PHYTOCHEMICALS

MECHANISMS OF ACTION

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CHAPTER 1

Absorption and Metabolism of Anthocyanins: Potential Health Effects

Ronald L. Prior

CONTENTS

ABSTRACT

This manuscript reviews literature on anthocyanins in foods and their metabolism and absorption and possible relationships to human health. Of the various classes of flavonoids, the potential dietary intake of anthocyanins is perhaps the greatest

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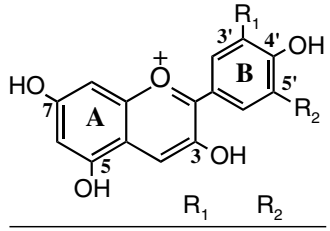
(100+ mg/day). The content in fruits varies considerably between 0.25 to 700 mg/100 g fresh weight. Not only does the concentration vary, the individual specific anthocyanins present also are quite different in various fruits. Anthocyanins are absorbed intact without cleavage of the sugar to form the aglycone. The proportion of the dose that appears in the urine is quite small (<0.1%). Plasma levels of anthocyanins are in the range of 1–120 nM following a meal high in anthocyanins, but fasting plasma levels are generally nondetectable. Information is limited as to possible metabolites of anthocyanins in the human. A number of antioxidant-related responses are reviewed in animal models as well as in the human. Anthocyanins can provide protection against various forms of oxidative stress in animal models, however, most of the health-related responses have been observed at relatively high intakes of anthocyanins (2-400 mg/kg BW).

INTRODUCTION

Anthocyanins (Figure 1.1) are water soluble plant secondary metabolites responsible for the blue, purple, and red color of many plant tissues. They occur primarily as glycosides of their respective anthocyanidin-chromophores. The common anthocyanidin aglycones are cyanidin (cy), delphinidin (dp), petunidin (pt), peonidin (pn), pelargonidin (pg), and malvidin (mv). The differences in chemical structure of these six common anthocyanidins occur at the 3′ and 5′ positions (Figure 1.1). The aglycones are rarely found in fresh plant material. There are several hundred known anthocyanins. They vary in 1) the number and position of hydroxyl and methoxyl groups on the basic anthocyanidin skeleton; 2) the identity, number and positions at which sugars are attached; and 3) the extent of sugar acylation and the identity of the acylating agent. Common acylating agents are the cinnamic acids (caffeic, ρ-coumaric, ferulic, and sinapic). Acylated anthocyanins occur in some of the less common foodstuffs such as red cabbage, red lettuce, garlic, red-skinned potato, and purple sweet potato.[1]



	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 1.1 Common anthocyanin structures. Sugar moieties are generally on position 3 of the C-ring.

This review will focus on the food content of anthocyanins, their absorption/metabolism, and reports of potential beneficial health effects. Other reviews have been published that deal more with the chemistry of anthocyanins.[2,3]

ANTHOCYANINS IN FOODS

The distribution of anthocyanins in 26 different common foods is presented in Table 1.1 and Table 1.2. This information is based upon our data as well as information obtained from Macheix et al.,[4] editors of a book on fruit phenolics. Cyanidin aglycone occurred in 23 of the 26 foods listed and, overall, seems to be present in about 90% of fruits[4] and is the most frequently appearing aglycone compared to all of the others. The glucoside form is present in 23 out of 26 of the foods listed in Table 1.1. The galactoside, arabinoside and rutinoside (6-O-α-L-rhamnosyl-D-glucose) were present in 30 to 40% of the foods in Table 1.1. The rutinoside seems to be present in those foods that do not contain either the galactoside or arabinoside.

Anthocyanin levels (mg/100g fresh weight (FW)) range from 0.25 in pear to 500 in blueberry[4] and more than 700 in black raspberry (Table 1.2). Fruits that are richest in anthocyanins (>20 mg/100 g FW) are very strongly colored (deep purple or black). Moyer et al.[5] surveyed genotypes of blueberries, blackberries, and black currants for their anthocyanin content. Means ± SEM and the range in mg/100 g fresh weight were 230 ± 89 (34–515), 179 ± 89 (52–607), and 207± 61 (14–411) for blueberries, blackberries, and black currants, respectively. The relative contribution of individual anthocyanins to the total anthocyanins in six fruits that are relatively high in total anthocyanins is presented in Table 1.2. Blueberry is unique in having a large number of individual anthocyanins (15–25). Lowbush blueberry has more of the acylated anthocyanins compared to cultivated blueberries (Highbush and Rabbiteye).[6] Black raspberry has one of the highest anthocyanin content of common foods (763 mg/100 g FW) (Table 1.2), with three anthocyanins contributing ~97% of the total anthocyanin content. Other foods that have been reported to contain anthocyanins include onion, red radish, red cabbage, red soybeans, and purple corn.[4]

In the U.S., the average daily intake of anthocyanins has been estimated to be 215 mg during the summer and 180 mg during the winter.[7] However, there are limited quantitative data available, but similar methodology indicates that the concentrations can be quite variable in any one food.[1,5] A recent report[8] demonstrated that increased childhood fruit intake, but not vegetable, was associated with reduced risk of incident cancer. Thus, childhood fruit consumption may have a long-term protective effect on cancer risk in adults. Because a major difference between fruits and most vegetables is the anthocyanin content, further study is needed to demonstrate a clear relationship between anthocyanin intake and cancer.

Table 1.1 Distribution of anthocyanins in common foods.[a]

	Glycosidic Pattern[b]											Anthocyanidins					
Food	3-Glu	3-Gal	3-Arab	3-Rut	3-Soph	3-Xyl-rut	3-Glu-rut	3-AGlu	3-CoGlu	3-CaGlu	Other	Cy	Dp	Pt	Pg	Pn	Mv
Blackberry	++	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-
Marionberry	+++	-	-	+	-	-	-	-	-	-	+	+++	-	-	-	-	-
Red Raspberry	+	-	-	+	++	-	-	-	-	-	-	++	-	-	+	-	-
Black Raspberry	+	-	-	+	-	+	-	-	-	-	-	+++	-	-	+	-	-
Cherry, sour	+	-	-	+	+	+	++	-	-	-	-	++	-	-	-	+	-
Cherry, sweet	+	-	-	++	-	-	-	-	-	-	-	++	-	-	-	+	-
Plum	++	-	-	+	-	-	-	-	-	-	-	++	-	-	-	+	-
Peach	++	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-
Apple	+	++	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Pear	-	++	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Strawberry	++	-	-	+	-	-	-	-	-	-	+	+	-	-	++	-	-
Chokeberry	+	++	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Black currant	+	-	-	++	-	-	-	-	-	-	-	+	+	-	-	-	-
Red currant	+	-	-	+	+	+	+	-	-	-	-	+	-	-	-	-	-
Grape	+	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	++
Chokeberry	+	++	+	-	-	-	-	-	-	-	+	++	-	-	-	-	-
Pomegranate	++	-	-	-	-	-	-	-	-	-	+	++	-	-	+	-	-
Fig	+	-	-	++	-	-	-	-	-	-	+	++	-	-	+	-	-
Cranberry	++	++	+	-	-	-	-	-	-	-	-	++	-	-	-	++	-
Bilberry	+	+	+	-	-	-	-	-	-	-	-	+	+	+	-	+	++
Blueberry	+	+	+	-	-	-	-	+/-	-	-	-	+	+	+	-	+	++
Elderberry	++	-	-	-	-	-	-	-	-	-	+	++	-	-	-	-	-
Mango	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Sweet Orange	+	-	-	-	-	-	-	+	+	-	+	+	-	+	-	+	-
Eggplant	+	-	-	+	-	-	+	-	+	-	+	-	+	-	-	-	-
Huckleberry	-	-	-	-	-	-	-	-	-	-	+	-	-	++	-	-	+

[a] Information summarized from our data and that of Macheix et al.[4]

[b] Glu = glucoside; Gal = galactoside; Arab = arabinoside; Rut = rutinoside (6-O-α-L-rhamnosyl-D-glucose); Soph = sophoroside (2-O-β-D-glucosyl-D-glucose); 3-Xyl-rut = 3-xylosylrutinoside; 3-Glu-rut = 3-glucosylrutinoside; AGlu = acetylglucoside; CoGlu = p-coumarylglucoside; CaGlu = caffeylglucoside; Others may include other diglucosides (i.e., sambubioside: 2-O-α-xylosyl-D-glucose), sambubioside-glucoside, or other forms of acylation, etc.; Cy = cyanidin; Dp = delphinidin; Pt = petunidin; Pg = polargonidin; Pn = peonidin; Mv = malvidin.

ANTIOXIDANT AND OTHER BIOLOGICAL EFFECTS OF ANTHOCYANINS *IN VITRO*

Like other flavonoids, anthocyanins have strong antioxidant capacity as measured by *in vitro* assays. Cyanidin glycosides tend to have higher antioxidant capacity than peonidin- or malvidin-glycosides,[9] likely due to the free hydroxyl groups on the 3′ and 4′ positions in the B-ring of cyanidin. Pool-Zobel et al. [10] compared anthocyanin extracellular and intracellular antioxidant potential *in vitro* and in human colon tumor (HT29 clone 19A) cells. Isolated compounds (aglycones and glycosides) and complex plant samples were powerful antioxidants *in vitro* as indicated by a reduction in H_2O_2-induced DNA strand breaks in cells treated with complex plant extracts; however, endogenous intracellular generation of oxidized DNA bases (comet test) was not prevented.[10] These data suggest that anthocyanins might not accumulate to sufficient concentrations intracellularly to have significant antioxidant effects. Youdim et al.[11] found that the incorporation *in vitro* of anthocyanins (1 mg/ml) from elderberry within the cytosol of endothelial cells (EC) was considerably less than that in the membrane. Uptake within both regions appeared to be structure dependent, with monoglycoside concentrations higher than those of the diglucosides in both compartments. Enrichment of EC with elderberry anthocyanins conferred significant protective effects against oxidative stressors such as (1) hydrogen peroxide, (2) 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), and $FeSO_4$/ascorbic acid. These findings may have important implications on preserving EC function and preventing the initiation of EC changes associated with vascular diseases.[11]

Hibiscus anthocyanins (HAs), a group of natural pigments occurring in the dried flowers of *Hibiscus sabdariffa* L., were able to quench free radicals from 1,1-diphenyl-2-picrylhydrazyl. HAs, at concentrations of 0.10 and 0.20 mg/ml (0.4-0.8 μM), were found to significantly decrease the leakage of lactate dehydrogenase and the formation of malondialdehyde in rat primary hepatocytes induced by a 30-min treatment of *tert*-butyl hydroperoxide (1.5 mM).[12] Wang and Mazza[13] demonstrated that common phenolic compounds found in fruits inhibited nitric oxide (NO) production in bacterial lipopolysaccharide/interferon-γ-activated RAW 264.7 macrophages. Anthocyanins/anthocyanidins, including pelargonidin, cyanidin, delphinidin, peonidin, malvidin, malvidin 3-glucoside, and malvidin 3,5-diglucosides in a concentration range of 60 to 500 μM, inhibited NO production by >50% without showing cytotoxicity. However, these concentrations are quite high (3–4 orders of magnitude higher) relative to concentrations measured in plasma.[14–17] Anthocyanin-rich crude extracts and concentrates of selected berries were also assayed, and the inhibitory effects of the anthocyanin-rich crude extracts on NO production were significantly correlated with total phenolic and anthocyanin contents.[13] Anthocyanins isolated from tart cherries exhibited anti-inflammatory activities as indicated by their ability to inhibit the cyclooxygenase activity of the prostaglandin endoperoxide H synthase I.[18]

The aglycones of the most abundant anthocyanins in food, cyanidin (cy) and delphinidin (dp), were found to inhibit the growth of human tumor cells *in vitro* in the μM range, whereas malvidin, a typical anthocyanidin in grapes, was less active. However, cyanidin-3-galactoside and malvidin-3-glucoside did not affect tumor cell

Table 1.2 Anthocyanins distribution (% of total concentration) and content in selected common fruits.[a]

Anthocyanin	Grape	Straw-berry	Cran-berry	Marion-berry	Blueberry	Black Raspberry
Delphinidin 3-galactoside	—	—	—	—	11.7	—
Delphinidin 3-glucoside	6.8	—	—	—	10.8	—
Delphinidin 3-arabinoside	—	—	—	—	7.1	—
Cyanidin 3-galactoside	—	—	27.1	—	4.1	—
Cyanidin-diglucoside	6.2	14.3	—	—	—	—
Cyanidin 3-glucoside	—	8.8	0.7	78.3	3.7	17.1
Cyanidin 3-arabinoside	—	—	0.3	—	3.3	—
Cyanidin-rutinoside-pentose	—	—	—	—	—	22.5
Cyanidin-2-rutinoside	—	—	—	19.5	—	57.7
Cyanidin-unknown	—	—	—	2.2	—	—
Pelargonidin-3-glucoside	—	67.0	—	—	—	—
Pelargonidin-3-rutinoside	—	9.9	—	—	—	—
Petunidin 3-galactoside	—	—	—	—	5.7	—
Petunidin 3-glucoside	5.5	—	—	—	7.0	—
Petunidin 3-arabinoside	—	—	—	—	3.6	—
Peonidin 3-galactoside	—	—	16.3	—	1.3	—
Peonidin 3-glucoside	21.4		33.4	—	8.5	—
Peonidin-3-arabinoside	—	—	2.6	—	—	—
Malvidin 3-galactoside	—		—	—	—	—
Malvidin 3-glucoside	31.1	—	—	—	12.7	—
Malvidin 3-arabinoside	—	—	19.6	—	4.7	—
Cyanidin 3-(6-acetyl)-galactoside	—	—	—	—	0.3	—
Delphinidin 3-(6-acetyl)-galactoside	—	—	—	—	3.7	—
Petunidin 3-(6-acetyl)-galactoside	—	—	—	—	—	—
Cyanidin 3-(6-acetyl)-glucoside	—	—	—	—	1.6	—
Cyanidin-hexose-coumarin	0.5	—	—	—	—	—
Malvidin 3-(6-acetyl)-galactoside	—	—	—	—	2.0	—
Petunidin 3-(6-acetyl)-glucoside	—	—	—	—	2.5	—
Petunidin-hexose-coumarin	1.5	—	—	—	—	—
Peonidin 3-(6-acetyl)-glucoside	0.3?	—	—	—	6.0	—
Malvidin 3-(6-acetyl)-glucoside	2.3?	—	—	—	—	—
Malvidin-hexose-coumarin	23.8	—	—	—	—	—
Unknown	—	—	—	—	—	2.2
Total concentration (mg/100 g)	18	36	188	237	450	763

[a] Concentrations expressed/100g of fresh weight.

growth up to 100 μM. The anthocyanidins (cyanidin and delphinidin) were potent inhibitors of the epidermal growth-factor receptor, shutting off downstream signaling cascades.[19] Whether these observations have meaning in an *in vivo* situation is not known, because the aglycones have not been observed in the plasma or urine of humans.

Anthocyanins and α-Glucosidase Activity

Anthocyanin extracts were found to have potent α-Glucosidase (AGH) inhibitory activity, with an IC(50) value of ~0.35 mg/mL, but anthocyanin extracts did not inhibit sucrase activity. In an immobilized assay system, which may more closely reflect the pharmacokinetics of AGH in the small intestine, the anthocyanin extracts were more potent in inhibiting maltase activities than those in the free AGH assay, with IC(50) values of 0.17 to 0.26 mg/ml. Since the anthocyanin extracts also inhibited α-amylase action, anthocyanins may have a potential function in suppressing the increase in postprandial glucose level following starch ingestion.[20] In further studies, Matsui et al.[21] found that anthocyanins acylated with caffeic or ferulic acids had the most potent maltase inhibitory activity (IC(50) = 60 μM). Furthermore, it appeared that the lack of any substitution at the 3′(5′)-position of the aglycone B-ring may be essential for inhibiting intestinal AGH action.[21]

ANTHOCYANIN ABSORPTION/METABOLISM

Anthocyanins can be absorbed intact as glycosides (Figure 1.1). The mechanism of absorption is not known; however, Passamonti[22] found that anthocyanins can serve as ligands for bilitranslocase, an organic anion membrane carrier found in the epithelial cells of the gastric mucosa, and suggested that bilitranslocase could play a role in the bioavailability of anthocyanins. Table 1.3 presents a summary of the research that has demonstrated absorption of intact anthocyanins in the rat, pig or human. At least 13 different anthocyanins from 7 different food sources have been observed to be absorbed intact and to be present in plasma or urine (Table 1.3). In

Table 1.3 Anthocyanins which have been observed to be absorbed intact and detected in plasma or urine following a meal.

Anthocyanins	Source	Species	Reference
Cyanidin-3-glucoside, Cyanidin-3-sambubioside	Elderberry	Human	56, 14, 15, 57, 26
Cyanidin-3-glucoside, Cyanidin-3-rutinoside, Cyanidin-3-sambubioside	Black raspberry	Pig	Wu and Prior, unpublished
Cyanidin-3-glucoside, Cyanidin-3-diglucoside	Red fruit	Rat, Human	24
Delphinidin-3-rutinoside, Cyanidin-3-rutinoside, Cyanidin-3-glucoside	Black currant	Rat, Human, Rabbit	16, 58, 59
Cyanidin-3-glucoside	Pure compound	Rat	25
Cyanidin-3-glucoside	Blackberry	Rat	28
Malvidin-3-glucoside	Wine	Human	17
Bilberry Anthocyanins[a]	Bilberry	Rat	37
Blueberry Anthocyanins[a]	Blueberry	Human	15, 27

[a] del-3-gal, del-3-glu, cyan-3-gal, del-3-arab, cyan-3-glu, pet-3-gal, peon-3-gal, pet-3-arab, mal-3-gal, mal-3-arab.

contrast to other flavonoids, the proportion of anthocyanins absorbed and excreted in the urine as a percentage of the intake seems to be quite small,[15] perhaps much less than 0.1% of intake. Maximum plasma levels of total anthocyanins were in the range of 1–120 nmol/L with doses of 0.7–10.9 mg/kg in human studies.[14,16,23,24] The clearance of anthocyanins from the circulation is sufficiently rapid that by 6 h, very little is generally detected in the plasma.[14,17]

In rats given cyanidin-3-glucoside (C-3-G) orally (0.9 mmol/kg body weight), C-3-G rapidly appeared in the plasma, but the aglycone of C-3-G (cyanidin) was not detected, although it was present in the jejunum.[25] Protocatechuic acid (PC), which may be produced by degradation of cyanidin, was present in the plasma of the rat at concentrations eightfold higher than that of C-3-G. We have not detected PC in the plasma of humans following anthocyanin consumption (prior, unpublished data), nor has it been reported in any of the other publications on anthocyanin absorption in humans. Although there are no data on the exact amount of anthocyanins that are absorbed, the plasma kinetic profile and the recovery of anthocyanins in the urine suggests that relatively small proportions are absorbed. However, urinary excretion does not provide an accurate measure of absorption, because metabolism and possible elimination in the bile may alter amounts excreted in the urine.

In studies by Cao and coworkers[14] the two major anthocyanins in elderberry (cyanidin-3-glucoside and cyanidin-3-sambubioside) were detected as glycosides in both plasma and urine of humans. Mulleder and Murkovic[26] observed a greater urinary excretion of cyanidin-3-sambubioside than cyanidin-3-glucoside (0.014 vs 0.004% of dose) and that addition of sucrose to the elderberry juice led to a reduced and delayed excretion of the anthocyanins. The reduced excretion of cyanidin-3-glucoside may be the result of increased degradation relative to cyanidin-3-sambubioside in the gastrointestinal tract (Wu and Prior, unpublished data). The complexity of the glycosidic pattern does not seem to noticeably affect absorption. Mazza and coworkers[27] suggested that acylated anthocyanins might be absorbed intact from blueberries, however, they have not been detected in plasma or urine in other reports. Most likely this is because they are present in low concentrations in the foods and current methods are not sensitive enough to detect them. Most anthocyanins were excreted in urine during the first 4 h. Total elderberry anthocyanin excretion in the first 4 h accounted for only 0.077% of the dose. Wu et al.[15] identified four additional anthocyanin metabolites from elderberry in the urine: (1) peonidin-3-glucoside, (2) peonidin-3-sambubioside, (3) peonidin monoglucuronide and (4) cyanidin-3-glucoside monoglucuronide. However, Miyazawa[24] was not able to detect conjugated or methylated anthocyanins in plasma of humans, but did observe the presence of peonidin-3-glucoside in the liver of rats following the consumption of red fruit anthocyanins (C-3-G; C-3-diglucoside). The formation of the peonidin metabolites likely takes place in the liver through the catechol-*O*-methyl transferase reaction. Delphinidin would be the only other anthocyanidin that might undergo this methylation reaction as malvidin and petunidin already are methylated in the 3′ position (Figure 1.1).

In an additional study reported by Wu et al.,[15] six women were given 189 g lowbush blueberry (BB), which provided a total of 690 mg of anthocyanins. In five of six subjects fed BB, urine samples contained five to eight different anthocyanins, all of which were identified as being present in the blueberries consumed. Plasma

anthocyanin levels were below detection limits (~5 ng) using 2 ml of plasma. Total urinary anthocyanin excretion during the first 6 h was 23.2 ± 4.8 μg or 0.004% of dose. Matsumoto et al.[16] reported that the cumulative urinary excretion of the four anthocyanins from black currant (delphinidin 3-*O*-β-rutinoside, cyanidin 3-*O*-β-rutinoside, delphinidin 3-*O*-β-glucoside, and cyanidin 3-*O*-β-glucoside) during the first 8 h after intake was 0.11 ± 0.05% of the dose ingested.[16]

Gut Metabolism of Anthocyanins

The metabolism of anthocyanins in the gut is an area that has largely been ignored up to this point. Felgines and coworkers[28] were among the first to report on anthocyanins in gut contents of rats after adaptation to consumption of a diet containing blackberry anthocyanins. The blackberries contained primarily cyanidin-3-glucoside with a small amount of malvidin-3-glucoside (~1.9% of C-3-G). Recovery of cyanidin plus C-3-G in the total cecal contents was ~0.25%. Interestingly, about the same amount of cyanidin products (~0.26%) was recovered in the urine. However, larger amounts of malvidin-3-glucoside were recovered in the cecum and urine (~1.3% and 0.67%, respectively). We have observed in the neonatal pig (Wu and Prior, unpublished) lower recoveries of C-3-G compared with other anthocyanins in black raspberry in all segments of the gut 4 h after consumption of black raspberry. More than 50% of all anthocyanins seem to be degraded within 4 h of consumption of a meal. Thus, it seems clear that more than 50% of the ingested anthocyanins are degraded or disappear within the gut in a few hours after ingestion, but the form of the metabolic products is not clear.

IN VIVO ANTIOXIDANT AND OTHER EFFECTS OF ANTHOCYANINS — ANIMAL STUDIES

Antioxidant Effects

Table 1.4 and Table 1.5 summarize both animal and human clinical studies and the biological responses observed following consumption of anthocyanins. Oral pretreatment with Hibiscus anthocyanins (HAs) (100 and 200 mg/kg) for 5 days before a single dose of *t*-butyl hydroperoxide (*t*-BHP) (0.2 mmol/kg, ip) significantly lowered the serum levels of alanine and aspartate aminotransferase, enzyme markers of liver damage, and also reduced oxidative liver damage in rats. Histopathological evaluation of the liver revealed that HAs reduced the incidence of liver lesions, including inflammatory, leucocyte infiltration, and necrosis induced by *t*-BHP in rats. Based on these results, the authors suggested that HAs may play a role in the prevention of oxidative damage in living systems.[12]

The decreased food intake and body weight gain, and increased lung weight and atherogenic index observed in rats in which paraquat was used to induce oxidative stress were clearly suppressed by supplementing acylated anthocyanins from red cabbage to the paraquat diet.[29] Paraquat feeding increased the concentration of thiobarbituric acid-reactive substances (TBARS) in liver lipids and decreased the

Table 1.4 Summary of *in vivo* models and dose of anthocyanins used in studies of biological effects of anthocyanins or foods rich in anthocyanins.

	Model	Source of Anthocyanins	Dose of Anthocyanins	Species	Reference
1.	Paraquat-induced oxidative stress	Red cabbage; acylated anthocyanins	N/A	Rat	29
2.	CCl_4-induced liver damage	Petals of *H. rosasinensis*	25, 125 or 250 mg; 5d/wk, 4 wk	Rat	30
3.	Hepatic ischemia reperfusion	Cyan-3-glu	2 g/kg diet	Rat	33
4.	Ischemia reperfusion	Cyan-3-glu	2 g/kg	Rat	34
5.	*t*-Butyl Hydroperoxide (0.2 mm/kg)-induced liver cytotoxicity	Dried flowers of *H. sabdariffa*	100 or 200 mg/kg orally for 5 d	Rat	12
6.	Vitamin E deficiency	Purified extract; glycosides of all aglycones	1 g/kg diet or ~25 mg/day	Rat	35
7.	Diabetic retinopathy		600 mg/day; 2 mo	Human	50
8.	*In vivo* antioxidant activity	Lowbush blueberry	1.2 g	Human	27
9.	*In vivo* antioxidant activity	Lowbush blueberry	690 mg; 10 mg/kg body wt)	Human	44
10.	*In vivo* antioxidant activity	Black currant concentrate	33 mg/kg; 1.7 mg D-3-Rut, 1.24 mg Cy-3-Rut, 0.5 mg D-3-G, 0.2 mg Cy-3-G/kg body weight	Human	46
11.	IUGR in pregnant women	Chokeberry	N/A	Human	49
12.	CCl_4-induced capillary permeability	Bilberry	25 to 100 mg/kg i.p. or 200 to 400 mg/kg orally	Rabbit, rat	36

Note: N/A = not applicable; IUGR = intrauterine growth retardation.

Table 1.5 Summary of *in vivo* models and biological effects resulting from consumption of anthocyanins or foods rich in anthocyanins.

	Model	Summary of Biological Responses to Anthocyanins	Reference
1.	Paraquat-induced oxidative stress	↑ TBARS; ↓ liver triacylglycerol levels; ↓ paraquat-induced liver NADP-Cyt-P450 reductase	29
2.	CCl_4-induced liver damage	↓ Hepatotoxicity as measured by serum ASPAT and ALAAT activities	30
3.	Hepatic ischemia reperfusion	↓ Liver TBA; ↓ liver damage based on marker enzymes; ↑ liver GSH	33
4.	Ischemia reperfusion	↓ Liver TBA; ↓ liver damage based on marker enzymes; ↑ liver GSH	34
5.	*t*-Butyl hydroperoxide (0.2 mm/kg)-induced liver cytotoxicity	↓ Serum ALAAT and ASPAT; ↓ leucocyte infiltration; ↓ liver necrosis	12
6.	Vitamin E deficiency	↑ Plasma antioxidant capacity; ↓ liver lipid hydroperoxides and 8-OOHdG	35
7.	Diabetic retinopathy	↓ Synthesis of polymeric collagen and glycoproteins	50
8.	*In vivo* antioxidant activity	↑ Plasma antioxidant capacity;	27
9.	*In vivo* antioxidant activity	↑ Hydrophilic and lipophilic plasma antioxidant capacity	44
10.	*In vivo* antioxidant activity	↑ Plasma antioxidant capacity;	46
11.	IUGR in pregnant women	↓ Autoantibodies to oxidized LDL	49
12.	CCl_4-induced capillary permeability	↓ Skin permeability; ↑ vascular resistance	36

Note: TBARS = thiobarbituric acid-reactive substances; IUGR = intrauterine growth retardation; LDL = low-density lipoprotein.

liver triacylglycerol level. These effects tended to be suppressed by supplementing acylated anthocyanins to the paraquat diet. In addition, catalase activity in the liver mitochondrial fraction was markedly decreased by feeding the paraquat diet; this decrease was partially suppressed by supplementing the paraquat diet with acylated anthocyanins. An increase in the NADPH-cytochrome-P450-reductase activity in the liver microsome fraction by paraquat was suppressed by supplementing the paraquat diet with acylated anthocyanins. These results suggest that acylated anthocyanins from red cabbage acted to prevent oxidative stress *in vivo* that may have been due to active oxygen species formed through the action of paraquat.[29] Anthocyanins obtained from the petals of *H. rosasinensis* were shown to prevent carbon tetrachloride-induced acute liver damage in the rat. Treatment of separate groups of rats with 2.5 ml of 1, 5, and 10% anthocyanin extract in 5% aqueous ethanol/kg body weight, 5 days/week for 4 weeks before giving 0.5 ml/kg carbon tetrachloride (CCl_4), resulted in significantly less hepatotoxicity than with CCl_4 alone, as measured by serum aspartate- and alanine-aminotransferase activities 18 h after CCl_4.[30]

Many flavonoids extracted from petals of higher plants and from fruit rinds, as well as purified flavonoids, have been reported to have antitumor effects *in vitro* and *in vivo*. Flavonoids extracted from red soybeans, but not red beans, were effective in inhibiting the growth of HCT-15 cells *in vitro*. Flavonoids extracted from both red soybeans and red beans were effective in prolonging the survival of Balb/C mice bearing syngeneic tumor-Meth/A cells, when the flavonoids were dissolved in drinking water and given at a dose of approximately 500 μg/mouse/day.[31] Flavonoids extracted from red soybeans were mostly the cyanidin aglycone conjugated with glucose and rhamnose, whereas flavonoids of red beans were cyanidin conjugated with rhamnose.

Feeding C-3-G significantly suppressed changes caused by hepatic ischemia-reperfusion (I/R) in rats fed 2 g/kg diet of C-3-G for 14 days. I/R treatment elevated liver TBARS and serum activities of glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, and lactate dehydrogenase, marker enzymes for liver injury, and lowered liver reduced glutathione concentration. Although liver ascorbic acid concentrations were also lowered by hepatic I/R, concentrations were restored more quickly in C-3-G fed rats compared with control rats. Feeding C-3-G also resulted in a significant decrease in generation of TBARS during serum formation, and serum also showed a significantly lower susceptibility to further lipid peroxidation provoked by AAPH or Cu^{2+} than that of the control group.[32] Under these feeding and oxidative stress conditions, C-3-G functioned as a potent *in vivo* antioxidant.[33,34]

In rats fed a vitamin E-deficient diet for 12 weeks and then repleted with a diet containing a highly purified anthocyanin-rich extract (1 g/kg diet), a significant improvement in plasma antioxidant capacity and a decrease in the vitamin E deficiency-enhanced hydroperoxides and 8-oxo-deoxyguanosine concentrations in liver were observed.[35] (The anthocyanin extract consisted of a mixture of the 3-glucoside forms of delphinidin, cyanidin, petunidin, peonidin, and malvidin.) Thus, it appears that anthocyanins can be effective *in vivo* antioxidants when included in the diet at 1 or 2 g/kg diet. These levels in the diet provide 20 to 40 mg per day, which are much higher amounts on a body weight basis than found in the typical diet of humans.

Vasoprotective Effects

Lietti[36] demonstrated significant vasoprotective and antiedema properties in experimental animals given an extract from bilberry that contained 25% anthocyanins. In rabbits, the increase in skin capillary permeability due to chloroform was reduced after both i.p. (25 to 100 mg/kg) and oral administration (200 to 400 mg/kg) of anthocyanins. Anthocyanins from *Vaccinium myrtillus* were effective both in a skin capillary permeability test as well as in a vascular resistance test in rats fed a diet devoid of rutin (quercetin rutinoside). In the former test, effective doses were in the range of 25 to 100 mg/kg (by oral route). Anthocyanins were twofold more active when compared with rutin. Orally administered anthocyanins from *V. myrtillus* also inhibited carrageenin paw edema in rats, and a dose–response relationship was observed. In the rat, elimination of anthocyanins occurs mainly through urine and bile, but the liver also extracts a small quantity of the anthocyanins.[37] Anthocyanins were found to possess a greater affinity for kidneys and skin than for plasma or other tissues. Interestingly, long-lasting activity of anthocyanins on capillary resistance was observed even when plasma levels of the anthocyanins were no longer detectable.[37] Cao and coworkers[38] demonstrated that hyperoxia in the rat induced a redistribution of low molecular antioxidants between serum and tissues and produced an increase in capillary permeability, which was alleviated by feeding a blueberry extract rich in anthocyanins. Early work of Mian et al.[39] suggested that anthocyanins protect capillary walls by (1) increasing the endothelial barrier-effect through a stabilization of the membrane phospholipids and (2) increasing the biosynthesis of the acid mucopolysaccharides of the connective ground substance. This may explain the marked increase of newly-formed capillaries and collagen fibrils induced by the anthocyanins. Whether these vasoprotective effects of anthocyanins are due to antioxidant effects is not clear.

Alterations in the capillary filtration of macromolecules are well documented in diabetic patients and experimental diabetes. Various flavonoids, including anthocyanins and ginkgo biloba extracts, have been shown to be effective against experimentally induced capillary hyperfiltration. Cohen-Boulakia et al.[40] demonstrated that anthocyanins were effective in preventing the increase in capillary filtration of albumin and the failure of lymphatic uptake of interstitial albumin in male rats with streptozotocin-induced diabetes. In an earlier study, Valensi and coworkers[41] demonstrated in a placebo-controlled trial that a purified micronized flavonoid fraction (Daflon 500 mg) can improve and even normalize capillary filtration of albumin in diabetic patients.

IN VIVO ANTIOXIDANT AND OTHER SIDE EFFECTS OF ANTHOCYANINS — HUMAN CLINICAL STUDIES

Antioxidant Effects

Studies in humans of antioxidant effects following consumption of anthocyanins are less definitive. Much of the early work on anthocyanins has resulted from studies

of bilberry or concentrated forms of anthocyanins from bilberry.[42,43] Much of the health-related effects reviewed in these publications focused on effects on the vascular system (vasorelaxant and vasomotor effects), effects on the eyes, antioxidant effects, and platelet aggregation effects.

Bub et al.[17] compared changes in plasma malvidin-3-glucoside (M-3-G) and its urinary excretion after ingestion of red wine, dealcoholized red wine and red grape juice in six healthy male subjects, who consumed 500 ml of each beverage on separate days. M-3-G was poorly absorbed and seemed to be differentially metabolized compared with other red grape polyphenols. Bub et al.[17] suggested that anthocyanins, such as M-3-G, may not be responsible for the observed antioxidant and health effects *in vivo* in subjects consuming red wine but rather are due to some other unidentified anthocyanin metabolites or other polyphenols in red wine.

We observed a small but significant increase in plasma hydrophilic and lipophilic antioxidant capacity following the consumption of a single meal of 189 g of blueberries (10 mg anthocyanins/kg).[15,44] Others[27,45] reported an increase in plasma antioxidant capacity (acetone fraction) after the consumption of approximately 1.2 g of anthocyanins (15 mg anthocyanins/kg) from blueberry. Matsumoto et al.[46] observed a rapid increase in plasma antioxidant activity, as indicated by monitoring chemiluminescence intensity, after oral administration of black currant anthocyanins (0.573 mg/kg). A small increase in antioxidant activity in plasma was observed in elderly subjects who consumed 1 cup of blueberries per day for a period of 30 days.[47] What is not known is if anthocyanins are accumulated in tissues when consumed over an extended period of time.

Factors that will impact *in vivo* antioxidant effects of anthocyanins and other flavonoids include (1) quantities consumed, (2) quantities absorbed or metabolized, and (3) plasma or tissue concentrations. Seeram et al.[48] demonstrated that cyanidin glycosides from tart cherries spontaneously degraded to protocatechuic acid, 2,4-dihydroxybenzoic acid, and 2,4,6-trihydroxybenzoic acid in solution at pH 7. Anthocyanins exist as the flavylium cation at pH <3, but at pH 3-6 they may exist as a quinoidall base and at pH 7–8 they may convert to the chalcone. Thus, in any cell or tissue culture study using anthocyanins, one must be aware that at pH 7, the anthocyanins may degrade. What happens to anthocyanins during the absorption process once they are inside the cell and in plasma where the pH will be above 7 is unknown. This instability of anthocyanins in tissue culture and in the body often tends to be overlooked and makes interpretation of *in vitro* data difficult because one does not know whether the effects observed are due to the anthocyanins or some breakdown product. Although anthocyanins can have antioxidant effects in cell culture and other *in vitro* systems at relatively high concentrations, it is not clear whether concentrations can be reached *in vivo* at the tissue level to produce antioxidant effects. Because of the instabilities of anthocyanins in the neutral pH range, it is not clear whether anthocyanins remain intact in tissues long enough to act as antioxidants.

Pawlowicz et al.[49] determined the influence of anthocyanins from chokeberry on the generation of autoantibodies to oxidized low-density lipoproteins in pregnancies complicated by intrauterine growth retardation (IUGR). Their results indicated that anthocyanins can be useful in controlling oxidative stress during pregnancies complicated by IUGR.[49]

Vascular Permeability

Diabetic retinopathy can lead to blindness because of an abnormally high synthesis of connective tissue to repair leaking capillaries and to form new capillaries. Twelve adult diabetics treated with 600 mg of anthocyanins per day for 2 months had a significant decrease in the biosynthesis of connective tissue, especially polymeric collagen and structure glycoproteins in gingival tissue.[50]

Effects on Vision

There have been early reports and some anecdotal information about anthocyanins improving night vision. Zadok et al.[51] assessed the effect of anthocyanins on three night vision tests. In a double-masked, placebo-controlled, cross-over study, 18 young, normal volunteers were randomly assigned to one of three different regimens of oral administration of either 12 or 24 mg anthocyanins, or a placebo, given twice daily for 4 days. No significant effect was found on any of the three night vision tests. However, based upon information presented earlier on dose and plasma levels, these doses would not be expected to produce measurable levels of anthocyanins in the plasma, plus the length of treatment may not have been sufficiently long to observe cumulative effects. Nakaishi et al.[52] studied the effects of oral intake of a black currant anthocyanin (BCA) concentrate on dark adaptation, video display terminal work-induced transient refractive alteration, and visual fatigue in a double-blind, placebo-controlled, crossover study with healthy human subjects. Intake of BCA at three dose levels (12.5-, 20-, and 50-mg/subject, n = 12) appeared to bring about a dose-dependent lowering of the dark adaptation threshold with a significant difference at the 50-mg dose ($p = 0.011$). In the assessment of subjective visual fatigue symptoms by questionnaire, significant improvement was recognized on the basis of the statements regarding the eye and lower back after BCA intake. Muth et al.[53] failed to find an effect of bilberry anthocyanins on night visual acuity or night contrast sensitivity in subjects given 120 mg of anthocyanins daily for 21 days.

In a randomized, double-blind, placebo-controlled study, bilberry fruit extract (160 mg twice daily for 1 month) resulted in improvements in confirmed retinal abnormalities in 79% of the patients with either diabetic or hypertensive vascular retinopathy.[54] Patients with Type II diabeties with retinopathy given 480 mg of bilberry anthocyanins daily for 6 months showed improvement by the end of the trial period as indicated by reduction of hemorrhage and alleviation of weeping exudates from the retina.[55] There is no consistent response in terms of vision based upon the studies presented. Other studies utilizing bilberry anthocyanins are reviewed by Upton.[43] Dose and length of feeding are clearly factors affecting outcomes. Positive effects have been observed at intakes in the range of 300–600 mg per day taken over a period of several months. However, consumption of these levels of anthocyanins from foods will be difficult unless one consistently consumes some of foods high in anthocyanin.

CONCLUSIONS

It is clear that under *in vitro* assay conditions, anthocyanins can function as antioxidants. However, *in vivo*, anthocyanin absorption appears to be low. In animal models, dietary anthocyanins at relatively high doses (1 to 2 mg/kg diet) are protective against oxidative stress induced in a number of models, including ischemia reperfusion, paraquat, CCL_4, and *t*-BHP. In humans, anthocyanins appear to have some vasoprotective effects, but whether these are the result of antioxidant mechanisms is not clear. It appears that in most of the studies reviewed, the dose of anthocyanins was well above that which might be normally consumed in the diet with natural foods, except for perhaps one study in which 1 cup of blueberries was consumed for 30 days and small increases in plasma antioxidant capacity were observed.[47]

Major limitations in many of the *in vitro* studies to date have been (1) the use of aglycones, when there is no evidence that the aglycone is absorbed and presented to the tissues, and (2) the use of concentrations well above those observed in plasma. Few studies to date have attempted to measure anthocyanin concentrations in different tissues. Research with anthocyanins has been slowed due to the lack of pure standard compounds, particularly of the anthocyanins and the availability of isotopically labeled anthocyanins, labeled so that the label is stable at different pH. Understanding any potential relationships to disease prevention has been limited because of the lack of availability of any database on the food content of anthocyanins. These data are being acquired in the U.S., allowing for estimation of daily intakes of anthocyanins from food intake data and for studying relationships to disease outcome in epidemiology studies.

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CHAPTER 2

Common Features in the Pathways of Absorption and Metabolism of Flavonoids

Gary Williamson

CONTENTS

INTRODUCTION

Interpretation of the *in vivo* biological activity of flavonoids from *in vitro* data requires an understanding of their bioavailability, which includes absorption and metabolism. The bioavailability of flavonoids depends on the chemical structure and whether the molecule is conjugated. Although the apparent bioavailability of flavonoids appears to be highly variable between types of flavonoid, from the very

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poorly absorbed anthocyanins to the well-absorbed isoflavones, the pathways involved in the absorption and metabolism are common to all flavonoids. The flux through metabolic pathways is determined by: (1) specificity and activity of transporters; (2) specificity and activity of metabolizing enzymes; and, (3) flavonoid stability. Figure 2.1 summarizes the current state of the art on the pathways that are known to impact bioavailability of flavonoids. Each step is considered individually here.

FLAVONOIDS GENERALLY REACH THE SMALL INTESTINE UNCHANGED FROM THE FORM IN THE FOOD

Flavonoids and isoflavonoids, including quercetin, kaempferol, genistein, daidzein, naringenin, and hesperidin, occur in plants and food almost exclusively as glycosides. Because flavonoid glycosides are stable to most normal cooking methods, stomach acid pH, and to secreted gastric enzymes, intact flavonoid glycosides reach the small intestine following ingestion. Although quercetin and isoflavone aglycones (sometimes consumed in supplement form) are absorbed in the rat stomach to a limited extent,[1] glycosides (of quercetin) are not.[2] The limited capacity of the stomach to

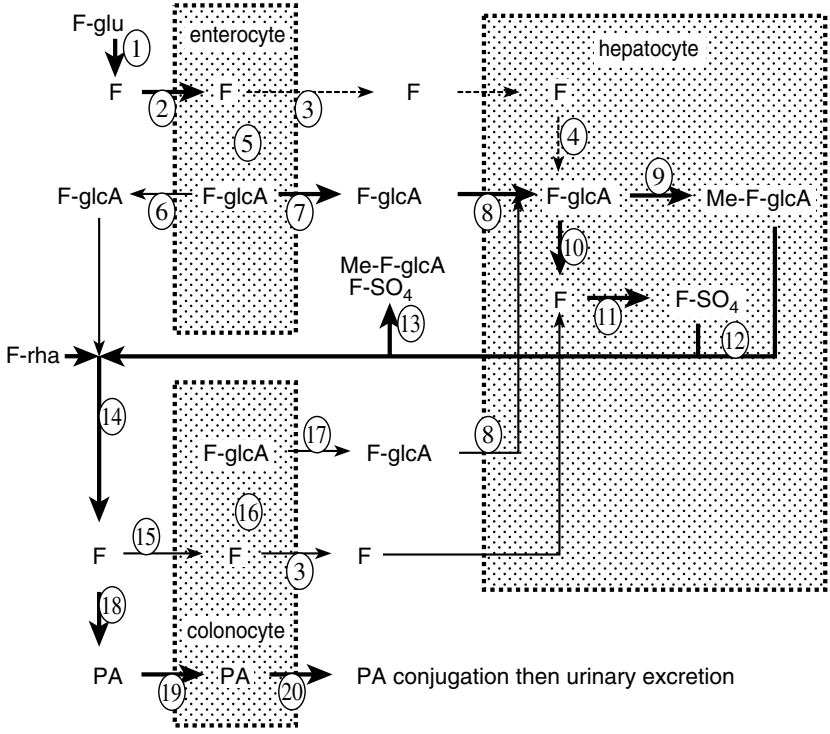

Figure 2.1 **Key step**: **Luminal deglycosylation by LPH**. For glycosylated flavonoids, this step determines whether the flavonoid is absorbed in the small intestine. Deglycosylation is catalyzed by the brush border hydrolase, lactase phlorizin hydrolase. The alternative of transport by a sugar transporter followed by hydrolysis by cytosolic β-glucosidase is a minor pathway for some flavonoids.
2. It is assumed that most aglycones diffuse into enterocytes. However, the partition coefficients of flavonoids vary widely depending on the structure, so it would be predicted that each flavonoid would diffuse at different rates.
3. Diffusion of aglycone into blood. Small amounts of isoflavone aglycones and larger amounts of galloylated catechins are found in plasma, suggesting that some aglycone diffuses across the basolateral membrane into the blood.
4. Hepatic conjugation of aglycone. The liver has a high capacity for conjugation of flavonoids, catalyzed by UDP-glucuronsyl transferase, sulfotransferases, and catechol-*O*-methyl transferase.
5. The enterocyte catalyses complete conjugation of many flavonoids. In the everted rat gut, the products are predominantly glucuronides, but the human small intestine may have a higher capacity to add sulfate than the rat gut.
6. **Key step**: **Export of glucuronides from the enterocyte**. This step determines if the flavonoid, after entering the enterocyte, is exported back to the lumen of the intestine or is transported into the blood. In humans, quercetin-3′-glucuronide is transferred back into the intestinal lumen in the jejunum.
7. Export of glucuronides from enterocytes into blood.
8. **Key step: Uptake of flavonoid glucuronides from blood into hepatocytes**. This step presumably at least partially determines the plasma half life of flavonoids.
9. Hepatic methylation of flavonoid glucuronides by catechol-*O*-methyl transferase leads to a more fully substituted molecule.
10. Hepatic β-glucuronidase is active on quercetin glucuronides in a liver cell model and was enhanced in the presence of methylation inhibitors.
11. Hepatic sulfation. Sulfotransferases are both active on quercetin and strongly inhibited by it.
12. **Key step**: **Biliary excretion of flavonoid conjugates**. This step controls the rate at which flavonoid conjugates are excreted into bile, and depends on the action of transporter(s), which probably includes MRP2.
13. Excretion of conjugates into blood. This is an uncharacterized step but presumably occurs, since flavonoids in the blood become increasingly conjugated with time.
14. Passage from small intestine to colon of conjugates that cannot be hydrolyzed in the small intestine, followed by deglycosylation by microflora.
15. Uptake of released aglycone into colonocyte, probably by passive diffusion.
16. Colonocyte glucuronidation. Uncharacterized.
17. Export of glucuronide from colonocyte into blood. Uncharacterized.
18. **Key step: Microbial conversion of flavonoid into phenolic acids**. This step determines the irreversible loss of flavonoid from the biological system.
19. Transfer of phenolic acids from colonocyte to blood. Unknown if this involves conjugation in the colonocyte, liver or both.
Note: F = flavonoid; glu = glucose; PA = phenolic acid; glcA = glucuronide; SO_4 = sulfate, Me = methyl; rha = rhamnose.

absorb aglycone flavonoids is due in part to the relatively small absorptive surface in stomach, 0.05 m^2, as compared with 200 m^2 in the small intestine.[3]

Flavanols, such as catechins and oligomeric proanthocyanidins, are largely unglycosylated and occur naturally as the aglycone form. Proanthocyanidins are stable in the stomach in humans *in vivo*[4] but break down *in vitro* at pH 2 over several hours to monomeric flavanols and unidentified compounds.[5] Most of the ingested proanthocyanidins and catechins therefore reach the small intestine intact.

IN THE SMALL INTESTINE, GLYCOSYLATED FLAVONOIDS MUST BE DEGLYCOSYLATED BEFORE ABSORPTION

Deglycosylation can potentially occur at several sites in the duodenum and jejunum: (1) within the intestinal lumen; (2) brush border hydrolases; or (3) intracellular hydrolases after transport of the flavonoid into the enterocyte. Deglycosylation is a prerequisite for conjugation by intestinal enzymes and transport to the serosal or mucosal sides.[6] Similarly, for isoflavones, the aglycone but not the glycoside form can be absorbed in the small intestine.[7] The luminal contents may contain glycosidase(s) capable of removing sugars from flavonoids. These glycosidases may arise from sloughed-off cells, intestinal secretions, as components of partially digested food, and a small number of microorganisms.

The initial step in the absorption process for glycosylated flavonoids and isoflavonoids is deglycosylation by lactase phlorizin hydrolase (LPH),[8] an enzyme that is located in the brush border of the small intestine and is responsible for lactose hydrolysis. The enzyme acts outside the epithelial cells so molecules can be deglycosylated in the lumen without first having to traverse the enterocyte membrane.[9] The product of the deglycosylation reaction is a free aglycone that can then diffuse into epithelial cells either passively or by facilitated diffusion. Using humans with ileostomy, the extensive absorption of quercetin in the small intestine as a result of β-glucosidase hydrolysis was shown.[10] Further evidence for luminal cleavage was obtained from incubations with rat everted gut sacs. Incubation with glycosides in the luminal side gave rise to aglycones on the same side,[11–13] but quercetin-3-rhamnoglucoside was not hydrolysed,[13] consistent with the specificity of mammalian β-glucosidases. Deglycosylation reactions are not only specific, they are high capacity. This conclusion follows the observation that absorption of flavonoid glycosides in human subjects is not affected by pretreatment with a microbial β-glucosidase, presumably because LPH in the small intestine catalyses the same reaction.[14]

Absorption of the aglycone released in the lumen is dependent on the presence of other components[15] and presumably on the solubility/partition coefficients of the flavonoid.

An alternative absorptive mechanism involves transport of the flavonoid glycoside into the enterocyte in an intact form via the function of a sugar transporter such as SGLT1.[16] Following flavonoid glycoside transport into the cell, it is then deglycosylated by cytosolic β-glucosidase.[17,18] A good substrate for the cytosolic-glucosidase is quercetin-4′-glucoside, and, by using rat everted intestine, it was shown that the sugar transporter/cytosolic β-glucosidase pathway accounted for 20% of the absorbed quercetin, whereas LPH accounted for the remaining 80%.[18] However, for quercetin-3-glucoside, which is not a substrate for cytosolic β-glucosidase, the LPH pathway accounted for 100% of the absorbed quercetin. Caco-2 cells are deficient in LPH, and hence the sugar transporter/cytosolic β-glucosidase pathway is the predominant one in these cells. Using this model, active transport of quercetin glucosides[19] has been shown.

Both pathways of absorption give rise to intracellular aglycone, and in fact transient intracellular free aglycone is found in rat small intestine tissue after perfusion *in vitro* with either quercetin glucosides[11,20] or isoflavone.[20]

THE SMALL INTESTINE IS THE MAJOR SITE OF FLAVONOID CONJUGATION

The intestine possesses considerable conjugating capacity, including glucuronosyl transferases (UGTs) and glutathione transferases. Although flavonoids do not appear to be glutathionylated, many reports have found glucuronide conjugates in various systems. The major site of the initial conjugation of flavonoids is the small intestine.[21–23] Absorption studies using models of the small intestine examining transfer of flavonoids from the mucosal (gut) compartment to the serosal (blood) compartment have found quercetin, catechin, and genistein predominantly in the glucuronidated form. The enzyme isoforms most likely to catalyze the conjugation in the human small intestine are UGT1A1 and 1A8,[24] although UGT1A9 may also play a role in the liver.[25]

A proportion of some flavonoids escapes intestinal conjugation. Evidence for this is derived from the presence of unconjugated flavonoids in the plasma. However, this has been shown only for galloylated catechins and isoflavones under certain conditions and is dose and time dependent, whereas unconjugated catechin and quercetin are not found in plasma in significant amounts.[1,26–31]

PLASMA CONJUGATES OF FLAVONOIDS ARE NOT GLUCOSIDES BUT ARE SULFATED, GLUCURONIDATED, OR METHYLATED DERIVATIVES AND ARE RARELY SUBSTITUTED

Following glucuronidation reactions during absorption, some flavonoids undergo further metabolism, at least in part. For those that are known, glucuronide residues are removed and replaced with a sulfate. The sulfation reaction is thought to occur predominantly in the liver. This conclusion stems from the observation that peripheral blood contains a mixture of glucuronide and sulfated flavonoid conjugates. Flavonoid glucuronides have been detected in the hepatic portal vein.[32] However, the exact nature of flavonoid conjugates in blood is known only for a limited number of compounds. After consumption of onions for example, quercetin occurs in blood as quercetin-3-glucuronide, quercetin-3′-sulfate, and methylquercetin-3-glucuronide.[27]

HEPATIC DECONJUGATION AND RECONJUGATION

The liver receives flavonoids from the blood, including blood from the small intestine during first pass metabolism. Based on *in vitro* and *in vivo* perfusion experiments on rats, flavonoids from the small intestine that reach the liver are almost entirely conjugates, especially of glucuronides.[11,32] Quercetin glucuronides from the small intestine are taken up into hepatic cells by a transporter, although quercetin-3-glucuronide and quercetin-7-glucuronide appear to be taken up by a different mechanism.[47] Following this uptake, the glucuronides from the small intestine are either deglucuronidated inside the cell by β-glucuronidase and then sulfated, or the intact

Table 2.1 "Pharmacokinetic" parameters of flavonoids in blood after consumption by volunteers.

Source	Flavonoid[a]	Tmax (h)	Half life (h)	Reference
Red wine	Catechin	1.4	3.2	71
Green tea	Epigallocatechin gallate	1.5	3.4	72
Green tea	Epigallocatechin	1.5	1.7	72
Green tea	Epicatechin	1.5	2	72
Green tea	4-O-Methyl epigallocatechin	1.7	4.4	72
Onion	Quercetin	0.7	11	39
Quercetin-4′-glucoside	Quercetin	0.5	22	36
Quercetin-4′-glucoside	Quercetin	0.7	12	39
Buckwheat tea	Quercetin	4.3	10	39
Quercetin-3-rutinoside	Quercetin	7	12	39
Quercetin-3-rutinoside	Quercetin	6	28	36
Soy	Genistein	8	8.4	73
Soy flour	Genistein	6	11.8	74
Soy flour	Genistein	8.4	5.7	75
Soy flour	Daidzein	6	6	74
Soy	Daidzein	8	5.8	73
Soy flour	Daidzein	7.4	4.7	75

[a] Measured as total aglycone after deconjugation.

glucuronides are methylated.[47,48] Catechin is also metabolized in a comparable way in rats.[32] These results imply that circulating flavonoid glucuronides are not necessarily inactivated. In fact, conjugation may even preserve the flavonoid in biological fluids. For an unstable aglycone like quercetin, the half life in cell culture medium is as short as 1 to 2 h, but the half life of quercetin (as conjugates) in human blood *in vivo* is around 10 to 22 h (Table 2.1). Conjugation, therefore, stabilizes quercetin and could act to deliver it to tissues. Other substances are also stabilized in blood in various ways, including conjugation.[49] A small proportion of some flavonoids may escape conjugation in the small intestine. These aglycones reach the liver, where they are conjugated with sulfate or glucuronide and may also be methylated. Cytochrome p450-catalyzed metabolism of quercetin also occurs *in vitro*,[50] but it is not known if this pathway occurs *in vivo*.

All flavonoid conjugates are ultimately exported, probably by MRP2,[51] into the bile and back to the small intestine. For example, 70 to 75% of the administered dose of genistein was secreted in the bile over a 4-h period as genistein-7-*O*-β-glucuronide.[52] Excretion into the bile then results in reentry into the small intestine lumen and, in the absence of further deconjugation, passage of the excreted conjugate into the colon. This is followed by deglucuronidation or sulfation by microbes in the ileum or colon, and reabsorption of the flavonoid leading to enterohepatic cycling.[53]

Although most flavonoids in the blood stream are conjugated, flavonoid aglycones, glucuronide, and sulfate conjugates are transported bound to albumin (Janisch, Plumb, and Williamson, unpublished results).[33,34] In the case of catechins, after 3 days consumption of green tea about 10% are present in blood in the lipoprotein

fractions: ~0.1 μM in very low-density lipoprotein and low-density lipoprotein, 0.2 μM in high-density lipoprotein, and 0.5 μM in other lipoprotein fractions.[35]

DECONJUGATION AND TISSUE UPTAKE

The blood delivers flavonoids to tissues throughout the body. If present in the plasma, aglycones could enter peripheral tissues by passive or facilitated diffusion. Glucuronide conjugates, however, presumably would need to be transported into peripheral tissues, because they are relatively hydrophilic and diffuse through membranes only very slowly. This may not be the case for the sulfate conjugates, because some may be relatively hydrophobic. For deconjugation in tissues, many cells possess β-glucuronidase activity, found both in the lysosomal fraction and in the lumen of the endoplasmic reticulum[54]; in liver cells, this enzyme is active on quercetin glucuronides.[47] Sulfatase activity is also present and acts on steroid and other sulfates inside the cell,[55–57] thereby producing intracellular aglycone. β-Glucuronidase activity may also be present in some extra-cellular fluids, such as aqueous humor, which is the interface between the blood and the lens.[58] Free genistein is found in endocrine-responsive tissues including brain, liver, mammary, ovary, prostate, testis, thyroid, and uterus.[59] Some free methyl-quercetin, catechins, and methyl catechin are found in liver but not in plasma.[60]

EXCRETION

The yield of flavonoids in the urine is dramatically dependent on the flavonoid under examination. For quercetin and anthocyanins, it is less than 1.5%, for isoflavones it is 2 to 20% and for catechins it is ~5% (reviewed in Scalbert and Williamson[61]). Flavonoids are found in urine as conjugated forms; for example, rat urinary catechins are (+)-catechin-5-*O*-β-glucuronide and (-)-epicatechin-5-*O*-β-glucuronide.[63] Generally, renal excretion is not a major pathway for intact flavonoids[62] and the urinary content of flavonoids cannot be used as a biomarker of bioavailability or dietary intake.

DEGLYCOSYLATION AND FURTHER BREAKDOWN OF FLAVONOIDS OCCURS IN THE COLON BY MICROFLORA

Certain flavonoids, such as rutin (quercetin-3-rhamnoglucoside), are not deglycosylated by human enzymes because rhamnose is not a substrate; these conjugates reach the terminal ileum and large intestine intact. In addition, biliary secretion is the major route of excretion of flavonoids, usually in the form of a glucuronide or sulfate conjugate. These conjugates are also predicted to reach the terminal ileum and colon intact in humans. Thus a high percentage of ingested flavonoids are available as

substrates for colon microflora, even though substantial amounts may have already circulated through the body.

The colon microflora enzymes have a very large capacity for deconjugation, including deglycosylation, deglucuronidation, and desulfation[64,65]; *in vitro*, the deconjugation is rapid, leading to production of the aglycone, and this is unlikely to be the rate-limiting step. The nature of the products from the breakdown of flavonoids in the colon is beyond the scope of this chapter, but the fate of the released aglycone depends on competition between pathways of colon absorption and microflora breakdown. For some aglycones, such as isoflavones, substantial absorption occurs in the colon,[66] presumably because genistein and daidzein are relatively resistant to degradation. In addition, a biological activation of daidzein occurs in some individuals to equol, and this step is known to increase phytoestrogen activity.[67,68] In marked contrast, quercetin is much less efficiently absorbed in the colon compared with the small intestine,[69] probably because quercetin is more readily broken down into low molecular weight phenolics by colonic microflora,[64] and the aglycone of quercetin is unstable.[70]

FLAVONOID PHARMACOKINETICS

Appearance and disappearance of flavonoids in the blood are described by pharmacokinetics and have been reported by several groups.[16,30,36–40] Table 2.1 summarizes some of the pharmacokinetic data for selected flavonoids. The time to reach maximum plasma concentration is an indicator of the site of absorption, and typically small intestine uptake is represented by values of <3 h and the colon by values of 5 to 10 h, although this depends on the meal size and transit times. For glycosylated flavonoids, the attached sugar is a major determinant of the Tmax. As described earlier, an attached glucose leads to absorption in the small intestine, whereas an attached rhamnose leads to absorption in the colon after microflora deconjugation. The half life represents both the rate of appearance within and the clearance from the bloodstream and, therefore, the time available for a biological effect to occur. For the half life, the flavonoid itself is the major determinant: Quercetin > isoflavones > catechins. It should be noted that most pharmacokinetics have been measured based on the determination of flavonoid aglycone after deconjugation. True pharmacokinetics are for plasma appearance of the administered compound only, but most flavonoids are conjugated with glucuronic acid, sulfate, or methyl groups making this impossible. The short half life of flavonoids makes the plasma concentration difficult to use as a biomarker of long-term flavonoid levels in the diet, and it has been shown, for example, that the plasma concentrations of hesperetin and naringenin are poor biomarkers of intake.[40]

Anthocyanin glucosides, however, are an exception for pharmacokinetic measurements, and low amounts of anthocyanin glucosides have been found in plasma and urine.[41–44] However, only very low levels have been measured in plasma, and the biological activity at these levels in likely to be low. It is also not clear whether proanthocyanidins are absorbed into plasma in an intact form. In rats, the procyanidin

dimer B3 is not found in plasma after consumption of either the purified dimer or a grape-seed extract,[45] whereas dimers have been detected at low levels in human plasma after consumption of cocoa.[46]

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CHAPTER 3

Pharmacokinetics and Bioavailability of Green Tea Catechins

Yan Cai and H-H. Sherry Chow

CONTENTS

INTRODUCTION

Tea is a beverage made from the leaves of the *Camellia sinensis* species of the Theaceae family. This beverage is one of the most ancient and, next to water, is the most widely consumed liquid in the world. Tea leaves contain specific polyphenols and polyphenol oxidase. Following harvesting, fresh tea leaves are subjected to a series of treatment steps that result in the manufacturing of different tea products: black tea, green tea, or oolong tea.

Green tea is made by steaming or frying fresh tea leaves at elevated temperatures to prevent polyphenol oxidation. The chemical composition of green tea is similar to that of the fresh leaves regarding the major components. It contains polyphenols, which include flavanols, flavandiols, flavonoids, and phenolic acids. Flavanols are the most abundant constituents and are commonly known as catechins. The major catechins present in green tea are (–)-epicatechin (EC), (–)-epicatechin-3-gallate

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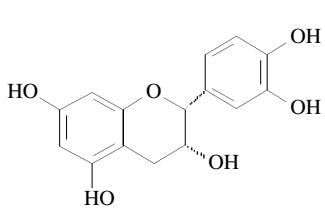
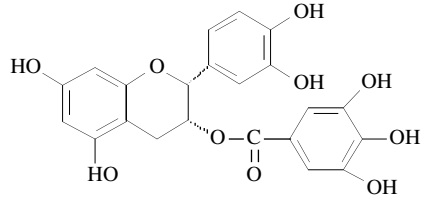
(ECG), (–)-epigallocatechin (EGC), and (–)-epigallocatechin-3-gallate (EGCG) (Figure 3.1). In addition, caffeine, theobromine, theophylline, and phenolic acids, such as gallic acids, are also present in green tea. Black tea is made by promoting enzymatic oxidation of fresh leaves. Most flavanols are converted to the oxidized form known as theaflavins and thearubigins (Figure 3.2). The total flavanol level is reduced from 35 to 50% in green tea to 10% in black tea. Theaflavins and thearubigins are present in black tea at a level of 3 to 6% and 12 to 18%, respectively.[1] All other components are virtually unchanged. Oolong tea is a half-fermented product. It contains monomeric catechins, theaflavins, and thearubigins, with a catechin level of 8 to 20% of the total dry matter.

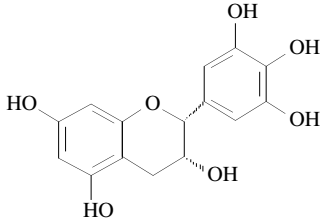
Tea consumption is not uniform throughout the world. Large segments of the world's population virtually consume no tea. Not only does tea consumption vary from country to country, but there is also enormous variation in any given population. Extensive laboratory research and epidemiological findings of the past 20 years have suggested that tea or tea components may reduce the risk of a variety of illnesses, including cancer and coronary heart disease. A number of review articles have summarized these findings.[2–5] Because the highly polymerized components in black tea are less well characterized, experimental studies showing the cancer preventive effects of tea have been conducted primarily with green tea or green tea components. Green tea, green tea extracts (GTEs), and EGCG have each been shown to inhibit



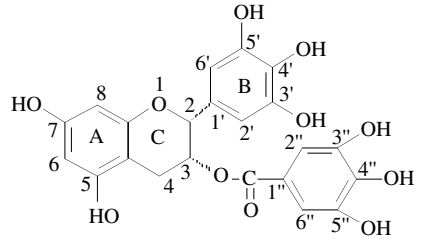
(-)-epicatechin (EC)



(-)-epicatechin-3-gallate (ECG)



(-)-epigallocatechin (EGC)



(-)-epigallocatechin-3-gallate (EGCG)

Figure 3.1 Chemical structures of major green tea catechins.

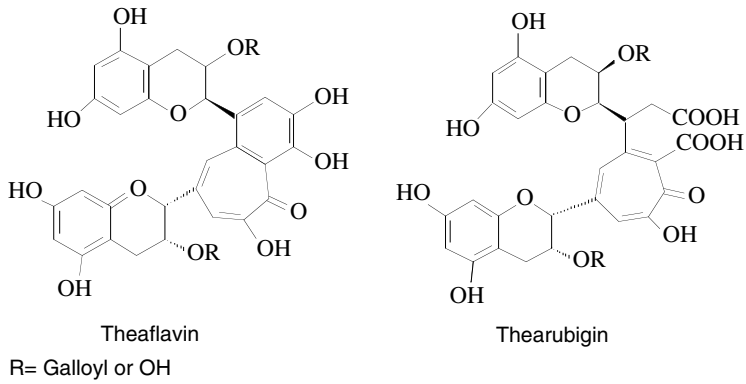


Figure 3.2 Chemical structures of major black tea polyphenols.

carcinogenesis induced by a wide variety of carcinogens in rodent cancer models. Cancer chemopreventive activity has been demonstrated in the following target organs: colon, duodenum, esophagus, forestomach, large intestine, liver, lung, mammary glands, and skin.[6,7]

The biochemical mechanisms responsible for the cancer-preventive effects of green tea have not been clearly defined. Laboratory studies have shown that green tea possesses antioxidant and free radical scavenger activities,[6,8–10] inhibits cell proliferation,[11,12] induces apoptosis,[13] modulates carcinogen-metabolizing enzymes,[14,15] and suppresses inflammatory responses,[16,17] all of which could contribute to the observed preventive effects.

The epidemiological evidence of the protective effect of tea consumption against the development of human cancers is not conclusive. Some studies have shown a protective effect of tea consumption against certain types of cancers,[18–23] while others found no association between tea consumption and cancer risk.[24–28] Understanding the pharmacokinetics of green tea constituents is important to help interpret epidemiological findings and to extrapolate preclinical data to human situations. The following sections summarize the pharmacokinetic information of green tea catechins.

IN VIVO PHARMACOKINETIC STUDIES

When a decaffeinated green tea extract (DGTE) was dosed to rats intravenously at a total dose of 50 mg/kg, containing 50% EGCG, 13% ECG, and 5% EC, tea catechins exhibited bi-exponential disposition.[29] The following pharmacokinetic parameters were observed for EGCG, ECG, and EC, respectively: terminal elimination half life ($t_{1/2}$) of 191, 362, and 45 min; systemic clearance (CL) of 9.0, 5.0, and 17ml/min/kg, and apparent volume of distribution (V_d) of 2.5, 2.8, and 1.1 L/kg. Urinary and fecal recovery of EGCG, ECG, and EC was 2.45% vs. 0.76%, 2.11% vs. 1.31%, and 14.2% vs. 1.01%, respectively. Compared with an average hepatic blood flow of 50 ml/min/kg in rats,[30] tea catechins can be considered to have low to moderate clearance.

The volumes of distribution of tea catechins correlate positively to their octanol/water partition coefficients (log $K_{o/w}$ of –0.3, 0.15, 1.2, and 1.38 for EGC, EC, EGCG, and ECG, respectively)[31] and are in ranges similar to those reported for polar drugs[32–34] and can be considered to have small distribution volumes. Pharmacokinetics of tea catechins were also studied in rats after intravenous dosing of DGTE at different dose levels (50 to 300 mg/kg of DGTE).[35] Systemic clearance and central volume of distribution (V_c) of tea catechins increased significantly when the dose was increased. Terminal elimination half life and volume of distribution at steady state (V_{ss}) did not change significantly over the dose range studied. Dose-dependent increase in the systemic clearance of tea catechins may be attributed to saturation of plasma protein binding of tea catechins at higher catechin concentrations.[35] When rats were administered with DGTE at a dose of 5g/kg orally, plasma concentrations of three tea catechins (EGCG, ECG, and EC) reached maximum levels (C_{max}) within 2 h postdose, and the C_{max} values were 112.0, 14.8, and 15.4 μg/ml, respectively.[29] The terminal half life of three tea catechins after oral dosing (449, 479, and 451 min for EGCG, ECG, and EC, respectively) was much longer than that after intravenous administration. Oral clearance (CL/F) and apparent volume of distribution (V/F) of three tea catechins were 61, 91, and 46 ml/min/kg, and 38.5, 63.0, and 29.7 L/kg for EGCG, ECG, and EC, respectively.

From the oral and intravenous data, oral bioavailability of EGCG, ECG, and EC in rats was found to be 14, 6, and 39%, respectively. However, the doses used in these studies are significantly larger than what could be achieved in humans. Extrapolation of these data to humans may be limited by saturation in the kinetic processes at higher doses.

When healthy human subjects ingested 1.2 g of DGTE in warm water (120 ml), containing 88 mg of EGCG, 82 mg of EGC, 33 mg of ECG, and 32 mg of EC, plasma catechin levels at 1 h after consumption were 46 to 268, 82 to 206, and 48 to 80 ng/ml for EGCG, EGC and EC, respectively.[36] After consumption of 1.5 g of DGT, the catechins in human plasma reached peak levels in 1.5 to 2.5 h.[37] The average C_{max} of EGCG, EGC, and EC was 326, 550, and 190 ng/ml, respectively. When the dosage was increased from 1.5 to 3.0 g, the C_{max} values increased 2.7- to 3.4-fold. However, increasing the dose to 4.5 g did not increase the C_{max} values significantly. It is likely that constituents in the GTE were not completely soluble in the gastrointestinal fluid when the dose was increased to 4.5 g. Terminal half lives of EGCG, EGC, and EC after oral administration of 1.5 to 4.5 g of DGT were 4.9 to 5.5, 2.5 to 2.8, and 3.2 to 5.7 h, respectively. Area under the plasma-concentration time curve (AUC) of EGCG, EGC, and EC after oral administration of 1.5 to 4.5 g of DGT was 0.90 to 2.64, 0.62 to 3.28, and 0.30 to 1.2 μg•h/ml, respectively. In both clinical studies, tea catechin levels were expressed as the sum of unchanged and conjugated forms, and pharmacokinetic parameters were calculated based on these hybrid values.

Chow et al.[38] studied the pharmacokinetics of green tea catechins in healthy human subjects following oral administration of purified EGCG or green tea catechin mixture (Polyphenon E, containing EGCG, EGC, and EC in the ratio of 1:0.185:0.155). Both unchanged and total of unchanged and glucuronide/sulfate conjugates of green tea catechins were measured in this study. In equivalent EGCG

doses, both formulations resulted in similar plasma levels of unchanged EGCG. The C_{max} values of unchanged EGCG were 73.7 ± 25.3, 111.8 ± 98.6, 169.1 ± 139.6, and 438.5 ± 284.4 ng/ml after 200-, 400-, 600-, and 800-mg dose of EGCG, respectively. The C_{max} values of unchanged EGCG were 72.7 ± 66.4, 125.3 ± 50.4, 165.7 ± 126.9, and 377.6 ± 149.8 ng/ml after 200-, 400-, 600-, and 800-mg dose of Polyphenon E, respectively. The Polyphenon E doses are expressed based on the EGCG content. A 200-mg dose of EGCG or Polyphenon E contains EGCG content equivalent to that in 2 to 4 cups of green tea. Figure 3.3 and Figure 3.4 illustrate

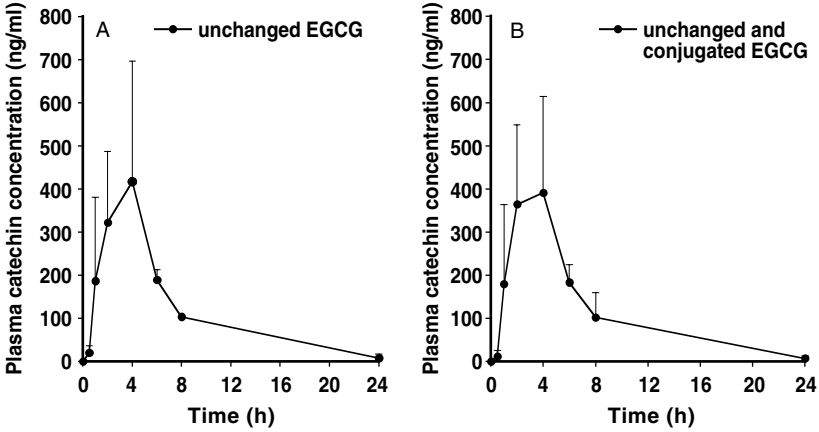


Figure 3.3 Average plasma tea catechin concentration vs. time profiles following an 800-mg dose of EGCG. A. Data obtained from plasma samples without subjecting to glucuronidase/sulfatase treatment. B. Data obtained from plasma samples treated with glucuronidase/sulfatase.

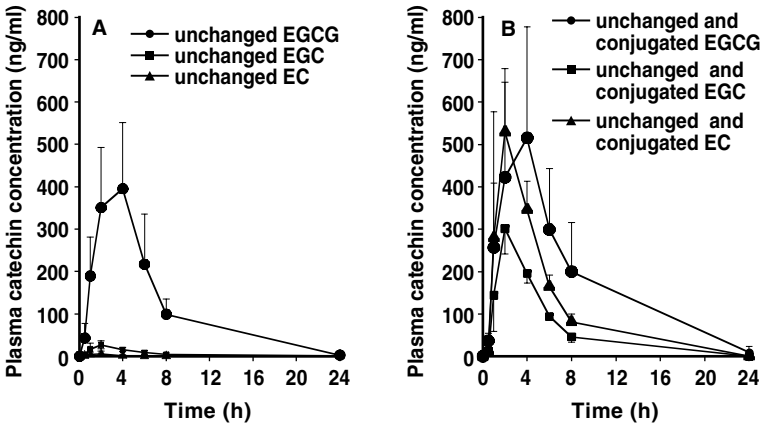


Figure 3.4 Average plasma tea polyphenol concentration vs. time profiles following an 800-mg dose of Polyphenon E. A. Data obtained from plasma samples without subjecting to glucuronidase/sulfatase treatment. B. Data obtained from plasma samples treated with glucuronidase/sulfatase.

plasma tea catechin concentration-time profiles following 800-mg dose of EGCG or Polyphenon E, respectively. Following EGCG administration, only EGCG was detected in human plasma. EGCG levels did not change significantly after the plasma samples were treated with deconjugating enzymes (β-glucuronidase/sulfatase), suggesting that EGCG is present in plasma mostly as the unchanged form. After Polyphenon E administration, EGCG levels were detected in human plasma, while EGC and EC levels were low or undetectable. After the samples had been treated with deconjugating enzymes, EGCG levels did not change much, whereas EGC and EC levels increased substantially. This observation was also reported in the study performed by Lee et al.[39] In their study, healthy human subjects were instructed to drink green tea in warm water containing 200 mg EGCG, 154 mg EGC, and 45 mg EC. A high percentage of EGCG (77%) was present in the free form, whereas 31% of EGC and 21% of EC were in the free form at 1 h after tea consumption.

Chow et al.[38] reported large inter-individual variations in the pharmacokinetics of unchanged EGCG and of the sum of conjugated and unchanged EGC and EC. The percent coefficient of variation of EGCG AUC ranged from 32.4 to 97.8% after oral administration of EGCG or Polyphenon E. Within-individual differences in the pharmacokinetics of the total of unchanged and conjugated EGCG were observed when GTE was given in drinking water, with the percent coefficient of variation of AUC ranging from 34.1% to 59.0%.[39] These inter- and intraindividual pharmacokinetic variations could be one of the factors contributing to the inconclusive epidemiological findings of the relationship of tea consumption and human cancers.

ORAL ABSORPTION AND BIOAVAILABILITY

Most of the absorption and oral bioavailability studies of green tea catechins were performed in laboratory animals. Chen et al.[40] dosed rats with EGCG through intravenous (10 mg/kg) and oral (75 mg/kg) administration and found that the oral bioavailability of EGCG was 1.6%. In this study, DGT containing 73, 68, and 27 mg/g of EGCG, EGC, and EC, respectively, was also dosed to rats via intravenous (25 mg/kg) and oral (200 mg/kg) routes. The C_{max} values of EGCG, EGC, and EC after oral dosing of DGT were 16.3, 1,432.8, and 685.4 ng/ml, respectively, and oral bioavailability was 0.1%, 13.7%, and 31.2% for EGCG, EGC, and EC, respectively. The authors suggested that the difference in oral bioavailability of EGCG after pure EGCG and DGT administration is due to the effect of other components in DGT on oral absorption of EGCG. In this study, the plasma concentration and oral bioavailability calculation were based on the combined unchanged and conjugated catechin concentrations. The bioavailability of the unchanged catechins could be lower than the reported values because more conjugated metabolites could be formed during presystemic first-pass metabolism after oral catechin administration.

By comparing the systemic exposure of green tea catechins in rats after intravenous and intraportal administration, Cai et al.[41] found that tea catechins do not undergo significant presystemic hepatic metabolism. Consistently, when EGCG was administered at a dose of 100 mg/kg to rats by intraperitoneal injection, much higher

plasma concentrations of free EGCG were observed (9.16, 0.92, 0.46, and 0.46 μg/ml at 0.5, 1, 2, and 24 h after dosing, respectively).[42] These studies suggest that presystemic intestinal loss/metabolism contribute more significantly to the low oral bioavailability of green tea catechins.

Intestinal absorption of tea catechins was studied via perfusion of EC through jejunum or ileum over 90 min in an isolated rat small intestine perfusion model.[43] A total of 56% of unchanged and conjugated EC was detected at the serosal side of the ileum, and 60% of that was in the unchanged form. Nevertheless, a low percentage (11.6%) of unchanged and conjugated EC was detected at the serosal side of the jejunum; about 7% was in the unchanged form. The study demonstrates that EC is absorbed more from the ileum than from the jejunum. The intestinal epithelial membrane transport of EC was studied recently using the human Caco-2 cell line.[44] EC was not absorbed from the apical to basolateral side, whereas efflux from basolateral to apical side with a high apparent permeability was reported. The efflux was inhibited by MK-571, a competitive inhibitor of the MRP2 transporter expressed in the epical membrane of Caco-2 cells. A *P*-glycoprotein inhibitor, verapamil, did not inhibit the efflux of EC from basolateral to apical side at the same concentration as the substrate. In the presence of MK-571, apical to basolateral absorption of EC could be observed, although rather low. This study suggests that intestinal efflux of green tea catechins may contribute to the low oral bioavailability of these phytochemicals.

Kim et al.[45] studied the effect of chronic dosing of green tea polyphenols (GTP) on systemic tea catechin levels in rats. GTP contains 590, 76, and 86 mg of EGCG, EGC, and EC per gram of solid, respectively. When 0.6% of GTP was administered through the drinking fluid for 8 days, plasma levels of EGC and EC were much higher than those of EGCG (984, 1,527, and 105 ng/ml for EGC, EC, and EGCG, respectively). When rats were dosed with 0.6% of GTP for 28 days, plasma levels of three tea catechins were increased, reached a maximum on day 14, and then decreased. A similar pattern of increase and then decrease in blood catechin levels was also seen in mice except that the decrease took place 4 days after treatment with tea.[45] In mice, the systemic exposure of EGCG was much higher than that in rats, whereas the EGC and EC levels were similar between the two animal species. This study suggests that treatment with 0.6% GTP in drinking water achieved plasma EGCG levels in rodents that can also be seen with GTP administration in humans. However, higher levels of EGC and EC were achieved at this dose level in rodents. Efficacy studies in rodent cancer models that employed doses higher than the tea catechin content used in this study may not be physiologically relevant.

TISSUE DISTRIBUTION

Studies on the tissue distribution of green tea catechins are limited. When EGCG was dosed intragastrically to rats at a dose of 500 mg/kg, the highest level of unchanged EGCG was found 1 h after dosing in small intestine mucosa (259 mg/g), followed by colon mucosa (31 mg/g), liver (22 mg/g), plasma (5.5 mg/g), and brain (0.2 mg/g).[46] The combined unchanged and conjugated EGCG levels were also found

to be the highest in the small intestine after intravenously administration of DGT (25 mg/kg) in rats.[40] The intestinal EGCG levels declined with a $t_{1/2}$ of 173 min. The highest levels of combined unchanged and conjugated EGC and EC were observed in the kidney, and the levels rapidly declined with a $t_{1/2}$ of 29 and 28 min, respectively. The area under the curve of EGCG in the intestine was fourfold higher than that in the kidney, while the areas under the curves of EGC and EC in the intestine were similar to those in the kidney. The levels of EGCG, EGC, and EC in the liver and lung were generally lower than those in the intestine and kidney.[40]

Tea catechin tissue distribution was also determined in rats when 0.6% of GTP was dosed for 8 days.[45] In this study, tea catechin concentrations were also presented as the total of unchanged and conjugated tea catechin levels. Substantial amounts of EGC and EC were found in the bladder (800 to 810 ng/g tissue), large intestine (300 to 930 ng/g tissue), kidney (400 to 500 ng/g tissue), lung (190 to 230 ng/g tissue), and esophagus (185 to 195 ng/g tissue). Levels of EGC and EC were low in the spleen (76 to 93 ng/g tissue), liver (44 to 50 ng/g tissue), thyroid (37 to 49 ng/g tissue), and heart (21 to 30 ng/g tissue). The amount of EGCG was higher in large intestine (488 ng/g tissue), esophagus (280 ng/g tissue), and bladder (201 ng/g tissue), and lower in kidney, prostate, spleen, liver, and lung. Tea catechin liver and lung distribution was also determined in mice when 0.6% of GTP was dosed for 12 days.[45] The concentration of EGCG was higher than EGC and EC in the lung, whereas EGC was slightly higher than EGCG in the liver. However, at any time, tea catechin concentrations in the lung were higher than those in the liver. In both tissues, tea catechins peaked on day 4 and then declined to day 12.

When [^{3}H]-EGCG was administered directly into the stomach of mice, radioactivity was found in the digestive tract, liver, lung, pancreas, mammary gland, and skin, as well as in brain, kidney, uterus, ovary, and testes.[47] Because the chemical nature of the radioactivity in these tissues was not determined, it is not known whether the radioactivity represents unchanged EGCG or its derived metabolites.

Zhu et al.[35] determined the plasma protein binding and red blood cell partitioning of green tea catechins in rats. The percentage of catechins bound to plasma proteins was 81% for EC, 97% for EGCG, and 100% for ECG at concentrations of 4, 36, and 9 μg/ml, respectively. A significant decrease in fraction bound to plasma proteins with increasing tea catechin concentrations was observed, indicating saturable plasma protein binding. The red blood cell partitioning of tea catechins was determined in the same study and was found to be 29 to 43%, 15 to 23% and 9 to 11% for EC, EGCG, and ECG, respectively. Distribution of catechins into red blood cells was minimally affected by the tea catechin concentration.

METABOLISM

Green tea catechins have been shown to undergo methylation and conjugation biotransformation. After DGT administration, the major conjugates appearing in rodent urine were identified as monoglucuronides and monosulfates of EGC and EC.[48] When EGCG was administered to rats intravenously or orally, EGCG conjugates and five methylated metabolites (3′-methyl-EGCG, 4′-methyl-EGCG, 3″-

methyl-EGCG, 4″-methyl-EGCG, and 4′,4″-dimethyl-EGCG) were detected in bile.[49–50] Most of the methylated EGCG metabolites were present as the glucuronide and sulfate forms in rat plasma after oral EGCG administration.[49–50]

Piskula and Terao[51] proposed that flavanol metabolism involves glucuronidation in the small intestine as the first step followed by *O*-methylation in the liver and kidney, because the activity of glucuronosyltransferase was the highest in the intestinal mucosa in rats, phenolsulfotransferase activity was the highest in the liver, and catechol-*O*-methyl transferase activity was the highest in the liver and kidney. When tea catechins were incubated with rat liver homogenates, tea catechin conjugates and methylated tea catechins were formed. The methylated metabolites include 4′-methyl-EGC, 4″-methyl-ECG, and 4″-methyl-EGCG from EGC, ECG, and EGCG, respectively.[52] When EC was incubated with rat liver homogenates, EC conjugates, 3′-methyl-EC, and 4′-methyl-EC were formed, the same metabolites as those observed after oral administration of EC in rats.[53] Among the products found in a 24–h urine collection in rats, 8% was EC and methylated EC and 16% was EC conjugates and methylated EC conjugates.[53] Among the EC metabolites, only 3′-methyl-EC conjugates appeared in bile after oral dosing in rats.[53] Consistently, when EC was perfused through jejunum and ileum over 90 min in an isolated rat small intestine perfusion model, three types of metabolites were detected; EC-glucuronides, methylated-EC (3′-methylated and 4′-methylated EC), and methylated-EC-glucuronides.[43]

Tea catechin derived metabolites were also detected in plasma and urine samples collected in humans after oral green tea administration (Figure 3.5). Glucuronides/sulfates of EGCG, EGC, and EC were detected in plasma and those of EGC and EC were present in urine after oral ingestion of DGT or green tea catechins in humans. [36,38]After oral administration of DGT, the major metabolites appeared in human urine included not only the monoglucuronides and monosulfates of EGC and EC, but also the *O*-methyl-EGC-*O*-glucuronides/sulfates and *O*-methyl-EC-*O*-sulfates.[54] The methylated EGC conjugates were present at high levels in human blood after oral DGT administration.[55] In addition, metabolites that seem to derive from microflora-mediated metabolic processes have been identified in human plasma and urine. These include 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone (M4) and 5-(3′, 4′-dihydroxyphenyl)-γ-valerolactone (M6).[48] They were found to be present in the conjugated form. M4 and small amounts of M6 were detected, and only M6 was detected in human urine after pure EGC or EC oral administration, respectively. The study results suggest that these metabolites are produced by intestinal microorganisms, with EGC and EC as the precursors of M4 and M6, respectively, and subsequently absorbed into the systemic blood and excreted in the urine. After oral administration in rats, EGCG has been shown to be metabolized by intestinal microflora to form EGC and 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone.[56] However, when EGCG was administered orally in humans, EGC was not found in blood or urine.[38]

At present, the biological activity of tea catechins has been determined mostly with the parent chemicals. EGCG, EGC, EC, and ECG have been shown to possess various biological activities, including antioxidative and free radical scavenger activity when studied in *in vitro* systems.[10–11,16,57–58] Pietta et al.[59] reported that free plasma catechins account for only about 20% of increase in the total antioxidant activity

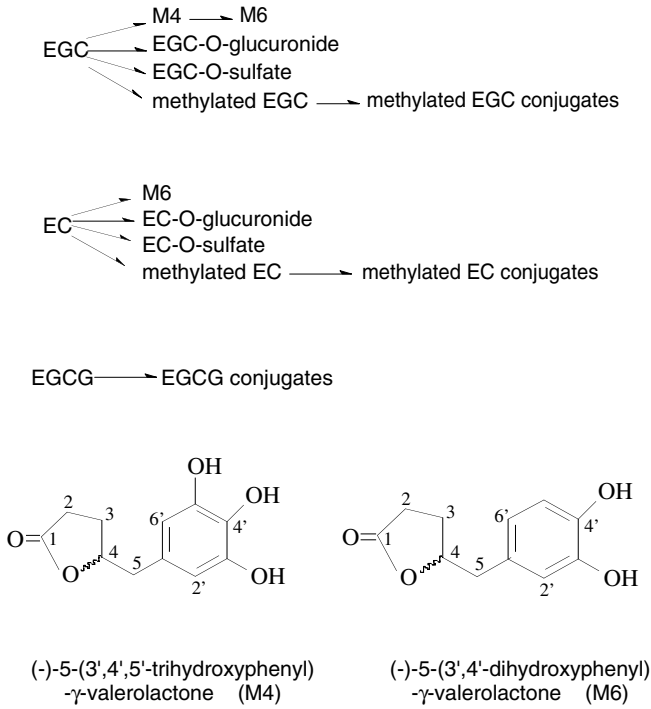


Figure 3.5 Green tea catechin metabolites identified in human plasma and urine.

after green tea intake in humans, suggesting that catechin-derived metabolites may contribute to the activity. Harada et al.[60] demonstrated that conjugated catechin (CT-glucuronide) and conjugated EC (EC-glucuronide) have antioxidant activity similar to that of CT and EC when measured by electron spin resonance (ESR) spectrometry. The methylated CT and EC conjugates were shown not to possess antioxidant activity. However, conjugated catechins are much more polar than the parent chemicals, thus the distribution of these metabolites to target tissues could be limited. Whether catechin-derived metabolites contribute to the observed *in vivo* activities is yet to be characterized.

EXCRETION

Green tea catechins and their derived metabolites are excreted via urinary and biliary routes. After intravenous dosing of GTE, urinary excretion of unchanged EC in rats was higher (21 to 31%) than that of EGCG and ECG (~ 2 to 5%), suggesting that nonrenal clearance for elimination of EGCG and ECG was more important than that of EC.[35] The fecal recovery was 3 to 5% for EC, 0.6 to 1% for EGCG, and 0.5 to 2% for ECG after i.v. dosing of GTE. After oral administration of tea catechins in rats, the urinary recovery was 4.72, 0.17, and 0.25% for EC, EGCG, and ECG, respectively, and the fecal recovery was 11.0, 7.89, and 5.80% for EC, EGCG, and

ECG, respectively.[29] Higher fecal recovery of tea catechins after oral administration in rats indicated incomplete absorption of green tea catechins. Intravenous administration of [4-^{3}H]-EGCG in rats resulted in 77% of the total radioactivity detected in bile within 48 h, and only 2.0% of that in the urine.[50]

When [4-^{3}H]-EGCG was dosed to rats orally, urinary and fecal recovery of total radioactivity within 72 h was 32.1 and 35.2%, respectively.[56] Increased urinary recovery and decreased fecal recovery of total radioactivity of EGCG after oral administration suggest that EGCG-derived metabolites may be formed presystemically after oral administration. They are subsequently absorbed into the systemic circulation and excreted into the urine. The major metabolite excreted into the urine after oral administration of EGCG in rats was the glucuronide of 5-(3′, 5′-dihydroxyphenyl)-γ-valerolactone, accounting for 68% of the total urinary recovery.[56] Conjugated EGC and 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone conjugates were excreted into the feces and accounted for 40.8 and 16.8% of the total fecal recovery, respectively.[56]

After oral administration of EGCG at a dose of 100 mg/rat, less than 5% of dose was excreted into the bile including 0.07% of unchanged EGCG, 2.65% of conjugated EGCG, 0.05% of methylated EGCG, and 0.63% of methylated EGCG conjugates.[49] When EC was dosed to rats orally, 8% of the total cumulative urinary recovery was in the form of unchanged EC and methylated EC and 16% of that was EC conjugates and methylated EC conjugates.[53] Only methylated EC conjugates were excreted into the feces.

EGCG and ECG were not found in the urine after oral administration of DGT or green tea catechins in humans.[36,38] Small amounts of unchanged EGC and EC were found in the urine. Conjugated EGC and EC; methylated EGC and EC conjugates; and microflora-mediated metabolites of EGC and EC, M4 and M6, were excreted in significant amounts into the urine after oral ingestion of DGT.[36,54] The maximum urinary excretion of EGC and EC conjugates occurred at 3 to 6 h after dosing and the cumulative urinary excretion accounted for 0.7 to 2.5% and 0.7 to 3.7% of the administered EGC and EC, respectively. Because the microflora mediated metabolites are formed until the catechins reach the distal end of the small intestine or colon, the urinary excretion of these metabolites peaked at 8 to 24 h after dosing. The total amount of M4 and M6 excreted into the urine accounted for 6 to 39% of the amount of tea catechin ingested.

SUMMARY

The absorption, distribution, metabolism, and excretion of green tea catechins have been studied in rodents. Limited studies have determined the pharmacokinetics of green tea catechins in humans. It was found that green tea catechins are available in the systemic circulation after oral administration of green tea or green tea catechins in animals and humans. Plasma levels of unchanged green tea catechins in humans after oral administration of GTE as a beverage or as an oral product are in the low-μM or sub-μM concentration range, which is much lower than the effective concentrations determined in *in vitro* studies.

More than 15 metabolites of green tea catechins have been identified after oral or i.v. administration. These include glucuronide/sulfate conjugates, methylated tea catechin conjugates, and conjugates of microflora-mediated metabolites. Some of these metabolites are present in systemic blood at levels much higher than those of the parent catechin. The contribution of catechin-derived metabolites to the biological effects associated with green tea is yet to be defined.

Large inter- and intraindividual variations in the pharmacokinetics of green tea catechins and their derived metabolites have been reported in humans. Green tea catechins are found to distribute widely into various tissues. High levels of green tea catechins or their derived metabolites are found in the gastrointestinal tract, kidney, and bladder. The tissue distribution pattern seems to coincide with the site of metabolism and the excretion pathway. Further understanding of the pharmacokinetics and metabolism of green tea catechins in humans is essential in interpretation of epidemiological findings, extrapolation of preclinical data to humans, and design of perspective intervention trials.

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CHAPTER 4

The Importance of *In Vivo* Metabolism of Polyphenols and Their Biological Actions

Stephen Barnes, Tracy D'Alessandro, Marion C. Kirk, Rakesh P. Patel, Brenda J. Boersma, and Victor M. Darley-Usmar

CONTENTS

ABSTRACT

Phytochemicals such as polyphenols are subject to the same principles of pharmacology and biochemistry as other xenobiotics and pharmaceuticals. Their conversion to forms capable of being absorbed from the small or large intestine is often an important first metabolic step. However, metabolism does not stop there — glucuronidation of polyphenols immediately occurs in the intestinal cells, resulting in very little of the polyphenol entering the blood stream and being distributed to the rest of the body. The low blood concentrations (in the low nM range) of the unconjugated polyphenols are often taken as a sign that they are not biologically active. However, many studies have shown that oral intake leads to well-defined anti-inflammatory responses and antioxidant events, suggesting that either the low polyphenol concentrations are adequate to induce biochemical changes, or that localized tissue metabolism to more active compounds occurs.

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We have recently carried out experiments with cell-based and animal models of inflammatory diseases. The experiments have revealed that human polymorphonuclear cells once activated with phorbol esters to cause an inflammatory response produce copious amounts of chlorinated polyphenol species. Rats treated with lipopolysaccharide (LPS) in an in vivo *model of inflammatory disease heavily nitrate polyphenols in tissues (e.g., lung and liver) where inflammatory cell invasion occurs. These modified polyphenols are better antioxidants than their parent compound. Using a luciferase reporter gene assay in COS cells, both chloro- and nitrogenistein were shown to have 1–2 orders lower estrogen receptor activation than genistein itself. In summary, metabolism of polyphenols is rampant, but not always inactivating.*

INTRODUCTION

The *polyphenols* are a series of phytochemicals synthesized by plants.[1] They include the bioflavonoids: anthocyanins, coumestanes, flavonoids, isoflavonoids, and stilbenes (Figure 4.1). Each bioflavanoid class is subdivided into other groups, e.g., flavones, flavans, flavanols, flavonols, and flavanones (Figure 4.2). Another class of polyphenol is the oligomeric polyphenols, such as the proanthocyanidins, found in the grape and various berries (black currant, blueberry, etc.).

Foods containing polyphenols or extracts of these foods are widely touted as preventing chronic diseases, such as atherosclerosis and cardiovascular disease, hormone-dependent cancers, arthritis, neurodegeneration, and osteoporosis.[2] Several mechanisms for their biologic activities have been proposed, varying from being estrogen-like,[3] inhibiting tyrosine kinases,[4] and having antioxidant activity.[5] However, the chemical form of the polyphenols in the plant extracts is frequently that of polar glycosidic conjugates (Figure 4.3).[6] These conjugates are generally poorly absorbed and therefore are usually inactive when incubated with cultured cells.[7] Cultures that consume foods containing polyphenols often subject the food to fermentation to increase its nutritional content. In doing so, the glycosidic polyphenols are hydrolyzed by enzymes from the fermenting microorganism.[8] Miso paste, a fermented food made from soybeans, is an example. The isoflavones in miso are mostly in the unconjugated form.[9]

INTESTINAL UPTAKE OF POLYPHENOLS

The polar nature of the glycosidic polyphenols minimizes their intestinal uptake by passive mechanisms. Although there is potential for uptake of the glycosidic polyphenols by intestinal glucose transporters (GLUTs and SGLTs), there is no evidence that this occurs *in vivo*.[9–12] Instead, the current view is that hydrolysis of the glycosidic polyphenols of the small intestine is catalyzed by lactose phlorizin hydrolase (LPH),[13] an enzyme in the apical membrane of the enterocyte (Figure 4.4). The active site of LPH is on the lumenal side of the enterocyte membrane.[14] The unconjugated polyphenol then diffuses through the unstirred water layer into the enterocyte. Passage through the enterocyte membrane is governed by the hydrophobicity of the polyphenol. Those with 1–2 hydroxyl groups are easily absorbed; trihydroxy flavonoids with 5-hydroxy groups are more hydrophobic than many dihydroxy flavonoids because of hydrogen bonding between the 4-keto oxygen atom and the

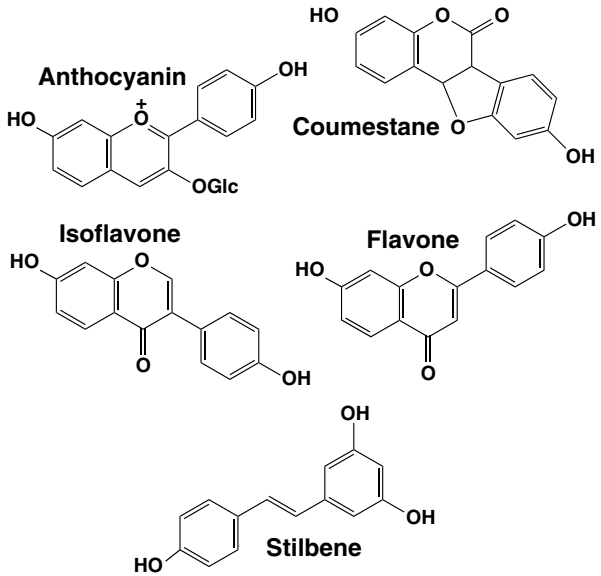

Figure 4.1 Chemical structures of the principal classes of polyphenols. The examples shown are of polyphenols with hydroxyl groups in the 7- and 4″-positions. *Anthocyanins* may also be found in oligomeric forms. The *coumestane* is coumestrol, The *isoflavone* (daidzein) differs from the *flavonoid* in the substitution of the phenolic group. For the flavonoid it is in the 2-position, whereas it is in the 3-position in the isoflavone.

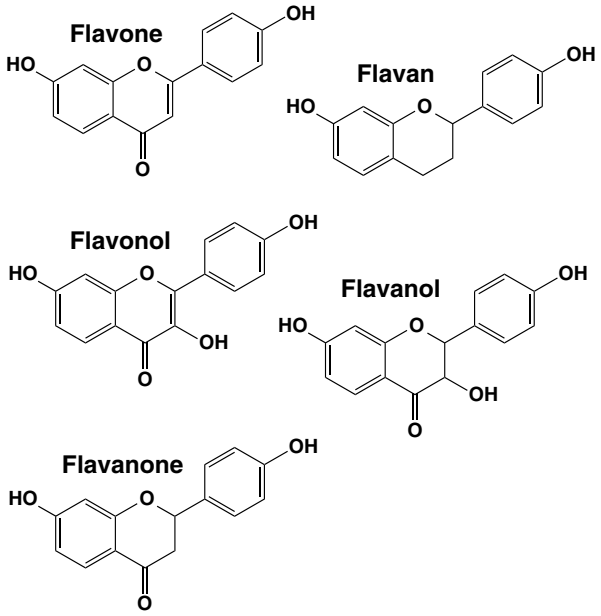

Figure 4.2 Chemical structures of various flavonoids The *flavone* heterocyclic ring can be reduced or oxidized in various ways. Reduction of the double bond leads to a *flavanone*. Additional loss of the carbonyl oxygen yields a *flavan*. The flavone can be hydroxylated to form a *flavonol*. This can be reduced to a *flavanol*. The flavan, flavanol, and flavanone each have a chiral center. The biological isomers are not known.

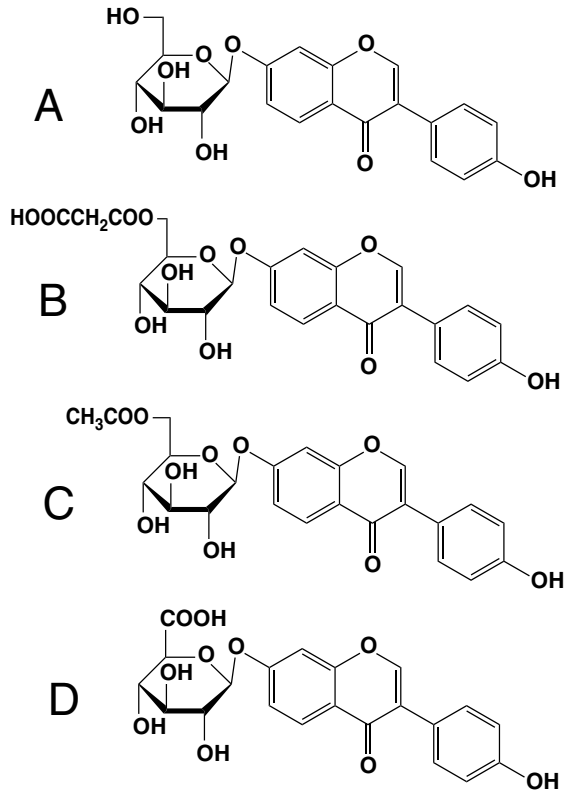

Figure 4.3 Chemical structures of isoflavone glycosides. In soy foods, isoflavones form 7-*O*-β-glucoside conjugates. **A** is the simple glucoside commonly found in soy milk, **B** is the 6″-*O*-malonyl-7-*O*-β-glucoside from the soybean, and **C** is the 6″-*O*-acetyl-7-*O*-β-glucoside, a product of toasting of soy flour. **D** is the 7-*O*-β-glucuronide that is the principal conjugate in the blood.

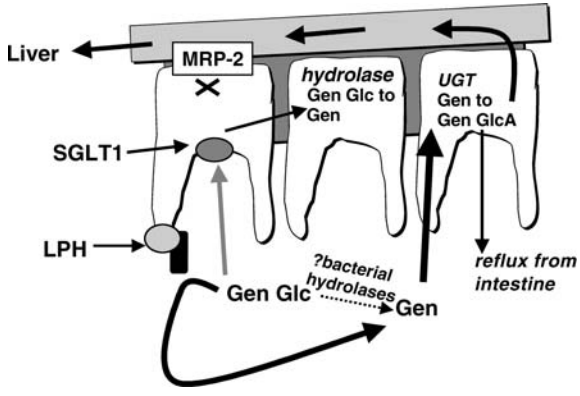

Figure 4.4 Schematic of the processes associated with intestinal hydrolysis and absorption of polyphenols.

proton of the 5-hydroxy group.[15] Flavonoids with 4–6 hydroxyl groups are sufficiently polar that their rate of transport is substantially reduced.[16] Polyphenols that are heavily methoxylated (such as nobiletin and tangeritin) are easily taken up by the enterocyte. However, the low aqueous solubility of these polyphenols (found as aglycones in the oil fraction) reduces their overall uptake.[17]

Once inside the enterocyte, some polyphenols can be transported back into the intestinal lumen by the multi-drug resistance transporter (MDR-2).[18] This is a particular issue when carrying out experiments with Caco-2 cells, a colon cancer cell line.[19] Chrysin[18] and isoflavone glycosides[20] undergo net transport in the serosal to lumenal direction, although this has been disputed.[21] In intact animal models, the uptake of genistein (as measured by its radioactivity appearing in the bile) approaches 80%,[22] suggesting that the Caco-2 cell model overestimates the role of MDR-2. This has been confirmed using an isolated perfused rat small intestine model.[23]

Both phase I and phase II detoxification enzymes are expressed in the enterocytes. The cytochrome P450s (CYPs) can demethylate methoxylated flavonoids,[24,25] as well as introduce new hydroxyl groups.[26] These metabolites may have greater biological/biochemical activities. In most cases, however, they are converted to polar β-glucuronides by the phase II UDP-glucuronyltransferases (UGTs). The net result of intestinal uptake of polyphenols is that polar conjugates make up >95% of total polyphenols appearing in the circulation. The process(es) by which polyphenols and their metabolites cross the basolateral membrane of the enterocyte and enter the venous drainage of the intestines is poorly understood. With the exception of the liver, circulating polyphenol conjugates are poorly taken up by most tissues.

POLYPHENOLS AND CHEMISTRY OF THE INFLAMMATORY RESPONSE

The common denominator among the different chronic diseases that are hypothesized to be ameliorated by polyphenol intakes is oxidative stress. Polyphenols are believed to have antioxidant activity both in plants[27] and in those that consume them.[2] Tissue oxidants are produced by inflammatory cells. The first oxidant is superoxide anion radical ($O_2^{\cdot-}$), generated by NADPH oxidase. This free radical is converted to hydrogen peroxide by superoxide dismutase. In activated neutrophils, hydrogen peroxide and chloride are converted to hypochlorous acid (HOCl) by the enzyme myeloperoxidase.[28]

Superoxide anion can also react with nitric oxide (NO) to form peroxynitrite, a powerful nitrating and oxidizing agent.[29] HOCl and peroxynitrite both react with protein tyrosine residues around the site of inflammation.[30–32] Given the similarity of the phenolic group in tyrosine with many polyphenols, it is not surprising that the isoflavones (daidzein and genistein) and the flavonoid quercetin are both chlorinated or nitrated when treated with HOCl or peroxynitrite (Figure 4.5).[33,34] When HL-60 cells are differentiated with DMSO to form neutrophil-like cells and are activated by phorbol esters, they convert the methoxylated isoflavone biochanin A to its monochloro- and dichloro-metabolites.[35] Recent experiments in rats treated with lipopolysaccharide to induce an inflammatory response

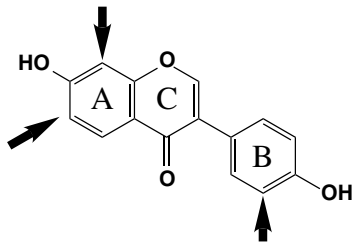

Figure 4.5 Sites of tissue modification of isoflavones. In the A-ring, chlorination and hydroxylation occur at the 6- and 8-positions. In the B-ring, hydroxylation and nitration occur at the 3′-position.

revealed that genistein taken up into the inflamed lung tissue is largely 3′-nitrogenistein (T. D'Alessandro et al., unpublished observations).

METABOLITES AND BIOCHEMICAL RESPONSE

The role of polyphenols as antioxidants could be to diminish the concentrations of the reactive oxygen species (ROS) and reactive nitrogen species (RNS) away from the site of the primary response. Because ROS and RNS are produced focally to attack an invading organism, the local concentrations around the activated inflammatory cell are very high (> 1 mM),[36] far above those of the polyphenols (nM and low μM). Thus, the polyphenols are very unlikely to be effective where ROS and RNS are produced but could be quantitatively more effective as antioxidants at lowering ROS and RNS concentrations in the surrounding uninvolved tissues. The introduction of the chloro- and nitro-groups into the polyphenol ring system would also be expected to enhance the antioxidant capacity of the parent polyphenol.

An alternative mechanism for the biological effects of polyphenols is based on the changed biochemical functions of the chloro- and nitro-polyphenols. These metabolites are new pharmacophores that are produced only in the region surrounding the site of inflammation. In this regard, they are analogous to prostanoids produced from long-chain polyunsaturated fatty acid precursors. Because the structure of the estrogen receptors (α and β) ligand binding sites are known,[37,38] it can be predicted that chlorination and nitration of isoflavones would interfere with activation of these receptors. With regard to tyrosine kinase inhibition, it is interesting that for the *Hck* kinase, a synthetic isoflavone, 3′-chloro-5,7-dihydroxyisoflavone, has a Ki in the nM range,[39] i.e., it is a far better inhibitor than genistein. Thus, the modification of polyphenols by the oxidant species may result in products with quite different properties from their precursors.

ARE SOME POLYPHENOLS REALLY ESTROGENIC?

One concern raised about isoflavones, coumestanes, and stilbenes is that they have *estrogenic activities*. Indeed, some of the health benefit claims for these compounds

are dependent on estrogenic action. However, in 2003 at the dawn of systems biology of the 21st century, the definitions of agonists and antagonists are undergoing revision. In the case of estrogens, the assumption has been that estrogen-like compounds interacting with estrogen receptors would modulate the same set of genes. Such categorization of compounds may be limited and unrealistic *in vivo*. The ability to simultaneously measure the expression of multiple genes by DNA microarray analysis is rapidly changing our perspective of estrogen-like activity that was previously based on single assays of gene expression of convenient reporter genes linked to estrogen.

Naciff et al.[40] recently showed that in the uterus of the developing rat treated with one of three different estrogens (17α-ethinyl estradiol (EE), bisphenol A and the isoflavone genistein), while EE and bisphenol A had very similar dose-dependent effects on gene expression (26 and 35 genes, respectively), genistein altered the expression of 188 genes, mostly in the direction of downregulation. To describe genistein as an estrogen, or even a phytoestrogen, is misleading — first and foremost it is an isoflavone, with additional effects including its minimal estrogen-like qualities.

ACKNOWLEDGMENTS

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CHAPTER 5

Cancer Prevention by Phytochemicals, Modulation of Cell Cycle

Diane F. Birt, Weiqun Wang, Nancy Paiva, Angela Au, Chilly Chung, Laura Schmitt, and Yu Jiang

CONTENTS

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ABSTRACT

The consumption of diets rich in fruits and vegetables has long been associated with reduced rates of cancer in people on self-selected diets. Cancer prevention has also been observed in laboratory animals fed vegetables, vegetable extracts, or numerous isolated components of vegetables and fruits.

Research in our laboratory has focused on cancer prevention in colon and skin by phytochemicals. We have recently studied apigenin, a tri-hydroxy flavone that is widely distributed in plants and resveratrol, a phytoalexin that is found in grapes, wine, peanuts, and Asian herbs. We initially found that topical apigenin inhibited chemically and ultraviolet (UV)-light induced skin cancer and that it was most effective in preventing cancer promotion.

Early investigations with cultured cells demonstrated cell cycle arrest at G2/M in cultured epidermal cells. More recent studies found that colon cancer cells were also arrested at G2/M and that this arrest coincided with a reduction of $P34^{CDC2}$ kinase activity and protein and reduced cyclin B1 protein. Apigenin was more effective in inducing G2/M arrest than several structurally related flavonoids and while weak additive effects were observed when apigenin was combined with low concentrations of other flavonoids, at high doses several other flavonoids blocked apigenin induction of cell cycle arrest. While apigenin was effective in preventing cellular proliferation in cultured cells and in animal models, it was not particularly effective in inhibiting colon carcinogenesis in chemically or genetically (mutant tumor suppressor) induced animal models.

Recent studies demonstrated that apigenin inhibited cell cycle at G2/M in colon cancer cell lines with mutant adenomotosis polyposis coli (APC) gene but not in the same colon cells with the wild-type APC gene expressed. These data suggest that apigenin may be selective against colon cancer cells with selected mutant genes and predicts that dietary apigenin may be a gene-specific therapy to prevent cancer.

Studies with resveratrol have demonstrated the inhibition of aberrant crypt foci (ACF) by purified resveratrol aglycone but not resveratrol glucoside in transgenic alfalfa. However, the addition of glucosidase enzyme to diet with transgenic resveratrol glucoside accumulating alfalfa resulted in fewer ACF. Resveratrol aglylcone reduced the growth of colon cancer cell lines and induced G2/M and S phase blockage in cell cycle. Studies of phytochemical modulation of cell cycle are helpful in optimizing cancer prevention strategies.

BACKGROUND

Fruit and Vegetable Consumption and the Prevention of Cancer

The consumption of diets rich in fruits and vegetables has been associated with reduced rates of human colon cancer. Case control studies have consistently suggested that diets rich in fruits and vegetables reduce the risk of cancer[1] and

prospective epidemiological studies have found reduced cancer rates in subpopulations, such as those people with high body mass index (BMI) and who are consuming diets high in linoleic acid.[2] Plant constituents have been identified that potentially contribute to cancer prevention: vitamins, A, C, E; minerals, Se, Zn; carotenoids; flavonoids; isothyocynates; terpenoids; and saponins.[3,4] Research in our laboratory has focused on apigenin, (4′,5,7-trihydroxyflavone), a plant flavonoid that is widely distributed in human foods, and resveratrol (3,5,4′-trihydroxystilbene), a natural constituent of grapes and red wine, as colon cancer preventive compounds.

Flavonoids and Cancer Prevention

Because the strongest association between fruit and vegetable consumption and cancer prevention appears to be for those individuals consuming a wide variety of these foods,[5] it appears that some of the phytochemicals that are responsible for cancer prevention must be widely distributed in our foods. One class of phytochemicals notable for its wide distribution in fruits and vegetables is the flavonoids. Flavonoids are composed of a number of related compounds with the general flavone structure. It is estimated that we consume approximately 1 gm of flavonoids daily, and the bulk of these compounds are in our diets as glycosides.[6] Studies in the Netherlands assessed the intake of particular flavonoids, and the daily consumption of quercetin was estimated to be 16 mg/day.[7,8] However, the analysis of foods for these compounds is problematic and estimates should be considered tentative. Many of the biological properties, including cancer prevention, of these compounds appear to be due to the aglycone, which is released in the intestine.

Our research in skin focused on apigenin (4′,5,7,-trihydroxyflavone) (Figure 5.1), a widely distributed flavone. Topical apigenin inhibited both ultraviolet light induced and 7,12-dimethylbenz(a)anthracene (DMBA) and 12-0-tetradecanoylphorbol-13-acetate (TPA) induced skin carcinogenesis.[9–11] Flavonoids have been studied in the prevention of cancer at several sites including skin, colon, breast, prostate, oral cavity, and liver.[4] In addition, our studies have demonstrated that apigenin induces cell cycle arrest in several types of cultured cells, including myeloid leukemia cells, cultured human diploid fibroblasts, cultured mouse skin tumor cells, and cultured human colon cancer cells.

Our results indicate that dietary apigenin at 0.1% inhibited colonic aberrant crypt foci (ACF) induction by azoxymethane (AOM) and suggest that colon cancer

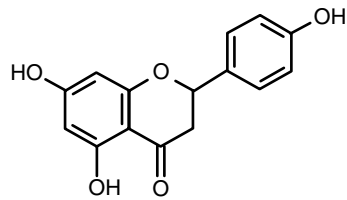

Figure 5.1 Structure of apigenin.

may be prevented by dietary apigenin. In tumor studies, however, only weak evidence for cancer prevention was observed. Our research also assessed the impact of apigenin on growth and cell cycle of cultured colon cancer cell lines: SW480, HT29, and Caco-2. Apigenin was observed to inhibit cell growth by blocking cell cycle at G2/M, and this was associated with a reduced p34cdc2 kinase activity. Differential sensitivities of these cell lines led us to hypothesize that dietary apigenin may be differentially effective against tumors with specific mutational spectra. Further research tested this hypothesis using colon cancer cell lines from parental lines that differ only in the presence or absence of wild-type APC genes, a critical tumor suppressor gene that is mutated in colon cancer. The expression of the wild-type APC reduced the sensitivity of cells to apigenin induction of G2/M arrest.

Resveratrol and Cancer Prevention

Resveratrol was first identified in grape vines in 1976 and in wine in 1992.[12] It was also found in itadori tea (used in Asia as herbal remedy, 0.008 to 2.17 mg/g), peanuts (0.0005 mg/g)[13,14] and in peanut roots (0.015-1.33 mg/g).[13,14] It was found primarily as the glucoside except in the case of wine where the cis- and trans-resveratrol aglycones are observed.[13,14] Dr. Nancy Paiva of the Samuel Roberts Noble Foundation developed and provided us with transgenic alfalfa that accumulates resveratrol glucoside (a compound not found in wild-type alfalfa). Our collaborative studies provide strong evidence for resveratrol aglycone in the prevention of ACF[15]; however, the transgenic alfalfa that accumulates resveratrol glucoside was effective in reducing ACF only when fed with a source of β-glucosidase activity. These results suggest that resveratrol aglycone may be effective in the prevention of colon cancer and that resveratrol glucoside accumulating alfalfa needs to be fed under conditions where the resveratrol glucoside can be hydrolyzed to resveratrol aglycone to be effective (Figure 5.2).

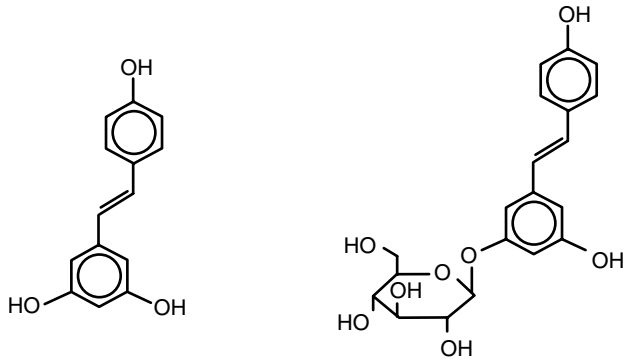

Figure 5.2 Structure of *trans*-resveratrol aglycone and resveratrol glucoside.

Our observations suggesting reduced bioactivity of the glucoside of resveratrol are consistent with earlier observations on glucosides of flavonoids that were less active than aglycones in studies with mouse epidermis treated topically with these chemicals or in colon cancer cell lines cultured with these agents. Further, in the parallel area of flavonoid prevention of colon carcinogenesis, many of the biological properties, including cancer prevention, of these compounds appear to be due to the aglycone that is released in the intestine.[16]

Dietary prevention of cancer by resveratrol aglycone was initially demonstrated in mouse skin.[17] Additional studies found inhibition of lung tumorigenesis induced by benzo(α)pyrene plus 4-(methylnitrosamino)-1-(3-pyridyl)-1-butone,[18] blockage of lung metastasis and neovascularization in Lewis lung carcinoma-bearing mice,[19] and inhibition of the growth of the Yoshida AH-130 ascities hepatoma.[20] The inhibition of the ascites hepatoma was associated with an increase in cells in G2/M and with the existence of an aneuploid peak, which were suggestive of the induction of apoptosis in this model.[20] Resveratrol inhibition of carcinogenesis has recently been reviewed.[21,22]

Mechanisms of Cancer Prevention by Components of Fruits and Vegetables

Several mechanisms have been studied (as reviewed)[23] for cancer prevention by phytochemicals, including modulating cell signaling, inhibiting inflammation, antihormone actions, modulating growth factors, enhancing detoxication and elimination (phases 1 and 2), antioxidant activity, enhancing apoptosis, and inhibiting cell cycle. Our research has focused on these latter two mechanisms because cell cycle aberrations and blocking apoptosis provide molecular markers of cancer and cell cycle and apoptosis modulators act as targets for cancer prevention. Indeed, a hallmark of cancer is the accumulation of cells with abnormal cell cycle regulation; cell division may be accelerated, cell death slowed, or a combination of these activities. Cell cycle regulatory targets are key cancer-therapy targets and numerous cancer therapies induce apoptosis. Finally, cell cycle is an endpoint that is related to cancer development and can be measured in cultured cells. Other endpoints, antioxidant, cellular signaling, etc., may result in changes in cell cycle.

Regulation of the Cell Cycle by Plant Constituents

The cell cycle in all eukaryotes is composed of five phases, beginning with G1 phase, followed by the DNA synthesis or "S" phase, then the G2 phase, then mitosis or "M" phase, and finally G0, the quiescent state.[24] Cyclin:cyclin-dependent kinase complexes control the two critical checkpoints in the cell cycle at the G1/S and G2/M transitions by phosphorylating a variety of proteins such as nuclear lamins and histones for nuclear membrane breakdown and chromosome condensation, as well as proteins leading to the transcription of genes required for proliferation.[25]

Passage through the cell cycle is governed by cyclin-dependent kinases (cdks) which complex with their "partner" cyclins.[24] The G1 cyclins and their associated cdks regulate the G1/S transition. Cyclins D1, D2 and D3 are expressed in early G1

phase, and cyclin E is expressed in late G1 phase. D-type cyclins complexed with cdk2, 4, or 6 promote cell cycle progression by phosphorylating a variety of substrates such as the retinoblastoma (Rb) protein, which is required for entry into S phase. The mitotic cyclins, first discovered as proteins with abundant oscillation in early invertebrate embryonic cell cycles, form complexes with cdks and regulate the G2/M transition.[24] In most organisms, B-type cyclins function as a subunit of the major mitotic protein kinase.[24]

Numerous plant constituents, including flavonoids and isoflavonoids, have been shown to modify the cell cycle. Our laboratory investigated the effect of the plant flavonoid, apigenin on the cell cycle of cultured human colon cancer cell lines, using SW480, (APC truncation, mutant p53 and mutant ras), HT 29 (APC mutation, mutant p53), and Caco-2 (APC truncation, wild-type p53) cells. Our results indicate that treatment of cells with apigenin produced G2/M arrest in a dose-responsive manner, with a doubling in the percent cells in G2/M observed at 10 μM apigenin for SW480 cells, at 50 μM apigenin for HT 29 cells and at 70 μM apigenin for Caco-2 cells. Thus, the cells with the mutations in three critical genes important in colon cancer development were the most sensitive to apigenin.[26] Cell growth experiments determined that apigenin inhibited cell growth in these cell lines in a parallel manner. Apigenin inhibited p34cdc2 kinase activity, a G2/M cyclin dependent kinase, in all three-cell lines. Recent studies using colon cancer cell lines from parental lines that differ only in the presence or absence of wild-type APC determined that the mutant APC gene in the HT-29 colon cancer cell line was necessary for sensitivity to apigenin cell cycle arrest at G2/M (Chung et al., unpublished).

We became interested in investigating the effect of resveratrol aglycone on the cell cycle after the recent reports in the literature on the effect of resveratrol aglycone on the growth and cell cycle progression of cells in culture.[27,28] Our results demonstrate that resveratrol aglycone inhibits the growth of cultured SW480 human colon cancer cells, decreases the proportion of cells in G1, and increases the proportion of cells in G2/M and S phases. Evidence that resveratrol induces cell cycle arrest and apoptosis came from studies with several cell lines. In human promyelocytic leukemia (HL-60) cells cultured with resveratrol aglycone, morphological, ultrastructural, internucleosomal DNA fragmentation, and flow cytometry data all suggested the induction of apoptosis.[27] Further, resveratrol aglycone treatment resulted in a decrease in the expression of antiapoptotic Bcl-2.[27] Studies from another laboratory using HL-60 cells noted that incubation with resveratrol aglycone concentrations as low as 30 μM resulted in the absence of the G2/M peak and accumulation of cells in G1 and S.[29] S-phase cell cycle arrest was also observed in prostate cancer[30] and histocytic lymphoma[31] cells, and one set of authors speculated that resveratrol's unique ability to induce a block in S-phase and inhibit DNA synthesis might account for its antiapoptotic and antiproliferative activities.[30]

Studies with JB6 mouse epidermal cells demonstrated that resveratrol aglycone-induced apoptosis in these cells was through p53 and that it was not dependent upon normal sphingomyelinase activity as the induction of apoptosis was observed in sphingomyelinase deficient and sufficient cell lines.[32] Studies by Lu,

et. al.,[33] using a resveratrol aglycone analogue, 3,4,5,4′-tetrahydroxystilbene, suggested that the induction of p53 and Bax was critical for the inhibition of growth in transformed diploid lung fibroblasts. However, investigations using HCT116 colon carcinoma cells with inactivated p53 indicated that resveratrol induced apoptosis independent of p53, potentially through mitochondrial proliferation and epithelial differentiation.[34] Indeed, in human acute lymphoblastic leukemia cells, resveratrol induced mitochondrial depolarization, activated caspase-9 and induced extensive apoptosis.[35]

In pancreatic cancer cells, resveratrol induced mitochondrial depolarization, cytochrome 3 release followed by caspase-3 activation in association with apoptosis.[36] Thus, resveratrol induces cell cycle arrest and apoptosis in numerous cancer cell lines using both p53-dependent and p53-independent pathways. In parallel with studies showing that resveratrol aglycone in the drinking water inhibited intestinal carcinogenesis in the Min mouse model,[37] the authors reported that resveratrol aglycone downregulated several genes that are involved in cell cycle progression and upregulated genes involved in immune responses.

RESULTS

Apigenin and Cancer Prevention

Studies on the Efficacy of Apigenin in the Prevention of Colon Carcinogenesis

We first assessed the effects of apigenin on ornithine decarboxylase (ODC) activity, a marker of cell proliferation, in cultured human colon cancer cells (Caco-2). ODC activity was measured by assessing the release of $^{14}CO_2$ from 1-C^{14}-ornithine. Apigenin treatment for 15 to 39 h at 10 or 30 μM inhibited the ODC in the cultured colonic Caco-2 cells by 26 ± 5 and 57 ± 12%, respectively, when compared with the vehicle ($p < 0.01$). Studies in CF-1 mice determined dietary apigenin effects on ODC and ACF, precursors of colon cancer. These studies in mice demonstrated that dietary apigenin fed at 0.1% for 1 week reduced the colonic basal ODC activity by 42 ± 23% in comparison with control diet ($p < 0.01$). Because ODC was inhibited by only 1 week of dietary apigenin at 0.1%, we used this level of apigenin and 0.025% dietary apigenin for a study on the prevention of ACF in carcinogen-treated mice. ACF were induced by single i.p. injection of azoxymethane at 5 mg/kg and counted in methylene-blue stained tissue. The formation of ACF was inhibited in mice fed a diet containing 0.1% apigenin (3.3 ± 2.8/mouse) for 6 weeks when compared with those fed a control AIN-93 diet (6.6 ± 2.1/mouse) ($p < 0.05$). We did not see significant changes in ACF with low doses (0.025%) of dietary apigenin.

In a subsequent colon carcinogenesis study 6-week-old CF-1 mice were separated into five treatment groups as follows: (1) AOM, control diet, 30 mice; (2) AOM, low-dose apigenin diet (0.025%), 30 mice; (3) AOM, high-dose apigenin diet (0.1%), 30 mice; (4) saline, control diet, 8 mice; (5) saline, high-dose

apigenin diet (0.1%), 8 mice. Mice were injected with AOM at 10 mg/kg BW or saline weekly beginning 1 week after arrival for 6 weeks. Mice were fed the assigned control and apigenin-containing diets beginning 3 days after the last injection and treatments were continued until 30 weeks after AOM. A modest decrease in tumor incidence was observed in the AOM-treated mice that were fed 0.025 or 0.1% apigenin.

In a second AOM tumorigenesis study, CF-1 mice received four weekly injections of AOM. Mice fed dietary apigenin at 0.025% had a significantly lower rate of tumor incidence (29%) when compared either with 0.1% apigenin (59%) or with basal diet (65%). A third study was conducted with Min mice that spontaneously develop colon adenomas due to a mutant APC gene. Tumors were found predominantly in the small intestine. The total number of tumors was 46 ± 5 and 39 ± 8 per mouse for the basal and apigenin-fed groups, respectively. Overall, dietary apigenin at 0.1% resulted in a 15% reduction in total number of tumors; however, the variability among animals was high and no statistical differences were observed. Collectively these tumor studies suggest weak cancer prevention by dietary apigenin in these animal models.

Studies Testing the Hypothesis that Apigenin Inhibits Colon Carcinogenesis by Inducing Cell Cycle Arrest

We have assessed the impact of apigenin treatment on cell cycle kinetics in colon cell lines representative of various genetic abnormalities in human colon cancer using flow cytometry. This study assessed the influence of apigenin on cell growth and cell cycle in three human colon carcinoma cell lines (SW480, HT29, and Caco-2). These colon cancer cell lines were selected because SW480 cells are known to be mutant in APC, P53, and ras; HT29 cells are mutant in APC and P53; and Caco-2 cells have mutant APC and questionable ras. In comparison with the controls, all the three cell lines treated with apigenin from 0 to 80 μM exhibited a dose response reduction in both cellular protein content as measured by sulforhodamine B staining and cell number. A maximum of 40 to 50% decrease in cell growth measurements was noted after 24 h for all three cell lines, but 65%, 55%, and 40% maximal decreases were observed after 48 h for SW480, HT29 and Caco-2, respectively.[38]

To determine whether the cell growth inhibition was due to the cytostatic effect of apigenin, the cell cycle was investigated by flow cytometry. Cells were cultured in 100-mm dishes to 50% confluency before adding 10 to 80 μM apigenin (final concentration in 0.2% DMSO) to the cell culture medium. Control cultures were treated with DMSO/medium alone and all dishes contained the same proportion of 0.2% DMSO/culture medium. Cells were exposed to apigenin for 24 or 48 h at which time they were harvested by trypsin release and resuspended in Vindelov's DNA staining solution containing propidium iodide. Cell cycle analysis was performed by measuring DNA content on the flow cytometer. Flow cytometry data were analyzed using the Cellfit computer program to calculate the percentage of cells in each phase. SW480 cells revealed a dose-dependent accumulation in G2/M phase from 16% (0 μM apigenin) to 64% (80 μM apigenin) after 48-h incubation (Figure

5.3). Treatment of HT29 cells with apigenin for 48 h resulted in an increase in the percent G2/M cells from 14% without apigenin to 42% with 80 μM apigenin. Caco-2 cells were the least responsive to cytostatic effect of apigenin with a maximal 26% increase in the percent G2/M cells (80 μM apigenin) after 48 h when compared with the controls at 15%. The proportion of S-phase cells was not altered by apigenin doses in the three cell lines. The increase of G2/M phase by apigenin was inversely correlated with the corresponding decrease of cell growth measurements in each of three cell lines ($r = -0.842 \sim -0.968$, $p < 0.004$).

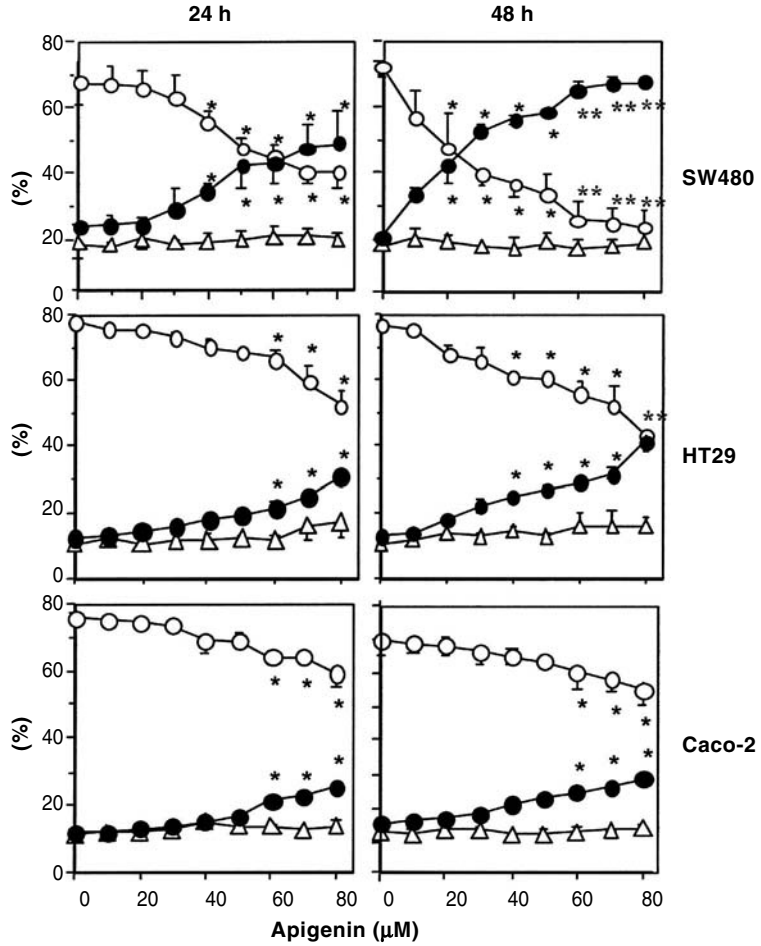

Figure 5.3 DNA flow cytometric analysis of cells treated with apigenin for 24 or 48 h. Each point represents the mean ± SD of three independent experiments. Cell cycle was monitored by a DNA flow cytometric analysis indicating as follows: ○,% of G1-phase cells; △,% of S-phase cells; ●;% of G2/M-phase cells. Means ± SD from three independent experiments are shown. *$p < 0.05$ and **$p < 0.01$ vs. the vehicle controls. (From Wang et al., *Molecular Carcinogenesis*, 28, 102–110, 2000. With permission.)

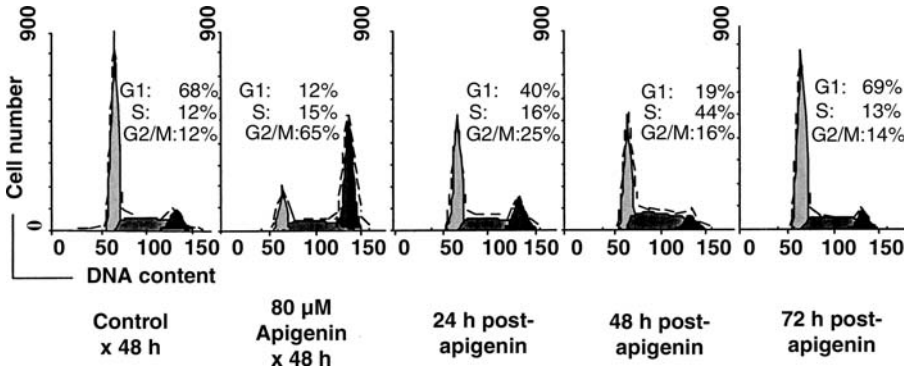

Figure 5.4 Reversibility of cell cycle arrest by apigenin. SW480 cells were cocultured with 80 μM of apigenin for 48 h, washed twice with PBS, and then recultured in fresh media without apigenin for up to 72 h. At the times indicated in the bottom of the panel, histograms of cellular DNA content were obtained by flow cytometry. (From Wang et al., *Molecular Carcinogenesis*, 28, 102–110, 2000. With permission.)

Investigation of the reversibility of cell cycle arrest induced by apigenin determined that the cells resumed cycling when apigenin was removed, suggesting that the apigenin was cytostatic and not cytotoxic in reducing cell number as shown in Figure 5.4.

We measured p34cdc2 kinase, a critical enzyme in G2 to M transition, by immune complex kinase assay in studies parallel to those described above with the three cell lines. Our results in Figure 5.5 demonstrate reduced p34cdc2 kinase activity in all three cell lines following treatment with apigenin between 50 to 80 μM.

These data indicate that apigenin inhibited colon carcinoma cell growth by blocking cell cycle in G2/M phase, and this was associated, at least in part, with a reduced p34cdc2 kinase activity. A strong cytostatic effect of apigenin in SW480 cells (mutant p53 and ras), intermediate effect in HT29 cells (mutant p53 and wild-type ras), and marginal effect in Caco-2 cells (wild-type p53) suggests that dietary apigenin may be differentially effective against tumors with specific mutational spectra.

This investigation led us to hypothesize that apigenin induces arrest at different cell cycle phases dependent upon the genetic background of the cell. This would mean that selected mutant genes in colon cancer cells might sensitize the cells to apigenin modulation of cell cycle. We have begun to test this hypothesis using colon cancer cell lines with regulatable wild-type genes that can be expressed in the mutant cell background. HT-29 APC cells have wild-type APC under a metalothionine promoter (Zn to induce). While HT-29 GAL control cells have β-galactosidase under the same promoter (cells were provided by B. Vogelstein, Johns Hopkins University). These studies demonstrated that apigenin inhibited cell growth in colon cancer cells with mutant or wild-type APC. Apigenin induced cell cycle arrest with mutant but not with wild-type APC. In addition, apigenin enhanced apoptosis in cells with wild-type but not with mutant APC.

We assessed the influence of structural features of apigenin on the inhibition of cell proliferation and cell cycle arrest at G2/M using SW480 cells. The analogues studied included acacetin, chrysin, kampherol, luteolin, naringenin, and quercetin.

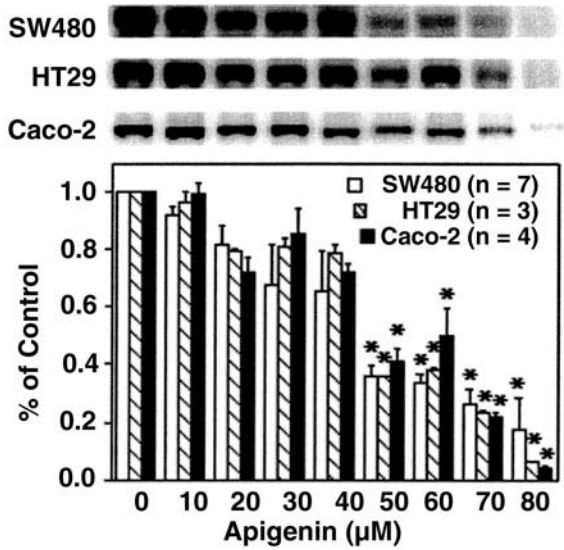

Figure 5.5 Inhibition of p34cdc2 kinase by apigenin in colon carcinoma cells. A representative image from the immune precipitation study is shown at the top. Each bar represents the mean±SD of three to seven experiments. *p < 0.05 vs. vehicle control.. *p < 0.05 vs. the vehicle controls. (From Wang et al., *Molecular Carcinogenesis*, 28:102-110, 2000. With permission.)

Apigenin was the most effective of the flavonoids studied in inducing G2/M cell cycle arrest. A modest arrest was observed with chrysin, acacetin, kampherol, luteolin, and quercetin. The unsaturation in ring C was necessary for arrest as was apparent from naringenin showing no evidence of cell cycle arrest. However, these analogues showed that a free hydroxyl in ring B was not necessary for G2/M arrest because both chrysin and acacetin did induce cell cycle arrest. Several flavonoids (acacetin, chrysin, luteolin, kampherol, or quercetin) were additive with apigenin at low dose but antagonistic at high dose in inducing cell cycle arrest. These studies validated that apigenin was the most effective naturally occurring flavonoid among those studied in inducing cell cycle arrest in cultured SW480 colon cancer cells.

Resveratrol and Cancer Prevention

Studies on Resveratrol and Transgenic Alfalfa that Accumulates Resveratrol Glucoside and Colon Cancer Prevention

Promising new technologies have been developed to genetically engineered plants for human and environmental benefit. Although the first generation of genetically modified crops was developed to address agronomic problems, such as weed control, a subsequent wave of genetically modified plants currently being developed has more direct consumer benefit and will potentially benefit human health. Our studies assessed alfalfa that was modified with genes that result in the accumulation of resveratrol glucoside and may provide both agronomic and human health benefits.

Alfalfa does not normally contain resveratrol so this system provides a nice model for testing the value of adding it. Resveratrol is known to have antimicrobial activity and this activity may contribute to reduced root and crown disease in transgenic alfalfa. In addition, resveratrol has human health benefits, including cancer and heart disease prevention. Furthermore, the technologies developed could be applied to other crops that are more commonly consumed as part of the human diet to achieve broader human health benefit.

Alfalfa is simple to transform, grows quickly, produces high yields of biomass, and is readily propagated by cuttings or by seed. In addition to using the leaves and stems directly in feeding tests, the transgenic tissues may possibly serve as an economical source of purified resveratrol glucoside for additional tests or dietary supplements. Juices extracted from these tissues could also be incorporated into animal diets, providing resveratrol glucoside, as well as other nutrients from alfalfa. We are conducting studies with alfalfa that accumulates resveratrol glucoside because the resveratrol is readily glucosylated in alfalfa.

Studies on the impact of resveratrol aglycone and transgenic alfalfa that accumulates resveratrol glucoside (80 to 100 μg/g dry weight) on AOM-induced ACF in mice have been conducted. We used control (nontransformed) alfalfa that does not contain resveratrol aglycone or resveratrol glucoside to assess the impact of alfalfa on ACF. Furthermore, in separate studies we added β-glucosidase activity (α-galactosidase enzyme) to the transgenic and control alfalfa diets to determine if increasing the likelihood for freeing the resveratrol aglycone from resveratrol glucoside might increase the potential of the transgenic alfalfa in colon cancer prevention. Data are shown in Table 5.1. In comparing groups with and without purified resveratrol aglycone, it is apparent that resveratrol aglycone reduced the number of ACF/mouse colon when given with control alfalfa and when given in the basal diet. The reduction of ACF in the rectal region of the colon is particularly relevant because this is the major site of colon carcinomas in AOM-induced animal models and in humans. Although alfalfa or transgenic alfalfa without β-glucosidase activity did not significantly reduce ACF, these groups tended to have lower ACF yield, suggesting cancer-preventive constituents in the alfalfa. Further, the transgenic alfalfa expressing resveratrol glucoside at the same level of resveratrol as was given in the above two groups as purified resveratrol aglycone did not inhibit colon carcinogenesis. None of the treatments altered the incidence of colon ACF. These results suggest that resveratrol glucoside may not be bioavailable and that it may need to be converted to the aglycone to allow bioavailability and bioactivity. It is also possible that other factors related to the resveratrol glucoside in the alfalfa, such as its cellular location, may block bioavailability. However, preliminary studies shown in Table 5.1 support the contention of poor bioavailability because the addition of glucosidase to the transgenic resveratrol glucoside accumulating alfalfa dramatically improved ACF prevention (experiment 2), whereas enzyme addition to the control alfalfa did not appreciably influence the result (experiment 3).

These studies demonstrated that feeding alfalfa with resveratrol glucoside accumulation did not inhibit ACF. However, glucosidase addition to alfalfa with

Table 5.1 Aberrant Crypt Foci in AOM-treated CF1 mice.[a]

	% Incidence	Total # ACF Per Colon Mean ± SE
Experiment 1 (N = 13 to 20 mice/group)		
Basal diet	80%	8.2 ± 1.5
Control alfalfa (CA)	77%	7.2 ± 1.8
Transgenic alfalfa (TA)	89%	6.6 ± 1.4
CA + resveratrol (resveratrol aglycone)	80%	4.2 ± 0.9[b,c]
Basal diet + resveratrol aglycone	89%	4.9 ± 1.0[b]
Experiment 2 (N = 10 mice/group)		
TA	80%	3.0 ± 1.1
TA + glucosidase act	40%	0.8 ± 0.4[d]
Experiment 3 (N = 10 mice/group)		
Basal diet (BD)	100%	6.9 ± 1.8
BD + glucosidase act[b]	100%	9.9 ± 2.1
CA	70%	3.3 ± 0.9
CA + glucosidase act[b]	90%	5.1 ± 0.9

[a] Single treatment of 5 mg/kg body weight.
[b] $p < 0.05$ compared with basal diet (AIN 93).
[c] $p < 0.05$ compared with control alfalfa.
[d] $p < 0.05$ compared with TA in experiment 2. Dried CA and TA were added in partial replacement of carbohydrate (20%). Resveratrol aglycone was added at 0.002% of the diet, the amount of resveratrol provided by the TA diet. Glucosidase activity was obtained from an α-galactosidase product (National Enzyme Company) that has been analyzed for β-glucosidase side activity and found to provide in 36,500 β-glucosidase units per gram and 0.0712 gram of enzyme preparation (2600 units β-glucosidase) was added per Kg diet.

resveratrol glucoside accumulation inhibited ACF. In addition, resveratrol aglycone inhibited ACF.

Studies Testing the Hypothesis that Resveratrol Inhibits Colon Carcinogenesis by Inducing Cell Cycle Arrest

Studies have assessed the impact of resveratrol aglycone on colon cancer cell growth and, in parallel studies, on cell cycle. We observed a dose-response inhibition of the number of SW480 colon cancer cells with resveratrol aglycone treatment (0 to 25 μM) for 24 or 48 h (50% and 70% reduction in the number of cells with 25 μM resveratrol aglcone at 24 and 48 h, respectively). The resveratrol concentration was assessed by HPLC in cells exposed to resveratrol aglycone (0 to 100 μM). Figure 5.6 shows the proportion of cells in G1, G2/M and S as measured by flow cytometry in SW480 colon cancer cells incubated for 24 h with resveratrol aglycone. The proportion of cells in G1 decreased with the concentration of resveratrol aglycone (0 to 25 μM), while the proportion of cells in G1/M and S phases of the cell cycle increased. Our cell cycle observations are in

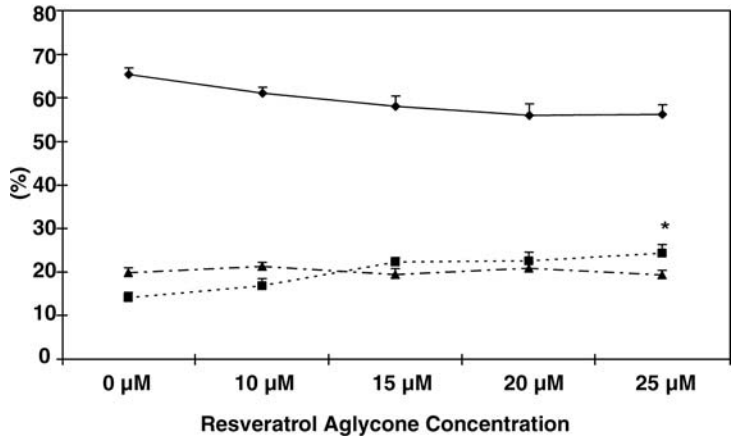

Figure 5.6 DNA flow cytometric analysis of cells treated with resveratrol aglycone for 24 h. Each point represents the mean ± SE of three to seven independent experiments. Cell cycle was monitored by a DNA flow cytometric analysis indicating as follows: ♦, % of G1-phase cells; ■, % of S-phase cells; ▲, % of G2/M-phase cells. *p < 0.05 vs. vehicle controls.

agreement with published reports using Caco-2 human colon cancer cells treated with 25 µM resveratrol aglycone.[28] These results suggest that resveratrol aglycone may prevent cancer in the colon.

CONCLUSIONS

The ability of phytochemicals to modulate cell cycle can be useful in evaluating and optimizing cancer prevention by these compounds. Studies reported here demonstrate that phytochemicals may differentially impact cell cycle and cell number depending upon the mutations in the cancer cell. In addition, cell cycle regulatory proteins are altered by phytochemicals in ways that may explain the cell cycle modulation. The fact that structural features of flavonoids impact cell cycle modulation suggests that cell cycle modulation may be useful in identifying the most active compounds for colon cancer prevention. Finally, cell cycle modulation studies can inform us on plant modifications that may be useful for nutritional enhancement.

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CHAPTER 6

Cancer Chemoprevention by Phytopolyphenols Including Flavanoids and Flavonoids through Modulating Signal Transduction Pathways

Jen-Kun Lin

CONTENTS

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ABSTRACT

Phytopolyphenols are widespread in the plant kingdom and are important in contributing the flavor and color of many fruits and vegetables. They are also important for the normal growth, development, and defense of plants. The important groups of phytopolyphenols in food are flavonoids and flavanoids, which consist mainly of flavones, flavanones, flavonols, flavanonols, isoflavones, flavanols, and anthocyanidines. Although flavanols, also called catechins, seem to be widely distributed in plants, they are rich only in tea leaves, where they may constitute up to 30% of dry leaf weight. The antioxidative, anti-inflammatory, and antitumor properties of green and black teas and their tea polyphenols (flavanoids) are intensively investigated.

Recent investigations with phytopolyphenols, including flavanoids (tea catechins, theaflavins, theasinensins, etc.), flavonoids (apigenin, myristin, genistein, etc.), and other polyphenols (curcumin, resveratrol, carnosol, garcinol, etc.), have provided important insights as to how these compounds modulate signal transduction pathways and the role of this modulation in executing their cancer chemopreventive actions. The induction of human cancer involves a multistep process, initiated with DNA damage by endogenous reactive oxygen species (ROS) and exogenous activated carcinogens, followed by oncogene activation and tumor suppressor gene mutations, and finally lead to the alteration of different signaling pathways.

Cell cycle arrest, apoptosis, cell proliferation, and cell differentiation are all mediated through signal transduction processes and appeared to be important executive targets for the operation of cancer chemoprevention. Several transducer proteins, such as cdks, cyclins, cdk inhibitors, ROS, cytochrome c, caspases, PARP, Bcl-2, Bax, p53, c-jun, c-fos, c-myc, PKC, PKB, PI3K, MAPK, NFκB, IKK, etc. are involved in these important cellular functions. Effects of the above mentioned phytopolyphenols on the activities of these transducer proteins were investigated by our laboratory and others, and the results will be selectively described and discussed.

INTRODUCTION

Phytopolyphenols are a major class of popular phytochemicals and secondary plant metabolites that commonly exist as multiple O- and C-glycosidic derivatives[1,2] but also may be present as aglycone.[3] They are an important part of human diet[4] and are also recognized as the active principle in a number of medicinal plants.[5]

The major groups of phytopolyphenols are the flavonoids and flavanoids, which are important in contributing the flavor and color of many fruits and vegetables. They play important roles in the normal growth, development, and defense of plants.[6] Recently, both flavonoids and flavanoids have been intensively investigated

because of their broad pharmacological activities, and their daily human intake has been estimated to be at gram level with the primary dietary source being vegetables and fruits.[7] It has been reported that diets rich in fruits and vegetables appear to protect against cardiovascular diseases, neurodegenerative disorders, and some forms of cancer.[4,8,9]

The most common phytopolyphenols in foods are flavanoids (Figure 6.1) and flavonoids (Figure 6.2), which consist mainly of flavone, flavanones, flavonols, flavanonols, isoflavones, flavanols, anthocyanidins, and their glycosides.[10] Flavonoids are ubiquitous in plants; almost all plant tissues are able to synthesize flavonoids. There is also a wide variety of types, at least 2000 naturally occurring flavonoids.[11] Flavonoids are present in edible fruits, leafy vegetables, roots, tubers, bulbs, herbs, spices, legumes, tea, coffee, and red wine.[10] The representative compounds of this group are apigenin, quercetin, tangeretin, nobiletin, kaempferol, myricetin, genistein, daidzein, and luteolin (Figure 6.2). Recent studies demonstrated that tangeretin induced tumor cell cycle arrest at G1 phase.[12] It is interesting to see that the widely consumed citrus flavonoid tangeretin exerted this tumor growth inhibitory effect.

Several polyphenols have been recognized as active chemopreventive agents (Figure 6.3). Curcumin, a yellow ingredient from tumeric (*Curcuma longae* L.) has been extensively investigated for its cancer chemopreventive potential.[13] 6-Gingerol (Figure 6.3) and 6-paradol are pungent ingredients of ginger (*Zingiber offincinale Roscoe*) also have antitumor promotional effects. Resveratrol (Figure 6.3) found in grapes and other dietary and medicinal plants[14] and tea polyphenols (Figure 6.1) from green, oolong, and black teas exert their striking inhibitory effects on diverse cellular events associated with multistage carcinogenesis.[14,15]

Although flavanols, also called catechins, seem to be widely distributed in plants, they are rich only in tea leaves, where catechins may contribute up to 30% of dry leaf weight. The antioxidative and antitumor properties of green and black teas and their tea polyphenols are extensively studied. It seems that tea polyphenols are important not only for plants but also for humans. Therefore, this brief review will discuss the current data with a particular emphasis on the effects of tea polyphenols on the cellular oxidative stress and cancer chemopreventive properties. The action mechanisms of several phytopolyphenols on cancer chemoprevention will be elaborated.

CANCER CHEMOPREVENTION OF SELECTED PHYTOPOLYPHENOLS

Tea Polyphenols

Inhibition of Carcinogenesis in Animals

In recent years, many studies demonstrated that topical application or oral feeding of a polyphenolic fraction from tea extract, or catechin derivatives, had anticarcinogenic effects in animal skin and other organs.[16]

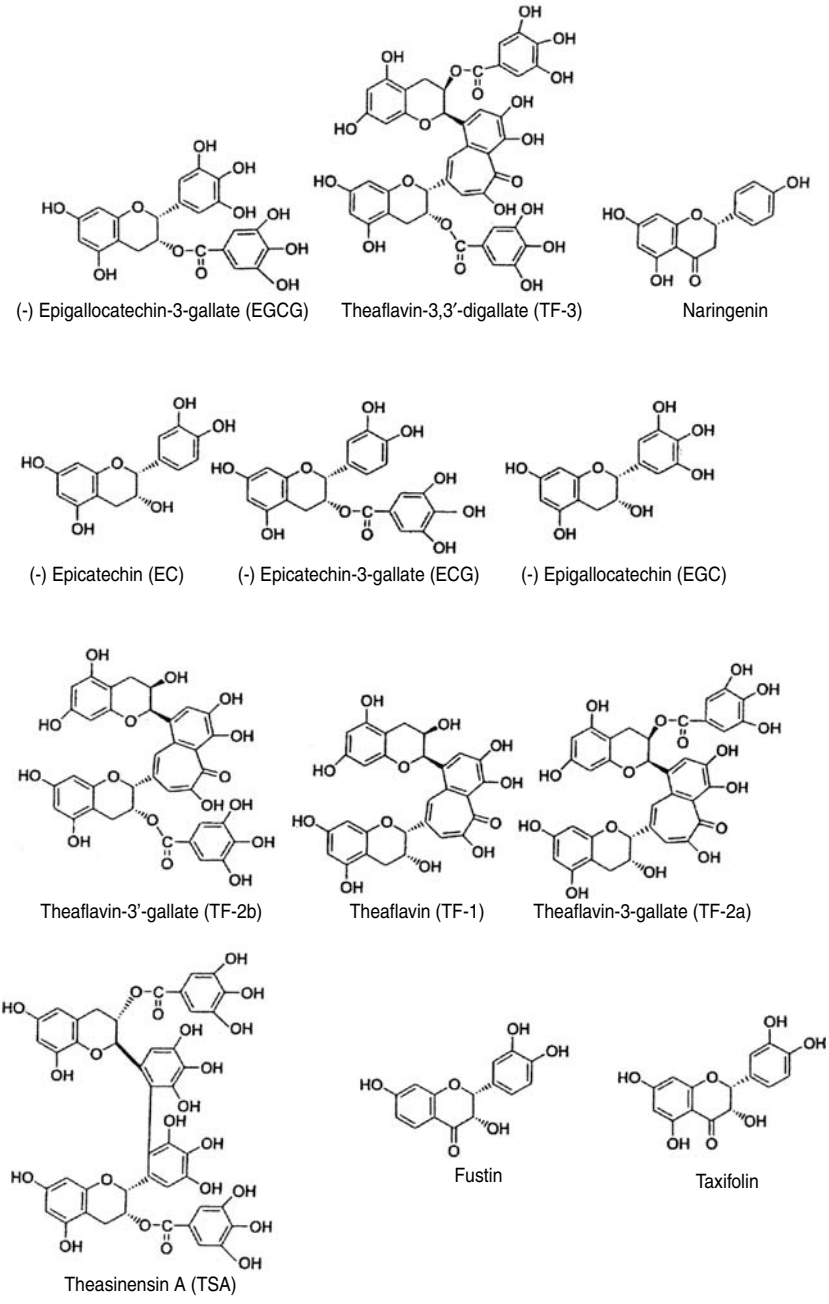

Figure 6.1 Chemical structures of flavanoids including catechins and theaflavins. These flavanoids consisted of two major groups: flavanones, including naringenin, taxifolin, and fustin, and flavanols including green tea polyphenols (EC, ECG, EGC, EGCG), black tea polyphenols (TF-1, TF-2a, TF-2b, TF-3), and oolong tea polyphenol (TSA).

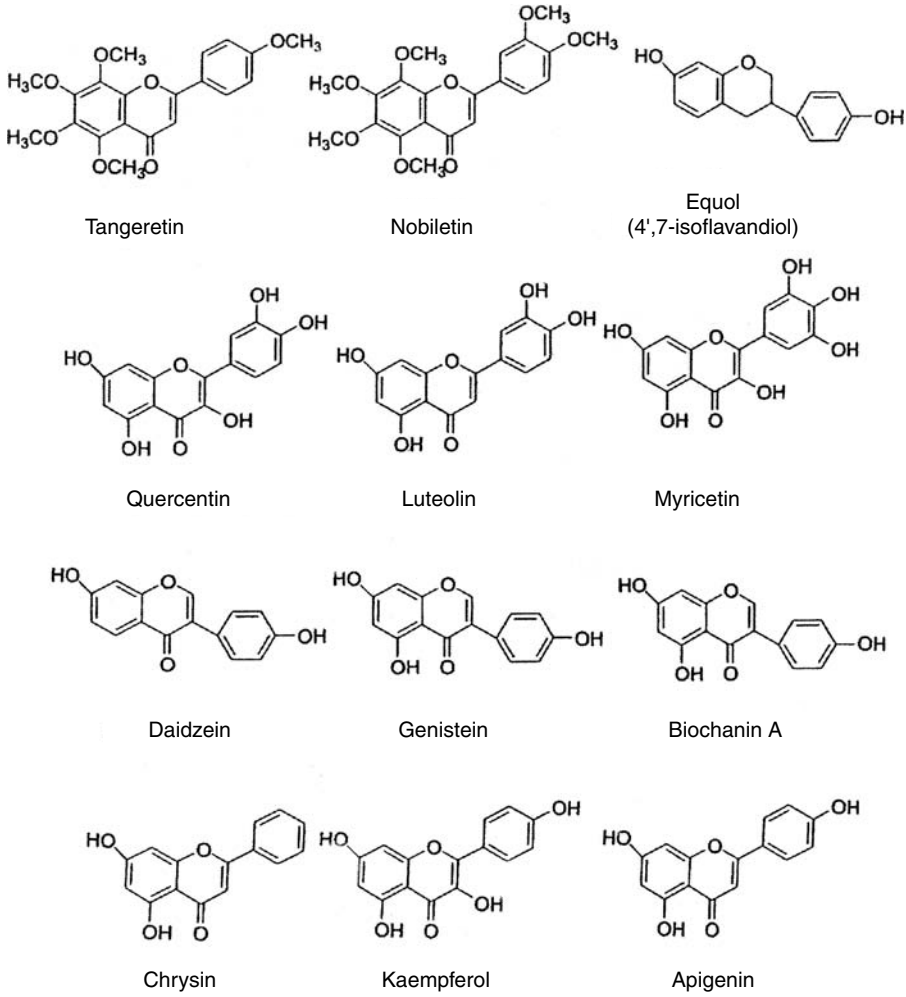

Figure 6.2 Chemical structures of flavonoids. These flavonoids consisted of three groups: flavone (apigenin and lutolin), flavonols (flavonol, kaempferol, quercetin, myricetin, tangeretin, and nobiletin) and isoflavones (daizein, genistein, biochanin A, and equol).

The antitumor effects of (–)-epigallocatechin-3-gallate (EGCG) and green tea extract on various organs, including skin, glandular stomach, duodenum, colon, liver, pancreas, and lung in rats and mice have been reported.[15–18] The antitumor effects of black tea extract on skin carcinogenesis and esophageal tumorigenesis in rodents were also reported.[19] Sugimura and his colleagues used a two-stage skin carcinogenesis mouse model to demonstrate that topical application of EGCG inhibited tumor promotion induced by teleocidin in DMBA-initiated mouse skin.[20] Studies by Mukhtar et al. further showed that green tea polyphenols exhibited strong inhibition on skin tumorigenicity in Sencar mice.[21]

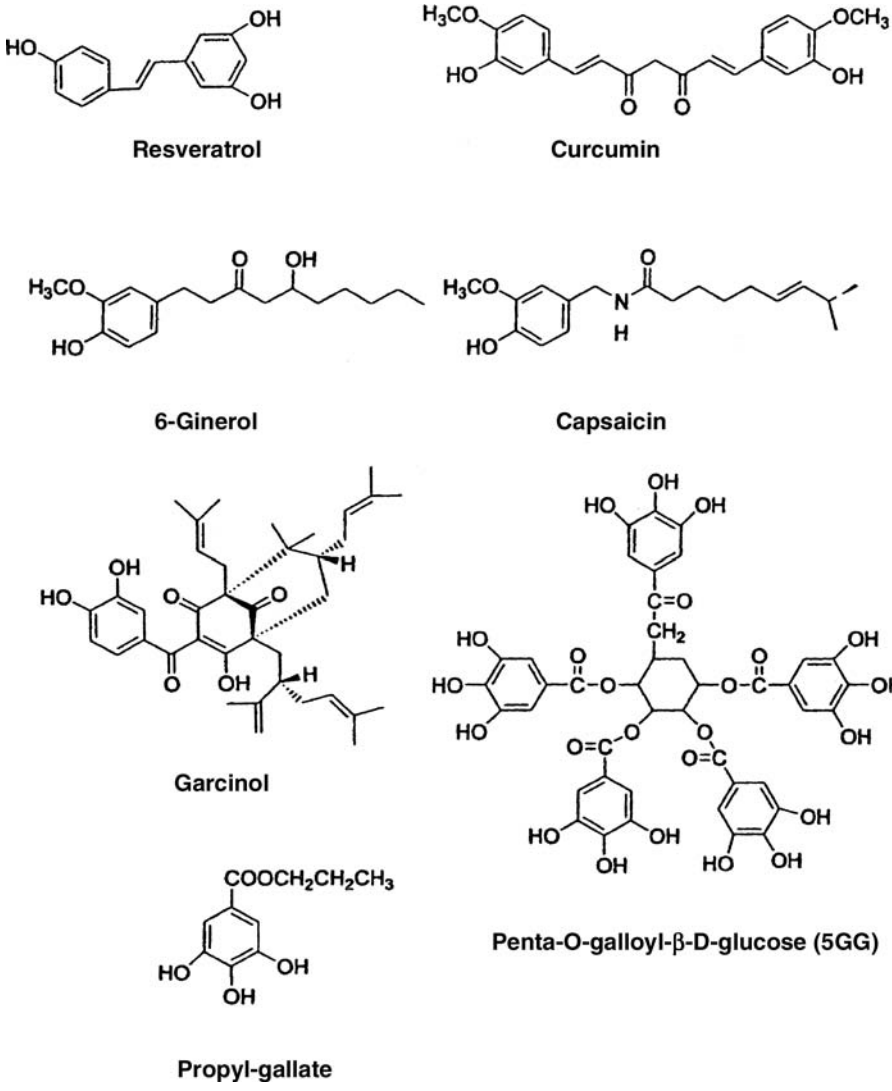

Figure 6.3 Chemical structures of curcuminoids, resveratrol, and gallates.

Wang et al.[17] reported that green tea polyphenols inhibited the growth of established skin tumors induced chemically or by ultraviolet (UV) light. Oral, subcutaneous, or intraperitoneal administration of EGCG or green tea polyphenols in mice also resulted in significant suppression of the growth of implanted tumor cells.[22,23] Oral administration of black tea in tumor-bearing mice inhibited proliferation and enhanced apoptosis in nonmalignant and malignant skin tumors. Sazuka et al.[24] and Taniguchi et al.[25] demonstrated that preoral administration of green tea infusion or EGCG inhibited lung metastasis in mouse melanoma and Lewis lung carcinoma cells. The mechanisms of antimetastatic effect of EGCG were associated with its

inhibition of cell spreading of tumor cells, suppression of matrix metalloprotease-9 (MMP-9) secretion, and serum-induced tyrosine phosphorylation of focal adhesion kinase (FAK). Sazuka et al.[26] also reported that the theaflavins and EGCG inhibited MMPs secretion from culture medium of LL2-Lu3 cells.

Landau et al.[27] showed that black and green tea infusion significantly decreased the spontaneous formation of lung tumors and rhadomyosarcoma in A/J mice. The suppression of azoxymethane-induced preneoplastic lesions and inhibition of cyclooxygenase-2 activity in the colonic mucosa of rats drinking a crude green tea extract has been described.[28] Recently, effects of tea polyphenols and tea pigments on the inhibition of precancerous liver lesions in rats have been reported.[29] Here, tea pigments are the oxidative products of tea polyphenols, which are primarily composed of theaflavins, thearubigins, and theabrownins.

Prevention of Human Cancer by Tea

In spite of the intensive studies of many investigators, the preventive effects of tea on the cancer development in humans have not been so conclusive. Studies in certain countries had reported no significant association,[30] in others, a positive association[31,32] was observed, and in still others, a negative association between tea consumption and cancer incidence was observed.[33,34] The discrepancy among these studies on the association of tea drinking with cancer incidence may arise from their different study subjects and different questionnaire designs. It is worth noting that the frequency and quantity of daily tea drinking in a population might affect the outcome of cancer prevention in such a population. There are many lifetime tea drinkers in Asian countries such as China, Korea, and Japan. They drink tea every day, even every hour during the daytime. This may be one of the reasons that more previous studies from Asian populations gave definitively positive preventive effects of tea on cancer incidence.[33,34] It is worth noting that most Asian people drink freshly prepared tea extracts, whereas some Western people drink formulated bottled tea. With respect to the air oxidation of tea polyphenols and other constituents, the chemical composition of these two types of tea might be significantly different.

A recent cohort study showed that the slowdown in increase of cancer incidence with age observed among Japanese females who consumed more than 10 cups a day is consistent with the finding that increased consumption of green tea is associated with later onset of cancer.[35]

Cancer Chemoprevention by Tea Polyphenols

It has been established that the pathological processes of multistep carcinogenesis comprises initiation, promotion, and progression.[36] The natural history of carcinogenesis and cancer provides a strong rationale for a preventive approach to the control of this disease and leads one to consider the possibility of active pharmacological intervention to arrest or reverse the process of carcinogenesis before invasion and metastasis occur. Such intervention is called chemoprevention.[37,38]

The inhibitory effects of tea against carcinogenesis have been attributed to the biologic activities of the polyphenolic fractions in tea. However, the molecular

mechanisms of cancer chemoprevention by tea extract and tea polyphenols are not fully elucidated. Some recent studies in our laboratory and others on this important issue are discussed here.

Antioxidative Effects

Tea polyphenols, including catechins and theaflavins, show profound antioxidative effects in various systems.[39] Tea polyphenols are strong scavengers against superoxide, hydrogen peroxide, hydroxyl radicals, nitric oxide, and peroxynitrite produced by various chemicals and biological systems. Chen and Ho[40] extensively investigated the antioxidant properties of various tea polyphenols. Their studies demonstrated that the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical-scavenging ability of various tea polyphenols was proportional to the number of –OH groups in the catechins or theaflavins. All the theaflavins showed the same capacity to inhibit the production of superoxide. Green tea, black tea, and EGCG were shown to block the production of oxygen free radicals derived from IQ in the presence of NADPH-cytochrome p-450 reductase.[41] A recent study shows that catechins of green tea are highly active in reducing the amount of oxidative damage sustained by DNA through •OH radical attack. Catechins, when compared with other classes of flavanoids, are found to be very active in reducing the amount of strand breakage and residual base damage by a mechanism other than direct scavenging of •OH radicals before they react with DNA. Pulse radiolysis data support the mechanism of electron transfer (or H-transfer) from catechins to radical sites on DNA.[42] These results support an antioxidant role of catechins in their direct interaction with DNA radicals.

The inhibitory effects of tea polyphenols on xanthine oxidase (XO) were investigated.[39] Theaflavins and EGCG inhibit XO to produce uric acid and also act as scavengers of superoxide. Theaflavin 3,3′-digallate (TF-3) acts as a competitive inhibitor and is the most potent inhibitor of XO among these compounds. TF-3 also inhibited the superoxide production in HL-60 cells. Therefore, the antioxidative activity of tea polyphenols is due not only to their ability to scavenge superoxides but also to their ability to block XO and relative oxidative signal transducers.[39]

Recently, several studies have found that black tea and green tea offered protection against oxidative damage to red blood cells induced by a variety of inducers, such as hydrogen peroxide, primaquine, 2,2′-azo-*bis*(2-amidinopropane) dihydrochloride (AAPH), phenylhydrazine, copper-ascorbic acid, and the xanthine/xanthine oxidase system.[43] Recently, we found that oral feeding of green tea leaves to rats resulted in enhanced superoxide dismutase (SOD) activity in serum and catalase activity in liver and an increased concentration of glutathione in the liver.[44]

Peroxynitrite is a cytotoxic species generated by the reaction between superoxide and nitric oxide. Catechin polyphenols could also decrease the peroxynitrite-induced nitration of tyrosine and protect apolipoprotein B-100 of LDL from peroxynitrite-induced modification of critical amino acids, which contribute to its surface charge.[45]

Recent studies have shown that EGCG has a neuroprotective effect against hippocampal neuronal damage following global ischemia in the gerbils.[46] Tea catechins protected the cultured newborn-mouse cerebral nerve cells from death induced

by glucose oxidase. Intracisternal injection of (–)-epicatechin improved the memory impairment induced by intracisternal glucose oxidase, and i.v. injection of (+)-catechin or (–)-epicatechin improved memory impairment induced by the cerebral ischemia.[47] These findings suggest that tea catechins ameliorate the injuries or impairments induced by active oxygens through scavenging intracellular oxygens.

The neuroprotective property of green tea and EGCG in *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-mice model of Parkinson's disease has been described.[48] MPTP neurotoxin caused dopamine neuron loss in substantia nigra concomitant with a depletion in striatal dopamine and tyrosine hydroxylase protein levels. These adverse effects could be suppressed by the green tea extract or EGCG.

Suppression of Tumor Proliferation and Mitogenic Signal Transduction

Tea polyphenols are known to inhibit a wide variety of enzymatic activities associated with cell proliferation and tumor progression. We have investigated the effects of the major tea polyphenols EGCG on the proliferation of human epidermoid cell line A431.[49] Using a tritiated thymidine incorporation assay, EGCG significantly inhibited the DNA synthesis of A431 cells. In *in vitro* assay, EGCG strongly inhibited the protein tyrosine kinase activities of EGF-R, PDGF-R and FGF-R and exhibited an IC_{50} value of 0.5 to 1 μg/ml. But EGCG scarcely inhibited the protein kinase activities of $pp60^{src}$, PKC, and PKA (IC_{50} >10 μg/ml). In an *in vivo* assay, EGCG could reduce the autophosphorylation level of EGF-R by EGF. Phosphoamino acid analysis of the EGF-R revealed that EGCG inhibited the EGF-stimulated increase in phosphotyrosine level in A431 cells. In addition, we showed that EGCG blocked EGF binding to its receptor. These results suggested that the inhibition of proliferation and suppression of the EGF signaling by EGF might mainly mediate dose-dependent blocking of ligand binding to its receptor, and subsequently through inhibition of EGF-R kinase activity.[49]

Recent developments in cellular biology have demonstrated the important role of mitogenic signal transduction in controlling the tumor proliferation. The induction of ornithine decarboxylase (ODC), PKC, protein kinase activities, and oxidative stress by the phorbol ester, TPA, is believed to be closely related to the tumor promotion activity of this compound.[36] Topical application of green tea polyphenols to mouse skin was found to inhibit TPA-caused induction of ODC activity in a dose-dependent manner.[50] Our studies demonstrated that EGCG and TF-3 inhibited TPA-induced transformation, PKC activation, and AP-1 binding activities in mouse fibroblast cells.[51,52]

Attenuation of PI3K Pathway

The inhibitory effects of tea polyphenols on UVB-induced phosphatidylinositol-3-kinase (PI3K) activation has been demonstrated in mouse epidermal JB6 C1 41 cells.[53] Pretreatment of cells with EGCG and TF-3 inhibited UVB-induced PI3K activation. Furthermore, UVB-induced activation of Akt and ribosomal p70S6 kinase (p^{70}S6-K), PI3K downstream effectors, were also attenuated by these tea polyphenols. In addition to LY294002, a PI3K inhibitor, pretreatment with MAP-

ERK kinase 1 inhibitor, U0126, or a specific p38 kinase inhibitor, SB202190, blocked UVB-induced activation of both Akt and p^{70}S6-K. It is worthy to note that UVB-induced p^{70}S6-K activation was directly blocked by the addition of EGCG or TF-3, whereas these polyphenols showed only a weak inhibition on UVB-induced Akt activation.[53]

Suppression of MAPK Signaling

It has been demonstrated that tea polyphenols inhibited PKC, MAPK, and AP-1 activities in NIH 3T3 cells[52] and mouse epidermal JB6 cells and the corresponding H-ras-transformed cell line 30.7b Ras 12.[54] The cells were incubated with EGCG or TF-3 (20 μM) for different times and the cell lysate was analyzed by immunoblotting. EGCG treatment decreased the levels of phospho-ERK1/2 time dependently by 30% at 30 min and 60% at 60 min, (from the presented data indicated that EGCG could slightly increased the phosphor-ERK by 10% at 15 min); TF-3 lowered their levels by 50% at 15 min, 40% at 40 min and 30% at 60 min. TF-3 effectively decreased total Raf-1 protein levels most likely through lysosomal degradation. On the contrary, EGCG did not affect protein levels or the activity of Raf-1 significantly but decreased its association with MEK 1 as determined by co-immunoprecipitation. In addition, EGCG and TF-3 (10 μM) inhibited the phosphorylation of Elk-1 by isolated phosphor-ERK1/2 *in vitro*.[54]

Exposure of normal human epidermal keratinocytes (NHEK) to UVB radiation induces intracellular release of hydrogen peroxide (oxidative stress) and phosphorylation of MAPK cell signaling pathways. Pretreatment of NHEK with EGCG inhibits UVB-induced hydrogen peroxide production and its mediated phosphorylation of MAPK signaling pathways.[55] Treatment of EGCG (20 μg/ml of media) to NHEK before UVB (30 mJ/cm^2) exposure inhibited UVB-induced hydrogen peroxide production (66 to 80%) concomitant with the inhibition of UVB-induced phosphorylation of ERK1/2 (57 to 80%), JNK (53 to 83%), and p38 (50 to 70%) proteins. These findings demonstrate that EGCG has the potential to inhibit UVB-induced oxidative stress-mediated phosphorylation of MAPK signaling pathways, suggesting that EGCG could be useful in attenuation of oxidative stress-mediated and MAPK-caused skin disorders in humans.[55]

Suppression of COX-2 and iNOS

Cyclooxygenase-2 (COX-2) plays an important role in carcinogenesis. Investigation of the suppressive action of 12 flavonoids of different chemical classes on the transcriptional activity using a reporter gene assay have revealed quercetin to be the most potent suppressor of COX-2 transcription (IC_{50} = 10.5 μM), whereas catechin and epicatechin showed weak activity (IC_{50} = 415.3 μM).[56] We have investigated the effect of various flavonoids and EGCG on the activities of COX-2 and iNOS in LPS-activated RAW 264.7 macrophages. Apigenin, genistein, and kaempferol were markedly active inhibitors of transcriptional activation of COX-2, with IC_{50} < 15 μM. In addition, apigenin, quercetin, and kaempferol were also

markedly active inhibitors of transcriptional activation of inducible nitric oxide synthase (iNOS), with $IC_{50} < 15$ μM, while EGCG was a weaker inhibitor. It appeared that of those compounds tested, apigenin was the most potent inhibitor of transcriptional activation of both COX-2 and iNOS.[57]

The small molecule nitric oxide (NO) has been shown to exhibit several profound physiological and pharmacological functions in the target cells. NO plays an important role in inflammation and multistep carcinogenesis. We have investigated the effects of tea polyphenols on the induction of NO synthase (iNOS) in thioglycolate-elicited and lipopolysaccharide (LPS)-activated peritoneal macrophages.[58,59] Gallic acid, EGC, EGCG, TF-1, TF-2, and TF-3 were found to inhibit nitrite production, iNOS protein, and mRNA in activated macrophages. Western blot, reverse transcription polymerase chain reaction, and Northern blot analyses demonstrated that significantly reduced 130-kDa protein and 4.5 kb mRNA levels of iNOS were expressed in LPS-activated macrophages with EGCG or theaflavins compared with those without tea polyphenols. Electrophoretic mobility shift assay indicated that EGCG blocked the activation of nuclear factor-κB, a transcription factor necessary for iNOS induction. EGCG and theaflavins also blocked the disappearance of inhibitor IκB from cytosolic fraction. These results suggest that EGCG and theaflavins decrease the activity and protein levels of iNOS by reducing the expression of iNOS mRNA and that the reduction could occur through prevention of the binding of NF-κB to the iNOS promoter, thereby inhibiting the induction of iNOS transcription.[58]

Inhibition of NFκB Activation through Down-Regulating IκB Kinase

Recently, we have investigated the inhibition of IκB kinase (IKK) activity in LPS-activated murine macrophages (RAW 264.7 cell line) by various polyphenols including EGCG and theaflavins.[60] TF-3 inhibited IKK activity more strongly than did the other polyphenols. TF-3 strongly inhibited both IKK1 and IKK2 activity and prevented the degradation of IκBα and IκBβ in activated macrophage cells. The results suggested that the inhibition of IKK activity by TF-3 and other tea polyphenols could occur by direct effect on IKKs or on upstream events in the signal transduction pathway. Furthermore, TF-3 blocked phosphorylation of IκB from the cytosolic fraction, inhibited NFκB activity, and inhibited increases in iNOS levels in activated macrophages. These results suggest that TF-3 and other polyphenols may exert their anti-inflammatory and cancer chemoprevention actions by suppressing the activation of NFκB through inhibition of IKK activity.[60]

In the JB6 mouse epidermal cell line, the tumor promoter TPA causes cell transformation at high frequency, marked induced NFκB activation. EGCG and TF-3 inhibited TPA-induced NFκB activity in a concentration-dependent manner. These tea polyphenols blocked TPA-induced phosphorylation of IκB at Ser 32 in the same concentration range. Moreover, the NFκB sequence-specific DNA binding activity induced by TPA was also inhibited by these polyphenols.[61] These results confirmed that inhibition of NFκB activation is also important in evaluation of the antitumor promotion effects of tea polyphenols.

Suppression of Cell Cycle Progression

It is generally accepted that the cell proliferation is intimately associated with cell cycle progression. EGCG potently inhibit cell proliferation and suppress tumor growth both *in vitro* and *in vivo*, but little is known regarding the cell cycle regulatory proteins mediating these effects. We have investigated the effects of EGCG and other catechins on the cell cycle progression.[62] DNA flow cytometric analysis indicated that EGCG blocked cell cycle progression at G1 phase in asynchronous MCF-7 cells. Over a 24-h exposure to EGCG, the Rb protein changed from hyper- to hypophosphorylated form and G1 arrest developed. The protein expression of cyclin D1 and E reduced slightly under the same conditions. Immunocomplex kinase experiments showed that EGCG inhibited the activities of cyclin-dependent kinase 2 (Cdk-2) and 4 (Cdk-4) in a dose-dependent manner in the cell free system. As the cells were exposed to EGCG (30 μM) over 24 h, a gradual loss of both Cdk2 and Cdk4 kinase activities occurred. EGCG also induced the expression of the Cdk inhibitor p21, and this effect correlated with the increase in p53 levels. The level of p21 mRNA also increased under the same conditions. In addition, EGCG also increased the expression of the Cdk inhibitor p27 protein within 6 h after EGCG treatment. These results suggest that EGCG either exerts its growth-inhibitory effects through modulation of the activities of several key G1 regulatory proteins such as Cdk2 and Cdk4 or mediates the induction of Cdk inhibitors p21 and p27. A dual combination of these two pathways is also possible.

Induction of Apoptosis by Tea Polyphenols

Apoptosis is induced by a variety of stimuli, such as genotoxic compounds, tumor necrosis factor, Fas ligand, and various environmental stresses. Despite the diversity of apoptosis-inducing agents, numerous experiments indicate that signals leading to the activation of members of the intracellular cysteine protease family, for instance, the caspase, may play a pivotal role in the initiation and execution of apoptosis induced by various stimuli.[63]

We have examined the growth-inhibitory effects of theasinensin A (from oolong tea), EGCG, and theaflavins in human cancer cells.[64] Theasinensin A TF-1 and TF-2 displayed strong growth inhibitory effects against human histolytic lymphoma U937 (IC_{50}, 12 μM) but were less effective against human acute T-cell leukemia Jurkat, whereas TF-3 and EGCG had lower activities. The molecular mechanisms of tea polyphenols induced apoptosis as determined by annexin V apoptosis assay, DNA fragmentation, and caspase activation were further investigated. Loss of membrane potential and ROS generation were also detected by flow cytometry. Treatment with tea polyphenols caused rapid induction of caspase-3, but not caspase-1, activity and stimulated proteolytic cleavage of poly(ADP-ribose)-polymerase (PARP). Pretreatment with a potent caspase-3 inhibitor, Z-Asp-Glu-Dal-Asp-fluoromethyl ketone, inhibited theasinensin A-induced DNA fragmentation. Furthermore, it was found that theasinensin A induced loss of mitochondrial potential, elevation of ROS production, release of mitochondrial cytochrome c into the cytosol, and subsequently induction of caspase-9 activity. Further experimental results indicate that theasin-

ensin A is effective in inducing DFF-45 (an inhibitor binding to DNAase) degradation, which allows caspase-activated DNAase to enter the nucleus and degrade chromosomal DNA.

Recent studies on apoptosis and cell cycle arrest in cancer cells by *in vivo* metabolites of teas have been described.[65] The tea extracts from green, oolong and black teas, the rat sera obtained after oral intubation of the tea extracts, and the tea polyphenolic compounds, EGCG, EGC, ECG and theaflavins were used in the related tests. The extracts, the sera from the treated rats and the polyphenolic compounds significantly inhibited the proliferation of a rat hepatoma cell line (AH109A) and murine B16 melanoma cells but not normal rat mesothelial (M) cells. These results suggest that the induction of apoptosis by theasinensin A and other tea polyphenols may provide a pivotal mechanism for their cancer chemopreventive function.[64] A commentary on the cancer chemoprevention by tea polyphenols through mitotic signal transduction blockade has been critically elaborated by Lin et al.[59]

Cancer Chemoprevention by Curcumin and Curcuminoids

In 1991, we found that the phorbol ester TPA-induced transcriptional factor c-Jun/AP-1 in mouse fibroblast cells is suppressed by curcumin (Figure 6.2).[66] Elevated expression of gene transcriptionally induced by TPA is among the events required for tumor promotion. Functional activation of transcriptional factor c-Jun/AP-1 is believed to play an important role in signal transduction of TPA-induced tumor promotion. Suppression of the c-jun/AP-1 activation by curcumin (10 μM) is observed in mouse fibroblast cells.[30] Curcumin also inhibits the TPA- and UVB light-induced expression of c-jun and c-fos in JB6 cells and in mouse epidermis.[67]

Treatment of NIH3T3 cells with 15 or 20 μM curcumin for 15 min inhibited TPA-induced PKC activity in particulate fraction by 26 or 60% and did not affect the level of PKC protein.[68] Curcumin (10 μM) inhibits EGF receptor kinase activity up to 90% in a dose- and time-dependent manner and also inhibits EGF-induced tyrosine phosphorylation of EGF receptors in A431 cells.[69]

Curcumin has been shown to suppress the expression of iNOS *in vivo*.[70] Recent studies in our laboratory have demonstrated that curcumin blocks the formation of iNOS through suppressing NFκB activity in macrophages. Furthermore, curcumin can suppress the NFκB activation through down-regulating IκB kinase activity in macrophages.[71] It has been demonstrated that iNOS is overexpressed in colonic tumors of human and also in rats treated with colon carcinogens. iNOS appear to regulate COX-2 expression and production of proinflammatory prostaglandins, which are known to play a key role in colon tumor development. Both iNOS activity and colonic aberrant crypt foci formation in male F344 treated with azoxymethane (AOM) were significantly inhibited by curcumin.[72]

The TPA-induced conversion of xanthine dehydrogenase to XO is reduced by curcumin to the basal level noted in untreated cells. The activity of XO is remarkably inhibited by curcumin *in vitro* but not by its structurally related compounds caffeic acids, chlorogenic acid, and ferulic acid.[73] When Colo205 colorectal carcinoma cells

were treated with curcumin (60 μM), the appearance of apoptotic DNA ladders was delayed about 5 h and G1 arrest was detected. The reduction of p53 gene expression was accompanied by the induction of HSP70 gene expression in curcumin-treated cells.[74] It is interesting to note that curcumin induces apoptosis in immortalized NIH3T3 and malignant cancer cells but not in normal embryonic fibroblast cells.[75] Curcumin also induces apoptosis in human leukemia cells HL-60 through suppressing Bcl-2 expression. Transfection of Bcl-2 gene into HL-60 cells was found to inhibit the apoptotic effects of curcumin.[76] Furthermore, curcumin exhibited synergistic effects on the induction of differentiation in HL-60 cells when it combined with all-trans-retinoic acid or 1α-25-dihydroxy-vitamin D_3.

Curcumin at 10 μM inhibited 17.4 and 70.6% of cellular migration and invasion of SK-Hep-1 cells, respectively. Compared with less invasive human cellular carcinoma cell line Huh 7, SK-Hep-1 showed much higher MMP-9 secretion. Furthermore, parallel with its anti-invasion activity, curcumin inhibited MMP-9 secretion in SK-Hep-1 in a dose-dependent fashion. It seemed that curcumin has a significant anti-invasion activity in SK-Hep-1 cells, and this effect is associated with its inhibitory action on MMP-9 secretion.[77]

The rhizomes of ginger contain 6-gingerol (Figure 6.3) and its homologs as pungent ingredients that have been found to possess many pharmacological and physiological activities, such as anti-inflammatory, analgesic, and antipyretic effects.[78] Gingerol inhibited the phospholipid peroxidation induced by the $FeCl_3$-ascorbate system.[79] Gingerol protects TPA-induced ear edema, epidermal ODC activity, and skin tumor promotion in female ICR mice.[80] Tropical application of the ginger extract prior to TPA led to dramatic protection against DMBA-initiated skin carcinogenesis in Sencar mice and suppressed TPA-induced epidermal ODC, lipoxygenase and cyclooxygenase activities.[81]

Capsaicin (Figure 6.3) is a principal pungent ingredient present in hot red and chili peppers that belong to the plant genus *Capsicum* (Solanaceae). Although topical application of capsaicin can initially induce ear edema in mice, subsequent applications of the compound suppressed the inflammatory response.[78] Capsaicin preferentially repressed the growth of some transformed cells of human origin, including HeLa, ovarian sarcoma, mammary adenocarcinoma and HL-60 cells in culture. The capsaicin-induced growth inhibition and apoptosis in these cells were correlated with inhibition of plasma membrane NADH oxidase activity.[82] Capsaicin abrogates the activation of NFκB by TPA, as well as by TNFα, which may provide a mechanistic basis for the possible antitumor-promoting activity of capsaicin.[83]

Flavonoids with Chemopreventive Activities

Most flavonoids are diphenylpropanoids that occur ubiquitously in plant foods that are important constituents of human diet. The major flavonoids are apigenin, quercetin, tangeretin, nobiletin, kaempferol, myricetin, genistein, daidzein, and luteolin (Figure 6.2). Although flavonoids are generally considered to be nonnutritive agents, interest in flavonoids has arisen because of their potential role in the prevention of human cancer.[4]

Tangeretin is a polymethoxylated flavone, 5,6,7,8,4′-pentamethoxyflavone, which is concentrated in the peel of citrus fruits.[84] Several biological activities have been shown for tangeretin itself, including the ability to enhance gap junctional intracellular communication,[85] to counteract tumor promoter-induced inhibition of intracellular communication, and to inhibit cancer cell proliferation.[12] DNA flow cytometric analysis indicated that tangeretin blocked cell cycle progression at G1 phase in colorectal carcinoma COLO 205 cells. The degree of phosphorylation of Rb was decreased after 12 h and G arrested developed. Tangeretin either exerts its growth-inhibitory effects through modulation of the activities of several key G1 regulatory proteins such as Cdk2 and Cdk4 or mediates the increase of Cdk inhibitors p21 and p27.[12]

Apigenin and other related flavonoids inhibited carcinogen-induced tumors in rats and mice.[86] Apigenin suppressed TPA-induced tumor promotion of mouse skin. Apigenin also reduced the level of TPA-stimulated phosphorylation of cellular proteins and inhibited TPA-induced c-jun and c-fos expression.[87,88]

Soy is a unique dietary source of isoflavones, namely genistein, biochanin A, and daidzein (Figure 6.2). Heavy consumption of soy in Southeast Asian populations is associated with reduction in the rate of breast and prostate cancer and cardiovascular disease. The potential chemopreventive efficacy of genistein appears to be related to its phytoestrogenic effects.[89] Genistein competes with estradiol for estrogen receptors, and the complex translocates to the nucleus, stimulating estrogen-related cellular events and accelerating cell differentiation. Some estrogen-independent mechanisms of genistein are important for its biological action. Genistein significantly inhibits tyrosine-specific protein kinase activity,[90] which may in turn inhibit cell proliferation and growth factor-stimulated responses and immune response as well as induce cell differentiation.

Induction of COX-2 and iNOS by Flavonoids through PPARγ Binding

Induction of COX-2 and iNOS have been implicated in the inflammatory processes. To examine the anti-inflammatory effects of apigenin, chrysin, and kaempferol, the reporter plasmids COX-2 or iNOS were transient transfected into RAW 264.7 cells. Both promoter activities were markedly increased when the cells were treated with LPS. Both promoters' activities were inhibited by concurrent treatment of the cells with apigenin, chrysin, and kaempferol. Moreover, transfection of PPARγ expression plasmid enhanced the inhibitory effects of these three flavonoids. The results suggested that these flavonoids exhibited anti-inflammatory effects and inhibited the promoter activities of COX-2 and iNOS genes partially through PPARγ pathways.[91]

PPARγ, a nuclear receptor and transcription factor that regulates the expression of many genes relevant to carcinogenesis, is now an important target for development of new drugs for the prevention and treatment of cancer. Ligands for PPARγ suppress breast carcinogenesis in experimental models and induce differentiation of human liposarcoma cells. By analogy to the selective estrogen receptor modulator (SERM) concept, it is suggested that selective PPARγ modulators (SPARMs), designed to have desired effects on specific genes and target tissues without undesirable effects on others, will be clinically important in the future for chemoprevention and chemotherapy of cancer.[92]

Cancer Chemoprevention by Limonene, Perillyl Alcohol, and Silymarin

The essential oils of many commonly consumed plants contain a wide variety of monoterpenes such as limonene and perillyl alcohol (Figure 6.4). These compounds have been known to exhibit a wide spectrum of anticancer activities.[93] They can detoxify carcinogens via the induction of phase II enzymes.

These compounds can also block the carcinogenic process at the promotion and progression stage. In addition to these chemopreventive activities, monoterpenes can be used to treat a variety of cancers.[94] The inhibition of protein isoprenylation has been demonstrated in monoterpene chemoprevention target tissue, i.e., the *in situ* mammary gland epithelial cells.[60]

Silymarin (Figure 6.4), a flavanoid compound isolated from milk thistle plant (*Silybum marianum* (L.) Gaertn) has been used clinically in Europe as an anti-hepatotoxic agent. Silymarin is a very strong antioxidant compound capable of scavenging both free radicals and ROS. It possesses strong inhibitory effects against the induction of ODC activity and mRNA expression in Sencar mice caused by TPA and several other known tumor promoters.[95] Silymarin also inhibits activation of erbB1 signaling and induces cyclin-dependent kinase inhibitors Cip/p21 and kip/p27, G1 arrest and anticarcinogenic effects in human prostate carcinoma DU 145 cells.[96]

Cancer Chemoprevention by Carnosol from Rosemary

Carnosol (Figure 6.4) is a naturally-occurring phytopolyphenol found in rosemary (*Rosmarinus officinalis* Labiatae). Carnosol functions as antioxidant and anticarcinogen. In the recent studies we have compared the antioxidant activity of carnosol and

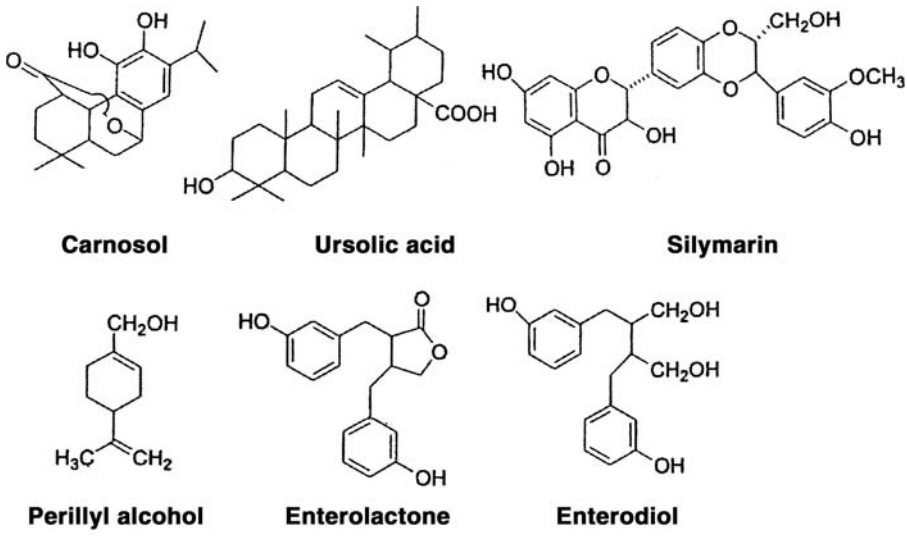

Figure 6.4 Chemical structures of carnosol, perillyl alcohol, and other polyphenols.

other compounds extracted from the rosemary. Carnosol showed potent antioxidative activity in α,α–diphenyl-β-picrylhydrazyl (DPPH) free radicals scavenge and DNA protection from Fenton reaction. Treatment of mouse macrophage RAW264.7 with carnosol markedly reduced LPS-stimulated NO production with IC_{50} of 9.4 μM. The results suggest that carnosol suppresses the NO production and iNOS gene expression by inhibiting NFκB activation, and provide possible mechanisms for its anti-inflammatory and chemopreventive action, since high concentration of NO are produced by iNOS in inflammation and multiple stages of carcinogenesis.[97]

GENERAL REMARKS ON THE POTENTIAL MECHANISMS OF ACTION OF PHYTOPOLYPHENOLS IN CHEMOPREVENTION

Growth Factors in Signal Transduction

It has been demonstrated that ROS, sulfhydryl oxidants, and reactive nitrogen species NO are thought to mediate signal transduction events in a number of cell lines.[98] NO plays a major role in the vascular homeostasis controlling the enzyme guanyl cyclase and modulating the activities of protein tyrosine kinases (PTK) and protein tyrosine phosphatase (PTP).[99] Tyrosine phosphorylation is associated with mitogenesis, cell transformation, and cell death. It is a dynamic and reversible process controlled by the opposing actions of PTK and PTP.[100]

Binding of a polypeptide growth factor (such as EGF, PDGF, and FGF) to its cognate receptor triggers the receptor tyrosine kinase activity, leading to a cascade of phosphorylation reactions. It is interesting to see that phytopolyphenols such as tea polyphenol EGCG or theaflavins effectively block the binding of EGF to its cognate receptor and inhibits the autophosphorylation of the receptor that leads to the downstream signal transduction blockade.[49,101]

High cell proliferation is the common characteristic of most cancer cells. This cellular property is well reflected in the up-regulation of signal transduction, cell cycle, and DNA-synthesis in the cancer cells. Cell growth is determined by extracellular conditions that act before the onset of DNA synthesis. The proliferation rate is initially determined by the probability of switching from the quiescent G0 to G1 phase of cell cycle.[102] Two major sites of control exist between G0 and S — competence and restriction point. Much is being learned about the molecules that regulate passage through these control points. Proto-oncogenes and tumor suppressor genes are of great interest in this regard. Some of these genes code for DNA-binding proteins such as fos and jun, which form a complex and bind to specific gene-regulating DNA sequences during the competence process. Other proto-oncogenes code for growth factor receptors such as the genes that encode the EGF receptor. Still others, such as c-raf, appear to have distinct functions, coding for protein kinases that act on substrates in the cascade involved in second messenger metabolism.

The cyclin proteins appear to play a major role in cell cycle regulation.[103] Cyclins are a family of proteins that accumulate in the G1 phase (G1-cyclins), and again during late S-G2 phases before mitosis (mitotic cyclins). After they reach a peak concentration, cyclin proteins are rapidly degraded.

Most phytopolyphenols, such as tea polyphenols, genistein, and silymarin, can produce cell arrest at G1 phase through inhibiting cyclin dependent kinase (cdk-2 and cdk-4) activities and inducing Cdk inhibitors p21 and p27.

Activation of NFκB by ROS through Protein Thiol

A great amount of evidence supports the contention that gene expression controlled by NFκB is redox regulated. At one level, its binding to DNA requires a key cysteine residue that must be reduced.[104] On the other hand, ROS appear to serve as common second-messenger-like molecules in the various pathways leading to NFκB activation. Several mechanisms have been proposed for the activation of NFκB. These include transformation of the cell to a more oxidized redox state, either by decreasing the concentration of reduced thiols or increasing the ration of glutathione disulfide to reduced glutathione (GSSG:GSH), or by generation of the hydroxyl radical or another reactive oxidant. It is generally assumed that this would result in oxidation of some critical protein thiol which is essential for NFκB activation.

Antioxidants, such as tea polyphenols, curcumin, and, carnosol, may be expected to work by increasing intracellular GSH or total thiols,[44] scavenging free radicals,[39] or iron chelations. Many of the compounds classified as antioxidants that have been shown to inhibit NFκB activation are phytopolyphenols that are good peroxidase inhibitors or substrates. Many are effective at concentration that may be too low to be compatible with a radical-scavenging role, and their effects might be better explained if they were acting on a more specific enzymatic process.

Blocking IκB Degradation by Phytopolyphenols

It has been demonstrated that incubation of cells with antioxidants, such as tea polyphenols, carnosol, curcumin, or garcinol, suppresses NFκB activation.[58,97,105] This has been interpreted as indicating that reactive oxygen intermediate (ROI or ROS) mediated NFκB activation, thus behaving as second-messengers in the cascade of transduction events leading to IκB degradation when inducers such as TNF, IL-1, or LPS are used.[58,106] Later studies suggested that there is no need to postulate a role of ROI in the mechanism of action of NFκB activators such as PMA (TPA) or TNF. It rather appears that an imbalance toward oxidation facilities, but does not directly mediate NFκB activation.[107] The production of ROI does not mediate NFκB activation after physiological stimulation is consistent with results showing that overexpression of catalase, a specific scavenger of hydrogen peroxide, does not block TNF-induced NFκB activation.[108]

Much remains to be learned about the redox control of the NFκB/IκBα system. This system is regulated in both a positive and negative way, and each step of that regulation may be subjected to control by oxidation and reduction. The effects of cellular reduction appear to differ in the cellular compartment considered, because reduction suppresses NFκB translocation from the cytoplasm, but may enhance NFκB in the nucleus. Because newly synthesized IκBα is expressed in the nucleus and terminates NFκB function, it will be important to know whether the nuclear redox status also controls this novel, negative function of IκBα. The reactive molecules involved in NFκB

activation are likely to be protein thiols such as thioredoxin and glutathione rather than ROI. It is suggested that ROI are capable of degrading IκBα and activating NFκB but are not required to execute the NFκB activating program triggered by physiological inducers.[109] The simplest explanation is that the reduction of cells induced by antioxidants such as phytopolyphenols suppresses one or more steps leading to activation of the important IκB kinase. Indeed, our recent results indicate that tea polyphenols, curcumin, and carnosol block LPS-induced degradation of endogenous IκBα.[58,97]

Signal Transduction in Tumor Promotion and Inhibition

Further insight into the anticarcinogenic mechanism of phytopolyphenols might be gained by looking at the individual steps in the multistage hypothesis of cancer development, which are all affected by these compounds,[110] namely initiation, promotion, and progression. Initiation either by directly acting carcinogens or after metabolic activation of procarcinogens can be prevented both by flavonoids and by other phytopolyphenols.[111] The inhibitory effect on metabolic activation may be related to the inactivation of drug-metabolizing cytochrome p450 isoenzymes.

Antitumor promoting activity of flavonoids is most often connected with the inhibition of the action of phorbol esters or the inactivation of ornithine decarboxylase. The possible correlation with oxidative events, indicated by the proposed involvement of lipoxygenase inhibition, has recently been brought into perspective.[112] The observations that the flavonol quercetin, flavone apigenin, isoflavone resveratrol, and tea polyphenols all arrest cell cycle progression in late G1 and G2/M stages are interesting in this context. These manifold interactions of various phytopolyphenols with tumor cells suggest that different mechanisms may occur, only some of them involving ROI (ROS). Compounds such as polymethoxylated flavonoid tangeretin effectively inhibit tumor cell proliferation.[12] These compounds have been shown to inhibit antioxidative capacity, but based on the effective concentrations, they probably do not react via direct radical scavenging.

The tumor-promotion stage is the rate-limiting step in carcinogenesis. Elucidating the molecular mechanisms of tumor promotion is, therefore, a prerequisite for cancer chemoprevention. Mitogenic stimulation for cell proliferation is likely to be an important pivotal force of tumor promotion. However, that alone is not significant for transformation, and additional changes in gene expression are required to escape from normal growth regulation or differentiation. In general, alterations in the transcription of a specific set of cellular genes are mediated by specific regulatory DNA-binding proteins or transcription factors that regulate gene expression directly by binding to specific DNA sequences in promoter regions.[113] The expression of genes induced by TPA and other tumor promoters such as UV irradiation are thought to be required in tumor promotion.

Studies conducted in the past decade, however, generated important insights into the mechanism of action of tumor promoters and led to unification of the field of tumor promotion with signal transduction. The best studied class of tumor promoters consists of phorbol esters (TPA) and related molecules. Phorbol esters are known to exert profound effects on cellular function including hormone release, blood cell activation, cell differentiation, mitogenesis, and tumor promotion. PKC appears to be the target

of action of TPA. More recently, two important additional links between tumor promotion and signal transduction have been established. Thapsigargin, a potent tumor promoter, acts by inhibiting calcium uptake/sequestration, thereby promoting elevation in calcium levels.[113] Okadaic acid, another tumor promoter, appears to inhibit serine-threonine protein phosphatases (PP1 and PP2A) and results in increased phosphorylation of protein substrates.[114] It appears that activation of calcium-dependent protein kinases or inhibition of protein phosphatases results in tumor promotion.

The products of most oncogenes appear to be integral members of signal transduction pathways. An understanding of their mechanism of action must involve dissection of their impact on signal transduction during normal cell function and determining how oncogenesis is related to disruption of these processes. It appears that when any essential component of a signal transduction pathway is rendered hyperactive autonomous, it may acquire the ability to drive the cell into unchecked proliferation or abnormal differentiation and finally lead to tumor promotion. Certain phytopolyphenols may block or attenuate the hyperactivity of these components of signal transduction as discussed below.

Inhibition of Carcinogenesis through Blocking Signal Transduction by Phytopolyphenols

During the previous discussion of the biological effects of phytopolyphenols, the potential interactions of these compounds with growth receptors, cytokines, and hormones have been alluded to and indirectly signal transduction processes.[49,59,101] The list of pertinent publications may be further expanded if we include the many reports of the inhibitory effects of phytopolyphenols with protein kinases or in tumor promotion/proliferation studies.

The detailed dissection of signaling pathways and their precise roles in cell regulation and cell transformation will offer a unique advantage in the development of specific preventive and therapeutic modalities of malignant disorders. It is expected that detailed biochemical and molecular analysis of biopsy material from individual tumors will generate insight into the cause and pathogenesis of individual tumors. This would then allow the development of specific therapeutic intervention agents. It is conceivable that modulation of receptors, coupling mechanisms, effectors, second messengers, protein kinases, protein phosphatases, and related substrates may also have profound therapeutic effects.

Accumulated evidence has demonstrated that signal transduction events, leading to the activation of the mitogen-activated protein kinase (MAPK) pathways (including ERK, JNK, and p38) and NF κB pathways, can result in cell proliferative, survival, differentiating, or apoptotic responses (Figure 6.5). The main theme of this scheme is to emphasize the action mechanisms of polyphenols that lead to the inhibition of survival gene expression (c-jun, c-fos, c-myc, etc.) and activation of apoptotic signal transduction pathways (caspase 8 and 9 cascades). In this scheme, we have tried to illustrate three important signaling events, namely the MAPK, NFκB inducing kinase (NIK), and caspase cascades (ICE/ced3 family proteases) pathways. Most phytopolyphenols could suppress the MAPK and NIK pathways but activate the caspase cascade pathways that lead to apoptotic response in the target cells.

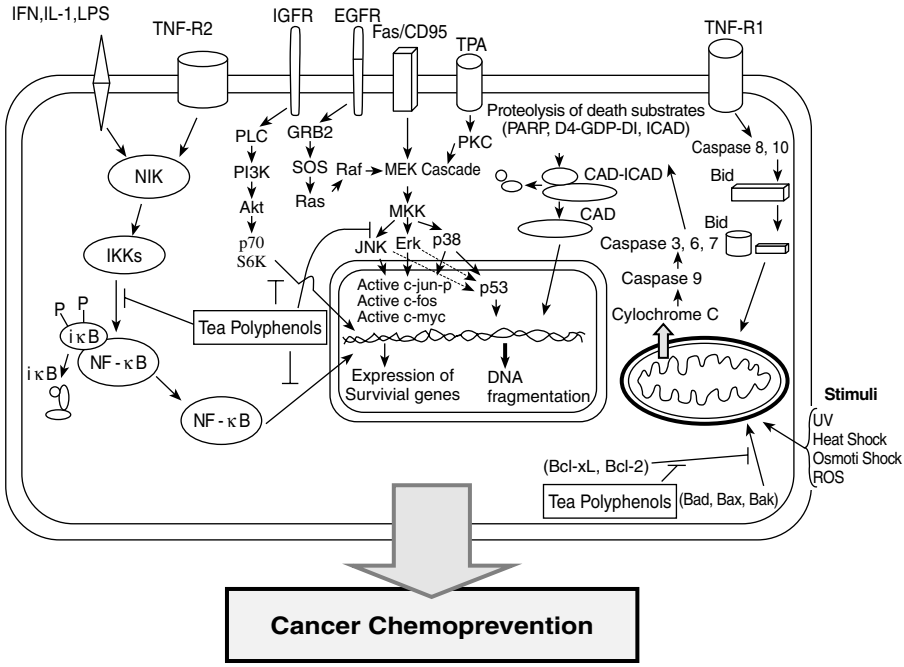

Figure 6.5 Multiple mechanisms of phytopolyphenols in cancer chemoprevention. The possible action mechanisms of phytopolyphenols for cancer chemoprevention and induction of program cell death (apoptosis) has been proposed and depicted. Extracellular growth factors (such as EGF, PDGF, TNF), cytokines (IL-1, IFN), or the phorbol ester tumor promoter TPA binds to membrane receptors such as EGFR, PDGFR, TNFR1, TNFR2, or PKC, resulting in the activation of a number of serine, threonine, or tyrosine kinases, which include ras, NFκB-inducing kinase (NIK), mitogen-activated protein kinases (MAPKKs, MAPKs, MKK, MEK), extracellular response kinase (ERK), p38 kinase, c-Jun N-terminal kinase (JNK), and IκB kinase (IKK). JNK is activated by MAPK kinase (MKK) and then to activate the c-Jun protein, which forms heterodimer with c-Fos protein, enhancing the activity of transcription factor AP-1. The EGFR can be activated by binding with its ligand EGF that leads to receptor dimerization and autophosphorylation. The growth signaling was transmitted to ras, raf(MAPKK) and MAPK (MEK cascade) through adaptor proteins GRB2 and SOS. Caspase pathways (caspases 9 and 3) are activated by polyphenols through down-regulation of Bcl-2. The inhibitor of caspase-activated DNAase (CAD) is cleaved by caspase 3 and leads to DNA fragmentation. Both IKK and PKC are important for activation of NFκB that leads to enhance the expression of c-myc, iNOS, and other cellular proliferation genes. Reactive oxygen species (ROS) are considered as endogenous mitogenic factors (or apoptotic factors in certain conditions) that can activate NFκB and other transcription factors in nucleus. Ultimately, activation of the MAPK family members causes activation of specific transcription factors, such as NFκB, AP-1, serum response factor (SRF), Bcl-2 and its family, p53, Rb, PCNA, Cdk-2, Cdk-4, cyclins, Cdk inhibitors, and others, which contribute to determine the cell fate, including proliferation, differentiation, inflammation, carcinogenesis, or apoptosis. Several polyphenols have been demonstrated to block several sites of these multiple-signal transduction pathways.

Most phytopolyphenols with cancer chemopreventive activities are antioxidants. It should be emphasized that, in addition to acting as ROS scavengers, these compounds can act through multiple mechanisms[59] to modulate the functions of receptors, effectors, protein kinases, protein phosphatases, and protein substrates in the mitogenic and differentiating signal transduction pathways that link to the process of tumor promotion (Figure 6.5). The detailed mechanisms that phytopolyphenols interact with these transducers deserve further investigation.

Nevertheless, we can already state with certainty that phytopolyphenols play a much wider role than acting merely as an antioxidant, which function was the first to be demonstrated for this extremely versatile class of plant secondary metabolites that appear to be important for human health. It is hoped that the extremely vigorous research activities involving the effects of phytopolyphenols on the signal transduction pathways *in vivo* will be initiated. We consider this topic one of the most exciting areas for future research in the field of traditional medicine and food science.

ACKNOWLEDGMENTS

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CHAPTER 7

Gene Regulation by Glucosinolate Hydrolysis Products from Broccoli

Kristin E. Stewart, Chu Won Nho, and Elizabeth H. Jeffery

CONTENTS

INTRODUCTION

Experimental and epidemiological studies suggest a correlation between increased fruit and vegetable consumption and lowering of cancer incidence. In many of these studies, the cruciferous vegetables are singled out as having a more significant beneficial effect than fruits and vegetables in general.

This suggests that cruciferous vegetables may provide some benefit not shared by all fruits and vegetables. Cruciferous vegetables, such as broccoli, Brussels sprouts, and cabbage, are a relatively unique dietary source of glucosinolates, a series of plant secondary metabolites derived from modified amino acids and including thioglucose and *N*-sulfate moieties (Figure 7.1). Glucosinolates have not been directly associated with anticarcinogenic activity. However, when the plant is

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Progoitrin	2-hydroxy 3,4-butenyl glucosinolate
Glucobrassicin	indol 3-yl methyl glucosinolate
Glucoraphanin	4-methyl sulfinyl butyl glucosinolate

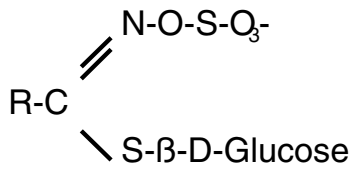

Figure 7.1 Major glucosinolates in broccoli.

chopped or crushed, the plant enzyme myrosinase comes into contact with the glucosinolate and hydrolyses it to release glucose. The resulting unstable thiono intermediate then rearranges, most frequently to form an isothiocyanate or a nitrile. It is the isothiocyante products of glucosinolate hydrolysis that have been associated most strongly with anticarcinogenesis, and it is their ability to trigger the up-regulation of a number of host-defense genes involved in destroying chemical carcinogens that is the focus of this chapter.

ANTICARCINOGENICITY OF BROCCOLI

A recent study shows three or more servings a week of cruciferous vegetables, such as broccoli, Brussels sprouts, or cabbage, can reduce risk for prostate cancer by 40%, compared with individuals eating only one or fewer servings per week.[1] Many more epidemiological studies suggest a role for cruciferous vegetables in prevention of cancers.[2–4] In particular, broccoli consumption has been associated with decreased incidence of cancers of the lung, colon, and prostate.[5]

In considering potential impact on the public health, we note that the Economic Research Service of the United States Department of Agriculture (USDA) reports that over the last 30 years, broccoli consumption has risen more than fivefold, to 7.7 lb/capita, while Brussels sprouts consumption remains unchanged over the same period, at 0.3 lb per capita.[6] This increased popularity of broccoli provides the means to translate basic science into improvement in the public health. Surveying a number of different varieties of cruciferous vegetables, one can identify four predominant glucosinolates: sinigrin, glucobrassicin, progoitrin and glucoraphanin.[7] The profile of the four glucosinolates is relatively similar for Brussels sprouts, cabbage, cauliflower and kale, all particularly high in sinigrin, Figure 7.2. The glucosinolate sinigrin, upon hydrolysis, releases allyl isothiocyanate, which is responsible for the spicy bite in mustard or the raw core of white cabbage. Many people in the U.S. prefer the less spicy flavor of broccoli, which has little or no sinigrin. However, the total glucosinolate level is similar among broccoli and the other cruciferous vegetables, because the lack of sinigrin is balanced by a larger amount of glucoraphanin, the hydrolysis products of which are not as pungent as those of sinigrin.

There are no reported feeding studies showing protection by broccoli against chemically induced cancers in animal models. However, there is an abundant

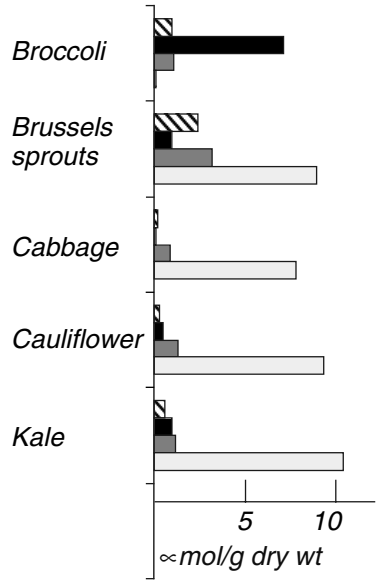

Figure 7.2 Distribution pattern of major glucosinolates in various vegetables belonging to *Brassica oleracea*. Fresh samples of the edible part of the vegetable were freeze-dried and analyzed for glucosinolate content by previously published methods.[7] Data are mean μmols glucosinolate/g dry weight vegetable: 50 varieties of broccoli, 4 varieties of Brussels sprouts, 6 varieties of cabbage, 3 varieties of cauliflower, and 2 varieties of kale.
Hatched bars = progoitrin; black-filled bars = glucoraphanin; grey-filled bars = glucobrassicin; open bars = sinigrin.

literature showing that diets containing other cruciferous vegetables, such as cabbage and Brussels sprouts, can protect animals against many chemically induced cancers.[5] Bioactive hydrolysis products of glucosinolates were first recognized as the active components by their ability to increase the levels of detoxification enzymes in the livers of test animals, whether given in purified form or as freeze-dried vegetables added to the diet.[8] Whereas broccoli feeding studies showing prevention of cancer have not been reported, many published studies show that purified sulforaphane, the isothiocyanate from glucoraphanin, can prevent chemically-induced cancer.[9] Data show that dietary broccoli, like its components and like other crucifers, is able to upregulate detoxification enzymes in rodents,[10] suggesting that broccoli is worthy of evaluation as a cancer preventative food. Furthermore, because it is a relatively popular food, broccoli is in a position to have a significant impact on the health of the public. There is a need for animal feeding studies to determine the efficacy of broccoli against chemically-induced cancers.

Glucobrassicin, progoitrin, and glucoraphanin are the three major glucosinolates found in broccoli, although the exact amount of each glucosinolate varies substantially across varieties and with growing and processing conditions.[11] Upon hydrolysis, these glucosinolates are converted to their biologically active hydrolysis products. Hydrolysis requires myrosinase (EC 3.2.3.1), a thioglucosidase that is absent from mammalian tissue but is present in cruciferous plant tissue, although

Table 7.1 Concentration ranges of the major glucosinolates in 50 varieties of broccoli and their bioactive hydrolysis products.[7]

	Concentration in Broccoli (μmol/g dry wt)	Bioactive Hydrolysis Product	Reference
Glucobrassicin	0.1–2.8	Indole-3-carbinol	24
Progoitrin	0.1–7.9	Crambene	38
Glucoraphanin	0.8–21.7	Sulforaphane	13

compartmentally separated from the glucosinolate substrates. When the plant is chopped or crushed, the enzyme and substrate come together and hydrolysis occurs. Although little detail is yet known, it appears that the microflora of the gut are also able to support glucoinolate hydrolysis, leading to the understanding that even if cooked broccoli no longer contains an active myrosinase, glucosinolate hydrolysis might occur in the colon.[12] Each of the three major glucosinolates in broccoli has bioactive hydrolysis products (Table 7.1).[13] Sulforaphane, an hydrolysis product from glucoraphanin, is present in the highest concentration (Table 7.1).

PHASE I AND PHASE II DETOXIFICATION ENZYMES

During the late 1950s, when detoxification enzymes were first identified and separated into two groups, phase I and phase II, to differentiate between those that chemically alter the substrate through oxidation, reduction, or hydrolysis, and those that conjugate the substrate to an endogenous molecule, such as glucuronide.[14] Since that time, many more xenobiotic metabolizing enzymes have been identified, and the categorization of phase II enzymes has broadened to include quinone reductase, epoxide hydrolase, γ-glutamyl-cysteine synthetase, and others that act to protect the cell from toxic or carcinogenic insult. In fact, the upregulation of quinone reductase has become a common biomarker for potential anticarcinogenic activity of natural products. In a cell culture system, quinone reductase can be rapidly measured and responds to many compounds that induce phase II enzymes.[15] In addition, induction of quinone reductase in cell culture appears to be a reliable predictor of induction in rodent organs. Furthermore, the induction of quinone reductase coordinates with the induction of many other detoxification enzymes, including glutathione-S-transferase, UDP-glucoronosyl transferase, and γ-glutamyl-cysteine synthetase.[16] Sulforaphane has been shown to be a potent upregulator of quinone reductase in cell culture, and this activity has led to an interest in developing diets high in sulforaphane-containing foods as cancer preventative diets.[17–19]

MONOFUNCTIONAL AND BIFUNCTIONAL INDUCTION

The upregulation of detoxification enzymes by glucosinolate hydrolysis products has been classified based on whether a compound activates enzymes from one or both of the phase I and phase II groups of detoxification enzymes. Monofunctional inducers

upregulate a number of phase II enzymes; bifunctional inducers cause upregulation of phase I as well as phase II enzymes.[20] Sulforaphane is considered a highly potent monofunctional inducer, upregulating a battery of enzymes, including quinone reductase, γ-glutamyl-cysteine synthetase, aldo-keto-reductase, and a number of glutathione-S-transferase isoenzymes.[21] Bifunctional inducers upregulate a few phase I cytochrome P450 monooxygenases, as well as a battery of phase II enzymes, overlapping, but not identical to those upregulated by monofunctional inducers. Metabolism of some bifunctional inducers by cytochrome P450 results in products that are monofunctional inducers and thereby up-regulating the full battery of phase II enzymes.[20]

THE XENOBIOTIC RESPONSE ELEMENT AND INDOLE-3-CARBINOL

The genes for CYP1A1/2, CYP1B1, and several phase II enzymes contain a 5′ regulatory sequence termed the xenobiotic response element (XRE), also termed the aryl hydrocarbon response element or dioxin response element (Table 7.2).[22] The core XRE sequence is GCGTC, although flanking sequences that vary from gene to gene are also important for activation of transcription. This regulatory region is activated through binding of a ligand-receptor complex. When a ligand enters the cell, it binds to a cytosolic receptor, the aryl hydrocarbon receptor (AhR). The AhR is a member of the per-arnt-sim family of basic-helix-loop-helix transcription factors. Under basal conditions, the AhR is found in the cytoplasm associated with heat shock protein 90. Ligand binding releases AhR from the heat shock protein, and AhR translocates to the nucleus. In the nucleus, ligand-bound AhR forms a heterodimeric complex with a transcription factor termed the aryl hydrocarbon nuclear translocator (ARNT). This complex subsequently binds to the XRE, increasing transcription of the target gene. The transcription factor ARNT is known to dimerize with at least one other transcription factor, hypoxia inducible factor-1β, and the resulting dimer binds to the hypoxia-response element found on a separate but overlapping battery of host-defense genes to those regulated by the XRE.[23]

Glucobrassicin is a glucosinolate derived from tryptophan, and when it undergoes hydrolysis, the major product is indole-3-carbinol. Diets containing indole-3-

Table 7.2 A partial list of genes containing an antioxidant response element (ARE) or a xenobiotic response element (XRE). More complete lists are reviewed in 60, 61.

ARE	XRE
NADPH:Quinone reductase	NADPH:Quinone reductase
Glutathione-S-transferase-Ya	Glutathione-S-transferase-Ya
Glutathione-S-transferase-A2	Glucuronosyl transferase
Glutathione-S-transferase Pi	Cytochrome P450 1A1/2
γ-Glutamylcysteine synthetase (H & L)	Cytochrome P450 1B1
Glucuronosyl transferase	Cytochrome P450 C
Heme oxygenase-1	Superoxide dismutase
Metallothionein	Xanthine oxidase/xanthine dehydrogenase
Apolipoprotein-A1	
Ferritin L	
Thioredoxin	
Thioredoxin reductase	

carbinol cause induction of detoxification enzymes via the XRE, producing bifunctional induction. Although only a weak ligand for the AhR itself, when subjected to the acidic environment of the stomach, indole-3-carbinol forms multiple acid condensation products.[24] These I3C acid condensates are capable of more potently ligating AhR and activating transcription through the XRE.[22] Two products, diindolyl methane and indole-3-carbazole, have been isolated and studied individually, and have been found to be agonists of the XRE on CYP1A genes.[25,26]

A controversy exists in the literature as to whether dietary bifunctional inducers can be considered healthy, based on the idea that P4501A, which is upregulated by bifunctional inducers via the XRE, is able to activate many polycyclic procarcinogens. However, it appears that the bifunctional inducers such as the indoles and some other dietary bioactive components may inhibit XRE-dependent pathways in carcinogenesis, possibly by acting competitively as antagonists to alternative XRE agonists,[25,26] possibly by disrupting cross-talk between the XRE and the estrogen response element. This latter idea is supported by the successful clinical trials on indole-3-carbinol in fighting breast cancer, which have led to the development of other potentially therapeutic XRE antagonists, also called selective AhR modulators.[27]

THE ANTIOXIDANT RESPONSE ELEMENT, SULFORAPHANE, AND CRAMBENE

Monofunctional inducers, which upregulate the phase II detoxification enzymes, have been found to be electrophiles, and transcriptional activation by these inducers has been traced to a cis-acting transcriptional enhancer termed the antioxidant response element (ARE) in rats and humans, and the electrophile response element (EpRE) in mice.[28,29] The molecular mechanism of ARE-dependent upregulation of phase II enzymes is not as completely understood as the mechanism of XRE-dependent bifunctional upregulation of enzymes. The ARE core sequence 5′-TGACnnnGC-3′ has been identified in the 5′ flanking sequence of several genes, including some rat, mouse, and human glutathione-*S*-transferase isoenzymes and quinone reductase (Table 7.2). Most recently the ARE has been identified in the regulatory region of thioredoxin and thioredoxin reductase genes.[30,31] These genes play an important role in regulating the sulfhydryl redox status of the cell.

When glucoraphanin is hydrolyzed by chopping broccoli in water, two products are formed, sulforaphane and sulforaphane nitrile.[32] In recent years, Talalay and colleagues have reported numerous studies on sulforaphane bioactivity and have shown that it is potent at upregulating the detoxification enzyme quinone reductase in mouse hepatoma cell cultures, and have suggested that anticarcinogenesis is related to this upregulation of detoxification enzymes.[13,17] They have shown that this activity is ARE-dependent.[33] Induction of detoxification enzymes is not the sole activity of bioactive hydrolysis products from broccoli. For example, in addition to upregulating phase II detoxification enzymes, sulforaphane is reported to inhibit mRNA synthesis and enzyme levels of several cytochromes P450, including CYP 1A1, a major cytochrome P450 involved in the bioactivation of procarcinogens.[21]

There is also considerable evidence showing that sulforaphane interacts with the regulation of the cell cycle to promote apoptosis.[34]

There are several compounds in broccoli unrelated to glucosinolates, such as dithiothiones and polyphenolics, that are capable of mediating ARE-dependent regulation of the phase II battery of detoxification genes (Table 7.2). Furthermore, whereas sulforaphane is present in substantial quantity in broccoli, it is a minor component in other crucifers. Other glucosinolate hydrolysis products, such as benzyl isothiocyanate, phenethylisothiocyanate, and allyl isothiocyanate, the predominant glucosinolate hydrolysis products of gardencress, watercress, and mustard, respectively, are not found in high amounts in broccoli. These compounds, like sulforaphane, upregulate quinone reductase and the battery of ARE/NF-E2 related factor (Nrf2) regulated genes.[35]

Another major glucosinolate of broccoli and other crucifers is progoitrin. Progoitrin hydrolysis products have been far less studied as potential anticarcinogens. This may be due to the toxicity seen when progoitrin-containing plant products were used as the sole protein source in animal feed.[36] *Crambe abysinnica* seed meal, when used as the sole source of protein in animal feeds, produces pancreatic atrophy due to the high level of nitrile in the diet.[36] The amount necessary to cause these effects is far greater than that found in any normal American diet. Chopping fresh broccoli produces the nitrile, termed cyanohydroxybutene, or crambene. Interestingly, low dietary levels of crambene administered orally to rats had a similar effect to that of sulforaphane, causing an increase in hepatic quinone reductase and glutathione-*S*-transferase without increasing P4501A activity.[37] Even the effective dose range for a single dose, 0.5 to 1.5 mmol/kg BW, was very similar to the effective oral dose range for sulforaphane.[32,37,38] It is interesting to note that crambene, unlike the other bioactive hydrolysis products, is a nitrile. Sulforaphane nitrile is essentially without activity.[32] One possible reason for this discrepancy is that crambene may rearrange to form an α, β-unsaturated ketone, an electrophile.

Like sulforaphane, crambene triggers a response at the ARE but not the XRE in *in vitro* reporter gene studies (Nho, personal communication). However, in contrast to the animal studies, in cell culture the dose required to cause a doubling of ARE reporter gene activity in human hepatoma cells is many times greater than the dose of sulforaphane required, 100 μM crambene compared with 0.6 μM sulforaphane. This low potency is consistent with the effect of crambene on quinone reductase enzyme induction in cell culture.[39] One possible reason for this is that conversion of the nitrile to a metabolite, such as the α, β- unsaturated ketone, is required for bioactivity, and that this may occur at a very slow rate in the hepatocyte. Alternatively, there may be a substantial difference in cellular accumulation between sulforaphane and crambene, because sulforaphane has been found to accumulate intracellularly in cell culture, reaching millimolar ranges following micromolar exposure.[40]

ACTIVATION OF TRANSCRIPTION FACTORS ASSOCIATED WITH THE ARE

Studies using knockout mice implicate the basic leucine zipper Nrf2 as part of the transcriptional complex directly involved in mediating ARE-dependent transcriptional

regulation of mouse glutathione-*S*-transferase and quinone reductase.[41–43] A recent publication utilized Nrf2 knockout mice and oligonucleotide array technology to identify genes regulated by Nrf2 for basal and sulforaphane-induced expression. The authors identified several previously unreported sulforaphane inducible genes under the control of Nrf2, including those involved in the inflammatory response, xenobiotic detoxification, and NADPH generation.[44] Nrf2 forms heterodimers with small Maf proteins and binds to the cognate ARE sequence. The exact Maf binding partner for Nrf2 may vary with stimulus or tissue, but MafG has been implicated as an activator and MafK as a repressor of transcriptional activation by Nrf2.[45] It has been suggested that in the cytoplasm Nrf2 may be largely bound to a protein, Keap1, which is anchored to the actin cytoskeleton.[46] Inducing agents like sulforaphane may oxidize critical sulfydryl groups of Keap1, disrupting the Keap1-Nrf2 complex and permitting Nrf2 to migrate to the nucleus, where it can interact with the 5′-upstream regulatory ARE of phase II genes and increase their transcription.[46]

Other posttranslational mechanisms may also regulate the activity of Nrf2. A role for protein kinase C and phosphatidyl inositol 3-kinase in this sequence of events may be to phosphorylate Nrf2 in response to inducer treatment, and this phosphorylation may drive release of the Nrf2 from Keap1, causing migration of Nrf2 to the nucleus and subsequent activation of ARE-driven gene trascription.[47–50] Alternatively, ERK2 and p38, members of the mitogen-activated protein kinase family, have been implicated as positive and negative regulators, respectively, in ARE-mediated induction of phase II detoxification enzymes.[51] Additionally, current studies suggest phosphorylation of Nrf2 by a protein kinase associated with the MAPK/ERK signaling cascade may lead to an increase in Nrf2 stability and transactivational activity.[52–54] Although the exact mechanism remains undetermined, the possibility exists that all or several of these mechanisms function in the release of Nrf2 from Keap1.

Recently, it has been found that, in addition to its detoxification function and its function as a biomarker for up-regulation of other phase II enzymes, up-regulation of quinone reductase by monofunctional inducers may play a role in the stabilization of p53, the protein product of a tumor suppressor gene, which induces growth arrest and apoptosis.[55] Sulforaphane has also been shown to mediate growth arrest and induce cell cycle arrest and apoptosis in many cancer cell lines, including those of human prostate, colon, and T-cell leukemia origin.[34,56,57] The exact mechanisms, and whether all the bioactivities of sulforaphane involve the ARE, are not yet understood.

QUINONE REDUCTASE IS REGULATED BY BOTH AN XRE AND AN ARE

The promoter regions of glutathione-*S*-transferase Ya and quinone reductase have been shown to possess both an ARE and an XRE.[58,59] A survey of additional genes, as they are recognized to be regulated by the ARE/Nrf2 system[60] or the XRE/AhR system,[61] will no doubt identify more genes that are regulated by both pathways. Interestingly, when rats are exposed through their diets to a mixture of the monofunctional inducer crambene and the bifunctional inducer precursor indole-3-carbinol, substantial synergism in phase II enzyme induction is seen.[39] Individual doses of crambene (50 mg/kg BW) and indole-3-carbinol (56 mg/kg BW) daily for

5 days each caused an approximate doubling of quinone reductase activity in rat liver. In contrast, quinone reductase levels in the livers of rats that were given both I3C and crambene were four- to fivefold greater than those of untreated rats.[39] These data support the possibility that a mixture of glucosinolate hydrolysis products, as found naturally in broccoli, may be more potent than the individual components.

SUMMARY

Epidemiological and animal studies suggest that a diet rich in crucifers protects against a number of cancers. These vegetables contain glucosinolates, which are hydrolyzed by a plant enzyme to form bioactive products able to upregulate detoxification enzymes. Whether or not this upregulation is causative in cancer prevention, the upregulation of detoxification enzymes serves as an excellent biomarker of exposure and effective dose of crucifers. Sulforaphane and crambene, two glucosinolate hydrolysis products from broccoli, are monofunctional inducers, up-regulating phase II enzymes through an ARE-dependent pathway. Brassica vegetables, including broccoli, all contain glucobrassicin, a glucosinolate that, upon hydrolysis, releases indole-3-carbinol. Passing through the acid stomach, indole-3-carbinol forms acid condensation products, ligands for the Ah receptor, causing bifunction induction and upregulation of both phase I and phase II detoxification enzymes through an XRE-dependent pathway. When rats were fed a mixture of crambene and indole-3-carbinol, both of which are present in broccoli, upregulation of the phase II detoxification enzyme quinone reductase in liver was not just additive but synergistic. Thus the metabolites of glucosinolates in broccoli are capable of serving as both mono- and bifunctional inducers and may have greater effects together than as isolated components. These data support the finding of epidemiological studies that a diet that includes broccoli may slow or prevent cancer. Further research into the underlying genetic regulation of detoxification enzymes will aid in determining how to optimize the health benefits of broccoli and other cruciferous vegetables.

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CHAPTER 8

Healthy Food Versus Phytosterol-Fortified Foods for Primary Prevention of Coronary Artery Disease

Douglas S. Lewis and Oksana Matvienko

CONTENTS

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ABSTRACT

High concentrations of plasma total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) accelerate atherogenesis in the teenage years, and 22-year-old men with high cholesterol levels are 5.6 times more likely to develop coronary artery disease (CAD), 6.0 times more likely to have a heart attack, and 9.6 times more likely to die during the next 40 years than those with lower cholesterol levels. Although the National Cholesterol Education Program Adult Treatment Panel recommends measuring blood cholesterol levels in all adults beginning at 20 years of age, the approach to be used to lower the cholesterol levels is still not clear. There is controversy about using hypocholesterolemic drugs as a means of primary prevention of atherosclerosis in young adults, and the American Heart Association (AHA) therapeutic lifestyle changes (TLC) diets have limited effectiveness in many individuals with hypercholesterolemia. Phytosterols are in the forefront of nutraceutical research on the development of food products. Experimental data clearly demonstrate that phytosterols in vegetarian-based diets and in fortified foods with phytosterols are highly effective at lowering plasma cholesterol in diverse populations including children, young adults and older adults. The use of phytochemicals such as phytosterols to alter the food supply appears to be one of the most promising avenues to significantly prevent and mitigate the prevalence and incidence of CAD in young adults.

INTRODUCTION

Coronary artery disease (CAD) is a major cause of morbidity and mortality leading to loss of quality of life and premature death with an economic burden of approximately $111.8 billion a year.[1] Advancements in the understanding of the pathogenesis of atherosclerosis in the 20th century resulted in the emergence of new treatments and medications,[2] which have decreased CAD mortality rates from 429/100,000 in 1963 to 173/100,000 in 1998.[3] Despite the continuing decline in CAD death rates, this disease remains an overall leading cause of mortality. Indeed, over the last few decades cardiac catheterizations and coronary angioplasty to treat CAD have increased by 355% and 285%, respectively.[1]

Strategies to prevent CAD are designed to prevent the progression of atherosclerosis in individuals without clinical symptoms of CAD (primary prevention) and to stabilize plaque lesions, to halt and reverse atherosclerosis in patients with CAD (secondary prevention). This article focuses on the primary prevention of CAD. The focus of primary prevention involves the use of statins and is aimed at "high risk" individuals in their 40s and 50s. The emphasis of primary prevention is on the reduction of either short term (< 10 years) or long term (< 20 years) absolute risk of developing CAD. However, younger adults (19- to 39-years of age) with higher relative risk due to hyperlipdemia or another risk factor are rarely considered as candidates for aggressive drug treatment, despite having a high absolute risk of

developing CAD sometime in their lives.[4] This article supports a role for phytosterol-fortified foods as a necessary part of aggressive lifestyle change in order to be effective in the primary prevention of CAD in young adults.

PREVENTION OF CORONARY ARTERY DISEASE (CAD)

Origins of CAD

Atherosclerosis is a slowly progressing inflammatory disease characterized by irregularly distributed lipid deposits in the intima of large and medium-sized arteries. Over a lifetime, atherosclerosis progresses from fatty streaks to more advanced and complicated lesions, which cause narrowing (stenosis) of the coronary arterial lumen culminating in the clinical horizon characteristics of CAD-including thrombosis and heart attack. Atherosclerosis begins in childhood[5] and is accelerated during young adulthood under the influence of multiple risk factors, many of which may be controlled to some extent with a healthy lifestyle.[6,7] Strong and McGill[8] examined the aortic and coronary arteries of 4737 males and females, aged 10 to 39 years. Early signs of atherosclerosis, fatty streaks, were found in the aortas of all subjects. Advanced lesions (i.e., fibrous plaques) were present in a few coronary arteries before age 20 but increased in number and extent in older subjects. Similar findings have been reported by others.[6,9,10]

Prevalence of Atherosclerosis in Young Adults

The prevalence of coronary atherosclerotic lesions in young people is remarkably high and increases substantially between 14 and 34 years of age. Indeed, the prevalence of significant coronary lesions with intimal thickness greater than 0.5 mm was 17%, 37% and 60% in heart transplant donors aged 13 to 19, 20 to 29, and 30 to 39 years, respectively.[11] Mahoney et al.[12] reported that 30% of men and 16% of women between the ages of 29 and 43 years had significant buildup of calcium deposits, a marker of the atherosclerosis process in coronary arteries. In contrast to atherosclerosis, the prevalence of CAD is low among 25- to 45-year-olds is 2% for males and 2.8% for women.[1]

Risk Factors for CAD

The major risk factors contributing to the progression of atherosclerosis and subsequently CAD are summarized in Table 8.1. Low plasma LDL-C results in slow advancement of atherosclerosis.[13] In contrast, elevated LDL-C plays a major role in the formation of atherosclerotic plague and subsequently is a target for prevention strategies.[4] Approximately 49% of men and 43% of women have plasma LDL-C greater than 130 mg/dl.[1] Recently, elevated plasma C-reactive protein, a marker for arterial inflammation, was reported to be another primary factor predicting the progression of coronary atherosclerosis as inflammation may accelerate the uptake of LDL particles into the intima of the arterial wall.[14] The overall risk for CAD is

Table 8.1 Risk factors for CAD, category of risk, and LDL-C target goals.[1]

A. Risk Factors that Affect the LDL-C Goal
Elevated LDL-C
Cigarette smoking
High blood pressure (140/90 mmHg or higher or on blood pressure medication)
Low HDL-C (less than 40 mg/dl)
Family history of early heart disease (heart disease in father or brother before age 55; heart disease in mother or sister before age 65)
Age (men 45 years or older, women 55 years or older)
B. Categories of Risk
I Highest risk: heart disease, diabetes, or risk score more than 20%[a]
II Next highest risk: two or more risk factors (A) and risk score 10-20%[a]
III Moderate risk: two or more risk factors and risk score less than 10%[a]
IV Low-to-moderate risk: 0 or 1 risk factor
C. LDL-C Target Goals and Recommended Therapy[b]
Category I, highest risk, LDL goal is less than 100 mg/dl
If LDL-C >100: TLC diet, maybe drug
>130: TLC diet + drug
Category II, next highest risk, LDL goal is less than 130 mg/dl
If LDL > 130: TLC diet
> 160: TLC diet for 3 months then drugs if necessary
Category III, moderate risk, LDL goal is less than 130 mg/dl
If LDL > 130: TLC diet
> 160: TLC diet for 3 months then drugs if necessary
Category IV, low-to-moderate risk, LDL goal is less than 160 mg/dl
If LDL > 160: TLC diet for 3 months then drugs if necessary

[a] Risk score calculated from Framingham Point Scores.[15]
[b] TLC = therapeutic lifestyle changes.

compounded when concentrations of LDL–C combine with arterial inflammation and other potentially modifiable (e.g., cigarette smoking, permanent weight loss) and nonmodifiable (e.g., age, gender, heredity) risk factors.

PRIMARY PREVENTION OF CAD IN YOUNG ADULTS

A candidate for aggressive lifestyle therapy is presented in Table 8.2. This young man was a member of a group of male volunteers that participated in a recent study that tested the hypothesis that soybean sterols added to ground beef as part of a daily lunch would significantly lower plasma total cholesterol and LDL-C concentrations in young mildly hypercholesterolemic men.[16] The case subject, according to the National Cholesterol Education Program (NCEP) guidelines, has a 1% 10-year risk of CAD because of a single major risk factor, family history of premature CAD, his elevated total blood cholesterol concentration and borderline, low HDL-C concentration. The risk of this subject would be categorized at Level IV-low risk. His LDL-C concentration (159 mg/dl) is below the goal of 160 mg/dl for Level IV subjects, and it is conceivable that this case subject would not receive dietary advice. Indeed, physicians are generally not compliant with the NCEP guidelines for risk

Table 8.2 Case Study: 24-year-old male with a body mass index (BMI) of 32 and family history of premature heart disease (male close relative before age 55).

Lipid Panel Results	**Percentile[a]**
Triglycerides 272 mg/dl	>95%
Cholesterol, Total 256 mg/dl	>95%
HDL-C 42.7 mg/dl	25%–50%
LDL-C (calculated)159 mg/dl	>90%
Risks	
Family History	
Risk Category	
IV. Low-to-moderate risk	
10-Year Risk Estimate	**Framingham Point Scores**
Age: 24 years,	–9
Total cholesterol: 256	+9
HDL: 42.7	+1
Nonsmoker	0
Systolic BP: < 120	0
Point total 1 = 10-year risk 1%	
Goal for LDL	
One risk factor: < 160 mg/dl	
Two or more risk factors: < 130 mg/dl	

[a] NCEP.[36]

factor assessment and counseling.[17] Even patients at highest risk are not being treated to reach the lower NCEP LDL-C goals: <15% of men and <10% of women of patients whose LDL-C was > 160 mg/dl received treatment.[18]

It is likely that, at best, a physician would instruct the subject to follow the Heart Healthy Diet, which is similar to the AHA Step 1 diet. However, there are several reasons why the case subject and others like him should be advised to make aggressive lifestyle and dietary changes to lower LDL-C to < 130 mg/d.[19,20] First, in addition to family history, the case subject has a body mass index (BMI) of 32 and plasma triglycerides (272 mg/dl), which may increase the risk of developing CAD.[21,22] Second, based on his plasma cholesterol concentration alone, the case subject is 5.6 times as likely to develop CAD, 6 times as likely to have a heart attack, and 9.6 times as likely to die by 64 years of age as similar aged individuals with plasma cholesterol < 160 mg/dl.[23] Third, two thirds of CAD-related mortality occurs in individuals who had plasma cholesterol > 200 mg/dl when they were young adults.[24] Finally, lowering LDL-C to below 130 mg/dl, or even lowering LDL-C by 20% would be expected to decrease substantially the risk of CAD events.

The significance of early effective primary intervention of atherosclerosis in young adults between 18 and 36 years of age is that the acceleration of atherosclerosis would be delayed, thereby preventing the occurrence of CAD, which is now 20.8% in men 44 to 65 years of age resulting in a subsequent decrease in

CAD-related deaths occurring before 65 years of age. There are approximately 78 million 20- to 39-year-olds in the U.S. today compared with 72 million in 1980.[25] More than 25% or 19.5 million have plasma LDL-C greater than 144 mg/dl, making this population a vast reservoir of future patients to burden health-care facilities and resources as they age into their 50s and 60s. Effective prevention strategies focused on the young adult age group could potentially decrease the incidence of advanced atherosclerosis or considerably delay its onset, improve quality of life at older ages, and ease the financial burden associated with atherosclerotic diseases, which is approximately $146.2 billion a year for coronary heart disease and stroke combined.[1]

DIETARY STRATEGIES FOR THE PREVENTION OF CAD

The current recommended diet options to lower LDL-C concentrations depend on the level of risk and the LDL-C goal. The available diet options include the Healthy Heart[26] and Therapeutic Lifestyle Change (TLC) diets. Usually these primary prevention strategies address older adults, well past the age span in which atherosclerosis and even significant lesion development has already occurred. The capability of dietary treatments to lower LDL-C concentrations will depend on three major factors: (1) one's compliance with the dietary treatment, (2) the extent to which one's habitual diet contributes to the elevated LDL-C level, and (3) the influence of genetics on LDL-C concentration and the extent to which one is able to lower his or her LDL-C level in response to the diet treatment (gene by diet interaction). The importance of genes is evident from the failure of AHA diets to lower plasma LDL-C concentrations in children with familial hypercholesterolemia[27] and the considerable individual variability in the change in LDL-C concentrations among patients with hyperlipidemia when fed a low-fat diet.[28] In response to a very low-fat diet 28% of 105 subjects had < 5% decrease in LDL-C and 18% actually increased LDL-C concentrations.[28] Additional barriers to adapting to cholesterol lowering diets include the lack of knowledge, lack of motivation, lack of access to care, and cultural and social factors.[18]

Heart Healthy Diet

The Heart Healthy Diet promoted by the National Heart, Lung, and Blood Institute (NHLBI) is similar to the AHA Step 1 Diet and is outlined in Table 8.3. The principle

Table 8.3 The Heart Healthy Diet Guidelines.

- 8–10% of the day's total calories from saturated fat
- 30% or less of the day's total calories from fat
- Less than 300 mg of dietary cholesterol per day
- Limit sodium intake to 2400 mg per day
- Just enough calories to achieve or maintain a healthy weight and reduce your blood cholesterol level

dietary feature of the Heart Healthy Diet is to consume no more than 8% of total energy as saturated fat. Using the equations developed by Hegstead et al.[29] and Mensink and Katan,[30] it was estimated that decreasing saturated fat intake from about 12% (estimated for young adults from NHANES, 1988 to 1994) to 8% of total energy would likely lower LDL-C by 5 to 7 mg/dl and lower HDL-C by about 2 mg/dl, consistent with clinical feeding trials with the AHA Step 1 diets.[31,32] In controlled human trials, low-fat and low-cholesterol diets in which saturated fat was replaced with mono- and polyunsaturated fatty acids decreased serum LDL-C concentrations by 7 to 15%.[33,34] However, placing free-living patients in controlled clinical experiments is not a practical primary prevention strategy to lower LDL-C. Tang et al.[35] concluded from 19 studies with free-living individuals that dietary advice for fat intake lowered total plasma cholesterol by 1.8 to 4.1%.

The case subject's habitual fat intake determined from a food frequency questionnaire was 34.8% of total energy intake as fat, 14% as saturated fat (51 g saturated fat), and <200 mg/d cholesterol. Major dietary sources of the subject's saturated fat intake were red meat (2 servings), diary (3.6 servings), and oil and fat (2.3 servings). The subject reported eating one and two servings of fruit and vegetables per day, respectively. In order to meet the Heart Healthy Diet recommendations for saturated fat the case subject would need to decrease his daily saturated fat intake by 22 g. Because a relatively small number of foods contribute to the majority of the atherogenic potential index,[37,38] the chief fat reduction strategies could include replacing whole milk with 2 or 1% or skim milk; fatty meats with lean meat; and all of the fat foods with either low-fat or fat-free foods. Computer modeling studies showed that in males only the more restrictive substitution of all the fat-containing foods with low- and fat-free foods was sufficient to decrease their saturated fat intake from 11.7% to 8%, whereas skim milk and lean meat substitutions were not.[39] This was confirmed in a study of 117 adults who used two and three fat-reduction methods to achieve a saturated fat intake of < 10%.[40] Not only do these strategies involve substantial changes in the case subject's diet, many recommended recipes involve considerable time because they originate from individual ingredients and often do not include the foods commonly consumed by young adults (i.e., pizza, hamburgers, processed meals).

If the case subject could significantly alter his habitual diet toward skim milk, lean meat, and low-fat products, it would be reasonable to expect about a 6% drop in saturated fat intake, which would lower his LDL-C from 159 mg/dl to about 146 mg/dl,[29,30] substantially short of the 130 mg/dl goal.

Therapeutic Lifestyle Changes (TLC) Diet

The National Cholesterol Education Program (NCEP)-recommended steps to lower the risk of CAD, collectively referred to as the TLC, are presented in Table 8.4. These guidelines are typically used for higher-risk individuals with elevated plasma cholesterol concentrations, established cardiovascular disease, and more than one major risk factor. The TLC dietary guidelines are relatively stringent, limiting daily total and saturated fat intakes to <30% and 7% of total energy and cholesterol

Table 8.4 Lowering cholesterol with therapeutic lifestyle changes (TLC).[a]

The TLC Diet
Low saturated fat (<7% calories)
Low cholesterol (< 200 mg/d)
Enough calories to maintain weight and avoid weight gain
Increase soluble fiber intake[b]
Eat certain foods with plant sterols[b]
Weight Management
Lose weight (if overweight)
Physical Activity
30 min of physical activity on most if not all days
Drug Treatment[c]
Statins
Bile acid sequestrants
Nicotinic acid
Fibric acids

[a] National Heart, Lung, and Blood Institutes, National Institutes of Health.[36,41]
[b] If LDL-C is not lowered enough (< 130 mg/dl).
[c] With two or more risk factors and diet fails to reach LDL-C target.

intake to <200 mg/d. Although the TLC diet is a modification of the previously recommended AHA Step 2 Diet, only a few studies have demonstrated the effectiveness of the TLC diet.[42,43] The AHA Step 2 Diet decreased plasma total cholesterol concentration by 10 to 15% in metabolic ward studies, but only by 6% in free-living subjects.[44] The discrepancy in cholesterol reduction between metabolic ward and free-living subjects was attributed to limited compliance with dietary advice by the free-living subjects. In other studies, the AHA Step 2 Diet lowered LDL-C concentrations by about 9% but also decreased HDL-C and increased plasma triglyceride concentrations to such an extent that the benefit of lowering LDL-C may have been mitigated.[31,32] Adoption of this diet by the case subject would likely lower his LDL-C concentrations by as much as 10%, still much higher than the 130 mg/dl target.

Recently, Jenkins et al.[45] demonstrated that a diet very similar to the TLC diet, which included soy protein in addition to phytosterols and soluble fiber, lowered plasma LDL-C by 29% with minimal change in HDL-C in individuals with hyperlipidemia who were already consuming a low-saturated fat, low-cholesterol diet. This TLC diet was described as satisfying and filling, and the foods could be purchased at grocery and health food stores. However, this extreme diet, consisting of 16 g of soluble fiber from oats, barley, and psyllium; 45 g of soy protein; 200 g of eggplant; and 100 g of okra daily, and 30 g of raw almonds, is not likely to lead young adults to the long term compliance that would be necessary to maintain low LDL-C concentrations sufficient enough to suppress atherosclerosis.

Vegetarian Diets

Adoption of a vegetarian lifestyle, although extreme, is another dietary approach to lower LDL-C levels. Although it is well established that vegetarians have markedly lower plasma LDL-C concentrations than nonvegetarians, few studies have examined the effect of an omnivore adopting a vegetarian diet to lower plasma LDL-C concentrations. Five hundred omnivore men and women with hypercholesterolemia who switched to a low-fat vegetarian diet providing 60 g of fiber and including both daily exercise and stress management lowered plasma cholesterol levels by 11% within 12 d. In addition, their HDL-C decreased to a similar extent as did LDL-C.[46] Clearly, a vegetarian lifestyle is effective in lowering total cholesterol and LDL-C cholesterol, especially if sustained for a long period of time. However, such a diet is highly restrictive and would require dedication and extensive modification of dietary habits for a lifetime. If the case subject had an average response comparable to the 500 individuals with hyperlipidemia studied by McDougall et al.,[46] the LDL-C would be lowered from 159 to about 141 mg/d. While suitable for certain individuals, this extreme approach would not be practical for primary prevention methods targeted to an at-risk population.

Food and Drug Administration (FDA) Health Claims

Food and Drug Administration (FDA) health claims[47] show that a relationship between a nutrient(s) or other substances and a disease or health-related condition, i.e., foods with soy protein, soluble fiber, and plant sterols, helps lower the risk of coronary heart disease (Table 8.5). These claims help individuals to select foods as either part of following recommended dietary guidelines, such as the TLC and Heart Healthy diets, or part of a self-treatment in response to "You need to eat less fat and cholesterol and more fiber."

Table 8.5 FDA health claims for lowering the risk of CAD.

Claim: May Reduce the Risk of Coronary Heart Disease	Typical Foods
Diets low in saturated fat (< 10% of calories) and cholesterol (300 mg/d)	Fruits, vegetables, skim and low-fat milks, cereals, whole-grain products, and pastas (not egg pastas)
Diets low in saturated fat and cholesterol and rich in fruits, vegetables, and grain products that contain fiber, particularly soluble fiber	Fruits, vegetables, and whole-grain breads and cereals
Diets low in saturated fat and cholesterol that include 3 g of soluble fiber from whole oats per day	Oatmeal cookies, muffins, breads and other foods made with rolled oats, oat bran, or whole oat flour; hot and cold breakfast cereals containing whole oats or psyllium seed husk; and dietary supplements containing psyllium seed husk
Diets low in saturated fat and cholesterol that include 25 g of soy protein per day	Soy beverages, tofu, tempeh, soy-based meat alternatives, and possibly some baked goods
Diets low in saturated fat and cholesterol that include two servings of foods that provide a daily total of at least 3.4 g of plant stanol esters in two meals	Spreads, salad dressings, snack bars, and dietary supplements in softgel form

Soluble Fiber

One of the oldest health claims is that food products containing fiber and soluble fiber lower the risk of coronary heart disease. However, recent evidence suggests that within the typical range of intakes (2 to 10 g/d), soluble fiber is only marginally effective,[48] transiently effective,[49] or not effective at all[50,51] in lowering plasma LDL-C concentrations. The consumption of 3 g of β-glucan, which is equivalent to 20 g of oat bran, did not decrease plasma LDL-C concentrations.[51,52] Foods containing soluble fiber appear to effectively decrease plasma LDL-C concentrations by 8 to 12% when they replace foods high in saturated fat and cholesterol.[48,53–56] The weak ability of typical soluble fiber intakes to lower cholesterol is one reason the NCEP recommends 10 to 25 g of soluble fiber per day. However, such intakes of soluble fiber would require substantial changes in the habitual diet, e.g., one has to eat 10 single-serve packets of oatmeal cereal to ingest the minimum 10 g of soluble fiber. It would be difficult to obtain 25 g of soluble fiber per day. Further, the value of adding fiber to diets is unclear because processing methods for fiber and fiber-rich foods can influence the viscosity properties of soluble fiber. Viscosity is thought to be a contributing factor to the mechanisms of action of soluble fiber on cholesterol metabolism.[53,57]

High-fiber diets including such foods as high-fiber cereals are associated with a lower risk of heart disease, but this lower risk may not be a result of lowering LDL-C.[58,59] Nevertheless, eating fiber-rich foods provides modest cardiovascular benefits by supplanting saturated and trans fats and cholesterol, but the magnitude of these effects is anticipated to be quite small (i.e., three apples, three bowls of oatmeal can decrease concentrations of total plasma cholesterol by 2% and LDL-C <2%). The current FDA health claims about soluble fiber appear to be overly optimistic because only very high intakes will have an appreciable effect on LDL-C concentrations, and such intakes would require a complete reconstruction of a diet with the likelihood of poor compliance. If the case subject incorporated oatmeal into his diet, plasma LDL-C concentrations would decline but fall far short of the 130mg/dl goal.

Soy Protein

Kerckhoffs et al.[60] recently reviewed the use of soy protein to lower cholesterol. Although soy protein is currently under investigation for possible hypocholesterolemic properties,[61,62] foods containing soy protein and carrying the FDA claim that "Diets low in saturated fat and cholesterol that include 25 grams of soy protein a day may reduce the risk of heart disease" are heavily marketed. Recent studies have clearly demonstrated that the consumption of 47 g of soy protein per day only modestly affects LDL-C concentrations.[63,64] Substitution of animal protein (casein) with 30 to 50 g of soy protein per day in diets that provided about 9% of energy from saturated fat and <300 mg cholesterol lowered plasma LDL-C by about 7% in subjects with hyperlipidemia.[64] Not only does this modest decrease fall short of the 130 mg/dl LDL-C goal, such a dietary change to accommodate up to 50 g of soy protein is not likely tenable for the great majority of young adults to maintain over a long period of time.

DIETARY PHYTOSTEROLS AND HYPERCHOLESTEROLEMIA

The use of phytosterols as part of diet therapy to lower LDL-C and risk for CAD is supported by both the NCEP guidelines and the FDA's health claim about the relationship between the consumption of plant sterol and stanol esters and reduced risk of coronary heart disease. This section briefly discusses the efficacy of the phytosterols as arguably the most effective dietary approach to lower plasma LDL-C in most types of subjects with the least impact on habitual diets.

Efficacy of Phytosterols to Lower Plasma Cholesterol Concentrations

Several excellent reviews have summarized the well-established ability of phytosterols to lower serum cholesterol concentrations in humans.[60,65–67] The consumption of food products supplemented with 1.6 to 3.5 g/d of phytosterols has been shown to consistently decrease serum total cholesterol and LDL-C concentrations by up to 15%, without affecting HDL-C and triacylglycerol concentrations (Table 8.6). About 1 g of mixed phytosterols per day is needed to decrease cholesterol by at least 5%, but phytosterol intakes exceeding 2.5 g/d decrease plasma cholesterol and LDL-C by up to 15%.

In many human trials, an effective daily dose of phytosterols was equally divided among three meals. Recently, two studies demonstrated that a single dose of phytosterols (~2.7 g/d) given once a day was also effective in lowering LDL-C concentrations by 15%.[16,70] Regular phytosterol consumption for 1 year maintained lower LDL-C concentrations in children[27] and adults.[73,78] Notably, phytosterols significantly lowered LDL-C concentrations in normal and hypercholesterolemic subjects, and significantly enhanced the LDL-C lowering effects of both low-fat and low-cholesterol diets[73,76,79] and statins.[80] Indeed, the effectiveness of phytosterols to exert effects beyond or in addition to statins has led to suggestions that consuming phytosterols could minimize statin doses in some patients.[81,82]

Mechanism of Action of Plant Sterols

Like cholesterol, most dietary phytosterols are 4-desmethylsterols with only small differences in the aliphatic side chain configurations. Phytosterols have one unsaturated bond in the steroid nucleus ring. Saturation of the bond with hydrogen results in the formation of the related compounds called stanols.[83] Stanols were once thought to be more effective than sterols in lowering cholesterol, especially when consumed in high doses, but at lower doses both phytosterols and phytostanols decrease LDL-C similarly.[77]

Phytosterols are partially absorbed in the small intestine. Human absorption efficiency is estimated to be approximately 10% for campesterol and campestanol, 4 to 5% for sitosterol and stigmasterol, and negligible for sitostanol.[83,85] The absorption of cholesterol ranges from 33 to 60%.[86,87] Structural features, including the length and configuration of the aliphatic side chain of phytosterols, are thought to contribute to the differences in the absorption efficiency between phytosterols and cholesterol.[83,84] Phytosterols apparently displace cholesterol by mass action and

Table 8.6 Effects of plant sterol or stanol treatment on plasma total (TC) and LDL-cholesterol concentrations in humans: results of selected randomized, double-blind studies of healthy normocholesterolemic (NC) and hypercholesterolemic (HC) males (M) and females (F).

Subjects	Study Design	Treatment	Decrease[a] in LDL (%)	Decrease[a] in TC (%)	Ref.
36 M HC	PC 4 weeks	2.7 g PSE/d in one dose/d ground beef	14.6	9.3	16
60 M, F NC 36 ± 14 years	PC 3 weeks	3 g PSNE/d in three doses/d in low-fat yogurt	13.7	8.6	68
42 M, F NC, HC 55 ± 9 years	PC, CO 4 weeks	2 g PSE/d in three doses in margarine	10	7	69
39 M, F NC, HC 18–65 years	PC, CO 4 weeks	2.5 g PSNE/d one dose/d 2.5 g PSNE/d in three doses/d in margarine	10 10.2	6.4 6.6	70
34 M, F HC 30–65 years	RM 4 weeks	2 g PSNE or PSE/d in three doses/d in margarine	7.3–9.2	10.4–12.7	71
22 M, F HC 50 ± 11 years	SB 4 weeks	0.8, 1.6, 2.4, 3.2 g PSNE/d in three doses in margarine	1.7–10.4[b]	2.8–11.3[b]	72
55 M, F HC 43 ± 8 years	8 weeks	~2.3 g PSNE/d in three doses/d in margarine	8.6–13.7	8.1–10.6	73
100 M, F NC, HC 37 ± 10 years	PC 3.5 weeks	0.83, 1.61, 3.24 g PSE/d in three doses/d in spread	6.7–10	5–6.8	74
23 F NC, HC 53 ± 6 years	5–6 weeks	2.43, 3.16, 3.18 g PSNE/d in three doses/d in margarine and butter	9–11	5–7	75
32 M HC 25–60 years	30 days	1.7 g PSNE-PSE mix/d in three doses/d in margarine	15.5	9.1	76
100 M, F NC, HC 45 ± 13 years	3.5 weeks	1.5–3.3 g PSE or PSNE/d in three doses/d in margarine	14	8	77

Note: PC = placebo controlled; PSE = plant sterol esters; PSNE = plant stanol esters; CO = cross-over; RM = repeated measures; SB = single blind.

[a] Significant at $p < 0.05$.

[b] Significant at $p < 0.05$ for sterol doses ≥ 1.6 g.

because they have a greater affinity for micelles than cholesterol.[65] The displacement of cholesterol from micelles can lower cholesterol absorption up to 50%.[65] The optimal ratio of phytosterols:cholesterol to maximally inhibit cholesterol absorption is not clear because of considerable variability in gut cholesterol levels as most gut cholesterol comes from endogenous sources (bile). Also, phytosterols may have a long residence time in the enteocyte and possibly inhibit cholesterol absorption in the enterocyte in addition to the gut lumen.[65] Clinical studies suggest that about 2 to 3.4 g of phytosterols per day maximally lower LDL-C (Table 8.6). The inhibition of cholesterol absorption by phytosterols leads to increased endogenous cholesterol

synthesis,[88] hepatic LDL receptor expression,[89] and decreased LDL production rates.[90] However, phytosterols do not appear to be as effective in lowering to LDL-C concentrations in subjects who have poor cholesterol absorption rates. Phytosterols are also not as effective in inhibiting cholesterol absorption when taken in forms that are not easily dispersed during digestion. For example, macrocrystals of phytosterols do not readily mix with bile salts or oil, which might lower the effectiveness of phytosterols.[65]

Potential Adverse Effects of Phytosterol Consumption

Phytosterols are safe when consumed in moderate amounts,[91] such as those used in human studies. In humans, phytosterol intakes of up to 25 g/d for several months were not associated with adverse health effects.[92] Phytosterol-fortified foods should be avoided by individuals with phytosterolemia, an extremely rare genetic disorder characterized by unusually high rates of intestinal absorption of phytosterols and an increased risk of premature atherosclerosis.[93]

A concern has been raised that phytosterol doses that are effective for cholesterol reduction may impair the absorption and lower blood concentrations of fat-soluble vitamins and antioxidants. A number of studies showed that phytosterols had no effect on plasma concentrations of vitamin D, retinol, or plasma-lipid-standardized alpha-tocopherol.[71,73,94,95] Moreover, the reports of the effect of phytosterols on concentrations of blood carotenoids (lutein, lycopene, and alpha-carotene) are controversial.[68,73,75,77,96] There seems to be general agreement that phytosterol doses >1 g/d significantly decrease LDL-C standardized beta-carotene concentrations; however, it remains to be determined whether a reported 15–20% reduction in beta-carotene due to phytosterol supplementation is associated with adverse health effects.[96] Noakes et al.[97] found that consumption of one or more carotenoid-rich vegetable or fruit servings a day was sufficient to prevent lowering of plasma carotenoid concentrations in 46 subjects with hypercholesterolemia treated with 2.3 g of either sterol or stanol esters.

ROLE OF PHYTOSTEROLS AND PHYTOSTEROL-FORTIFIED FOOD IN THE PRIMARY PREVENTION OF CAD IN YOUNG ADULTS

Most experts recommend a food-based dietary approach to lower plasma cholesterol levels. Indeed, the FDA begins all claims about the role of diet and heart disease with "…a diet low in saturated fat and cholesterol…." Such a diet normally would include many vegetables, fruits, and limits on animal products and can also include 25 g of soy protein along with 3 to 6 g of soluble fiber. However, the role of such advice should be evaluated in terms of compliance with the advice as well as the potential efficacy of the recommended nutrition changes. The experimental evidence supporting dietary approaches to lower plasma LDL-C concentrations comes from controlled feeding trials devoid of many practical barriers[18] that are likely to reduce the effectiveness of prescribed diets in real-life situations. Further, the efficacy of recommended diets depends on compliance and the extent that

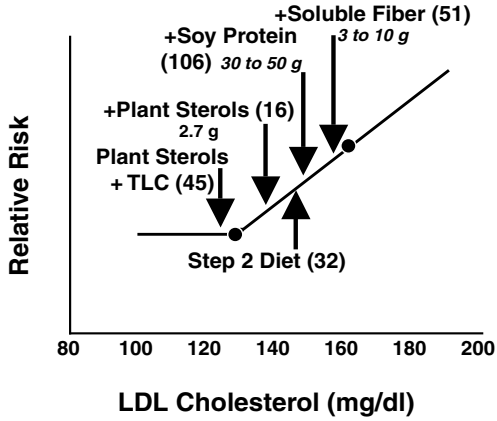

Figure 8.1 Typical expected changes in plasma LDL-C concentrations in response to diet and statin therapy based on cited references. Step 2 diet effects;[31,32] soluble fiber;[48,51,52] soy protein;[64] plant sterols;[16] and plant sterols and TLC.[45]

elevated baseline LDL-C concentrations were caused by diet. The estimated effectiveness of the common dietary approaches, including general diet recommendations, TLC diet, soy protein, soluble fiber, and phytosterols alone and in combination with diet on plasma LDL-C concentrations for the case subject, are summarized in Figure 8.1. Clearly, phytosterols can both provide leverage to cholesterol lowering diets and be quite effective alone with little perturbation to the habitual diet. For these reasons, phytosterols should be paramount in any dietary advice and complementary to pharmaceutical approaches to lower CAD risk by lowering LDL-C concentrations.

The unique role of phytosterols is that they can be incorporated into diets in much smaller quantities than soluble fiber, soy protein, or any other nutrient. Phytosterols are more effective at lowering LDL-C, can be put into foods without detection by subjects,[16] and can be delivered in foods such as spreads that are consumed in small quantities. An argument is put forth that phytosterols should be foremost in the TLC diet recommendations (not at last resort) because of likely greater compliance and because of the effectiveness of relatively small quantities of phytosterols to lower LDL-C without affecting HDL-C or triglycerides concentrations.

Barriers to Primary Prevention of CAD

The practical complexity involved in incorporating soluble fiber, soy protein, and phytosterols into a low-fat and low-cholesterol diet may limit compliance and adherence to meet various nutrition counseling guidelines.[98,99] Individuals develop food preferences within a complex sociocultural environment and exhibit innate preferences for highly palatable foods rich in sugar and fat,[100,101] with taste as the main influence on food selection.[100,101] Moreover, one study demonstrated that young adult food preferences depend more on taste than healthiness.[102] All these factors compound the problem of compliance to NCEP recommendations that emphasize replacing highly palatable, habitually consumed foods with less palatable but healthier

starches and grains.[103] Indeed, only 20% of 13,777 individuals with hypercholesterolemia reported intakes of fat, saturated fat, and cholesterol consistent with the NCEP Guideline,[104] and in a smaller study compliance with NCEP recommendations in free-living hypercholesterolemic subjects was poor.[105]

Compliance with the nutrition guidelines is increased in individuals who have a college degree and/or had a previous cardiovascular event.[106] However, the presence of cardiovascular disease in young adults is rare, only about 31% of young adults are attending college, and only one of four Americans older than 25 years has completed a bachelor's degree.[25] Considering these and other barriers involving physician compliance,[18] it is not surprising that primary prevention programs that use education, special diets, and drugs to lower cardiovascular risk factors in adults are largely ineffective.[107]

Drewnowski[103] suggested that dietary interventions should consider the sensory pleasure response to foods in addition to sociocultural variables. Phytosterols added to foods, such as lean ground beef, were imperceptible to subjects,[16] suggesting that phytosterols can be incorporated into a wide variety of palatable foods subsequently increasing the likelihood of compliance.

Phytosterol-Fortified Foods

The intake of phytosterols in the Western diet is comparable with that of cholesterol and ranges from 160 to 360 mg/d, with vegetarians consuming 400 to 500 mg/d.[83] The variability in phytosterol intakes has been suggested to contribute to variability in cholesterol absorption among individuals.[108] The most common naturally occurring phytosterols (beta-sitosterol, campesterol, and stigmasterol) are principally found in vegetable oils, seeds, nuts, cereals, beans, and some fruits and vegetables.[109] Naturally occurring plant stanols are minor components in plants compared with the sterols.

For food applications, stanols are synthetically derived from plant sterols. Although the naturally occurring amounts of phytosterols in 30 g of corn oil are sufficient to inhibit cholesterol absorption,[110,111] it is not clear whether natural intakes of phytosterols significantly lower plasma LDL-C concentrations. The relatively greater intake of phytosterols by vegetarians may contribute to the lower LDL-C levels commonly observed in this population. It would, however, be nearly impossible for most individuals to obtain more than 1 g of phytosterols from food without ingesting large quantities of food. Corn oil is a rich source of phytosterols, yet one would have to consume about 100 g (900 kcal) of corn oil per day in order to obtain 1 g/d of phytosterols.

An alternative approach entails increasing the consumption of phytosterol-fortified foods. Indeed, phytosterols have been added in gram quantities to experimental regular and reduced-fat margarines, salad dressings and low-fat yogurt. A landmark study published in the *New England Journal of Medicine* demonstrated that the stanol esters in a spread product decreased plasma LDL C concentrations by 14%.[78] After reviewing 15 human clinical trials, the FDA concluded that there was sufficient evidence for a consistent, clinically significant effect of plant sterol and stanol esters on blood total and LDL-C in both mildly and moderately hypercholesterolemic populations.[47] Subsequently, Lipton, a subsidiary of Unilever, and McNeil Consumer

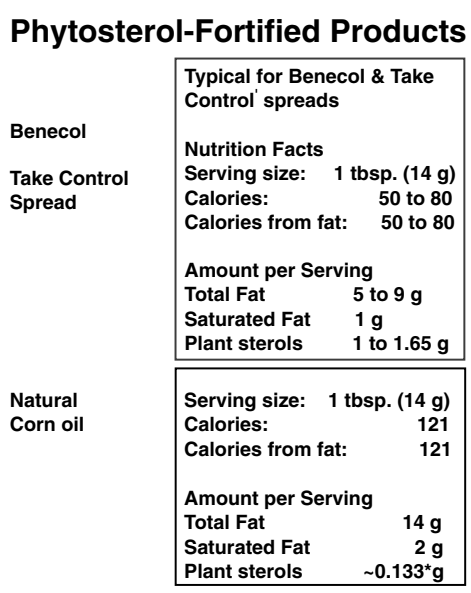

Figure 8.2 Most commonly available sources of phytosterols that would provide relatively large doses of phytosterols known to lower LDL-C concentrations in humans.

Healthcare, a division of Johnson & Johnson, manufactured and marketed Take Control®[112] and Benecol®[113] spreads, respectively. Current Benecol products include Benecol Buttery Taste Spread, Benecol Light Spread, Benecol Olive Spread, Benecol Light Cream Cheese Style Spreads, Benecol Low Fat Bio Yogurts, Benecol Snack Bars, and, as a supplement, Softgels® (a nonfood supplement).

The nutritional content of the most commonly available phytosterol-fortified spreads and corn oil, one of the richest natural sources of phytosterols, is depicted in Figure 8.2. One problem with adopting the TLC diet is the limited selection of phytosterol-fortified foods that is available to consumers. Another problem is that young adults eat less butter and margarine than do older adults[37] and thus may not include the phytosterol-fortified table spreads in their diet. The third problem is that the currently available phytosterol-fortified table spreads are high in fat and low in nutritional value (Figure 8.2). Consumption of the recommended three servings of the normal fat versions of Take Control or Benecol would provide 22 to 45% of the recommended daily amount of fat (67 g) for an individual consuming a 2000 kcal diet. Adding phytosterols to healthier foods that are lower in fat and high in nutrition value would offer more options for consumers, better nutrition, and perhaps greater compliance with dietary guidelines.

Value of Phytosterol-Fortified Lean Beef for the Primary Prevention of CAD

Lean ground beef is an excellent food vehicle for delivering an effective dose of phytosterols as part of a population-based primary prevention of CAD. Beef and

beef products are the major source of protein for young adult Caucasian men.[114,115] Unfortunately, beef is also a major source of saturated fat in young adults,[37,114] and young adults who consume more red meat were more likely to have elevated plasma LDL-C concentrations than those who consume little red meat.[116] However, the consumption of lean red meat was not associated with elevated LDL-C concentrations,[115–117] suggesting that the meat fatty acids are the likely nutrients in red meat that are associated with elevated LDL-C. Dietary advice to replace fatter cuts of meat with leaner cuts has been effective in lowering LDL-C concentrations.[117] According to the AHA, lean ground beef (15% fat by weight) as part of 6 ounces of cooked meat daily can be part of a healthy diet.[118] Second, free-living subjects with hypercholesterolemia who switched to lean cuts of red meat to meet dietary guidelines better lowered their LDL-C concentrations by 1 to 5%.[117]

The consumption of lean ground beef fortified with 2.7 g of phytosterols for 4 weeks lowered LDL-C concentrations by 15% (from 156 to 135 mg/dl) in 17 mildly hypercholesterolemic young men.[16] In contrast, 17 subjects fed nonfortified lean ground beef lowered their LDL-C levels by only 1% (not significant). In addition to leveraging a very modest effect of lean beef on LDL-C levels, the phytosterols added to beef were delivered as part of a more complete nutrition package that provided 29 g of protein and heme iron, vitamin B_{12}, niacin, and zinc.[115] The phytosterol-fortified lean ground beef has less total fat and is considerably more nutritious than the currently marketed phytosterol-fortified spreads. A greater variety of foods containing phytosterols will increase compliance to recommendations. Benecol now includes a variety of products including low-fat versions, and adding phytosterols to diverse foods, such as bread and sausage, is also effective at lowering LDL-C concentrations.[119]

In summary, lean ground beef is an excellent vehicle for delivering a dose of phytosterols to effectively lower plasma total cholesterol and LDL-C concentrations, while also providing 29 g protein and only 13 g of fat and 6 g saturated fat.[16] Lean ground beef supplemented with phytosterols could become part of a variety of hypocholesterolemic-functional foods that can comprise part of the dietary recommendations to lower the risk of heart disease in young adults with mild hypercholesterolemia. The use of phytosterol functional foods is consistent with a recent quote of Dr. Ernest Shafer[34] about changing the food supply: “In our view, diet assessment is best done with food records, and the most effective way to change dietary intake is to alter the food supply, by providing specific foods or supplements, or to provide prepared meals to the consumer.” The use of phytochemicals such a phytosterols to alter the food supply appears to be one of the most promising avenues to significantly prevent or mitigate the prevalence and incidence of CAD in the young adult to older adult. The strength of effectiveness to lower plasma cholesterol by relatively small quantities of phytosterols, combined with the minor change to the diet needed portend a high level of compliance. The use of phytosterols in a wide variety of foods should be foremost in any diet approach to lower LDL-C concentrations and not a third or fourth option as is currently the case with the NCEP guidelines.

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CHAPTER 9

Vascular Effects of Resveratrol

Joseph M. Wu, Xiaohua Lu, Junqiao Guo, and Tze-chen Hsieh

CONTENTS

ABSTRACT

Atherosclerosis (AS) is a leading cause of coronary heart disease (CHD) in most developed countries. Over the past 3 decades, significant progress has been made in reducing CHD-related mortalities through concerted efforts to modify diet and life-

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style factors that increase the risk of CHD. Whereas the past focus has primarily been on reducing saturated fat and cholesterol intake, there is mounting evidence that other dietary factors may play a supporting role in the prevention of heart disease. These include dietary fiber, whole grains, plant sterols, and a host of phytochemicals.

Epidemiological studies show an association between low to moderate consumption of red wine and reduced risk for CHD. This phenomenon is known as the French paradox and may be mediated, in part, by the phytochemical resveratrol. The mechanism underlying the cardiomodulatory effects of this polyphenol has not been completely elucidated but may be partly attributable to the broad spectrum of biological responses it elicits.

This chapter summarizes our recent studies on resveratrol, with regard to its effects on (1) oxidation of low-density lipoprotein (LDL), (2) cells affecting development of the AS, and (3) experiments using hypercholesterolemic rabbits. Furthermore, we report preliminary results of the effects of resveratrol in human aortic endothelial cells (HAEC). In these cells, RT-PCR analysis shows that resveratrol elicits an increase in p38 MAP kinase, concomitant with significant induction of heat shock protein 27 (Hsp27). We hypothesize that resveratrol may confer cardioprotection by functioning as a pleiotropic cellular effector.

INTRODUCTION

Coronary heart disease (CHD) is the leading cause of death in the U.S., contributing to more deaths than all other causes combined. The pathogenesis of atherosclerosis (AS) is integrally linked to the interplay between structural integrity of the arterial wall and hemodynamic, thrombotic, and lipid variables.[1,2] Since the 1970s, immense strides have been made in reducing CHD-related mortalities as a result of efforts to modify diet and lifestyle factors known to increase the risk of CHD. These efforts have principally been directed at reducing cigarette smoking and lowering consumption of saturated fat and cholesterol in the diet. In more recent years, focus has shifted to dietary fibers, whole grains, plant sterols, and a host of phytochemicals, owing to the recognition and acceptance that they also may play a supporting role in heart disease prevention (Figure 9.1).[3–5]

Epidemiological Evidence of the Association between Alcohol (Red Wine) Use and Risk of CHD

Epidemiological studies have shown an association between low to moderate consumption of red wine and reduced risk for CHD.[6–10] The evidence of apparent compatibility between consumption of a high-fat diet and low incidence of CHD is best illustrated with data from certain regions of France (the French paradox), which has led to the suggestion that consumption of red wine may confer protection against CHD.[11–13] A negative correlation between CHD and alcohol consumption was noted more than two decades ago. Numerous recent studies have validated a statistically significant inverse relationship between the two factors.[14–16] Interestingly, *in vivo* studies have also revealed that consumption of wine, particularly red wine, may have

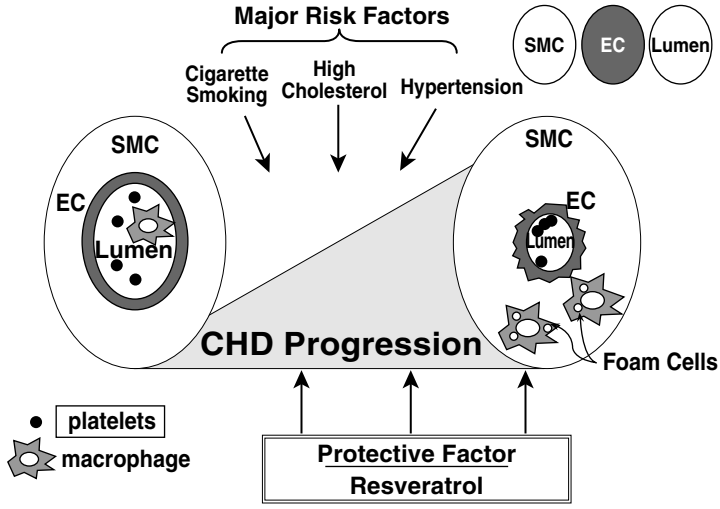


Figure 9.1 The progression of CHD as a continuum, affected by a host of risk factors (with cigarette smoking, high cholesterol, and hypertension as the three major risk factors) and protective factors including resveratrol. The scheme also shows that multiple cell types, such as endothelial and smooth muscle cells, and platelets and macrophages, are involved in the pathogenesis of CHD.

an additional advantage over use of other forms of alcoholic beverages in the prevention of CHD.[12,13]

Wine contains a variety of compounds, including naturally occurring fungicides, anthrocyanins, and polyphenolics.[17] The proposition that trans-resveratrol, a trihydroxy stilbene in red wine, may be the principal polyphenol responsible for cardioprotective properties of wine consumption[18] has prompted significant research efforts to elucidate its biological activities. Because structural and functional derangements of multiple cell types contribute to clinical CHD, we reasoned that a systematic investigation of the effects of resveratrol in these cells might provide some clues on the mechanism of cardioprotection conferred by this grape-skin polyphenol and other dietary agents implicated in the modulation of atherogenesis.

In the first part of this paper we shall summarize our previous studies of the effects of resveratrol on LDL oxidation, on cells participating in atherogenesis, and on aspects of hypercholesterolemia using rabbits as a model. In the second part, we shall report our preliminary studies of the effects of resveratrol on cultured human aortic endothelial cells (HAECs). The implications of our findings will also be discussed briefly.

SUMMARY OF PREVIOUS STUDIES FROM THIS LABORATORY

Inhibition of LDL Oxidation by Resveratrol

Oxidation of LDL is considered a key event in the initiation of AS. Because resveratrol has antioxidant properties, we tested whether it affected LDL oxidation. LDL isolated from normolipidemic adults was oxidatively modified using Cu^{++}, with and

Table 9.1 Resveratrol inhibits oxidation of LDL catalyzed by Cu^{++}.

LDL	Relative Electrophoretic Mobility	Degradation Mediated by Macrophages (μg/5h/mg)
Unmodified	1.00	1.15 ± 0.14
Modified 18 h with Cu^{++}	2.33	10.84 ± 1.07[a]
+ DMSO (1%, v/v)	2.17	9.92 ± 0.42[b]
+ 10 μM resveratrol	2.00	9.34 ± 1.02[b]
+ 50 μM resveratrol	1.50	3.72 ± 0.92[c]
+ 100 μM resveratrol	1.33	2.27 ± 0.58[c]

[a] $p < 0.01$ vs. native LDL.
[b] $p > 0.05$ vs. Cu^{++}.
[c] $p < 0.01$ vs. Cu^{++}.

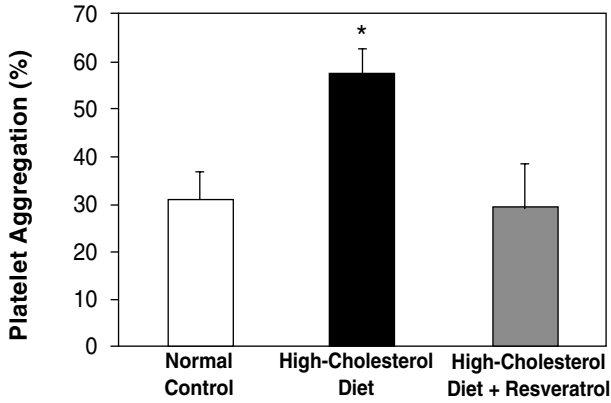
without the addition of resveratrol. The status of LDL modification was monitored by reactivity to thiobarbituric acid and agarose gel electrophoresis. Results of these studies show that ≥50 μM resveratrol significantly inhibited LDL oxidation.[19,20] Inhibition of LDL oxidation by resveratrol was additionally tested by examining its uptake and degradation. Human LDL was labeled with $Na^{125}I$ and purified. To measure uptake and processing, ^{125}I-LDL (100 μg/ml) modified with 40 μM freshly prepared $CuCl_2$ with or without resveratrol was diluted to 10 μg/ml, followed by 5 h incubation with cultured mouse peritoneal macrophages. Degradation of iodinated LDL by macrophages was determined as described.[21] Results in Table 9.1 show that oxidized LDL was degraded 10–fold more rapidly than native LDL. In contrast, LDL oxidized in the presence of resveratrol was degraded to a lesser extent by peritoneal macrophages, suggesting that the wine polyphenol suppressed LDL modification by $CuCl_2$.

We also investigated the effects of resveratrol on changes in rabbit plasma tissue-plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI) activities. In these experiments, rabbits were fed regular feed, with and without concurrent gastric feeding of resveratrol (2 or 4 mg/kg/day or DMSO as the carrier vehicle), for 1 week. Plasma was prepared from various groups of animals. Levels of t-PA and PAI were measured. Administration of high doses of resveratrol significantly decreased plasma PAI activity and increased the activated t-PA levels (Table 9.2).

Table 9.2 Effect of resveratrol on t-PA and PAI activities in rabbit plasma (X ± SD, n = 6).

	t-PA (IU/ml)	PAI (AU/ml)	Activated t-PA[a] (%)
Normal	1.72 ± 0.17	11.44 ± 0.56	13.1 ± 1.23
DMSO	1.75 ± 0.52	11.33 ± 0.50	13.3 ± 3.49
Low-dose resveratrol	1.75 ± 0.21	10.31 ± 0.85	14.6 ± 1.92
High-dose resveratrol	2.09 ± 0.67	9.17 ± 0.52[b]	18.4 ± 4.58[c]

[a] Activated t-PA = [t-PA]/[t-PA + PAI] × 100%.
[b] $p < 0.01$ compared with normal.
[c] $p < 0.05$.



* P=0.003 compared with normal control
P=0.001 compared with high-cholesterol diet + resveratrol group

Figure 9.2 Effect of resveratrol on platelet aggregation in rabbits (X ± SD, n = 8). Twenty-four male New Zealand rabbits were randomly divided into three groups: control, fed normal forage (open bars); hypercholesterolemic, fed forage containing 1.5% cholesterol (filled dark bars); resveratrol treated, fed forage containing 1.5% cholesterol and 4 mg/kg/day resveratrol introduced intragastrically (filled gray bars). Treatment was continued for 12 weeks. Blood (9 part) was drawn from each animal following a 12 h fast, mixed with sodium citrate (1 part), and rate of platelet aggregation was measured using 10 μM ADP as the inducer.

Effects of Resveratrol on Platelet Aggregation

Platelets are actively involved in the process of hemostasis, by which any break in the vascular endothelium is rapidly repaired without compromising the fluidity of the blood. In response to injury, platelets adhere to the subendothelial matrix of a damaged vessel, spread over the surface, and recruit additional platelets within a developing platelet aggregate or thrombus. Whereas hemostasis is a normal physiological response to endothelial wound repair, improper regulation or overreactivity of this system can lead to the pathological condition of thrombosis.

Thrombus formed within either the venous vasculature or the arterial vasculature is a major cause of morbidity and mortality in Western civilizations, with at least 5 million adults in the U.S. alone suffering from related symptoms.[22] We therefore assessed the effects of resveratrol on platelet aggregation. Platelets isolated from healthy subjects were induced to aggregate *in vitro* using collagen (5 μg/ml), thrombin (0.33 units/ml), and ADP (4 μM). Platelet aggregation was dose-dependently inhibited by 10 to 1000 μM resveratrol. To test the physiological significance of this observation, we also evaluated the platelet aggregation modulatory effects of this polyphenol in hypercholesterolemic rabbits. Animals were fed a high-cholesterol diet, with and without concurrent gastric feeding of resveratrol (4 mg/kg/day). After 12 weeks of treatment, platelets were isolated from various animal groups and the average platelet aggregation rate (PAR) was determined. Results in Figure 9.2 show PAR was significantly elevated by the high-cholesterol diet (61.0 ± 7.0%, compared

to 39.5 ± 5.9% in normals, n = 8, $p < 0.001$). This diet-induced increase in PAR was reduced to control levels by resveratrol (35.7 ± 6.3%, n = 8, $p < 0.001$, when paired against the high-cholesterol-fed group). Thus, *in vitro* and *in vivo* experiments provide additional evidence that this polyphenol can inhibit platelet aggregation.[23–25]

These results are consistent with previous data showing that flavonoids in wine and grape juice decreased platelet aggregation *in vitro*,[26–28] as well as induced preformed platelet thrombi disaggregation *in vitro*.[29] Rotondo et al.[30] reported that resveratrol also inhibited the release of platelet serotonin *in vitro* in a concentration-dependent manner. Kirk et al.[23] provided evidence that resveratrol suppressed signaling pathways and aggregation in washed platelets but had less effect in whole blood platelet aggregation experiments.

Effects of Resveratrol on Smooth Muscle Cell Proliferation

Because migration and proliferation of smooth muscle cells (SMCs) in the intima of susceptible vessels are also considered a requisite for atherogenesis, we investigated the effects of resveratrol on SMC proliferation and cell cycle control. Resveratrol reduced SMC proliferation in a dose-dependent manner, with 50 to 100 μM resveratrol resulting in 70 to 90% growth reduction, in response to mitogens such as endothelin and platelet-derived growth factor (PDGF) (Tables 9.3 and 9.4). The antimitogenic effects of resveratrol were not due to induction of apoptosis but appeared related to $G_1 \rightarrow S$ block in cell cycle traverse.[31]

In other studies, we also showed that resveratrol effectively suppressed proliferation of cultured bovine pulmonary aortic endothelial cells (BPAEC).[32] These results imply that resveratrol, delivered to endothelial cells in sufficient concentrations to inhibit proliferation *in vivo,* could facilitate rapid and efficient repair of damages on the endothelium, with the overall effect of decreasing the probability of sustained endothelial injury and exposure of the subendothelial matrix, which would trigger the formation of atherosclerotic plaques and the development of CHD.[32,33]

Table 9.3 Effect of resveratrol on cell proliferation in cultured calf SMC (X ± SD, n = 6).

Group	Cell Number ($\times 10^4$)	Group	Cell Number ($\times 10^4$)
Control	17.14 ± 1.47	Control	14.19 ± 1.55
DMSO	16.08 ± 3.01[a]	ET	18.79 ± 1.46[c]
Resveratrol 1 μM	14.67 ± 4.40[a]	DMSO	12.46 ± 1.81[d]
Resveratrol 5 μM	14.30 ± 3.40	ET + DMSO	15.63 ± 0.69
Resveratrol 10 μM	10.30 ± 3.40[b]	Resveratrol 10 μM + ET	11.54 ± 0.78[e]
Resveratrol 50 μM	9.75 ± 3.50	Resveratrol 50 μM + ET	5.70 ± 0.91[f]
Resveratrol 100 μM	8.00 ± 1.72[b]	Resveratrol 100 μM + ET	3.42 ± 0.72[f]

[a] $p > 0.05$.
[b] $p < 0.01$ compared with control.
[c] $p < 0.01$.
[d] $p > 0.05$ compared with control.
[e] $p < 0.05$.
[f] $p < 0.01$ compared with ET.

Table 9.4 Flow cytometric analysis on cell cycle phase distribution in calf SMC, induced by PDGF with and without resveratrol.

Group	Cell Cycle Phase (%)	
	G1	S + G2/M
Control	59.14	40.86
DMSO	56.59	43.41
PDGF-AB	29.55	70.45
PDGF + resveratrol 10 μM	59.80	40.20
PDGF + resveratrol 50 μM	77.80	22.80
PDGF + resveratrol 100 μM	82.00	18.00

Effects of Resveratrol on Morphology and Histopathology of Intimal Lesions Associated with Endothelial Denudation: Animal Studies

To further elucidate the mechanism of action of resveratrol, we performed animal experiments using hypercholesterolemic rabbits to test whether resveratrol modulates intimal hyperplasia resulting from endothelial denudation.[34] Rabbits were given resveratrol intragastrically, at 2 and 4 mg/kg/day, respectively, for a period of 5 weeks beginning 1 week before denudation. To validate intimal hyperplasia, 2-cm segments of the injured iliac artery were excised from control, and treated animals, fixed, embedded in paraffin, and sectioned at 5-mm intervals. Sections were stained and analyzed. Photomicrographs of stained sections showed intimal thickening in denuded but not control artery sections. A significant increase in intimal area and decrease in cross-sectional area of lumen in the injured denuded arteries were observed, which was substantially reduced in the 4 mg/kg/day resveratrol treated groups (Figure 9.3). These findings provide convincing evidence that resveratrol, as an orally administered dietary agent, has the potential of affecting cardiovascular structure and function in experimental animals.

Modulation of Endothelial Cell Signaling by Resveratrol[33]

The endothelial cell lining of the blood vessel is extremely sensitive to damage from reactive oxygen species (ROS), the results of which are losses of both microvascular metabolic function and barrier properties.[35] To minimize such oxidant damage, cells rely on the production of nitric oxide (NO) by the enzyme NO synthase (eNOS). Biological functions attributed to NO include vasodilation,[36] inhibition of platelet adhesion and aggregation,[37,38] reduction of expression of adhesion molecules and chemokines,[39,40] and suppression of cell growth and migration.[41,42] These multifaceted activities of NO prompted us to ask whether resveratrol affects the expression of NOS. BPAEC were treated with varying concentrations of resveratrol. Cells were harvested at different times after treatment and eNOS levels were measured by Western blot analysis. These studies show that resveratrol induced eNOS expression as early as 6 h; the increase was maintained over a 4-day period. These observations raise the possibility that resveratrol consumed in the diet could provide a gradual yet sustained increase in NO production. The stimulation of NO production could possibly represent a major mechanism of the cardioprotective effects of resveratrol.

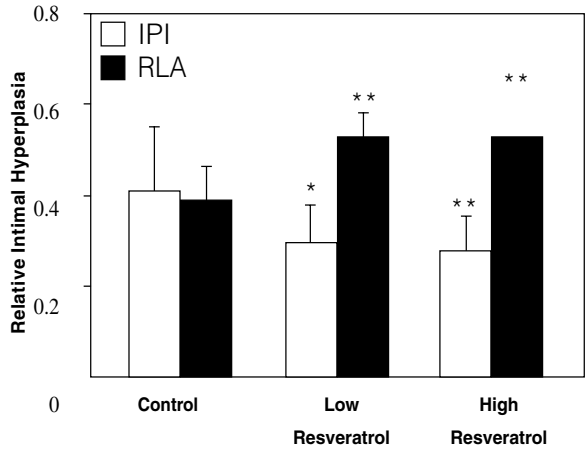


Figure 9.3 Inhibition of intimal hyperplasia by resveratrol in rabbits subjected to endothelial injury by denudation. Groups of eight New Zealand white rabbits, weighting 2.2 to 3.6 kg, were assigned randomly to control (untreated) (M), low (2 mg/kg/d) (L), and high dose (4 mg/kg/d) (H) resveratrol treatment, which was administered intragastrically for 5 weeks beginning 1 week before surgery. A 2-cm segment of injured iliac artery was excised, fixed in 4% paraformalin, embedded in paraffin, and sectioned at 5-mm intervals from the proximal to the distal end. Representative sections were stained with hematoxylin/eosin. The external and internal elastic lamina were manually identified. Intimal proliferation index (**IPI**) was defined as the ratio of intimal area to [intimal+medial] area; relative luminal area (**RLA**) was defined as the ratio of luminal area to [luminal+intimal+medial] area.

A prevailing theme of atherogenesis, known as the "response to injury" hypothesis, posits that AS is initiated by some inciting events injuring the endothelium, leading to increases in endothelial permeability and exposing the subintimal and medial layers to the blood elements. We therefore asked whether resveratrol can affect endothelial cell structure. Analysis of morphology based on F-actin staining with rhodamine-conjugated phalloidin revealed a distinct change in growth pattern in resveratrol-treated cells, resembling EC exposed to shear stress (Figure 9.4). To gain information on the possible involved signaling pathways, the experiment was repeated using the following inhibitors: quin2-AM (10 μM), herbimycin A, chelerythrine (2 μM), cytochalasin D (40 nM), and nocodazole (3.3 μM). In the inhibitor studies, most inhibitors were applied 24 h prior to the addition of resveratrol (100 μM), except for quin2-AM, which was added 1 h prior to resveratrol treatment. Control and treated cells were cultured for an additional 48 h, and the cell morphology was assessed by staining with rhodamine-conjugated phalloidin. Results showed that the resveratrol-induced change in cellular morphology was coupled to intracellular calcium and tyrosine kinase activities and also to assembly of actin microfilaments and microtubules but was unrelated to PKC activity.[33]

Modulation of Endothelial Cell Response to Shear Stress by Resveratrol

The endothelial cell lining of the blood vessel is continuously exposed to the shearing forces created by flowing blood, leading to the development of specialized shear-

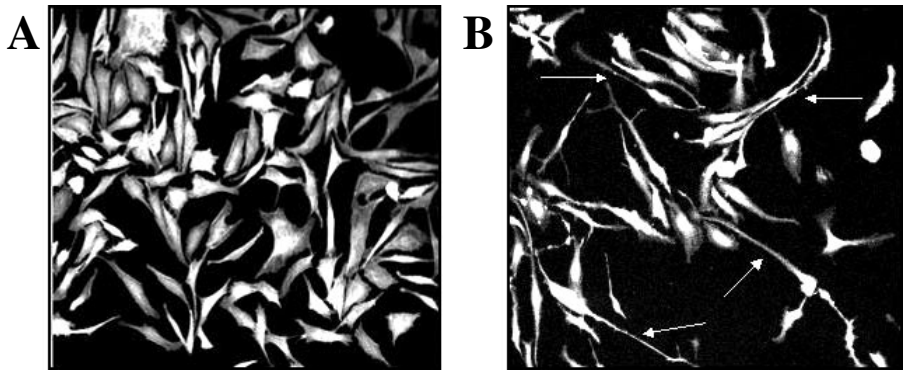


Figure 9.4 Resveratrol induces morphological changes in cultured BPAEC. Panel A. Microscopic illustration of the stellar, cobblestone-like morphology characteristic of cultured normal BPAECs. Panel B. Illustration of elongated, spindle-shaped morphology characteristic of 100 μM resveratrol-treated cells. Cells are stained with rhodamine-phalloidin and viewed with a 20X objective. Note the long, tortuous projections.

induced cellular features.[43] Long-term cellular adaptations include reorganization of the actin cytoskeleton such that cells become elongated and flattened. Such morphological changes reorient cells in the direction of flow and are accompanied by the expression of new genes, including those coding for vasoactive substances such as the NO. Elicitation of these changes involves a process known as mechanotransduction, by which mechanical stimuli are converted into chemical signals. This process begins with the activation of specific cytoskeleton-associated cell-surface proteins that act as primary flow sensors.[43] At the same time, force transmission may occur via cytoskeletal elements from the local flow-exposed cell surface to various remote sites within the cell, including the nucleus, focal adhesion sites on the basal cell surface, and cell-cell adhesion proteins.[44] Once the shear sensors are activated, short-term intracellular signaling cascades are initiated, leading to the activation of transcription factors, resulting in long-term gene expression changes.

To test the idea that resveratrol modulates EC response to shear stress, ECs were cultured in sterilized plastic coverslips and treated with 100 μM resveratrol 24 h later. Two days afterward, the coverslips were exposed to 0 (control), 2, or 5 min of simulated arterial shear stress using a parallel plate perfusion chamber. The coverslips with remaining attached cells were then fixed, stained with rhodamine-phalloidin, mounted onto slides, and viewed under confocal microscopy. Pictures were taken of the confocal microscopy fields located directly in the center of the coverslip region exposed to the flow. Representative confocal microscopy fields were counted for total number of cells. As can be seen in Figure 9.5, while the number of treated ECs remaining on the plastic (uncoated coverslips) were fairly constant under both flow durations, the number of untreated cells adhering to the coverslips dropped off dramatically. These results suggest that treatment with resveratrol altered the ECs so that they became resistant to simulated arterial flow conditions. Such a resistance to detachment would make endothelial cells less likely to dislodge and become part of a growing thrombotic plug, thus contributing to yet another possible

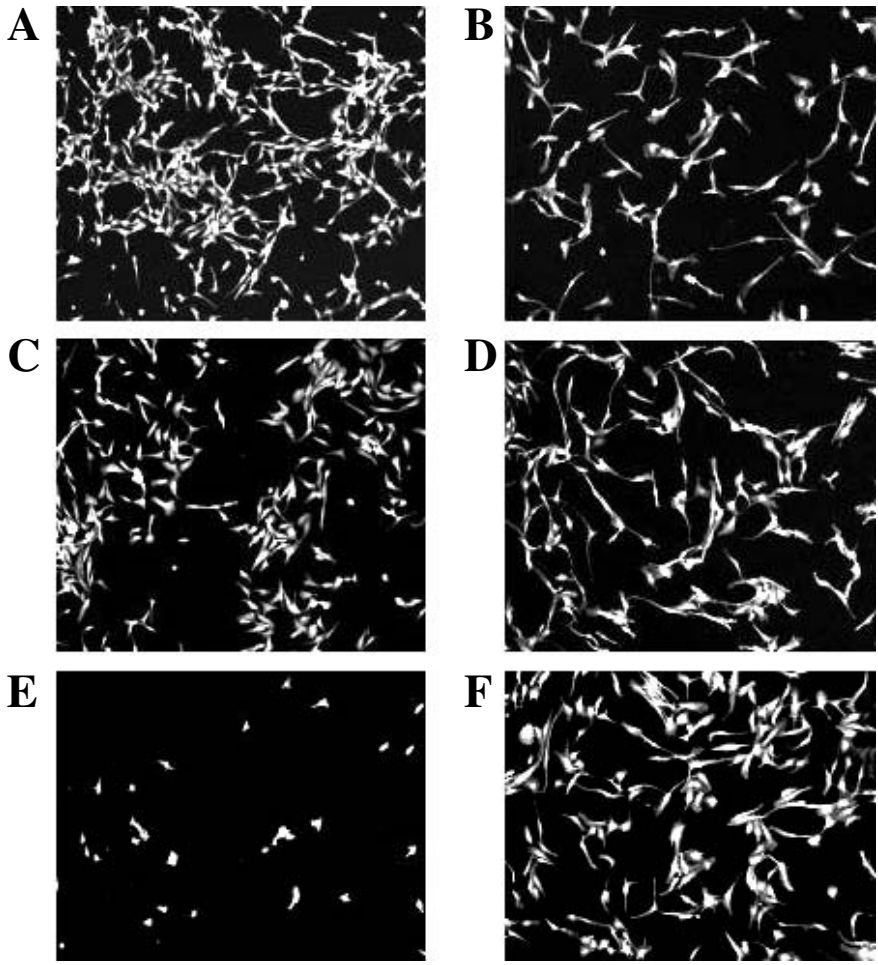


Figure 9.5 Passage 6 BPAEC were treated with resveratrol and subjected to simulated arterial flow conditions (shear rate of 650 s^{-1}), respectively. Panels A, C, and E. Control cells subjected to flow of 0, 2, and 5 min durations. Panels B, D, and F. Resveratrol (100 μM)-treated cells subjected to flow of 0, 2, and 5 min duration.

mechanism of prevention of AS by resveratrol. The exact manner by which resveratrol treatment induces such changes is not totally known but may involve increases in the number of focal contact adhesion sites, a more effective redistribution of the focal contacts, or increased production of the α_{II}/β_{III} integrin complex.

PHARMACOKINETICS AND METABOLISM OF RESVERATROL

The primary dietary source of trans-resveratrol is red wine. Although the concentration of resveratrol in red wine is variable, it has been found to be much higher than in white wines. Using chromatographic analysis, several studies have investigated

the resveratrol level of wines. The resveratrol contents for red wine vary from 0.65 mg/l in New York Chardonnays[18] to 7.17 mg/l in wines from Cabernet Sauvignon in Italy.[45] Whether the human body can absorb resveratrol in biologically significant amounts remains questionable.[46] Juan et al.[47] showed that rats administered orally with transresveratrol (2 mg/kg) were able to absorb it to a great extent, as a plasma concentration of 0.175 mg/l became detectable at 15 min after administration. Bertelli et al.[48] reported that dosages corresponding to the amount of resveratrol in red wine were able to produce a pharmacological effect on platelet aggregation in tested animals. They concluded that even moderate drinkers of red wine can absorb sufficient resveratrol to gain beneficial effects on health. Furthermore, Blache et al.[49] reported that resveratrol was able to be incorporated into blood cells and lipoprotein after *in vitro* incubation with plasma, lipoproteins, and cells. Clearly, more work needs to be done in this area in order to determine more definitively whether the biological effects of resveratrol observed *in vitro* may have significance *in vivo*.

STUDIES WITH HUMAN AORTIC ENDOTHELIAL CELL (HAEC)

Most of the biological effects of resveratrol, in the context of cardioprotection and chemoprevention, have come from experiments using transformed cells and, in limited cases, animals. Relatively few studies have examined the effects of this stilbene in normal human cells. For instance, whether resveratrol affects human endothelial cells has yet to be addressed. Accordingly, we investigated the response of HAEC to resveratrol, with the initial focus on underlying molecular changes that may be relevant to the morphological changes seen in cultured BPAEC (Figures 9.4 and 9.5).

We based our exploratory experiments on the premise that living cells are equipped with various defense mechanisms to meet the challenges imposed by physiologically relevant as well as undue stressful events. Oftentimes, these defense mechanisms are activated to restore homeostasis. Prominent among these is the synthesis of a specific set of proteins called heat shock proteins (Hsp).[50–52] Two families of Hsp have been identified according to their apparent molecular weight.[53] The small Hsps (sHsp) are induced by a variety of stimuli including heat shock, oxidative stress, exposure to anticancer drugs and inflammatory mediators. A particular sHsp member, hsp27, has been shown to confer tolerance to cells.[54,55] For example, induction of thermoresistance may involve a cytoskeletal rearrangement, arising in part owing to the participatory role of hsp27 to regulate actin microfilament dynamics. Because phosphorylation of hsp27 is linked to the activation of MAPK, we monitored changes in the RNA expression of these genes in control and 1-day resveratrol-treated HAEC cells. By using specific forward and backward primer sets, RNA expression was assayed using RT-PCR. Expression of both p38 MAPK and p27 was increased by resveratrol (data not shown).

Mechanism of Cardioprotection by Resveratrol

Taking the published data of resveratrol on various aspects of the cardiovascular system as a whole, we propose that resveratrol acts as a pleiotropic cellular modulator

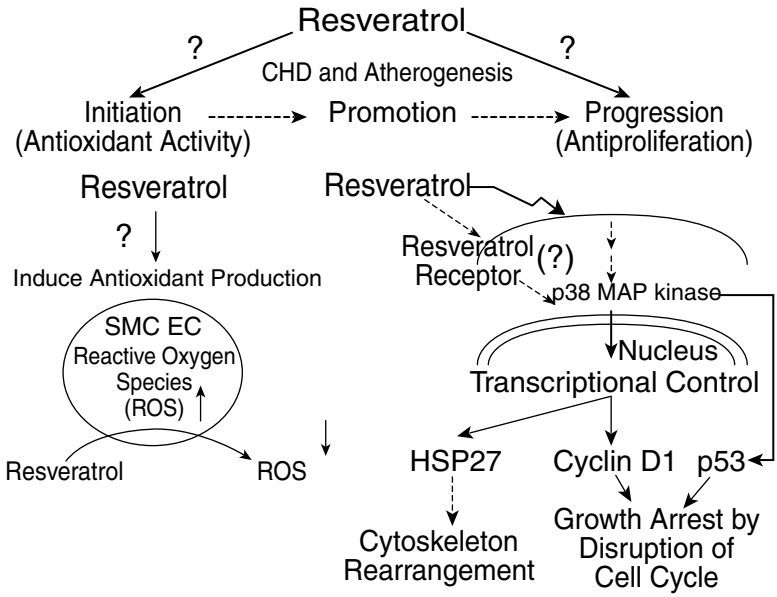


Figure 9.6 Proposed mechanism of cardioprotection by multiple activities of resveratrol in target cells: Resveratrol may act as an antioxidant to modulate reactive oxygen species (ROS) production and foster the establishment of cellular redox homeostasis. This would reduce mitogenic signaling in SMC and, by restricting the establishment of an oxidative state, reduce the oxidation of LDL and, in sequence, disrupt propagation of oxidized LDL-elicits events. Conceivably, resveratrol could act by physically binding to a hypothetical cellular receptor. Ontogenic changes and tissue-specific expression of the presumed receptor could in turn affect the biological activity of resveratrol. Resveratrol could additionally function by activating the p38 MAPK signaling events, which, in turn, catalyzes the phosphorylation of target proteins such as hsp27, cyclin D1, and p53.

the ability of which confers cardioprotection via several possible mechanisms (Figure 9.6). First, resveratrol may simply be functioning as an antioxidant that attenuates LDL oxidation and modulates ROS equilibrium in such a manner as to establish cellular redox homeostasis. This could be a significant dampening factor in the mitogenesis of SMC and EC; intracellular ROS such as superoxide and hydrogen peroxide are known to function as signaling molecules and may act as potent molecular drivers for proliferation of aforementioned cells that play an integral role in atherogenesis.

Second, resveratrol could function as a signal coupler to directly or indirectly activate signaling pathways, e.g., p38 MAPK. Our current hypothesis is that resveratrol affects the expression and function of p53, secondary to activation of the p38 MAPK. Specifically, we surmise that resveratrol, through p38 MAPK activation, elicits site-specific phosphorylation changes of the p53, leading to its stabilization and eliciting an increase in the cdk inhibitor p21, with the overall effect of inhibiting cell proliferation. This mechanism is supported by data from other systems showing that p38 MAPK phosphorylates p53 at serine-15, which results in stabilization of p53 and its nuclear accumulation.[56] The proposed activation of p38 MAPK by

resveratrol could affect SMC and EC proliferation through other gene targets. For instance, activated p38 MAPK has been shown to inhibit the cyclin D1 promoter.[57] Also, the cyclin D1 protein has been shown to be phosphorylated by p38 MAPK and targeted for ubiquitin-dependent degradation.[58] Similarly, the G_1 inhibitor p21 is a p38 MAPK substrate. In this case, however, phosphorylation by MAPK stabilizes p21.[59] These effects previously reported of the p38 MAPK in other systems are directly applicable to the molecular sequence we hypothesize to be elicited by resveratrol, particularly in the context of its ability to inhibit proliferation of SMC and EC. Pilot studies with HAEC show p38 MAPK and hsp27 to be induced by resveratrol. As mentioned, hsp27 plays an integral role in the regulation of actin microfilament dynamics. This could indirectly impinge on cytoskeletal arrangement and cell adhesion. In the case of ECs, adhesion to the subendothelial matrix is also crucial for cellular survival and organization. Cell adhesion is controlled by functional complexes of extra-cellular matrix components, transmembrane adhesion molecules, and cytoskeletal proteins. Focal adhesions represent such functional complexes, and are comprised of integrins, integral membrane proteoglycans, associated cytoplasmic proteins such as vinculin and paxallin, and several protein kinases. The assembly and disassembly of the focal adhesions is a dynamic process under complex regulation as the cell converts from the adhesive to the migratory phenotypes.[60] Whether and how these events are controlled or impacted by resveratrol must await further investigations in the future.

SUMMARY

We suggest that resveratrol diminishes thrombotic episodes through its ability to reduce several independent and interactive events involved in the initiation, progression, and establishment of atherogenesis. A key control by resveratrol may involve attenuation of SMC proliferation and flow-mediated EC damage. Further elucidation of the nature of gene responses elicited by resveratrol is currently underway using gene arrays, in combination with cellular-biochemical-molecular approaches.

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CHAPTER 10

Development of a Mixture of Dietary Carotenoids as Cancer Chemopreventive Agents: C57BL/6J Mice as a Useful Animal Model for Efficacy Studies with Carotenoids

Frederick Khachik, Nitin Telang, Fumio Enjo, and Martin Lipkin

CONTENTS

INTRODUCTION

One class of phytochemicals that has been widely studied for their preventive effect against cancer is carotenoids. To date, as many as 600 carotenoids have been isolated from various sources, and their chemical structures have been characterized. The evidence for nutritional prevention of cancer, heart disease, and macular degeneration (an age-related degenerative eye disease) by carotenoids has been obtained from various interdisciplinary studies; these may be classified as (1) epidemiological

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studies, (2) studies of carotenoid distribution in fruits, vegetables, human serum, milk, and tissues, (3) *in vitro* studies of chemopreventive properties, and (4) *in vivo* studies with rodents.

For more than two decades epidemiological studies have associated the high consumption of carotenoid-rich fruits and vegetables with a lower risk for certain types of human cancers.[1] Consumption of fruits and vegetables rich in specific carotenoids such as lutein, lycopene, α-carotene, and β-carotene in a human intervention study has been shown to significantly reduce the oxidative deoxyriboneucleic acid (DNA) base damage as was detected in the peripheral blood lymphocytes.[2] For a recent review of the case-controlled epidemiological studies and the role of carotenoids in disease prevention in humans, see the publications by van Poppel[3] and Mayne.[4]

More recently, dietary intakes of carotenoids from fruits and vegetables, as well as of individual carotenoids, have been inversely associated with the risk of stomach, lung, breast, and prostate cancers.[5–8] For example, the evidence from one epidemiological study indicates that premenopausal breast cancer risk is inversely associated with intake of fruits and vegetables and specifically greens such as spinach in which the concentration of lutein is much higher than β-carotene.[9] In addition, a strong association has been observed between carotenoid intake, plasma lutein, and estrogen receptor status, linking a lutein-rich diet with improved prognosis after diagnosis of breast cancer.[10]

The observations from the epidemiological studies conducted in the early 1980s resulted in a number of randomized clinical trials with pharmaceutical doses of β-carotene that showed either no effect or an increased risk of lung cancer in smokers.[11-13] Although these studies were well designed and conducted, they were based on the assumption that the main active carotenoid component in fruits and vegetables was β-carotene. This was primarily due to the lack of commercial availability, as well as the knowledge of fundamental aspects of the absorption, metabolism, and transport of other dietary carotenoids and their metabolites in humans.[14-17]

To date, a wide range of carotenoids have been isolated, identified, and quantified from the extracts of fruits and vegetables commonly consumed in the U.S.[18–23] These studies have revealed that 40 to 50 carotenoids may be available from the diet and absorbed, metabolized, or utilized by the human body.[21] However, among these, only 13 *all-E (trans)*- and 12 *Z (cis)*-carotenoids are routinely found in human serum and milk.[24–28] In addition, there are one *Z (cis)*- and eight *all-E (trans)*-carotenoid metabolites resulting from two major dietary carotenoids, namely, lutein and lycopene that have also been characterized.[24,25,27,28] This brings the total number of carotenoids and their metabolites detected in humans to 34.[28] These dietary carotenoids include lutein, α- and β-cryptoxanthin, lycopene, ζ(zeta)-carotene, α- and β-carotene, phytofluene, and phytoene which have been detected in μg-ng/g quantities in human lung, liver, breast, and cervical tissues.[29]

The correlation between dietary carotenoids and the carotenoids routinely found in the extracts from human serum/plasma has revealed that only selected groups of carotenoids make their way into the human bloodstream. Some of these carotenoids are absorbed intact and others such as lutein, zeaxanthin, and lycopene are presumably converted to several metabolites.[30–33] In addition to their antioxidant mechanism

of action, carotenoids may also exert their biological activity in prevention of cancer by other mechanisms. These are (1) enhancement of the expression of the intercellular communication proteins,[34] (2) induction of the phase 2 enzymes (detoxification enzymes),[32] (3) anti-inflammatory effects, and (4) antitumor-promoting properties.[32] These mechanisms have also revealed that various carotenoids and their metabolites, depending on their chemical structures, exhibit different degrees of activity. Furthermore, in some cases, these activities are more pronounced with carotenoid metabolites than with dietary carotenoids.

Due to the intense focus on β-carotene, most of the *in vivo* studies with other dietary carotenoids involving rodents were not conducted until mid-1990s. These studies have been carried out with several prominent food carotenoids such as lycopene and lutein that have become commercially available in the course of only the past few years. In a 1996 publication by Narisawa et al., the inhibitory effect of four dietary carotenoids, α-carotene, β-carotene, lycopene, and lutein, prevalent in human blood and tissues against the formation of colonic aberrant crypt foci, has been reported in Sprague-Dawley rats.[35] The rats received three intrarectal doses of *N*-methylnitrosourea in week 1 and a daily gavage of de-escalated doses of carotenoids during weeks 2 and 5. Lycopene, lutein, α-carotene, and palm carotenes (a mixture of α-carotene, β-carotene, and lycopene) inhibited the development of aberrant crypt foci (ACF) quantitated at week 6. In contrast, β-carotene was not effective. The study investigators concluded that lycopene and lutein in small doses might lower the risk for colon carcinogenesis.

In another study, application of α-carotene and lycopene in drinking water at a concentration of 0.05% has been associated with strong inhibition of mouse lung carcinogenesis induced by 4-nitroquinoline-1-oxide.[36] In contrast, a tendency for an increase in lung tumor development was noted in female mice given β-carotene.[36,37] The results from this study are in agreement with the paradoxical effects of β-carotene observed in recent clinical trials involving humans.[12,13]

The chemoprevention of mouse lung neoplasia by lycopene, administered during postinitiation stage in a multiorgan carcinogenesis model, has also been investigated.[38] One hundred eighteen $B6C3F_1$ mice of both sexes were subjected to combined treatment with diethylnitrosamine (DEN), *N*-methyl-*N*-nitrosourea (MNU), and 1,2-dimethylhydrazine (DMH) from day 11 after birth to week 9 (DMD = DEN plus MNU plus DMH treatment) (groups 1 and 2) or their vehicle (group 3). Then group 1 was given lycopene 25 or 50 ppm in drinking water for 21 weeks from weeks 11 to 32. Group 2 served as carcinogen-alone control and group 3 was given only lycopene (25 or 50 ppm). All surviving animals were sacrificed at week 32 and the major organs, including the liver, lung, kidney, and colon, were subjected to histological examination. The incidence and multiplicities of lung adenomas plus carcinomas combined in male mice in group 1 receiving 50 ppm lycopene were significantly decreased as compared to the DMD alone or DMD and 25 ppm lycopene groups. No such effect was observed for females. Although hepatocellular carcinomas were lacking in the DMD and lycopene groups while two cases were found in the DMD alone group, the investigators did not find these results statistically significant. The values for ACF and tumors in the colon and kidney did not show any significant variation among the carcinogen-treated subgroups. The investigators of

this study concluded that lycopene exerts a chemopreventive effect limited to male lung carcinogenesis when given in the post-initiation stage to mice. Based on the epidemiologic and experimental evidence described earlier, it appears that a mixture of prominent dietary carotenoids (multicarotenoid) might be expected to serve as a more effective chemopreventive agent than a single carotenoid. To investigate this hypothesis, the cancer chemopreventive efficacy of a multicarotenoid mix needs to be evaluated in an appropriate animal model for studying cellular abnormalities associated with target organ carcinogenesis.

Rodent models have provided important clinically relevant leads for colon, breast, and prostate carcinogenesis and its prevention by natural and synthetic compounds. C57BL/6J Strain of mouse exhibits low incidence of spontaneous breast and colon cancer. Administration of Western-style diet (WD) (high fat, low calcium, and low vitamin D) results in accelerated growth of precancerous lesions in colon and breast of these mice.[39] In a study by Risio et al., it has been shown that feeding WDs to mice for 2 years without any chemical carcinogenesis leads to the development of gross colonic lesions that can be histologically classified as dysplastic crypts and focal hyperplasias with or without atypical nuclei.[39] This study has revealed that among the early biological events contributing to the development of colonic neoplasia was a significant and transient increase in mitotic activity in the basal and intermediate portions of the colonic crypts in young mice after feeding them WDs. This was accompanied by diffuse activation of apoptosis of the colonic epithelial cells. In the middle of the rodent's life span, after administration of both the WD and control diet, the rodents developed a marked depletion of apoptotic epithelial cells in the mid-region of the colonic crypts; this was followed by the expansion of an epithelial cell population containing atypical nuclei and the emergence of the gross lesions noted earlier. Therefore, with this sequence of events, prolonged feeding of WDs to mice produces single-crypt dysplastic lesions and focal hyperplasias indicative of tumorigenesis.

Here, we report on the bioavailability of a supplemental mixture of seven prominent dietary carotenoids (multicarotenoid) in colon, breast, brain, and liver in C57BL/6J mice fed a WD with and without multicarotenoid. The objective of this study was to evaluate the suitability of the above rodent model for studying cellular abnormalities relevant to organ site carcinogenesis.

DESIGN AND FORMULATION OF MULTICAROTENOID MIXTURE (MCM)

In a 1997 publication, we reported the relative distribution of 13 major dietary carotenoids in the serum of 10 healthy human subjects with a high intake of fruits and vegetables.[40] The concentrations of the 12 dietary *cis*-carotenoids were combined and reported together with 13 of their corresponding all-*trans*-compounds. The average distribution of serum carotenoids for these subjects were lutein (20%), lycopene (20%), β-carotene (10%), ζ-carotene (10%), β-cryptoxanthin (8%), phytofluene (8%), α-carotene (6%), α-cryptoxanthin (4%), phytoene (4%), zeaxanthin

Table 10.1 Relative distribution and source of carotenoids in multicarotenoid mixture (MCM).

Carotenoids	Relative Distribution (%)	Source
Lutein	25	Kemin Foods, LC, Des Moines, IA
Zeaxanthin	1	Isolated and purified from Chinese wolfberries (Chinese Lycium Mill)
α-Carotene	11	Isolated and purified from concentrated red palm oil by crystallization
all-E-β-Carotene	16	
9Z-β-Carotene	4	
13Z-β-Carotene	3	
Lycopene	25	Isolated and purified by flash chromatography from commercially available tomato oleoresin produced by LycoRed, Beer Sheva, Israel
ζ-Carotene	1	
Phytofluene	4	
Phytoene	4	
γ-Carotene	5	
Neurospoene	1	
Total	100	

(3%), anhydrolutein (3%), γ-carotene (2%), and neurosporene (2%). This study, as well as our detailed analyses of the serum of several hundred subjects with various dietary habits, has revealed that the relative distribution of carotenoids in human serum is, to a large extent, reflective of the dietary habits of individuals.[33,41–44] Furthermore, these published reports have also demonstrated that in supplementation studies with purified carotenoids, the interindividual variability in the serum carotenoid profile of human subjects can be significantly reduced. Therefore, the composition of the multicarotenoid supplement was based on the relative ratio of serum carotenoids from the above study. Some of the carotenoids employed in this study were commercially available and others had to be isolated and purified from various natural sources. The source, method of purification, and relative distribution of the carotenoids in the multicarotenoid mixture (MCM) employed in the present study are shown in Table 10.1. The chemical structures of these carotenoids are shown in Figure 10.1.

PREPARATION OF WESTERN-STYLE DIET WITH AND WITHOUT MULTICAROTENOID MIXTURE (MCM)

The multicarotenoid (30 g) was shipped on dry ice to Research Diets, Inc. (New Brunswick, NJ), where it was mixed with the AIN-76AWD diet. A mixture of MCM (30 g), α-tocopherol (0.3 g), and Tween 80 (75 g) in ethanol (300 ml) was homogenized at room temperature for 30 min. Corn oil (2000 g) was added to the suspension and the mixture was stirred at room temperature for 15 min. This was blended with 20 kg of the ingredients of AIN-76A diet (Research Diets, Inc.), and the remaining 2000 g of corn oil was added. The pellets of the diets were prepared from the mixture and dried by air flow at room temperature for 2 days and stored in a freezer at –70°C. The ingredients of the AIN-76AWD diet to which MCM has been added are shown in Table 10.2. The control diet (AIN-76AWD) was prepared exactly the same as the

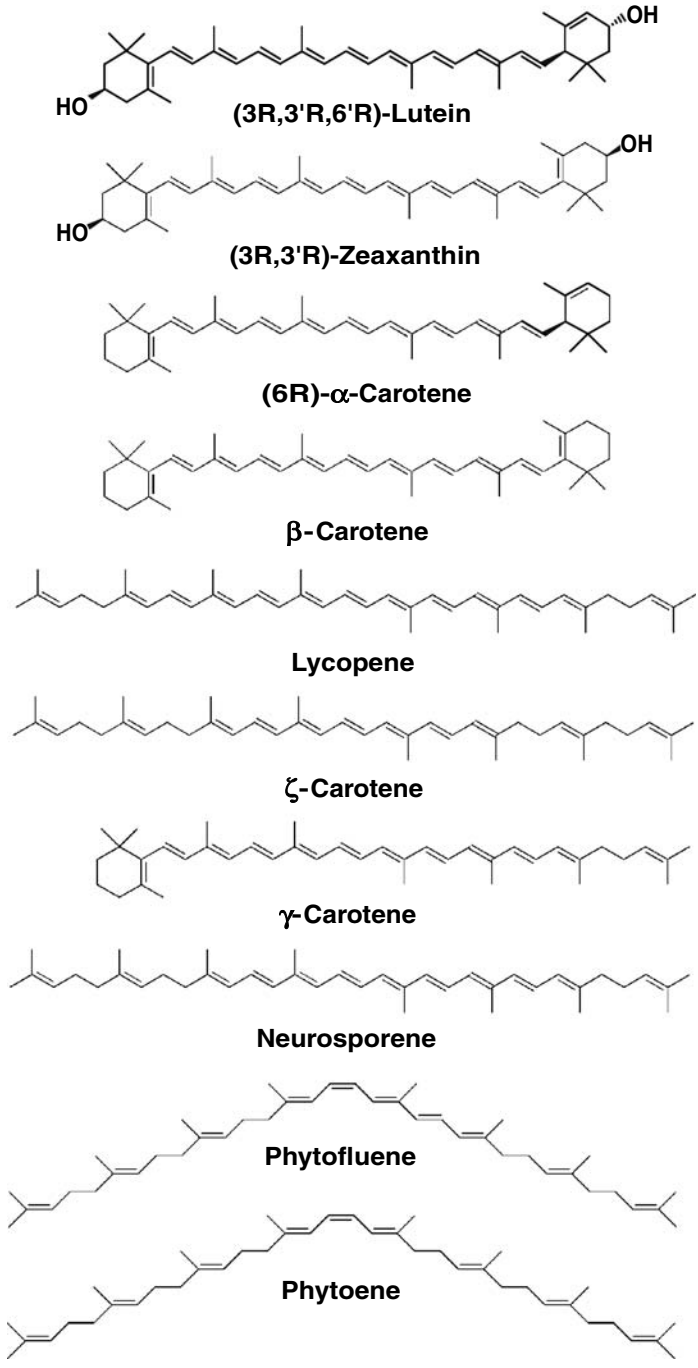

Figure 10.1 Chemical structures of dietary carotenoids formulated into multicarotenoid mixture (MCM).

Table 10.2 Composition of Western-style diet (AIN-76AWD) plus multicarotenoid mixture (MCM).[a]

Western-style Diet Plus MCM	Composition
Base	**Gram%**
Protein	24.40
Carbohydrate	45.10
Fat	20.00
Kcal/g	4.48
Ingredients	**Gram/Kg**
Multicarotenoid Mixture (MCM)	1.50
Corn oil	200.00
Tween 80	3.75
Vitamin E, 1,100 IU/g	0.015
Vitamin D_3 100,000 IU/g	0.0012
Vitamin mix V13201	12.00
Casein, 80 mesh	240.00
dl-Methionine	3.60
Ethoxyquin	0.01
Corn starch	150.00
Sucrose	288.95
Cellulose, BW200	60.00
Monosodium phosphate	7.98
Monopotassium phosphate	7.91
Calcium carbonate	0.88
Choline bitartrate	2.40

[a] Western-style diet (AIN-76AWD) is prepared from AIN-76A diet and is high in fat and low in calcium and vitamin D.

above with the exception that it did not contain MCM. The diet was prepared in bulk for the duration of the study (6 months) and was kept in a refrigerator. Based on stability studies on carotenoids in this preparation, diets were thawed and provided fresh to the animals every 3 days (Table 10.3).

STUDY DESIGN

Twenty female C57BL/6J mice at 4 to 5 weeks of age were randomized by weight and allocated into two treatment groups of 10 each. Group I (control) received AIN-76AWD diet without MCM and group II received AIN-76AWD diet plus MCM. Freshly stored diets (slightly in excess of 5 g with or without MCM) at –70°C were thawed and provided to each animal for 3 days of their supplies (2.5 mg total carotenoid/day). After 6 weeks, five animals from each group were sacrificed by carbon dioxide asphyxiation and all the remaining animals were similarly sacrificed after 24 weeks. Tissues (liver, colon, breast, and brain) were excised and fixed for histopathology and frozen at –70°C until analysis.

Table 10.3 Concentration of carotenoids in freshly prepared diet and the diet stored for 3 days at ambient temperature.

Carotenoids in Multicarotenoid Mixture (MCM)	Concentration (mg/g)	
	Fresh Diet	Diet after 3 Days at Room Temperature
Lutein	190.7	140.6
Zeaxanthin	10.3	7.6
α-Carotene	95.2	72.2
β-Carotene	170.5	132.9
9Z-β-Carotene	23.7	17.6
13Z-β-Carotene	12.2	12.3
Lycopene	157.7	133.9
ζ-Carotene	7.0	7.1
Phytofluene	28.1	28.6
Phytoene	35.5	35.0
γ-Carotene	37.9	27.9
Neurosporene	7.0	7.7
Total	775.8	623.4

TISSUE EXTRACTION OF CAROTENOIDS

Tissues (liver, colon, breast, brain) from mice were pooled, weighed, and extracted three times with tetrahydrofuran (THF, 3 × 10 ml) containing 0.1% butylated hydroxytoluene (BHT) in the presence of anhydrous sodium sulfate (20% by weight of tissue) by sonication at 5°C to 10°C for 30 min each time. The combined extracts were evaporated to dryness on a rotary evaporator under reduced pressure below 40°C. The residue was dissolved in dichloromethane (2 ml) and was saponified with 1 ml of 1% potassium hydroxide in methanol (weight/volume) for 30 min at room temperature. The mixture was transferred into a separatory funnel and washed with water (4 × 5 ml) until the pH of the aqueous layer was 7, and then dried over anhydrous sodium sulfate. The solution was evaporated to dryness on a rotary evaporator under reduced pressure below 40°C. The residue was dissolved in dichloromethane (4 ml) and filtered through a 0.45-μm disposable Acrodisc polyvinylidene fluoride filter assembly (VWR Scientific Products, Bridgeport, NJ) into a 5-ml graduated microsample vial. The solvent was evaporated under nitrogen, and the extracts were redissolved in the HPLC injection solvent consisting of a mixture of acetonitrile (85%), dichloromethane (2.5%), hexane (2.5%), and methanol (5%). The vials were centrifuged at ≈ 2000 *g* to remove the minor amounts of insoluble solid particles; 50 μl samples were injected onto the HPLC system.

HPLC ANALYSIS OF CAROTENOIDS

The HPLC analyses were performed on an Agilent Technology Model 1100 HPLC system equipped with a quaternary solvent delivery system, 1100 autosampler,

thermostat-controlled column compartment, and 1100 diode array detector. The data were stored and processed by an HP Kayak XM600 computer on Windows NT with HP Chem-Station software (version A.08.01), HP 19-in. color display monitor, and an HP Laserjet 4050 printer. HPLC separations were carried out with eluent A on a Microsorb (25-cm length × 4.6 mm i.d.) C_{18} (5-μm spherical particles) column (Rainin Instrument Co., Wouburn, MA), which was protected with a Brownlee guard cartridge (3-cm length × 4.6 mm i.d.) packed with spheri-5-C_{18} (5-μm particle size). A combination of isocratic and gradient HPLC employing a two-pump solvent module was used with this eluent. Pump A pumped a mixture of acetonitrile/methanol (9/1, v:v), and pump B pumped a mixture of hexane/dichloromethane/methanol/DIPEA (*N*,*N*-diisopropyl-ethylamine) (4.5/4.5/0.99/0.01, v:v:v:v:v). At time zero, an isocratic mixture of acetonitrile (85.5%), methanol (9.995%), dichloromethane (2.25%), hexane (2.25%), and DIPEA (0.005%) (95% pump A, 5% pump B) was pumped for 10 min. After 10 min, a linear gradient was run for 30 min resulting in a final composition of acetonitrile (40.5%), methanol (9.95%), dichloromethane (24.75%), hexane (24.75%), and DIPEA (0.055%) (45% pump A, 55% pump B). The column flow rate was 0.70 ml/min. The HPLC injection solvent consisted of a mixture of acetonitrile (85%), dichloromethane (2.5%), hexane (2.5%), and methanol (10%). At the end of the gradient, the column was equilibrated under the initial isocratic conditions for 15 min. The column temperature was maintained at 25°C and the monitoring wavelengths were 446, 470, 400, 350, and 290 nm. Carotenoids were identified by comparison of their HPLC retention times, UV-visible absorption spectra, and co-injection with those of synthetic or isolated standards. Details regarding the UV-visible absorption maxima of carotenoids can be found in our previous publications.[24,28] Because the use of an internal standard could possibly interfere with the presence of unknown carotenoids, no internal standard in the extraction of the various samples was used. To monitor the accuracy and reproducibility of the HPLC analysis of carotenoids, a solution containing known concentrations of lutein, lycopene, ζ-carotene, α-carotene, β-carotene, phytofluene, and phytoene was routinely analyzed by HPLC. The recovery and reproducibility of the HPLC analysis for carotenoids was greater than 95%.

HISTOPATHOLOGY

Histopathological analysis was performed according to a published procedure by Lipkin.[45] The entire cecum, colon, and rectum of two animals, one each from the control group and the MCM-treated group, were removed and fixed with 10% buffered formalin (12 h), 80% ethanol (12 h), and 95% ethanol (12 h). Representative sections were taken, paraffin embedded, and 4-μm sections cut, mounted into glass slides, and stained with hematoxylin and eosin. Five slides were prepared from each tissue, each slide containing five serial sections. The number of epithelial cells per intestinal crypt over 50 intestinal crypts was counted. The number of crypts containing dysplastic epithelial cells per 50 intestinal crypts was counted.

RESULTS AND DISCUSSION

No carotenoids were detected in tissues of animals sacrificed after 6 weeks. However, as shown in Table 10.4, after 24 weeks, nearly all carotenoids (lutein, zeaxanthin, lycopene, γ-carotene, ζ-carotene, α-carotene, β-carotene, phytofluene, phytoene) were bioavailable in colon and liver of the animals that received MCM. A typical HPLC profile of carotenoids in a pooled extract from mouse liver is shown in Figure 10.2. The major carotenoids in brain were lycopene, lutein, and β-carotene. ζ-Carotene predominated in the breast tissues, while lutein, lycopene, γ-carotene, and α-carotene were detected in low concentrations. Carotenoids were not detected in tissues of the mice on WD without MCM.

Table 10.4 Average (n = 3) distribution of carotenoids in C57BL/6J female mice after 24 weeks of multicarotenoid supplementation.

Carotenoids	Tissue Concentration (μg/g)			
	Liver	Colon	Breast	Brain
Lutein and zeaxanthin	0.045	0.707	N.D.	0.171
Lycopene	1.189	3.388	N.D.	0.596
γ-Carotene	0.244	N.D.	N.D.	N.D.
ζ-Carotene	0.345	N.D.	N.D.	N.D.
α-Carotene	0.261	0.881	0.019	0.051
β-Carotene				
all-E (trans)	0.389	1.44	0.032	0.092
9Z (cis)	0.172	0.545	N.D.	0.009
13Z (cis)	0.088	0.717	N.D.	0.006
Phytofluene	1.110	N.D.	N.D.	N.D.
Phytoene	1.269	N.D.	N.D.	N.D.
Total	5.112	7.678	0.051	0.925

Note: N.D. = Not detected.

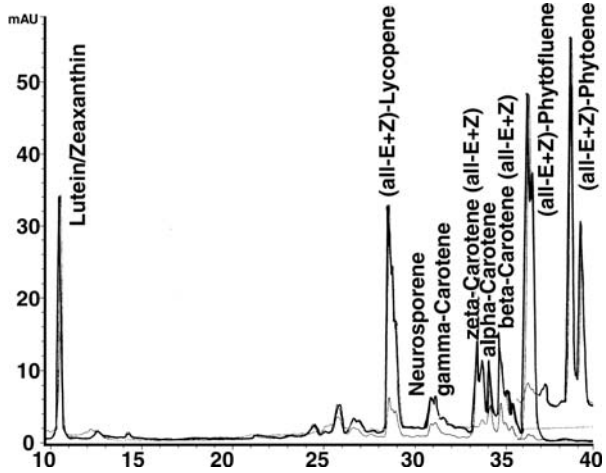

Figure 10.2 Carotenoid HPLC profile of pooled extract from liver of mice supplemented with multicarotenoid mixture for 24 weeks; HPLC conditions described in text.

No dysplastic cells or crypts were evident within the rectal or distal colonic mucosa for either diet group. Dysplastic crypt epithelium was evident within the cecum of both diet groups, but fewer total number of dysplastic crypts were observed in the MCM-treated group.

Epidemiologic and animal studies have shown that specific dietary factors can inhibit the induction and development of a wide variety of cancers.[45] In addition, a recent epidemiologic study concludes that a high consumption of carotenoid-rich foods (greens, tomatoes, oranges, carrots) may help reduce the risk of developing colon cancer.[46] The inhibitory effects of a mixture of natural carotenoids (lycopene, lutein, α-carotene, β-carotene) on the development of ACF in Sprague-Dawley rats suggests that supplementation with small doses of these carotenoids may prevent colon carcinogenesis.[35] Here we have shown that supplementation of C57BL/6J female mice with AIN76AWD (Western-style diet) containing MCM results in the absorption of a wide range of carotenoids in various tissues examined. Although nearly all carotenoids were highly bioavailable in colon and liver, the major carotenoids in brain were lycopene, lutein, and β-carotene. ζ-Carotene predominated in the breast tissues while lutein, lycopene, γ-carotene, and α-carotene were detected only in low concentrations. Therefore, C57BL/6J female mice appear to serve as an appropriate model for investigating the efficacy of individual or purified mixtures of dietary carotenoids on cellular abnormalities relevant to target organ carcinogenesis. Future studies with this animal model should provide mechanistic and phenomenological leads to understand the efficacy of multicarotenoid supplementation in cancer chemoprevention.

Although there is some evidence that individual or certain combinations of dietary carotenoids may protect specific organs against carcinogenesis, supplementation with a wide range of carotenoids would be expected to provide a collective protective effect and may prove to be a more effective chemoprevention strategy. This is also supported by the epidemiological studies that have associated the high consumption of carotenoid-rich foods with a lower risk of cancer. Therefore, future studies should inevitably examine the protective effect of all the serum carotenoids and their metabolites rather than focussing on an individual carotenoid.

The efficacy studies with multicarotenoid-involving rodents are undoubtedly the first step in establishing the biological properties of carotenoids as chemopreventive agents. However, at the same time, clinical studies with multicarotenoid involving humans can focus on prevention and treatment of cancers that have been successfully treated with certain individual carotenoids. An example of this is a clinical trial involving patients with oral leukoplakia in which treatment with β-carotene has, to some extent, been successful.[47] Preliminary data from a recent phase II randomized clinical trial with lycopene and several other minor carotenoids (purified from a concentrated tomato extract) in patients before radical prostatectomy have also shown promising results in treatment of early stage prostate cancer.[48] Colon and prostate cancers and oral leukoplakia are ideal models for investigating the chemopreventive efficacy of multicarotenoid in humans because the known intermediate and end-point biomarkers for these cancers can be used to determine the study outcome within a relatively short time.

ACKNOWLEDGMENTS

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CHAPTER 11

Chemoprevention of Colon Cancer by Curcumin

Bandaru S. Reddy and Chinthalapally V. Rao

CONTENTS

INTRODUCTION

Cancer is a major cause of death throughout the world, and in the developed world, it is generally exceeded only by cardiovascular disease. Cancer of the large bowel, which is the fourth most common cancer in the world, is one of the leading causes of cancer death in both men and women in Western countries, including the U.S. where about 150,000 new cases of this cancer and about 56,000 related deaths were estimated in 2002.[1] Marked international differences in the incidence and mortality of colon cancer and increase of risk in populations migrating from low- to high-risk

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areas, such as from Japan to the U.S., suggest that environmental factors, notably dietary habits, play an important role in etiology of this disease. It is important to note that in Japan there is an upward trend in colon cancer risk, which cannot be attributed to genetic differences.[2,3] The dietary habits in Japan have dramatically changed since the 1960s and are rapidly becoming similar to those in the U.S. and Canada. Importantly, nutritional epidemiological studies conducted in Japan suggest that increases in colon cancer in Japan can be attributed to westernization of Japanese food habits.[2,3] Several epidemiological and preclinical studies also suggest a relationship between colorectal cancer and dietary factors;[2–6] thus, diet modification is a logical preventive strategy.

There is increasing evidence that consumption of certain kinds of vegetables and fruits and intake of certain nonnutrients present in foods do, in fact, reduce the risk of colon cancer, and there are many reasons why this is biologically plausible.[7] Although the protective mechanisms underlying the effects of fruit and vegetable consumption are complex, it is likely that many food items contain significant levels of phytochemicals, some of which have chemopreventive potential. An attempt to identify chemopreventive agents present in fruits and vegetables should lead to new strategies for cancer prevention. These agents include curcuminoids, carotenoids, terpenoids, tocopherols, isothiocyanates, allium compounds, and plant sterols, to cite a few. Phytochemicals are present in human diets in substantial quantities. They give little concern for toxicity and are relevant not only for primary prevention of colon cancer in the general population but also for secondary prevention in patients with colonic polyps.

Basic research in carcinogenesis has identified many enzymes, genetic lesions, and other cellular constituents associated with initiation and promotion of precancerous lesion to invasive disease. As our understanding of the mechanisms of carcinogenesis has increased, we envision more possibilities for intervening at multiple points along the multistep process of carcinogenesis, namely during initiation, promotion, and progression. The concept of cancer prevention before occurrence of clinically detectable tumors is receiving increasing attention as an attractive and plausible approach to cancer control.[8,9] It has the potential to be a major component of cancer control. Growing knowledge about the mechanisms by which chemopreventive agents, both naturally occurring and synthetic compounds, act defines opportunities to use specific agents or combinations at critical stages of cancer initiation, promotion, and progression. It should be recognized that phytochemicals and their bioactive metabolites act as modulators of molecular pathways of cancer progression.

CURCUMIN AS CHEMOPREVENTIVE AGENT

It is noteworthy that use of medicinal plants or their crude extracts in the prevention and treatment of several chronic diseases has been traditionally practiced in various ethnic societies worldwide. In South and Southeast Asia, including India, turmeric, the powdered rhizome of *Curcuma longa* L., has been used extensively in food preparations and to treat inflammatory conditions and chronic diseases. It is also

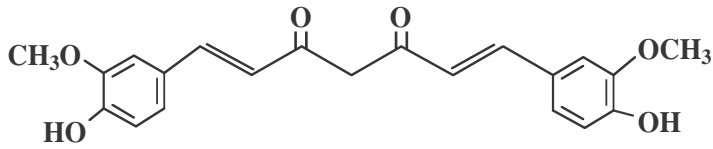

Figure 11.1 Chemical structure of curcumin.

used as a coloring and flavoring additive to foods.[10,11] Curcumin (Figure 11.1; diferuloylmethane; 1,7-*bis*-[4-hydroxy-3-methoxyphenyl]-1,6-heptadine-3,5-dione), the major pigment in turmeric, has both anti-inflammatory and antioxidant properties.[12–15] Antioxidant/anti-inflammatory activities include scavenging of reactive electrophiles and oxygen radicals, modulation of eicosanoid production, and induction of apoptosis. Preclinical efficacy studies have demonstrated that dietary administration of curcumin inhibits carcinogen-induced tumors in the skin, colon, mammary gland, and oral cavity in model assays. The chemopreventive efficacy of curcumin is based on multiple mechanisms, including inhibition of lipid peroxidation, free radical formation, lipoxygenase (LOX) and cyclooxygenase (COX), and protein kinase C.

This paper focuses on the chemopreventive efficacy of curcumin in preclinical models of cancer prevention with a focus on cancers of the colon, skin, mammary gland, prostate, and oral cavity and discusses the results of mechanistic studies thus far conducted in our laboratory.

Skin Carcinogenesis

Earlier studies have indicated that antioxidants and anti-inflammatory agents inhibit polycyclic aromatic hydrocarbon-induced initiation, as well as promotion by 12-*O*-tetradecanoylphorbol-β-acetate (TPA) of mouse skin carcinogenesis. For example, Huang et al.[16–18] conducted a series of studies in CD mice on the chemopreventive efficacy of curcumin against skin carcinogenesis. Topical application of 1, 2, or, 10 μmol of curcumin together with TPA twice a week for 20 weeks inhibited the number of skin tumors per mouse by 39, 77, and 98%, respectively, and reduced tumor incidence (percentage of animals with skin tumors) by 21, 66, and 82%. In another study, application of 10 μmol of curcumin twice a week for 19 weeks completely inhibited 7, 12-dimethylbenez[a]anthracine (DMBA)-induced and TPA-promoted skin tumor incidence and multiplicity. Furthermore, topical application of 3 or 10 μmol of curcumin prior to application of benzo(a)pyrene or DMBA and TPA reduced skin tumor multiplicity by 58 and 62%, respectively. Soudamini and Kuttan[19] showed that topically applied curcumin significantly suppressed DMBA-induced and croton oil-promoted skin carcinogenesis in Swiss mice. These studies provided convincing evidence that topical application of curcumin is an effective inhibitor of skin tumorigenesis in preclinical models. In most of these studies, curcumin was found to be less than 85% pure.

The mechanisms by which curcumin inhibits skin carcinogenesis are not completely understood, but it is known that inhibition of skin tumorigenesis is associated

with modulation of TPA-induced DNA synthesis and ornithine decarboxylase activity in the epidermis and also with TPA- and arachionic acid (AA)-induced edema of mouse ears.[20] It has also been shown that curcumin strongly inhibits epidermal COX and LOX activities *in vitro*.[21,22] This suggests that curcumin may inhibit the enzymes that metabolically activate benzo[a]pyrene (B[a]P) and DMBA. Thus, the chemopreventive properties of curcumin against skin carcinogenesis could be related to its potent inhibitory effect on (AA)-induced inflammation and on AA metabolism through both the COX and LOX pathway in mouse epidermis.[21,22]

Forestomach Carcinogenesis

Curcumin has also been tested as a chemopreventive agent against chemically-induced forestomach carcinogenesis in preclinical models.[23] Administration of commercial-grade curcumin at 0.5 or 2.0% in the diet during the initiation period (2 weeks before, during, and for 1 week after carcinogen treatment) suppressed B[a]P-induced forestomach tumors in A/J mice by 51 to 53%.[23] In the diet given at 0.5 and 2.0% levels during the postinitiation period (1 week after carcinogen treatment) curcumin inhibited B[a]P-induced forestomach tumors in A/J mice by 47 to 67%. In these experiments dietary curcumin also reduced tumor size and the multiplicity of papillomas and squamous cell carcinomas of the forestomach. Curcumin appears to influence the metabolic activation and detoxification of B[a]P and/or the metabolic activation of B[a]P to DNA adducts, and it appears to inhibit AA metabolism *via* COX and LOX activities in inhibiting the formation and preventing the progression of tumors of the forestomach in this model assay.[21–24]

Colon Carcinogenesis

Efficacy Studies

Curcumin has been tested as a chemopreventive agent against azoxymethane (AOM)-induced colon carcinogenesis in preclinical models. Aberrant crypt foci (ACF) are putative preneoplastic lesions that occur in the colon of both animals and humans. Lesions in humans resemble those induced in rodents with carcinogens. ACF express mutations in the APC gene and ras oncogene that appear to be biomarkers of colon cancer development. Because ACF are induced specifically by carcinogens that predominately elicit colonic tumors, they are considered to be precursors of colon cancer. The multiplicity of ACF increases over time and reliably to be a predictor of colon tumor outcome. We assessed the potential colon cancer chemopreventive effect of curcumin using AOM-induced ACF as a biomarker. Our results indicate that 0.2% curcumin in the diet significantly inhibited AOM-induced colonic ACF in rats (Figure 11.2).[25] Other investigators reported that oral administration of 2% curcumin inhibited AOM-induced colonic ACF in rats[26] and AOM-induced focal areas of dysplasia in the colons of mice.[27] Also, dietary administration of 0.2% and 0.5% tetrahydrocurcumin, a derivative of curcumin, significantly inhibited 1,2-dimethylhydrazine-induced colonic ACF in mice.[28] BrDU labeling indices were also decreased in animals given the curcumin.[27]

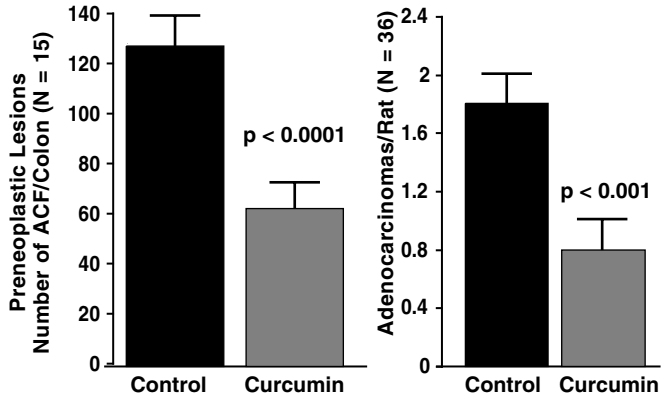

Figure 11.2 Effect of curcumin on azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) and adenocarcinoma multiplicity in male F344 rats.

The described studies provided an impetus to investigate the chemopreventive efficacy of curcumin against colon carcinogenesis with tumors as the endpoint. We observed that dietary administration of curcumin during the initiation and postinitiation stage at 0.2% level significantly suppressed the incidence and multiplicity of AOM-induced adenocarcinomas (invasive and noninvasive) of the colon (Figure 11.2) and also curtailed tumor volume in male F344 rats.[29] The results of this study conducted with synthetic (99.9% pure) curcumin are of great interest because long-term dietary administration produced no gross changes in the liver, kidney, stomach, intestine, and lungs of F344 rats. Huang et al.[23] found that dietary administration of 0.2, 2.0, or 4.0% curcumin during initiation and/or postinitiation suppressed AOM-induced colon adenomas and adenocarcinomas in CF-1 mice by 50 to 66%. Pereira et al.[26] reported that 0.8 and 1.6% curcumin when administered in the diet during initiation and postinitiation inhibited both the incidence and multiplicity of adenomas in a dose-dependent manner.

All the above studies clearly demonstrate the chemopreventive potential of curcumin during the initiation and postinitiation period of colon carcinogenesis. We have also evaluated the efficacy of curcumin during the promotion/progression stage when premalignant lesions would have developed[30] and found that 0.2 and 0.6% of curcumin in the diet given during promotion/progression period (14 weeks after carcinogen treatment) inhibited AOM-induced colon adenocarcinomas by 33 to 56% (Figure 11.3). This suggests that curcumin may effectively retard growth and development of existing neoplastic lesions in the colon and bodes well for the potential use of this agent in secondary prevention of colon cancer in high-risk individuals, such as patients with colonic polyps.

Mechanistic Studies

Curcumin's chemopreventive activities in the colon may be mediated, at least in part, by modulation of key signaling pathways involved in cell proliferation, including

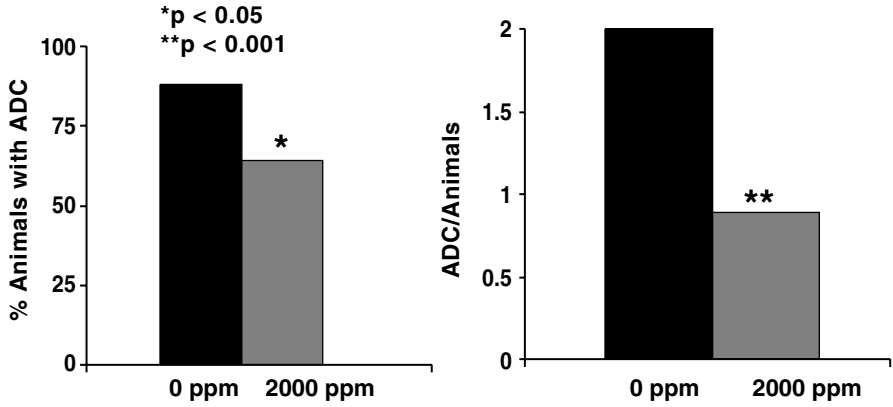

Figure 11.3 Effect of curcumin administered during promotion/progression stage of colon carcinogenesis. *Note:* ADC = adenocarcinomas.

ornithine decarboxylase (ODC), a rate-limiting enzyme in polyamine biosynthesis, and tyrosine protein kinase (TPK), an enzyme that has been found to be highly active in proliferating normal and neoplastic cells.[31,32] Based upon this, lowering ODC and TPK activities may prevent the early proliferation of neoplastic cells. In order to answer the question whether inhibition of colon carcinogenesis by curcumin is mediated through the inhibition of colonic ODC and TPK, we determined the activities of these enzymes in colon mucosa of male F344 rats treated for colon-specific carcinogen, AOM, and fed 0.2% curcumin in the diet. Dietary curcumin significantly suppressed the AOM-induced ODC and TPK activities in the colonic mucosa ($p < 0.0001$) of animals fed the 0.2% curcumin in the diet as compared with controls.[25] Curcumin may also exert chemopreventive activities by decreasing the overexpression of COX-2, which is thought to play an important role in colon carcinogenesis.[33] Tsujii and DuBois[34] have implicated COX-2 activity in the regulation of apoptosis of rat intestinal epithelial cells and have shown that overexpression of COX-2 can lead to the suppression of apoptosis. Elevated levels of COX-2 have been observed in human colon tumors and chemically induced colon tumors in rats.[35,36]

The inducible form of nitric oxide synthase (iNOS) can be expressed in response to proinflammatory agents. Nitric oxide at high and sustained levels, or its oxidative product peroxynitrite, may activate COX-2 and induce DNA damage and gene mutations. Importantly, increased iNOS expression and activity have been observed in human and rat colon tumors.[37,38] Also, increased 12-LOX mRNA expression has been observed in human and rat colon tumors. LOX-catalyzed products, leukotrienes, and hydroxyeicosatretraenoic acids (HETEs) have been shown to be involved in the progression of cancer.[39,40] 12(*S*)-HETE and 5(*S*)-HETE, implicated in progression of colon tumors, elicit biological action of growth factors and cytokines.[39,40] Of some interest, additional evidence supporting the role for COX-2 and iNOS comes from our studies, which show a marked reduction in colon carcinogenesis in rodents with highly selective COX-2 and iNOS inhibitors.[41,42]

Other mechanisms by which curcumin may inhibit colon carcinogenesis are via decreasing activities of colonic mucosal and tumor phospholipase A_2 (PLA_2) and phos-

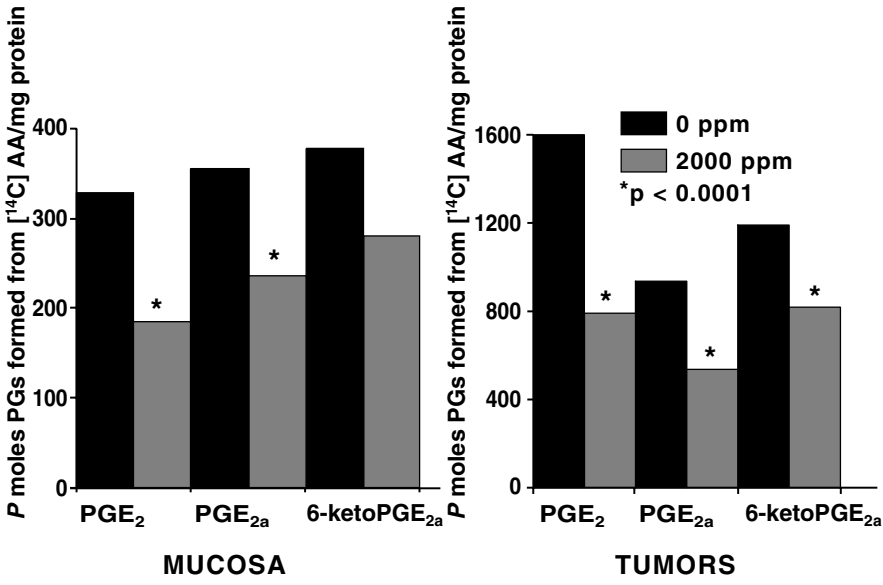

Figure 11.4 Effect of dietary curcumin on catalytic activity of cyclooxygenase (COX) in colon.

pholipase Cγ1 (PLCγ1) and levels of PGE_2.[25,29] PLA_2 and PLCγ1 are dominant pathways for AA release, an essential first step in the production of inflammatory eicosanoids.

It is possible that curcumin not only modulates PLA_2 and $PLC\gamma_1$, altering the endogenous AA available as a substrate for production of COX and LOX metabolites, it may also affect COX and LOX pathways. The effects of dietary curcumin on catalytic activities of COX and LOX enzymes in the colonic mucosa and tumors of animals treated with AOM was determined in order to understand the possible modulating role of this phytochemical in colon carcinogenesis.[29] The catalytic activity of LOX was determined by measuring the ^{14}C-HETES formed after incubation of cytosol fraction of colonic mucosa and tumors with ^{14}C-arachidonic acid. COX catalytic activity was determined in the microsomal fraction of colonic mucosa and tumors by measuring the ^{14}C-prostaglandins formed from ^{14}C-arachidonic acid. The results summarized in Figure 11.4 indicate that markedly increased levels of prostaglandins (three- to fivefold) were observed in the colonic tumors of animals fed the control diet when compared with the colonic mucosa of animals fed the curcumin diet. Animals fed the 0.2% curcumin in the diet significantly suppressed the COX catalytic activity (25 to 50%) in the colonic mucosa and tumors compared with those fed the control diet. As summarized in Figure 11.5, the levels of HETEs were significantly higher in colonic tumors compared with colonic mucosa. Dietary administration of 0.2% curcumin significantly inhibited the formation of colonic mucosal and tumor 5(*S*)-, 8(*S*)-, 12(*S*)-, and 15(*S*)-HETES by 27 to 49% as well as PGE_2, $PGE_{2\alpha}$, and 6-keto $PGE_{2\alpha}$, PGF_1, suggesting that dietary curcumin suppressed the catalytic activity of LOX in colonic mucosa and tumors.

The inhibition of colon tumorigenesis was also associated with increased apoptosis.[30,43] Results generated in our laboratory indicate that continuous administration

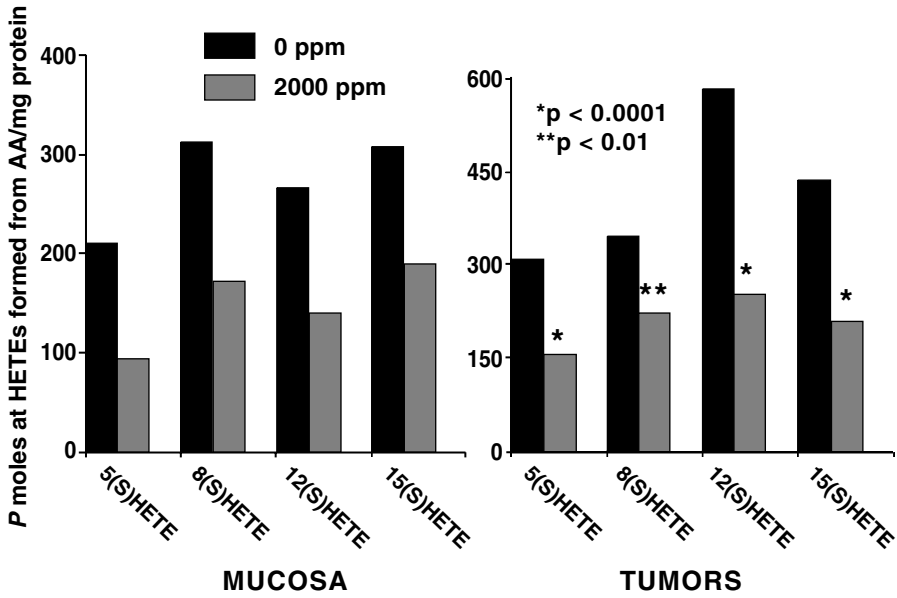

Figure 11.5 Effect of dietary curcumin on catalytic activity of lipoxygenase (LOX) in colon.

of 0.2% curcumin in the diet significantly increased the apoptotic index (17.7%) in the colon tumors as compared with that in tumors of rats given the control diet (8.3%) ($p < 0.0001$). Curcumin has also been reported to inhibit cell growth and induce apoptosis of human colon carcinoma cells *in vitro*.[44]

Curcumin and related phytochemicals may also exert chemopreventive activity via molecular mechanisms. Our results indicate that curcumin-mediated suppression of colonic COX-2 and iNOS enzyme activities in rats (Figure 11.6). was accompanied by suppressed activation of the transcription factor, nuclear factor-kappa B (NF-KB) involved in the regulation of COX-2 and iNOS expression.[45,46] Li and Lin-Shia[47] demonstrated that curcumin is also a potent inhibitor of protein kinase C, EGF-receptor tyrosine kinase, and I kappa B kinase. Also, curcumin inhibits the expression of the c-jun, c-fos, c-myc, and iNOS genes, suggesting that it may suppress tumor promotion by broadly interfering with cell growth-related signal transduction pathways in the target cells. A full understanding of the mechanism(s) by which curcumin inhibits colon tumorigenesis remains to be determined; however, it is likely that the chemopreventive action may, at least in part, be related to the modulation of AA metabolism, signal transduction pathways, and apoptosis.

Mammary Carcinogenesis

There is growing evidence that dietary curcumin possesses chemopreventive potential against mammary cancer. Dietary administration of 1% turmeric or 0.5% ethanolic extract of turmeric inhibited DMBA-induced mammary carcinogenesis during the initiation as well as the postinitiation phase of carcinogenesis.[48] Singletary

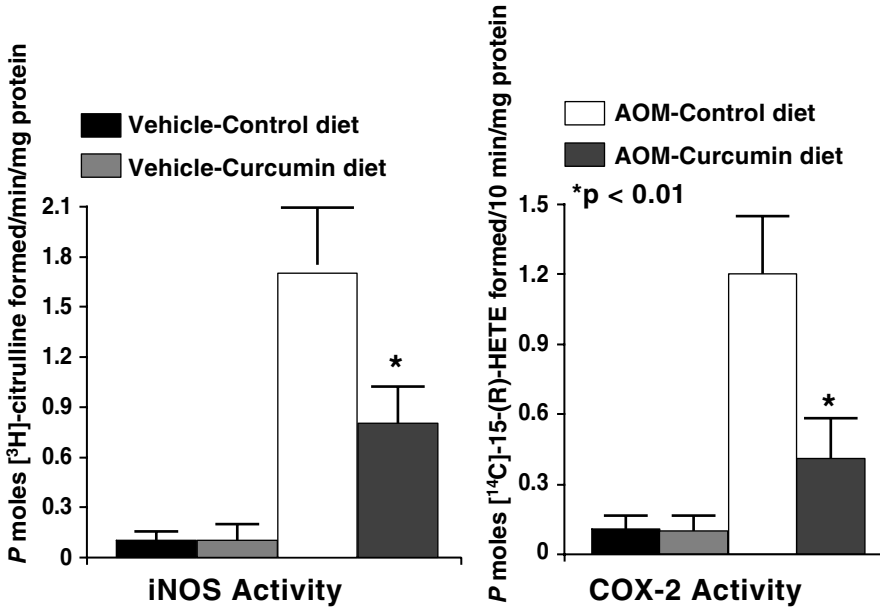

Figure 11.6 Effect of curcumin on AOM-induced colonic mucosal inducible NOS and COX-2 activities during early stage of colon carcinogensis.

et al.[49] demonstrated that i.p. administration of 100 and 200 mg curcumin/kg body weight significantly inhibited the number of DMBA-induced mammary tumors in female rats. Inhibition of mammary tumors in this model system was associated with a decrease in the formation of mammary DMBA-DNA adducts. In radiation-induced mammary carcinogenesis, dietary administration of 1% curcumin to pregnant rats during the initiation phase significantly inhibited mammary adenocarcinomas.[50] There was no change in litter size or in body weights of pups born from curcumin-fed pregnant rats, suggesting that curcumin at these doses was not toxic. Also, administration of 1% curcumin in the diet significantly inhibited radiation-induced mammary adenocarcinomas and ER (+) PgR (+) tumors in rats during the promotion phase.[51] Mehta et al.[52] examined antiproliferative effects of curcumin in several breast cancer cell lines, including hormone-dependent and hormone-independent cell lines, as well as multidrug-resistant cell lines. All these cell lines were highly sensitive to curcumin. The growth-inhibiting effect of curcumin was correlated with its inhibition of ornithine decarboxylase activity, suggesting that curcumin is a potent antiproliferative agent in breast tumor cells. Another *in vitro* study of transformed human breast epithelial cells (MCF10A) showed that curcumin inhibits H-ras-induced invasive phenotype in these cells and downregulates matrix metalloprotease in a dose-dependent manner.[53] Curcumin-induced cell death in H-ras MCF10A cells was mainly due to apoptosis in which a downregulation of Bcl-2 and upregulation of BAX were involved, suggesting that curcumin inhibits invasion and induces apoptosis.

Prostate Cancer

Another area of research focuses on the chemopreventive properties of curcumin against prostate cancer. Curcumin decreased the proliferative potential and induced apoptosis in both androgen-dependent and androgen-independent prostate cancer cells *in vitro*.[54,55] Dietary administration of 2% curcumin also caused a marked decrease in cell proliferation, a significant increase in apoptosis, and a significant decrease in angiogenesis in nude mice treated with LNCaP prostate cancer cells.[54] A study by Dorai et al.[55] showed that curcumin inhibits tyrosine kinase activity in the epidermal growth factor receptor and can induce apoptosis in both androgen-dependent and androgen-independent prostate cancer cells. Thus curcumin could be a therapeutic agent and potentially prevent the progression of prostate cancer to its hormone refractory state.

Other organs

There is limited but promising evidence that curcumin inhibits esophageal and oral carcinogenesis. Inhibitory effects of curcumin administered during the initiation or postinitiation stage of *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal carcinogenesis in male F344 rats have been described by Ushida et al.[56] Administration of 500 ppm curcumin in the diet significantly suppressed the incidence and multiplicity of NMBA-induced esophageal preneoplastic lesions and neoplasms during both initiation and postinitiation phases of carcinogenesis. This inhibition was associated with suppression of NMBA-induced cell proliferation. Tanaka et al.[57] showed that dietary curcumin inhibits 4-nitroquinoline-1-oxide-induced oral carcinogenesis (primarily in the tongue) in rats. Also, curcumin at 0.1-, 1.0-, and 10-μm doses induced significant dose-dependent inhibition of both cell growth and cell proliferation of the human oral squamous carcinoma cell line, SCC-25.[58]

Curcumin was also effective against diethylnitrosamine (DEN)-induced murine hepatocarcinogenesis.[59] Administration of 0.2% curcumin in the diet 4 days before DEN treatment and until death, i.e., at 42 weeks of age, suppressed the incidence (by about 62%) and multiplicity (by about 81%) of hepatocellular carcinomas. This inhibition of hepatocarcinogenesis was associated with the modulation of ras p21 and provided proliferating cell nuclear antigen (PCNA).

PRECLINICAL TOXICITY STUDIES

Although there are only limited preclinical toxicity studies on curcumin, the toxicity of turmeric oleoresin has been extensively studied as part of the National Toxicology Program (NTP). Commercial turmeric is composed of approximately 75% curcumin by weight. In rats, the LD_{50} of curcumin was found to be >3,500 mg/kg body weight. Single *ig* dose between 1,380 and 3,500 mg/kg body weight produced no adverse effects in rats.[60] In another study, single *ig* doses up to 5,000 mg/kg body weight gave no clinical symptoms and did not affect relative organ weights in male and female rats.[61] In the 90-day rat study, curcumin at 1,995 mg/kg body weight/day

and above given by *ig* intubation to male rats decreased reticulocyte counts and increased corpuscular hemoglobin (MCH). The effects were not considered biologically significant.[60]

In dogs, 250, 500, and 1,000 mg curcumin/kg body weight/day in a gelatin capsule significantly increased MCH levels at higher doses; however, this is not considered to be biologically relevant because overt anemia was not detected.[60] The safer dose or no observed adverse effects (NOAE) for curcumin appears to be >1,000 mg/kg body weight/day in male and female dogs.

There are several short- and long-term studies on curcumin in the literature that confirm the lack of significant toxic effects from this agent as a food component on body weight, hematology, serum chemistry, or histology of the gastrointestinal tract, liver, spleen, or kidney.[62] Also, oral administration of curcumin to rats at doses up to 1,000 mg/kg body weight/day for 3 months and to monkeys at doses up to 800 mg/kg body weight for 3 months remained without evidence of adverse effects on growth, behavioral, biochemical, and histopathological parameters.[63,64]

Absorption, distribution, and metabolism studies using radiolabelled curcumin suggested poor gastrointestinal absorption and very limited extra hepatic metabolism of this agent.[65,66] Dietary curcumin at dose levels of 2,000 to 50,000 ppm (2-year NTP study) failed to show evidence of carcinogenesis in rats and mice.[67] Curcumin was not mutagenic in the Ames *Salmonella* typhimurium assay with or without metabolic activation or in the mouse-dominant lethal assay.[68,69] In contrast, positive results were obtained in some assays of clastogenicity depending on the dose, length, and route of exposure to curcumin.[70] Overall, curcumin, even at high dose levels, is free of adverse effects on growth, biochemical, histopathological, mutagenic, carcinogenic, and reproductive toxicities in preclinical studies.

SUMMARY AND CONCLUSIONS

Curcumin is a major pigment in turmeric, the powdered rhizome of *Curcumia longa Linn*, which has been widely used as a coloring and flavoring agent in foods and also has been traditionally used for the treatment of certain inflammatory conditions and chronic diseases. There is convincing evidence in preclinical models and *in vitro* assays that curcumin inhibits tumorigenesis in the oral cavity, skin, forestomach, colon, prostate, and mammary gland when administered during initiation and post-initiation stages of carcinogenesis.

Earlier studies have demonstrated that topical application of low doses of curcumin markedly inhibited skin carcinogenesis in mice. In the rat, dietary administration of curcumin during the promotion/progression stage of colon carcinogenesis led to retardation of growth and of existing preneoplastic lesions in the colon. Several studies have also provided strong evidence that the modulation of AA metabolism through COX and LOX activities and the induction of apoptosis may be related to its chemopreventive properties.

The inhibition of tumorigenesis by curcumin may also be mediated through modulation of a signal transduction pathway(s) associated with tumor promotion. Preclinical efficacy studies and the lack of toxicity and side effects, as well as the

availability of curcumin as a natural product that has been used in population groups for several centuries, are compelling factors to schedule this agent for human clinical trials for chemopreventive efficacy. Curcumin has several advantages over other synthetic agents with similar modes of action and efficacy that are already being tested in human clinical trials.

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