate is then converted to pyruvate, which is converted to acetyl-CoA by the pyruvate dehydrogenase complex. Acetyl-CoA is an important component of several biosynthetic pathways, one of which can lead to the formation of cellular phospholipids and fatty acids. ADH and aldehyde dehydrogenase are inhibited (shown by X) by pyrazole and cyanamide, respectively.

ways including phospholipid synthesis (fig. 1) [15]. The presence and activity of different isoforms of ADH in rat and in human blood vessels have been studied, thus suggesting that blood vessels themselves may contribute to extrahepatic ethanol metabolism [1].

Vascular endothelial cells (ECs) play an important role in mediating the effects of various substances that are introduced into the body through the circulation. Quiescent EC have the important physiological function to facilitate blood flow by providing an antithrombotic, profibrinolytic surface that inhibits adhesion of various types of circulating blood components. EC contribute to vasoregulation by releasing compounds such as nitric oxide, prostacyclin (PGI_2), and platelet-activating factor (PAF) in response to changes in their external cellular environment [6]. Vascular EC are continuously exposed to ethanol circulating in blood; therefore, ethanol can profoundly affect ECs. One pertinent issue is the incorporation of this ethanol into specific phospholipid pools, some of which have roles in cellular signalling responses.

It is well known that ethanol affects fatty acids and glycerolipid metabolism in liver. The formation of the phospholipid phosphatidylethanol (PEth) during ethanol exposure has been implicated in the pathogenesis of alcohol-induced organ damage [7]. Hepatic triacylglycerol accumulations have been described in chronic alcoholism [12]; however, reports on the effects of ethanol administration on the biosynthesis of cellular phospholipids are few and contradictory [4, 10, 16]. Several researchers have found that ethanol affects the incorporation and distribution of various exogenously supplied substrates in different ways. To our knowledge, there has been no study of the metabolic fate of ethanol into individual phospholipids. We have addressed this issue using EC.

Methods

Materials

Fetal bovine serum, pyrazole, cyanamide, wortmannin and the NAD-ADH assay kit were purchased from Sigma (St. Louis, Mo.). Minimum essential media, penicillin, streptomycin and glutamine were purchased from Gibco BRL (Grand Island, N.Y.). The [^{14}C]-ethanol and [^{3}H]-acetic acid were purchased from American Radiochemicals (St. Louis, Mo.). Propranolol and Ro-31-8220 were purchased from BioMol (Plymouth Meeting, Pa.). The compound UO126 was purchased from Calbiochem (San Diego, Calif.). Precoated silica gel G thin-layer chromatography (TLC) plates were purchased from Analtech (Newark, Del.).

Cell Culture

The experiments were conducted using calf pulmonary artery endothelial (CPAE) cells (ATCC CCL289), a bovine (calf) pulmonary artery EC line, between passages 17 and 27. Stock cultures were routinely maintained in 75-cm^2 flasks in a 37°C incubator with an atmosphere of 95% air/5% CO_2. The cells were grown to confluence in 60-mm culture dishes in a medium of 20% fetal bovine serum and 80% minimum essential medium supplemented with 100 U/ml penicillin, 10 μg /ml streptomycin and 2 m*M L*-glutamine.

Metabolism of Ethanol by CPAE Cells

CPAE cells (2.5×10^5) were seeded and grown to confluence ($\sim 1 \times 10^6$ cells) in 60-mm culture dishes in modified Eagle's medium (MEM) supplemented with 20% FBS. The 20% FBS MEM was aspirated, the cells were washed with PBS, and 1% FBS MEM containing 100 m*M* ethanol was added to the plates. Cell-free dishes with the ethanol-containing media were used as controls. The dishes were sealed with parafilm to prevent loss of ethanol due to evaporation. Media (10 μl) were collected at the specified time points and the concentration of ethanol was determined using an NAD-ADH assay kit.

Incorporation of [^{3}H]-Acetate into CPAE Cell Phospholipids

Prior to cell stimulation with agonists, the 20% FBS MEM was aspirated, the cells were washed with PBS, and 1% FBS MEM containing ethanol was added to the dishes. The dishes were then sealed with parafilm to prevent evaporation of ethanol. After 8 h of treat-

ment with ethanol, the media were again aspirated and the cells were washed with PBS. Next, 1% FBS MEM containing 100 μCi [^{3}H]-acetate was added to each dish for 10 min. ATP (10 μ*M*) was added to each dish for 10 min. Control dishes were incubated for 10 min without ATP. Cell stimulation was stopped by washing the cells with ice-cold PBS, adding ice-cold methanol containing 50 m*M* acetic acid, and placing the dishes on ice. The cells were scraped from the dishes and transferred to glass tubes maintaining ice-cold conditions.

Incorporation of [^{14}C]-Ethanol into CPAE Cell Phospholipids

The [^{14}C]-ethanol was acquired from ARC (St. Louis, Mo.) in a pressure-sealed glass tube. The tube was kept cold with dry ice when it was opened in order to minimize evaporation. The [^{14}C]-ethanol was then diluted with ice-cold 3% BSA-saline for a final activity of 1 μCi/μl. For the [^{14}C]-ethanol experiments, 5 μCi of [^{14}C]-ethanol was added along with varying amounts of unlabelled ethanol to bring the final concentration of ethanol to 0, 50 or 100 m*M*. The dishes were sealed with parafilm during the incubation period with ethanol. After 8 h of treatment, 10 μ*M* ATP was added to each dish for 10 min. Control dishes were incubated for 10 min without ATP. Cell stimulation was stopped by washing the cells with ice-cold PBS, adding ice-cold acidified methanol as described above, and placing the dishes on ice. The cells were scraped from the dishes and transferred to glass tubes as above.

Extraction and Analysis of Phospholipids

The phospholipid samples were extracted from the cells using a chloroform-methanol-water system [3]. The samples were centrifuged and the organic phase was collected and dried under N_2 stream. Ten percent of the extracted total lipids were subjected to base catalyzed methanolic hydrolysis to analyze the moiety where the radiolabel was incorporated. The remaining 90% of the extracted total lipids were fractionated by TLC on precoated silica gel G using several different systems and visualized by spraying the plates with 2-*p*-toluidinylnaphthylene 6-sulfonate (TNS) and their positions compared to known R_f values of standard lipids run in parallel. The phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol/phosphatidylserine (PI/PS) and sphingomyelin (Sph) were fractionated using either chloroform/methanol/water (65:35:6; R_f values PC = 0.77, PE = 0.90, PI/PS = 0.67, Sph = 0.63) or chloroform/methanol/water/concentrated NH_4OH (65:35:4:2; R_f values PC = 0.82, PE = 0.86, PI/PS = 0.72, Sph = 0.59). PAF was fractionated using two sequential TLC systems. First, it was separated in a system of chloroform/methanol/water (65:35:6). The band comigrating with the PAF standard (R_f PAF = 0.4) was cut and scraped off the plate, the phospholipid extracted from the silica and dried. The PAF was then purified from the eluted sample using a second TLC system of water and methanol (80:40). PEth and phosphatidic acid (PA; R_f values 0.13 and 0.53, respectively) were fractionated using benzene/chloroform/pyridine/formic acid (45:38:4:2.2). The appropriate bands were then cut and scraped off the plates, eluted, dried, and the radioactivity of each band was determined by liquid scintillation in a Beckman LS-1801 counter. Incorporated radioactivity is reported as cpm per 1×10^6 cells.

Statistical Analysis

ANOVA followed by Student's t test was used to determine statistical significance at $p < 0.05$.

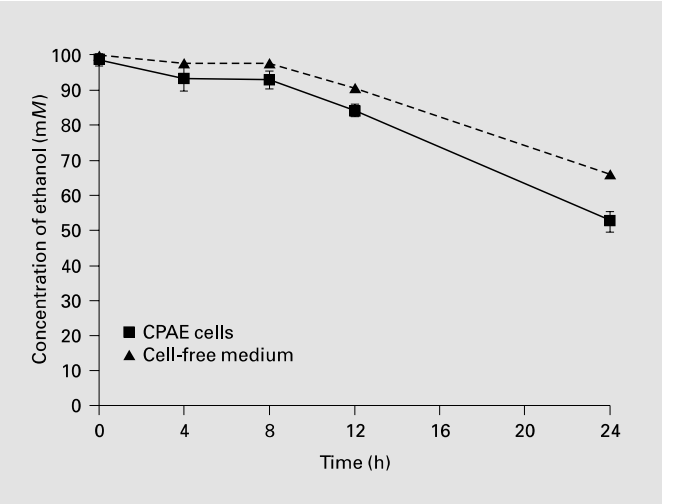


Fig. 2. Metabolism of ethanol by CPAE cells. CPAE cells were grown to confluence in 60-mm culture dishes in 20% FBS media. Cells were incubated in 100 m*M* ethanol for a total of 24 h. Media were collected at specific time points and the concentration of ethanol was determined using an NAD-ADH assay kit. Cell-free dishes were used as a control to correct for loss due to evaporation of ethanol. Values presented are means ± SEM of two experiments.

Results

Metabolic Fate of [^{14}C]-Ethanol in CPAE Cells

One of the first questions to assess in our study was whether EC could metabolize ethanol, and the extent of its incorporation into lipids. Confluent CPAE monolayers were incubated with 100 m*M* ethanol and media were sampled at the indicated time points and analyzed for alcohol content using an NAD-ADH assay kit. Cell-free control dishes were used to correct for loss of ethanol due to evaporation. As shown in figure 2, between 0 and 12 h, only 5% of the ethanol was metabolized by the CPAE cells. However, by 24 h, the cells had metabolized 15% of the ethanol in the media. Figure 3 shows incorporation of [^{14}C]-ethanol into CPAE cell total lipids. In one set, cells were stimulated with ATP (10 min) after 8 h of labelling. The graph indicates that with an increasing concentration of unlabelled ethanol, incorporation of the [^{14}C]-ethanol was decreased, suggesting that ethanol was incorporated into the lipid pool and the observed decrease in incorporation was due to dilution of the [^{14}C]-ethanol with unlabelled ethanol. Stimulation with ATP had no effect on the lipid radioactivity.

Next, incorporation of [^{14}C]-ethanol was monitored under stimulated conditions using ATP as an agonist.

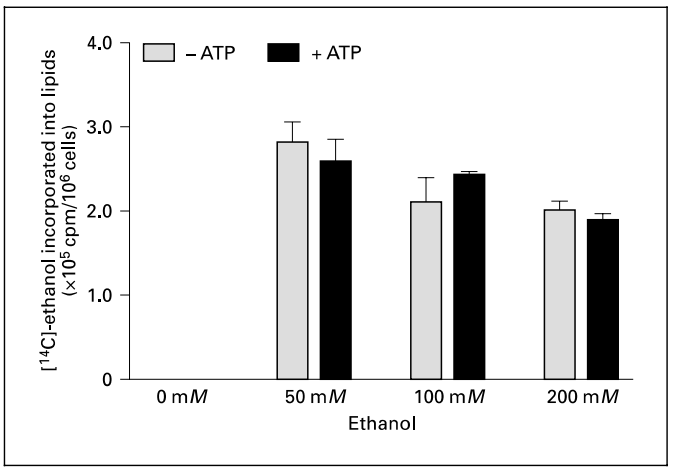


Fig. 3. Total [^{14}C]-ethanol incorporated into CPAE cell lipids. CPAE cells were incubated with the indicated concentrations of cold ethanol +5 μCi of [^{14}C]-ethanol for 8 h. In one set, cells were stimulated with ATP for 10 min at the end of labelling. Lipids were extracted from the cells and the amount of [^{14}C]-ethanol incorporated into total lipids was determined by liquid scintillation counting. The values are presented as cpm ± SEM of three experiments.

Table 1. Distribution of [^{14}C]-ethanol into CPAE cell phospholipids

[^{14}C]-labelled lipid	Ethanol		
	50 m*M*	100 m*M*	200 m*M*
PC	56.2 ± 0.377	47.5 ± 2.73	48.0 ± 2.30
Neutral lipids	16.0 ± 0.586	17.4 ± 0.312	18.3 ± 0.417
Sph	14.7 ± 0.279	15.6 ± 0.294	15.1 ± 0.320
PI/PS	3.6 ± 0.226	8.8 ± 2.59	8.3 ± 1.89
PE	5.0 ± 0.125	5.0 ± 0.202	4.9 ± 0.082
PEth	4.0 ± 0.707	5.3 ± 0.255	4.9 ± 0.271
PAF	0.5 ± 0.040	0.4 ± 0.065	0.6 ± 0.133

Confluent CPAE monolayers were incubated for 8 h with ethanol at the concentration shown +5 μCi of [^{14}C]-ethanol, and then incubated with or without 10 μ*M* ATP for 10 min. Total cell lipids were extracted and then fractionated by TLC. Bands corresponding to comigrating standards were scraped and radioactivity determined by liquid scintillation counting. Results are presented as the level of [^{14}C] radiolabel in a particular fraction as a percentage of total radioactivity recovered from each sample lane on the TLC plate. Values are the mean percentages ± SEM of two experiments.

Table 1 shows the relative distribution of [^{14}C]-ethanol into various phospholipids as percentages of the total recovered [^{14}C] radioactivity from TLC fractionation and purification. At each concentration of ethanol, approximately half of the recovered radioactivity was found in the PC fraction. Incorporation of [^{14}C]-ethanol into neutral lipids and Sph was approximately 17 and 15%, respectively. About 5% of the radioactivity was in the [^{14}C]-PEth fraction. Other phospholipids had a much lower incorporation. Interestingly, a small but reproducibly significant fraction, 0.5%, of the [^{14}C]-ethanol was incorporated into PAF (table 1). However, there was no change in the distribution of cellular lipids with ATP stimulation (data not shown).

Figure 4a shows the incorporation of [^{14}C]-ethanol into total CPAE cell lipids after pretreatment of the cells with pyrazole (2 m*M*) and cyanamide (200 μ*M*), inhibitors of ethanol metabolism (see fig. 1). In the presence of each of these inhibitors, incorporation of [^{14}C]-ethanol into CPAE cell total lipids was reduced by about 30%. However, when these inhibitors were used combined at the above concentrations, the CPAE cells detached from the culture plates during the incubation period, indicating that such treatment was toxic (data not shown). Inhibitors of various intracellular signalling components [wortmannin for PI-3 kinase, Ro-31-8220 for protein kinase C (PKC), UO126 for mitogen-activated protein kinase, propranolol for phosphatidate phosphohydrolase] were also used. These inhibitors individually had no effect on the incorporation of [^{14}C]-ethanol into total CPAE lipids (data not shown). However, incorporation of PEth was increased in the presence of wortmannin (fig. 4b). Again, ATP stimulation had no effect on the incorporation.

Effect of Ethanol on [^{3}H]-Acetate Incorporation in CPAE Cell Phospholipids

Ethanol is metabolized to acetaldehyde, then acetate, which is subsequently incorporated into cell phospholipids. In the case of PAF biosynthesis, exogenous acetate can be used for acetyl-CoA production, which then transfers its acetate to the *sn2*-acetyl group on PAF via an acetyl-CoA transferase enzyme. We, therefore, investigated whether directly supplied [^{3}H]-acetate would also be incorporated into phospholipids and PAF in these cells. Duplicate samples of CPAE cells were incubated for 10 min in low serum medium with 100 μCi [^{3}H]-acetate followed by 10 min stimulation with 10 μ*M* ATP. Control cells, which were not exposed to ATP, were incubated for a total of 20 min with [^{3}H]-acetate. Total cell lipids were then isolated and radioactivity was determined. Figure 5 shows that with increasing concentration of ethanol, there was a decrease in the incorporation of [^{3}H]-acetate into CPAE cell phospholipids, likely due to dilution of the [^{3}H]-acetate pool by the acetate derived from the ethanol

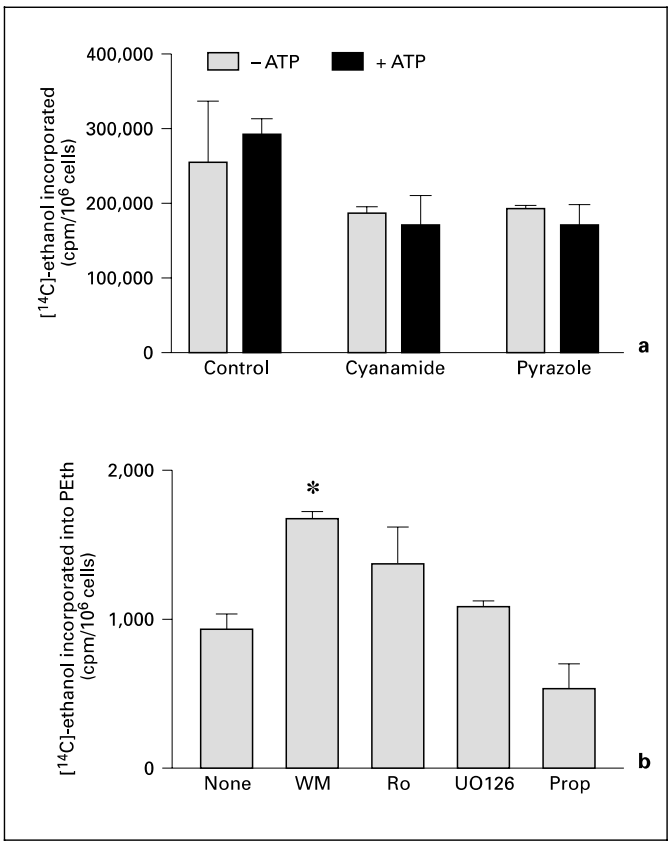


Fig. 4. Effect of inhibitors on the incorporation of [^{14}C]-ethanol into CPAE cell lipids. Confluent monolayers of CPAE cells were pretreated with either 200 μ*M* cyanamide or 2 m*M* pyrazole (**a**) or 10 μ*M* wortmannin (WM), 10 μ*M* Ro-31-8220 (Ro), 10 μ*M* UO126 or 100 μ*M* propranolol (Prop) (**b**) for 1 h before incubation in 50 m*M* ethanol +5 μCi ^{14}C-ethanol for 8 h. Total cell lipids were extracted and incorporation of [^{14}C]-ethanol was determined by liquid scintillation counting. Data are represented as cpm ± SEM of two experiments. *$p < 0.05$.

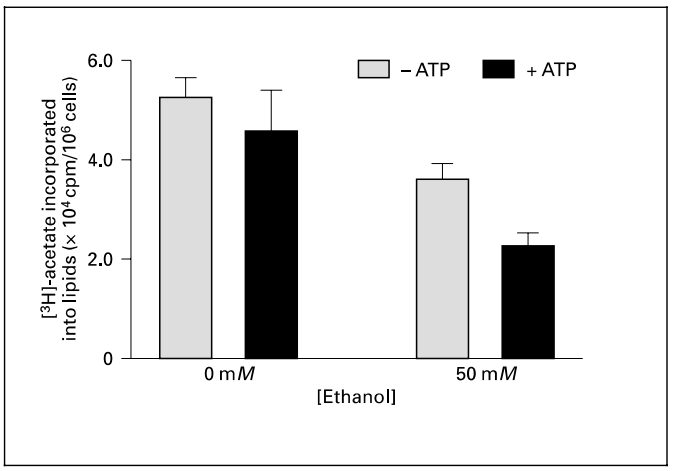


Fig. 5. Effect of ethanol on the incorporation of [^{3}H]-acetate into CPAE cell lipids. CPAE cells were incubated with the indicated concentrations of ethanol for 24 h. The cells were then incubated in media containing 100 μCi [^{3}H]-acetate for 10 min, and incubated for an additional 10 min with or without 10 μ*M* ATP. Lipids were extracted from the cells and the amount of [^{3}H]-acetate incorporated into phospholipids was determined by liquid scintillation counting. The values are presented as cpm ± SEM of three experiments.

metabolism. Thetotal cell lipids were then analyzed by TLC fractionation to determine incorporation of [^{3}H]-acetate into individual phospholipids (table 2). In contrast to the observed distribution of [^{14}C]-ethanol into phospholipid classes, a majority (55%) of the [^{3}H]-acetate was incorporated into neutral lipids and 35% into PC. The other phospholipids showed relatively small percentages of distribution. Again, a small fraction of [^{3}H]-acetate was incorporated into PAF. Ethanol treatment did not affect percent incorporation of [^{3}H]-acetate into phospholipids. Stimulation of the CPAE cells with ATP decreased the incorporation of [^{3}H]-acetate into neutral lipids and increased the incorporation into Sph.

Analysis of Incorporation of [^{14}C]-Ethanol or [^{3}H]-Acetate in Fatty Acid and Glycerophosphoryl Moieties

We next investigated the extent of incorporation of [^{14}C]-ethanol or [^{3}H]-acetate into the phospholipid head group-containing glycerol backbone and into the fatty acid side chains. Table 3 shows the results of methanolic hydrolysis of total cell lipids. The basal level of distribution of [^{3}H]-acetate was 13% in the water-soluble fraction and 87% in the fatty acid moiety. Stimulation of the cells with ATP slightly increased incorporation into the water-soluble fraction with a concomitant decrease in distribution into the lipid-soluble fraction. Increasing the concentration of alcohol also increased the incorporation of [^{3}H]-acetate into the water-soluble fraction. However, in the case of [^{14}C]-ethanol, approximately 95% of the [^{14}C]-ethanol was incorporated into the fatty acid side chains. Neither increasing the concentration of alcohol nor stimulation of the cells with ATP significantly altered the pattern of distribution of [^{14}C]-ethanol between the water-soluble and lipid-soluble fractions.

Production of PAF by CPAE Cells

Since PAF is a potent lipid mediator involved in EC interactions with other cells (e.g. neutrophils), we investigated more closely the PAF production by these cells. The

Table 2. Effect of ethanol and ATP on distribution of [3H]-acetate into lipids in CPAE cells

[3H]-labelled lipid	No ethanol		50 m*M* ethanol	
	–ATP	+ATP	–ATP	+ATP
Neutral lipids	55.6 ± 0.4	49.5 ± 2.4	56.3 ± 0.8	42.9 ± 4.4
PC	35.1 ± 2.7	39.9 ± 2.0	35.3 ± 2.3	32.5 ± 1.9
PE	4.4 ± 2.5	2.6 ± 0.5	2.2 ± 0.1	2.1 ± 0.1
PI/PS	2.1 ± 0.1	1.65 ± 0.7	1.9 ± 1.5	1.1 ± 0.5
Sph	1.5 ± 0.2	4.6 ± 1.2	3.3 ± 0.2	17.0 ± 6.8*
PAF	1.4 ± 0.1	1.7 ± 0.6	0.9 ± 0.1	4.4 ± 3.6

Confluent CPAE cell monolayers were incubated for 24 h in ethanol at the concentrations shown, incubated for 10 min with 100 μCi [3H]-acetate and then for an additional 10 min with or without 10 μ*M* ATP. Total cell lipids were extracted and subjected to TLC fractionation. Each lipid band was identified by its R_f value, scraped, and incorporated [3H]-acetate was determined by liquid scintillation counting. Results are reported as the [3H]-acetate activity in a particular TLC fraction as a percentage of the total [3H]-acetate activity recovered from each sample lane on the TLC plate. Values are percent distribution ± SEM for two experiments. * $p < 0.05$.

Table 3. Effect of ethanol on distribution of [3H]-acetate or [14C]-ethanol into fatty acyl or water-soluble fraction of phospholipids

Ethanol	3H-acetate incorporation				14C-ethanol incorporation			
	water-soluble		lipid-soluble		water-soluble		lipid-soluble	
	–ATP	+ATP	–ATP	+ATP	–ATP	+ATP	–ATP	+ATP
0	13.4	15.1	86.6	84.9	–	–	–	–
50 m*M*	14.6	17.1	85.4	82.9	4.8	5.2	95.2	94.8
100 m*M*	–	–	–	–	3.6	2.7	96.4	97.3
200 m*M*	–	–	–	–	2.6	3.5	97.4	96.5

Distribution in CPAE cell phospholipids into fractions after methanolic hydrolysis. Confluent CPAE cell monolayers were incubated with or without ethanol and with either 100 μCi 3H-acetate or 5 μCi 14C-ethanol. The cells were then incubated for 10 min with or without ATP and then total cell lipids were extracted. Ten percent of the extracted total lipids were subjected to base catalyzed methanolic hydrolysis to analyze the moiety where the [3H]-acetate or [14C]-ethanol was incorporated.

PAF pool was labelled by both [14C]-ethanol and [3H]-acetate. Figure 6 indicates that treatment of the [3H]-acetate-exposed cells with ATP for 10 min stimulated a 4-fold increase in formation of [3H]-PAF. A 24-hour pretreatment of the cells with either 50 or 100 m*M* ethanol decreased basal [3H]-PAF; however, stimulation of the ethanol-treated cells with 10 μ*M* ATP for 10 min stimulated only a 1- and 2-fold increase, respectively.

We also investigated the pattern of [14C]-ethanol incorporation into PAF. Figure 7 shows that increasing time of incubation with 50 m*M* [14C]-ethanol resulted in increased [14C]-PAF. However, unlike the incorporation of [3H]-acetate, treatment of the cells with 10 μ*M* ATP for 10 min did not result in increased incorporation of [14C]-ethanol into [14C]-PAF.

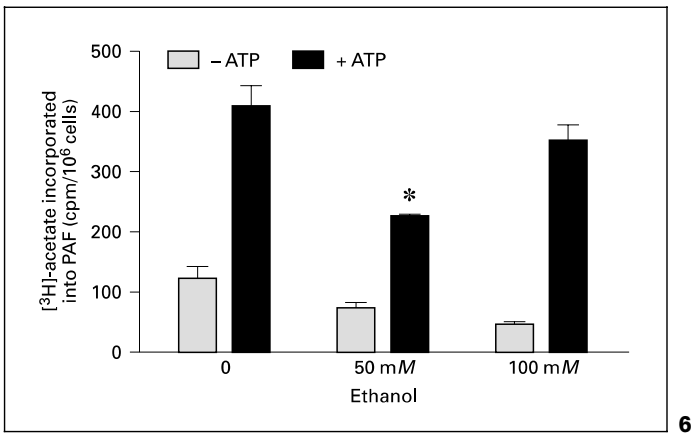
Discussion

As far as it can be ascertained, this is the first report of metabolic incorporation of [14C]-ethanol into various cellular phospholipids of EC. Bioactive lipids in EC play a central role in vascular homeostasis and the response to injury. Studies suggest that alcohol acts mainly on the

acyltransferase activities involved in the remodeling of membrane phospholipids. Since the incorporation of radioactivity into each phospholipid is an index of the metabolic turnover of its acyl chains and head groups, we determined the effect of ethanol on the distribution of radioactivity among different phospholipids (PA, PAF, PE, PEth, PS, PC, Sph) as well as in neutral lipids. In examining the incorporation of [^{14}C]-ethanol and of [^{3}H]-acetate into phospholipids, there was a striking contrast in the pattern of distribution. Whereas [^{14}C]-ethanol was mostly incorporated into PC with neutral lipids accounting for much less, [^{3}H]-acetate was incorporated mostly into neutral lipids with PC being second. Also, the effect of increased concentration of ethanol caused a dramatic decrease in the total incorporation of [^{3}H]-acetate while showing little effect on the distribution of [^{14}C]-ethanol. It can be inferred that even though the [^{14}C]-ethanol is being metabolized to acetate, it is possibly contributing to a separate acetate pool than that of the directly supplied [^{3}H]-acetate. Also, cyanamide and pyrazole caused decreases in [^{14}C]-ethanol incorporation into lipids and this is consistent with the metabolic conversion of ethanol into acetate and then to lipids.

PAF is not constitutively synthesized nor is it stored in resting, unactivated cells [17]. Rather, it is rapidly synthesized in response to agonists [5, 11, 14]. Previous research has demonstrated that EC synthesize PAF in response to stimulation with ATP [13]. In our present studies, we investigated the effect of alcohol on ATP-induced PAF accumulation. The acetate arising from ethanol metabolism was subsequently incorporated into various EC lipids. There was a marked difference in the response of the cells. Cells incubated with [^{3}H]-acetate showed increased incorporation of [^{3}H]-acetate into PAF upon cell stimulation with ATP. In the cells incubated with [^{14}C]-ethanol, treatment with ATP did not result in increased incorporation of [^{14}C]-ethanol into PAF. These data suggest that the acetate (directly supplied [^{3}H]-acetate or [^{14}C]-ethanol-derived acetate) may exist in distinct pools that are differently affected by ATP stimulation.

PC is the preferred phospholipid hydrolyzed by phospholipase D (PLD) to produce PA and choline. In the presence of ethanol, the enzyme PLD catalyzes transphosphatidylation to form PEth [18]. In [^{14}C]-ethanol-treated cells, PEth constituted approximately 5% of the recovered activity in phospholipids. This value is much higher than that reported before where PEth concentration was found to reach 0.5–1% of total cellular lipids [7]. In EC, this may represent a substantial fraction in microdomains of membrane phospholipids and may effect changes seen in intracellular signalling in ethanol-exposed cells. Inhibition of PI-3 kinase, PKC, mitogen-activated protein kinase, and modulation of PLD activity had no effect on the incorporation into lipids other than PEth. In this case, it can be proposed that in EC, inhibition of the PI-3 kinase path-



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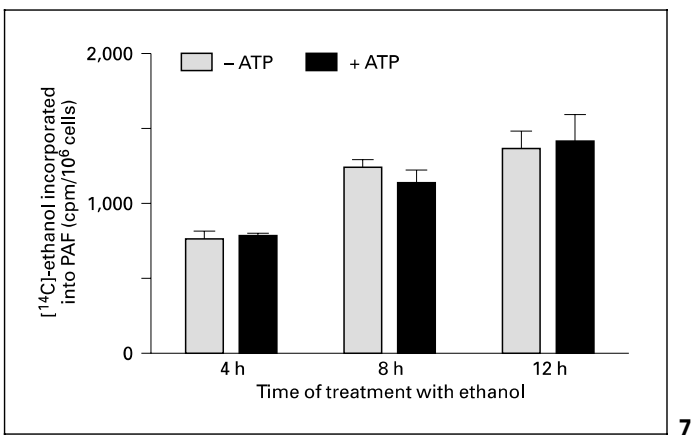

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Fig. 6. Effect of ethanol and ATP on the incorporation of [^{3}H]-acetate into PAF in CPAE cells. Confluent CPAE cells were incubated in ethanol containing media for 24 h. Following that incubation period, the cells were then incubated in media containing 100 μCi [^{3}H]-acetate for 10 min and then stimulated for 10 min with 10 μ*M* ATP. Total cell lipids were extracted and fractionated in two sequential TLC steps to purify PAF. The amount of [^{3}H]-acetate incorporated into PAF was determined by liquid scintillation counting of the eluted lipid. Results are presented as cpm ± SEM of two experiments. *$p < 0.05$.

Fig. 7. Time course of incorporation of [^{14}C]-ethanol into PAF in CPAE cells. Confluent CPAE cells were incubated in media containing 50 m*M* ethanol and 5 μCi [^{14}C]-ethanol for the times indicated. Following this incubation, the cells were then incubated with or without 10 μ*M* ATP for 10 min. Total cell lipids were extracted and fractionated in two sequential TLC steps to purify PAF. The amount of [^{14}C]-ethanol incorporated into PAF was determined by liquid scintillation counting of the eluted lipid. Results are presented as cpm ± SEM of two experiments.

way, in some manner, has a negative influence on basal PEth formation in the presence of ethanol. The formation of PEth brings to bear its effect on membrane characteristics. It has been demonstrated that for at least one PKC isoform, PEth can specifically take the place of PS in activating PKC [2]. This observation raises the interesting question of the role of PEth, in addition to PS, in EC surface interactions with neutrophils and platelets [6].

In the CPAE cells incubated with [^{3}H]-acetate, there were changes seen in Sph formation both with ethanol treatment and upon stimulation with ATP. There is accumulating evidence that sphingolipids can affect steady state concentrations of choline-containing glycerolipids such as PC. It is well established that glycerophospholipids and their metabolic products such as diacylglycerol, inositol 1,4,5-trisphosphate (IP_3), PAF and eicosanoids function as mediators in signal transduction and cellular responses. More recently it has been hypothesized that membrane sphingolipids could serve in signal transduction pathways. A Sph cycle has been described in which activation of a sphingomyelinase leads to the breakdown of Sph and the generation of phosphocholine and ceramide [8]. This cycle is thought to be analogous to the generation of IP_3 and diacylglycerol from the phospholipase C-mediated hydrolysis of inositol phospholipids. Ceramide has been shown to modulate a number of downstream events such as protein phosphorylation, phosphatase activation, downregulation of the c-myc protooncogene, and apoptosis. The changes seen in incorporation of [^{3}H]-acetate into Sph with ethanol treatment, as well as under stimulated conditions (+ATP), suggest that activation of the Sph cycle may be involved in the altered signal transduction observed in cells exposed to ethanol.

The vascular endothelium is not only a structural barrier between circulation and organs, it also plays a pivotal role in responding quickly to environmental changes that influence the regulation of blood flow. In this regard, the maintenance of adaptive processes in the EC is of great importance in both normal and disease states. We have shown in this study that vascular EC actively metabolize alcohol and that a portion of this alcohol is incorporated into different cell lipids, some of which are biologically active, such as PAF. Signalling by PAF is closely linked to adhesive reactions between cells involved in inflammatory responses and the vascular endothelium [6]. Thus the effect of alcohol on PAF and other lipids (e.g. PEth, Sph) may be critical determinants in the pathophysiological responses of the alcoholic endothelium.

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