Antiatherogenic Properties of Naringenin, a Citrus Flavonoid

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INTRODUCTION

An inverse association between flavonoid intake and coronary heart disease (CHD) has been suggested by a number of epidemiological studies (42,44,54,78). As a consequence, there is considerable interest in investigating the antiatherogenic nature of these compounds. Flavonoids are naturally occurring molecules abundant in fruit, vegetables, nuts, seeds, and beverages, such as tea and wine. Over 4000 different flavonoids have been identified in the human diet, where the daily intake ranges from levels as high as 1 g (59) to a more conservative estimate of 23 mg (43). Flavonoids are characterized by their polyphenolic structure (Fig. 1). Variations in this structure give rise to the major classes of flavonoids, including the flavonols, flavones, flavanones, catechins, anthocyanidins, isoflavones, dihydroflavonols, and chalcones.

Naringenin belongs to the class of flavonoids called the flavanones. The flavanones are abundant in citrus fruits such as grapefruit (Citrus paradisi) and the oranges (Citrus sinensis). The role of naringenin and the related citrus flavanone hesperetin in the prevention and treatment of disease has recently received considerable attention (71), with particular interest in the use of these flavanones as anticancer (36) and antiatherogenic (85) compounds. This review focuses on the potential antiatherogenic roles of citrus flavonoids, with particular focus on naringenin.

CHEMISTRY

The chemical name of naringenin is 2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one (Fig. 1), and it has a molecular weight of 272.26 (C_{15}H_{12}O_{5}). Naringenin is almost insoluble in water and is soluble in organic solvents such as alcohol. Naringenin is derived from the hydrolysis of glycone forms of this flavanone, such as naringin or narirutin (67). Naringin (naringenin-7-rhamnoglucoside), the bitter principle

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of grapefruit (Citrus paradisi), is found in the juice, flower, and rind of the fruit and constitutes up to 10% of the dry weight. Unlike the aglycone naringenin, naringin is relatively soluble in water (1 mg/mL in water at 40°C) (67). Naringin is present in grapefruit juice at concentrations of up to 800 mg/L (81) and occurs as a mixture of chiral isomers that vary markedly in proportion depending on the maturity of the fruit and the method of purification. Naringin and other naringenin glycosides can be found in a variety of other sources including propolis (73) and Prunus davidiana (14). Monotes engleri contains a prenylated form of naringenin (6-(1,1-dimethylallyl)naringenin) (86).

A number of flavonoids including hesperetin, are structurally related to naringenin. Hesperetin, S-2,3-dihydro-5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-1-benzo-pyran-4-one (Fig. 1), has a molecular weight of 302.28 (C_{16}H_{14}O_{6}). Like naringenin, hesperetin is relatively lipophilic, being soluble in organic solvents and only slightly soluble in water (67). Hesperetin, which differs from naringenin by the substitution of a methoxy group at position R6 and the addition of a hydroxy group at position R5, is derived from the hydrolysis of its glycone form hesperidin (hesperetin 7-rhamnoglucoside) (67). Hesperidin is the predominant flavonoid in lemons and sweet oranges. As a citrus flavanone, hesperetin might possess antiatherogenic actions in common with the structurally related naringenin. Therefore, this review will also briefly describe the antiatherogenic properties of hesperetin.

**PHARMACOKINETICS**

As naringenin is generally present in foods bound to sugars as β-glycosides (i.e., naringin), it was originally thought that absorption from the diet would be negligible.
However, a number of studies have detected naringenin in human urine (3,29,50,64,92) and plasma (3,29) following oral doses of pure naringin (3,50) or grapefruit juice (3,29,64,92). Hesperetin has been detected also in human urine (3,92) and plasma (3) following doses of pure compound or orange juice. These results showed that naringenin and hesperetin can be absorbed from the diet. In fact, studies using 3-[14C]-hesperetin in rats indicate that intestinal absorption of aglycone flavanones may be greater than 90% (47).

The glycoside form of naringenin, naringin, is not detected in either human or animal urine suggesting that naringin is deglycosylated prior to intestinal absorption. It has been shown that the intestinal microflora of rats (9,34) and humans (29,46) can cleave the glycosidic bonds of naringin, liberating the aglycone form naringenin. Studies by Furh et al. (29) have shown large interindividual differences in the ability of humans to convert naringin to naringenin as evident in feces, suggesting that the presence or absence of certain bacterial strains in the gut may explain the interindividual variability observed in studies examining grapefruit-juice–drug interactions (6) (discussed later). In a recent study, Kim et al. (46) identified a number of bacteria in the human intestine that are capable of transforming naringin to naringenin and hesperidin to hesperetin, including Fusobacterium K-60, Eubacterium YK-4, and Bacteroides JY-6.

Intestinal microflora have been shown to further metabolize naringenin. Booth et al. (9) originally showed that oral doses of naringin administered to rats yielded 4-hydroxyphenylpropionic acid, 4-hydroxycinnamic acid, and 4-hydroxybenzoic acid sulfate in the urine. Griffiths and Smith (34) showed that rat intestinal microflora resulted in the generation of 4-hydroxyphenylpropionic acid and naringenin only, suggesting that 4-hydroxycinnamic acid and 4-hydroxybenzoic acid may be metabolites that are generated by hepatic enzymes. Honohan et al. (47) showed that the major intestinal metabolite of 3-[14C] hesperetin in rats was 3-phenylpropionic acid, while hepatic enzymes mediated further breakdown to benzoic acid and CO₂. More recently, Kim et al. (46) have shown that human intestinal bacteria can metabolize naringin to naringenin and then to 4-hydroxybenzoic acid, phloroglucinol, 2,4,6-trihydroxybenzoic acid, and 4-hydroxyphenylacetic acid, demonstrating species differences in the intestinal metabolism of naringin and naringenin.

A large percentage of naringenin absorbed in humans appears in the urine as naringenin glucuronides (29,50,64), indicating that conjugation, presumably within the intestine or liver (29), may play a major role in the metabolism of this compound. Naringenin has also been reported to be a substrate for a UDP glucuronosyl transferase (33). Furh et al. (29) showed that excretion of naringenin glucuronides in humans reaches levels more than 100-fold higher than the concentration of naringenin excreted in the urine. Hackett et al. (38) have shown that a major route for flavonoid metabolism in rats is excretion in the bile. This generally occurs following conjugation of flavonoid polar hydroxyl groups with glucuronic acid, sulfate, or glycine. Naringenin present in the bile may either be excreted or reabsorbed, therefore raising the possibility of enterohepatic recycling of naringenin.

In addition to conjugation, naringenin may be further metabolized by hepatic enzymes. Nielsen et al. (74) have recently examined the metabolism of naringenin and hesperetin in rat liver microsomes. The major metabolite observed for both naringenin and hesperetin was the flavonoid eriodictyol. Eriodictyol was generated by the addition of a hydroxy group to the R6 position of naringenin and the demethylation of the R6 methoxy of hesperetin. The hydroxylation of naringenin was shown to be mediated by hepatic cytochrome P450 1A (CYP1A) (74).
Naringenin has been detected in the plasma following oral administration of naringin or grapefruit juice but is generally reported to be below accurate detection limits (3,29) and has not been reported to exceed 4 μM (29). However, due to the lipophilic nature of naringenin, it is possible that it accumulates within tissues, particularly membranes, and eventually reaches greater concentrations than those observed in the plasma. This accumulation would most likely occur in tissues such as the liver and intestine. In support of this, it has been demonstrated that approximately 40% of a dose of hesperetin\textsubscript{-3-}[\textsuperscript{14}C] given either orally or intraperitoneally to rats, was recovered as \textsuperscript{14}CO\textsubscript{2}, a by-product of hepatic metabolism (47). This suggests that at least 40% of the hesperetin dose reached the liver. Similar experiments using radiolabelled naringenin in either humans or rats have not yet been reported. Clearly further studies are required to examine the tissue distribution of naringenin.

TOXICITY

Toxicity studies of naringenin are scarce; however, flavonoids are generally considered to have low toxicity. In one study (9), a single, 2-g oral dose of naringin was administered to a human volunteer with no deleterious effects. Naringin has also been administered to humans at oral doses of 500 mg with no adverse responses (3,50). Kim et al. (46) have reported on the \textit{in vitro} cytotoxicity for various flavonoids in a number of cells lines. The IC\textsubscript{50} (50% inhibition concentration) for cell growth was >1 mM for both naringenin and hesperetin in the human hepatoma cell line HepG2, the Macacus\textsuperscript{\textregistered} rhesus monkey kidney cell line MA-104, and the human lung cancer cell line A549. The results of these studies indicate that these flavonoids have relatively low toxicity in cell culture.

POTENTIAL CARDIOPROTECTIVE ACTIVITIES

An association between flavonoid consumption and reduced cardiovascular mortality was first revealed in the Zutphen Elderly study, in which 805 men from the Netherlands, aged 65–84 years, were studied over 5 years (42). Flavonoid consumption (with quercetin and kaempferol representing 95% of the measured flavonoid intake) was inversely associated with mortality from CHD. Relative risks for CHD mortality and first myocardial infarction were approximately 50% lower in the highest tertile of flavonoid intake (mean intake 41.6 mg/day) compared to the lowest tertile (mean intake 12.0 mg/day) (p = 0.015, 95% CI 0.20–0.88). Further results from the Zutphen Elderly study (54), showed an inverse association between dietary flavonoid intake and the incidence of stroke. Similar associations between flavonoid intake and reduced CHD were reported in men and women from Finland (57) and men from the Seven Countries Study, which included cohorts from Finland, Greece, Yugoslavia, Japan, Netherlands, Italy and the United States (44). The relationship between intake of flavonols and flavones and the risk for fatal and nonfatal CHD was examined in the Health Professionals Follow-up Study (78), which involved 34,789 male health professionals. The intake of flavonoids was not associated with risk for total CHD; however, a nonsignificant trend for an inverse association with CHD mortality and flavonoid intake was observed in men with previous CHD. In contrast to these reports, a recent study by Hertog et al. (45) revealed increased mortality from ischemic heart disease in Welsh men consuming high amounts of flavonols, mainly from tea. Therefore,
although inconclusive, the epidemiological evidence suggests a protective effect that flavonoids have against cardiovascular disease. To date, no study has specifically examined the association between the intake of flavanones, the group of flavonoids to which naringenin belongs, or citrus flavonoids and CHD.

**ANTIOXIDANT, ANTITHROMBOTIC, ANTIINFLAMMATORY, AND VASODILATORY ROLES IN ATHEROSCLEROSIS**

Much of the observed association between flavonoid intake and reduced CHD mortality has been attributed to their antioxidant properties (25,76,84). Cells of the arterial wall including macrophages, smooth muscle cells, and endothelial cells can oxidize or otherwise modify low density lipoproteins (LDL) (40,83). Modified LDL can be a ligand for receptor-mediated processes leading to significant accumulation of cholesteryl esters (CE) in macrophages and smooth muscle cells (40,83). These CE-rich cells, known as foam cells, are the hallmark of the early atherosclerotic lesion. Central to this issue is whether flavonoids are present within the subendothelial space of the arterial wall in concentrations sufficient to protect lipoproteins such as LDL from oxidation. There is some evidence to suggest that flavonoids can be incorporated into lipoproteins within the liver or intestine and subsequently be transported within the lipoprotein particle (30,55). Naringenin has been shown also to associate with and penetrate lipid membranes (84). Therefore flavonoids, including naringenin, may be ideally located for protecting LDL from oxidation.

Flavonoids have been shown to inhibit the oxidation of LDL in vitro (18,19,27,39, see ref. 5 for review). Furthermore, the addition of the flavonoids quercetin and catechin to the diet have been shown to reduce LDL oxidation ex vivo in rats (27) and was found to decrease atherosclerotic lesion area in apoE-deficient mice (39). The mechanisms whereby flavonoids inhibit LDL oxidation are unclear. They may protect α-tocopherol in LDL from oxidation, possibly by being preferentially oxidized themselves, or they may reduce the formation or release of free radicals. Flavonoids can react with superoxide anions (1), hydroxyl radicals (49), and lipid peroxy radicals (91). These compounds may also act by chelating iron (1,72) which is thought to catalyze processes leading to the appearance of free radicals.

A number of studies have investigated the ability of flavonoids to act as antioxidants. Saija et al. (84) found that naringenin was able to inhibit Fe$^{2+}$-induced linoleate peroxidation with an IC$_{50}$ of 565 μM and autooxidation of rat cerebral membranes (ARCMs) with an IC$_{50}$ of 322 μM. The inhibitory activity of naringenin was relatively weak compared to the structurally-related flavanone hesperetin, which inhibited Fe$^{2+}$-induced peroxidation and ARCM, with IC$_{50}$s of 17 μM and 148 μM, respectively. Interestingly, this study demonstrated also that naringenin and hesperetin interact with liposomes of dipalmitoylphosphatidyl-choline. This may result from an ability of naringenin to anchor to the polar head groups of phospholipids or to penetrate and associate with the lipid bilayer. This suggests that some physiological effects of naringenin may be caused by its interaction with biological membranes.

Naringenin has been shown also to inhibit microsomal lipid peroxidation (IC$_{50}$ of 465 μM) (63), nonenzymatic lipid peroxidation (33% inhibition at a concentration of 1 mM)
(76), and ascorbic acid-induced malondialdehyde (MDA) formation (by 21% at a concentration of 100 μM) (Fig. 2). Hesperetin, at the same concentration, showed a similar level of inhibition. Naringenin, however, had no effect on ferrous sulfate–induced MDA production (76). In the studies mentioned earlier, neither naringin nor hesperidin were able to inhibit lipid peroxidation, suggesting that the effect was specific to aglycone flavonoids.

In addition to direct oxidant scavenging, flavonoids may inhibit enzymes involved in generating pro-oxidant molecules. For example, flavonoids have been shown to inhibit the generation or release of free radicals derived from lipoxygenase (LOX) (18). It has been suggested that LOX is involved in the early events of atherosclerosis by inducing plasma LDL oxidation in the subendothelial space of the arterial wall (18). Laughton et al. (63) investigated the effects of the glycoside naringin on 5-LOX and cyclooxygenase (COX) in rat peritoneal leukocytes, which generate products of both LOX and COX pathways when activated. Naringin was found to be a poor inhibitor of 5-LOX (IC₅₀ > 500 μM) and COX (IC₅₀ = 320 μM). In a related study, Corvazier and Maclouf (17) demonstrated that naringenin (500 μM) is an irreversible inhibitor of both LOX and COX pathways. Furthermore, naringenin has been shown to inhibit myeloperoxidase (MPO) (approximate IC₅₀ = 150 μM) (22). MPO secreted by macrophages and activated neutrophils is a potent catalyst of LDL oxidation in vitro and colocalizes with macrophages in human atherosclerotic lesions (40). MPO generates a range of reactive species, including the potent oxidizing agent hypochlorous acid (40). Therefore, naringenin in addition to direct antioxidant roles, may inhibit the oxidation of LDL by mechanisms involving inhibition of LOX, COX and MPO.

Recent studies by Hayek et al. (39) demonstrated that cholesteryl ester accumulation in cultured macrophages incubated with LDL derived from mice consuming the flavonoids

![Graph](image_url)

**FIG. 2.** Effect of naringenin and hesperetin on malonaldehyde (MDA) formation induced by 1.0 mM ascorbic acid in rat brain mitochondrial suspensions. The flavonoids were examined at concentrations of 0.1 mM, 1 mM, and 4 mM and the values are expressed as percentage inhibition relative to controls. Adapted from ref. 76.
catechin and quercetin was significantly lower than the accumulation observed in the presence of LDL from control mice. This effect has been attributed to the reduced oxidative modification of LDL. Whether a similar mechanism occurs with the intake of flavonoids, such as naringenin, is unknown. Furthermore, it has not been determined if naringenin has a direct effect on foam cell formation, independent of an effect on LDL. As discussed later, we have recently shown that naringenin inhibits cholesterol esterification in cultured hepatocytes (94). It is possible that a similar mechanism exists in macrophages and smooth muscle cells.

Flavonoids have been shown to have a number of antithrombotic actions (17,62). The effect of flavonoids on the oxidative metabolism of arachidonic acid (AA) in human platelets and neutrophils was investigated by Corvazier and Maclouf (17). Naringenin was found to inhibit thromboxane B₂ production in platelets stimulated with either thrombin or AA with an IC₅₀ of approximately 175 to 200 μM, whereas the glycoside form naringin was inactive. Naringenin also inhibited the formation of oxygenated metabolites in platelets stimulated with thrombin and inhibited AA-induced platelet aggregation with an IC₅₀ of 500 μM. Landolfi et al. (62) demonstrated that naringenin is a weak inhibitor of AA- or collagen-induced platelet aggregation (IC₅₀ of 90 μM and > 200 μM, respectively), but it had no effect on the production of thromboxane B₂, 12-hydroxyheptadecatrienoic acid (HHT), or hydroxycisatetraenoic acid (HETE) from AA. Nevertheless, naringenin appears to be a relatively weak inhibitor of these processes when compared to the flavonol quercetin, which inhibits platelet aggregation with an IC₅₀ of 25 μM and thromboxane B₂ production with IC₅₀ of 44 to 55 μM (17).

Flavonoids have also been shown to have antiinflammatory activities (68). The anti-inflammatory roles of naringenin may be of particular interest with respect to atherosclerosis, as this disease is increasingly being viewed as one with a strong inflammatory component (for review see ref. 83). The earliest form of the atherosclerotic lesion, the fatty streak, is an inflammatory lesion consisting of monocyte-derived macrophages and T lymphocytes. It is tempting to speculate that flavonoids may be useful in inhibiting a wide range of inflammatory responses, including those proposed to play roles in the progression of CHD. Middleton and Kandaswami (68) have reviewed the effect of flavonoids on immune and inflammatory functions. Flavonoids can effect the function of T cells, B cells, NK cells, macrophages, mast cells, basophils, neutrophils, eosinophils and platelets, each of which are involved in immunity and inflammation. Cytokines and growth factors secreted by these cells may control processes involved in atherosclerosis, such as macrophage activation, scavenger receptor expression, smooth muscle proliferation, and nitric oxide production (80). Habtemariam (37) examined the effect of flavonoids on TNFα-induced cytotoxicity in murine fibroblast L-929 cells. Although eriodictyol, a hepatic metabolite of naringenin, offered protection against TNF-induced cytotoxicity with an EC₅₀ (50% effective concentration) of 4–6 μM, naringenin itself was not protective and actually potentiated the effect when examined at concentrations up to 250 μM. Some flavonoids also exert antiinflammatory effects by inhibiting cytokine-induced gene expression (31). Apigenin (25 μM), the flavone equivalent of naringenin, blocks the cytokine-induced upregulation in human endothelial cells of intercellular adhesion molecule (ICAM-1) and vascular adhesion molecule (VCAM-1), both of which have been implicated in inflammation and atherosclerosis. However, at concentrations up to 50 μM, naringenin did not inhibit the expression of these molecules (31). Therefore, although
related flavonoids may affect cytokine-induced gene expression, this has not yet been demonstrated for naringenin.

Flavonoids may have beneficial effects on cardiovascular diseases involving vasodilation. Herrera et al. (41) examined the effects of flavonoids on the noradrenaline-, KCl-, and phorbol myristic acid (PMA)-induced contractions of rat aortic rings ($10^{-5}$M, $80\mu$M, and $10^{-7}$M, respectively). Naringenin ($IC_{50} = 46\, \mu$M to 96 $\mu$M) and hesperetin ($IC_{50} = 88\, \mu$M to 139 $\mu$M) displayed a concentration-dependent inhibition of the agonist-induced contractile responses. Sodium nitroprusside potentiated the vasodilation by naringenin, therefore suggesting the potential involvement of cGMP-specific phosphodiesterases. The ability of naringenin to block the effect of PMA suggests that naringenin may inhibit contraction through inhibition of protein kinase C (PKC).

**LIPID AND LIPOPROTEIN METABOLISM**

While the majority of research has focused on the antioxidant roles of flavonoids, a number of reports have suggested that these compounds may also influence atherogenesis through an effect on lipid and lipoprotein metabolism (4,8,10,14,15,51,88,94,96). Investigation of naturally occurring compounds as regulators of triglyceride and cholesterol metabolism has particular therapeutic importance, as evidenced by the discovery of the first HMG-CoA reductase inhibitors derived from fungal fermentation products, which are now widely used for the treatment of hyperlipidemia. Flavonoids might represent another beneficial group of naturally occurring hypolipidemic compounds. Studies in rats have shown that the flavonoids quercetin (8), hesperidin (70), marsupin (51), pterosupin (51), liquiritigenin (51), biochanin A (88), formononetin (88), and pratensein (88) cause significant reductions in serum total cholesterol (TC) and triglyceride (TG). In nonhuman primates, dietary genistein (Figure 1), the isoflavone analog of naringenin, significantly reduces plasma LDL and VLDL cholesterol levels (4).

Studies in hyperlipidemic rats (14) fed high-fat diets showed that i.p. administration of a methanolic extract from Prunus davidiana and its flavonoid components catechin, naringenin 7-O-glucoside (prunin), and hesperetin 5-O-glucoside for 3 days resulted in a significant reduction in blood TG and TC. Furthermore, naringenin 7-O-glucoside or hesperetin 5-O-glucoside, when administered alone at doses of 20 mg/kg and 10 mg/kg, respectively, showed significant hypocholesterolemic effects. In a related study by the same investigators (15), the effects of naringenin 7-O-glucoside (prunin) in streptozotocin-diabetic rats was examined. Single i.p. administration of prunin (10 mg/kg) caused a significant decrease in plasma glucose, TG, and TC (Fig. 3).

In a recent study Kurowska et al. (61), reported that replacing the drinking water of rabbits with either grapefruit or orange juice resulted in significant reductions in the elevation of serum LDL cholesterol and hepatic cholesteryl ester (CE) levels induced by feeding a semipurified, cholesterol-free, casein-containing diet. This model is characterized by an overproduction of hepatic apoB-containing lipoproteins (56). It was hypothesized that the hypocholesterolemic effects of the juices were due to their flavonoid components (naringenin in grapefruit juice and hesperetin in orange juice). Subsequently, the effects of these flavonoids on the secretion of apolipoprotein B(apo B)-containing lipoproteins by the human hepatoma cell line HepG2 was examined. Naringenin and
Hesperetin dose-dependently reduced the accumulation of apoB in the culture media (76–81% at 200 μM) over 24 hours (10). Reduced hepatic secretion of apoB-containing lipoproteins would be expected to contribute to a hypocholesterolemic effect of naringenin in vivo.

Recent studies by Wilcox et al. (94) have further investigated the effect of naringenin on lipid and lipoprotein metabolism in HepG2 cells. Significant reductions in apoB secretion were observed (83% reduction at 200 μM, p < 0.002) (Fig. 4) after a 24-hour incubation with naringenin. Importantly, we demonstrated that naringenin (200 μM) reduced the secretion of newly synthesized CE, TG, and phospholipid (PL) (Fig. 5). Naringenin (200 μM) significantly reduced intracellular CE mass, which was largely due to a dose-dependent inhibition of CE synthesis (up to 89% at 200 μM of naringenin) (Fig. 4). The mass of free cholesterol and TG were unaffected. This effect on hepatic CE synthesis may be explained, in part, by an inhibition of hepatic ACAT as evidenced by a reduction in ACAT activity in isolated porcine hepatic microsomes (IC50 = 200 μM).

Previously, we have shown that inhibition of ACAT reduces the hepatic production of apoB-containing lipoproteins (11), possibly by limiting the availability of newly synthesized CE for association with apoB during assembly of the lipoprotein. Therefore, inhibition of hepatic ACAT may be the mechanism whereby naringenin exerts its hypocholesterolemic effects. Inhibition of hepatic ACAT has also been demonstrated for a flavonoid structurally different from naringenin, baicalein (IC50 of approximately 100 μM in isolated rat hepatic microsomes) (96). Whether reduced apoB secretion and inhibition of ACAT activity are general features of flavonoids remains to be determined.

Inhibition of ACAT activity and apoB secretion by naringenin might occur via a direct inhibition of ACAT, following receptor-mediated signalling events or following direct...
interaction with cellular kinases (68). Another possible mechanism may be through interaction with the plasma membrane transporter, multidrug resistance p-glycoprotein (p-gp). Recently, Conseil et al. (16) have shown that flavonoids can bind mouse p-gp and modulate its activity. Flavonoids are thought to have bifunctional interactions at the vicinal ATP-binding site and steroid-interacting regions of p-gp (16). Naringenin was shown to bind purified p-gp with 50% binding at 28.5 μM (16). Binding at the ATP-binding site is thought to antagonize ATP binding and thus inhibit the ATPase activity of this protein. This may contribute to the ability of naringenin to inhibit the activity of p-gp (87). Inhibition of p-gp activity has been shown to inhibit cholesterol esterification in cell lines, including HepG2 (20) and the intestinal cell line CaCo-2 (20). The secretion of apoB in CaCo-2 cells was also found to be reduced following inhibition of p-gp. It is possible that this mechanism contributes to the inhibition of cholesterol esterification and apoB secretion by naringenin in HepG2 cells.

In further studies from our laboratory, we discovered a potentially novel mechanism for the regulation of apoB secretion by naringenin. Naringenin (200 μM) caused a significant 50% decrease in the mRNA for the microsomal triglyceride transfer protein (MTP) (94), a protein known to be essential for the assembly and hepatic secretion of apoB-containing lipoproteins (93). Together with the inhibition of ACAT (discussed earlier), these results suggest potential mechanisms whereby naringenin may reduce the plasma concentrations

FIG. 4. A. Effect of naringenin (10–200 μM) on apoB secretion and cholesterol esterification by HepG2 cells. The accumulation of apoB in the media was measured using a noncompetitive ELISA assay following a 24 h incubation with naringenin. B. [14C]Oleic acid (0.08 μCi) incorporation into cholesteryl esters was determined after a 5 h incubation in the presence of [14C]oleic acid and naringenin. Adapted from ref. 94.
of apoB-containing lipoproteins, such as VLDL and LDL. Whether naringenin produces similar effects in vivo remains to be determined.

ESTROGENIC ROLE

Flavonoids which contain a phenolic group and 6-membered rings with different degrees of desaturation, such as naringenin, structurally resemble estradiol and other steroid hormones, thyroid hormone, retinoic acid, nucleosides, and folic acid. This structural similarity raises the possibility that flavonoids or their metabolites could bind steroid receptors. Although specific flavonoid receptors have not been identified, naringenin and other flavonoids can bind to the estrogen receptor (7,60,69). Using an estrogen receptor reporter construct, Balaguer et al. (7) showed that naringenin elicits a dose-dependent induction in activity with maximal activity at 50 μM and an EC_{50} of 1 μM. Furthermore, Kuiper et al. (60) showed that naringenin can bind to both estrogen receptors, ERα and ERβ. Importantly, naringenin competed more effectively with 17-β-estradiol for binding to ERβ (IC_{50} for competition = 590 nM) than for ERα. Interaction of naringenin with ERβ may be relevant for cardiovascular effects as this receptor is present in significant amounts in arterial tissue (77). In fact, estrogen has been reported to have a number of beneficial cardiovascular effects. Estrogen has been shown to protect against foam cell formation (90), and estrogen replacement has also been shown to reduce CHD risk (21) and plasma TC and LDL cholesterol in postmenopausal women (12,95). This reduction has been attributed, in part, to increased clearance of LDL (12,95). This is consistent with

**FIG. 5.** Effect of naringenin (200 μM) on the incorporation of [14C]acetic acid (0.5 μCi) into secreted cholesteryl ester, triglyceride, phospholipid, and free cholesterol from HepG2 cells. Values are given as the mean ± SEM from experiments with triplicate samples. From ref. 94.
the observation that estrogen induces an increase in hepatic LDL receptor expression (58). Whether naringenin affects clearance of apoB-containing lipoproteins by mechanisms similar to those mediated by estrogen is unknown.

Flavonoids, including naringenin, have been shown to bind to the estrogen receptor with binding affinities of 1000- to 10000-fold less than that of 17-β-estradiol (60). Therefore, the question arises as to whether the in vivo concentrations can reach sufficiently high concentrations to exert a biological effect. However, flavonoids may accumulate in fatty tissue and reach concentrations sufficient to activate steroid nuclear receptors. Alternatively, the flavonoid could be metabolized to a form with enhanced estrogen activity. Naringenin may also influence estrogenic actions by altering the metabolism of the steroid. Kao et al. (53) have shown that naringenin competitively inhibits human aromatase (estrogen synthetase), the enzyme that converts androgen to estrogen, with a Kᵢ of 5.1 μM (53). Huang et al. (48) report that naringenin inhibits estrone sulfatase, a key enzyme in 17-β-estradiol synthesis, with an IC₅₀ < 10 μM in human hepatic microsomes. Naringenin and hesperetin were found to inhibit rat hepatic microsomal glucuronidation of estrone and estradiol with an IC₅₀ of approximately 25 μM (97). Relative to other flavonoids, naringenin and hesperetin were potent competitive inhibitors of estrone glucuronidation at a flavonoid concentration of 10 μM and noncompetitive inhibitors at a concentration of 50 μM.

Ruh et al. (82) showed that, in addition to its weakly in vitro estrogenic effects, (Fig. 6), naringenin exhibits antiestrogenic activity in vivo. In rats, naringenin (30 mg/rat) was shown to inhibit the 17-β-estradiol–induced increase in uterine weight, induction of progesterone receptor binding, [³H]thymidine uptake, and uterine peroxidase activity. Naringenin also attenuated the estrogen-induced increase in cell proliferation in MCF-7 human breast cancer cells. These studies showed that, in addition to estrogenic effects, naringenin may significantly attenuate estrogenic activity. The balance between these two actions in vivo remains to be determined.

In addition to the estrogenic actions discussed above, naringenin may also act as a weak progestin. Rosenberg et al. (79) have shown that naringenin (10 μM) acts as a weak progestin in mammary cancer cell lines. Naringenin was approximately 10⁴-fold less potent than the agonist norgestrel. In contrast, the related flavanone hesperetin acts as an antiandrogen and antiprogestin at concentrations of 10 μM. The reason for the difference between naringenin and hesperetin, which are structurally quite similar, is unclear.

SIGNAL TRANSDUCTION PATHWAYS

The citrus flavonoids may interact directly with intracellular signal transduction pathways. Since second messenger signalling plays an important role in the development and progression of atherosclerosis (75), it can be predicted that the modulation of these signalling pathways may alter the progression of the disease. For example, the interaction of compounds such as flavonoids with the proteins involved in signal transduction could modulate the expression of inflammatory molecules within the lesion and/or modulate the activity and/or expression of proteins involved in cholesterol trafficking in smooth muscle and immune cells within the lesion, as well as in hepatocytes. The growth factor- and cytokine-mediated signalling involved in the atherogenic process was recently reviewed in detail by Pomerantz et al. (75). Briefly, ligand activation of receptor tyrosine kinase or
membrane-associated G-protein–mediated signalling pathways can lead to the formation of inflammatory mediators such as prostaglandins and leukotrienes via phospholipase A2 (PLA2). Signalling pathways can also regulate the expression and/or activity of a number of proteins involved in cholesterol trafficking, including the LDL-receptor, the scavenger receptor, HMG CoA-reductase, ACAT, and HDL-receptors.

Recently, a number of flavonoids, including hesperetin, have been shown to inhibit PLA2 activity in vitro (66). In general, hydroxyl groups at positions 5, 6, and 7 on the A ring have been implicated in the PLA2-inhibitory activity of flavonoids (13,32,66). This is of interest since naringenin, like hesperetin, has hydroxyl functional groups at positions 5, and 7 (which correspond to R2 and R3 in Figure 1): however, the ability of naringenin to inhibit this enzyme has not been examined. Neither naringenin nor hesperetin have been shown to significantly alter phosphatidylinositol 3-kinase (PI3 kinase) activity (2) or PKC activity (2,26,89). The role of naringenin as an inhibitor of rat brain PKC was investigated by Ferriola et al. (26). Naringin and hesperetin showed only a small (approximately 10%) and nonsignificant inhibition of PKC activity. Although the effect of the active metabolite of naringenin, naringenin, was not investigated, it has been reported that naringenin inhibits PKC activity by less than 20% in human breast cancer cells (89). Similar results have been observed with hesperetin (89). Collectively, these observations indicate that naringenin is not an effective inhibitor of PKC activity. The effects of the citrus flavonoids on other

FIG. 6. Effect of naringenin on pS2-luciferase reporter vector activity in MCF-7 human breast cancer cells. Estradiol induction of pS2 gene expression is mediated by a palindromic estrogen responsive element-like sequence that acts as an enhancer for the nuclear estrogen receptor. Values are the means ± SEM of four measurements for each treatment and are reported as a percentage of induction relative to 1 nM estradiol. Adapted from ref. 82.
signal transduction enzymes, such as phospholipase C (PLC) and mitogen-activated protein kinase (MAPK), have not yet been examined. Further research is also required to determine whether the observed inhibition of ACAT activity by naringenin (94) could be partially due to modulation of signalling pathways involved in the regulation of expression of this enzyme.

NARINGENIN DRUG INTERACTIONS

Naringenin-drug interactions and the effects of naringenin on cytochrome P450 enzymes were first suggested in studies that investigated the effects of grapefruit juice on the metabolism of dihydropyridine calcium channel blockers (6). It was subsequently shown that grapefruit juice increased the oral bioavailability of felodipine (6) and cyclosporin (23). Inhibition of selected cytochrome P450 isozymes by citrus flavonoids may explain the altered bioavailability of some coadministered drugs. More direct evidence to indicate that naringenin effects drug metabolism was provided by studies that demonstrated a direct inhibition of specific cytochrome P450 enzymes (24,35) by the aglycone naringenin. The glycoside naringin (the primary form of naringenin in grapefruit juice) did not affect human cytochrome P450 activities in vitro (28,35). Fuhr et al. (28) reported that naringenin is a potent competitive inhibitor of CYP1A2-mediated caffeine 3-demethylation in hepatic microsomes. Guengerich et al. (35) have shown that naringenin competitively inhibits CYP3A4 in vitro (approximate IC_{50} 100 \mu M), in human liver microsomes. Hesperetin, although less effective than naringenin, was also shown to possess CYP3A4-inhibitory activity. Therefore, naringenin may increase the oral bioavailability of drugs with marked first-pass metabolism that involve metabolism by CYP3A4 or CYP1A2.

Naringenin-drug interactions may have important consequences in relation to the potential antiatherogenic uses of flavonoid compounds in hyperlipidemic individuals. Grapefruit juice has been shown to increase the serum concentrations of simvastatin (65) and lovastatin (52), two HMG-CoA reductase inhibitors commonly used for the treatment of hypercholesterolemia. This is thought to occur by inhibition of CYP3A4-mediated first-pass metabolism of the HMG-CoA reductase inhibitors in the small intestine, as discussed earlier.

SUMMARY

There is significant experimental evidence to suggest that naringenin and other citrus flavonoids may be potentially useful as pharmacological agents in the treatment or prevention of atherosclerosis. However, due to the substantial metabolism of naringenin by intestinal and hepatic mechanisms, it is not clear whether orally administered naringenin will reach the systemic circulation and other tissues in sufficient concentrations to influence the many metabolic pathways involved in atherogenic processes. While orally administered naringenin might influence apoB-containing lipoprotein production from the intestine, as we have shown in hepatocytes, the effects of orally administered naringenin on hepatic apoB production in vivo requires further investigation. In light of the inhibitory effects of naringenin on intestinal CYP450 enzymes, oral administration of naringenin as an antiatherogenic drug may have important implications in individuals currently receiving drugs such as calcium channel blockers and statins.

Clearly naringenin possesses a number of antiatherogenic activities (Table 1), the majority of which have been investigated in vitro. If these effects occur in vivo, naringenin
represents a potentially useful antiatherogenic agent by interfering with processes directly related to the early events of atherosclerotic lesion formation including foam cell formation. However, more extensive investigation of the \textit{in vivo} action of naringenin in animal models of atherosclerosis and in humans are required to further elucidate the usefulness of this flavanone.

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