Chapter 14

Modulation of Cholesterol and Triacylglycerol Biosynthesis by Citrus Polymethoxylated Flavones

Cholesterol-Lowering Properties of Citrus Flavonoids

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Citrus polymethoxylated flavones modulate the biosynthesis of cholesterol and triacylglycerols via multiple mechanisms. Tangeretin inhibits the activities of diacylglycerol acyltransferase and of microsomal triglyceride transfer protein, as well as activates the membrane peroxisome proliferator-activated receptor in human hepatoma *HepG2* cells. These modulatory effects subsequently inhibit the assembly and secretion of apolipoprotein B-containing lipoproteins, such as the very low-density lipoprotein (VLDL) and the low-density lipoprotein (LDL). Nobiletin, but not tangeretin, inhibited macrophage acetylated LDL metabolism linked to the action of the specific class A scavenger receptor. This inhibitory effect blocked the formation of macrophage foam-cells, which are essential to atherosclerotic plaque formation. In hamster feeding trials the polymethoxylated flavones dramatically lowered serum total cholesterol, LDL+VLDL cholesterol, as well as the levels of serum triacylglycerols. Total liver concentrations of tangeretin derivatives corresponded to hypolipidemic concentrations of intact tangeretin in earlier *in vitro* studies. If similar actions occur in humans, these compounds may be viable alternatives to the statin drugs to combat elevated cholesterol and triacylglycerols.

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Citrus fruit are rich in polyphenols, particularly flavonoids and hydroxycinnamates, and in certain species, coumarins and psoralens (*1*). The flavonoids in citrus include several main classes of compounds including numerous flavanone glycosides, flavone glycosides and the highly methoxylated flavones, termed polymethoxylated flavones (PMFs) (Figure 1). These latter compounds lack glycosidation and contain few, if any, free phenolic hydroxyls. These chemical properties strongly influence the metabolism and oral bioavailability of the PMFs, as well as their interactions with other biological molecules. As lipophilic, planar molecules, PMFs are likely to exhibit significantly greater permeabilities across biological membranes than most glycosidated flavonoids and, as a consequence, are likely to exhibit a wide range of biological activities. Extensive biological testing of the PMFs in numerous enzyme inhibition assays, cell culture studies, and small animal trials has borne this out (*2*). However, with long-term dietary intake, many of the glycosidated flavonoids in citrus, *i.e.* hesperidin, naringin, diosmin, *etc.*, also show many similar biological actions, particularly pertaining to anticancer and anti-inflammation activities (*2*).

An important biological action for both the citrus flavanone glycosides, as well as for the PMFs, is the lowering of blood serum cholesterol (*3-11*). Hypolipidemic effects have also been observed for the nonglycosidated hesperetin and naringenin flavanone aglycones (*3*). These effects were first investigated in human hepatoma *HepG2* liver cells, using an assay measuring the production of atherogenic apolipoprotein B (apoB)-containing lipoproteins (apoB-Lp), such as the very low-density lipoprotein (VLDL) and the low-density lipoprotein (LDL). *HepG2* cells incubated with either hesperetin or naringenin exhibited lowered net secretion of LDL-associated apoB (*3*). The decreased apoB secretion occurred with a reduction in the levels of cellular cholesterol esters, whereas the cholesterol esterification decreased dose-dependently at flavonoid concentrations up to 200 μM (*3,4*). The IC_{50} values for the inhibition of apoB secretion were 43 μg/mL or 142.2 μM (hesperetin) and 48 μg/mL or 178.1 μM (naringenin) (*5*). Further studies also showed that naringenin and hesperetin inhibited the activities of the acyl CoA:cholesterol acyltransferases, ACAT1 and ACAT2, along with a selective inhibition of gene expression for ACAT2, and an inhibition of the microsomal triglyceride transfer protein (MTP) (*4*). Both hesperetin and naringenin increased the expression of the LDL receptor, an effect likely to increase cellular uptake and degradation of LDL (*4*). Naringenin was subsequently shown to inhibit the lipid assembly and subsequent secretion of apoB lipoproteins, primarily by decreasing triacylglycerol (TG) accumulation in the endoplasmic reticulum (*4*). The observed decreases in TG accumulation were linked to inhibition of the cellular MTP activity and gene expression (*6*).

Effects of the flavanone glycosides hesperidin and naringin on the levels of serum cholesterol and TGs have also been characterized in animal trials. Naringin fed to hypercholesterolemic rabbits did not significantly affect plasma cholesterol, but decreased the area of fatty streaks in thoracic aorta, and reduced

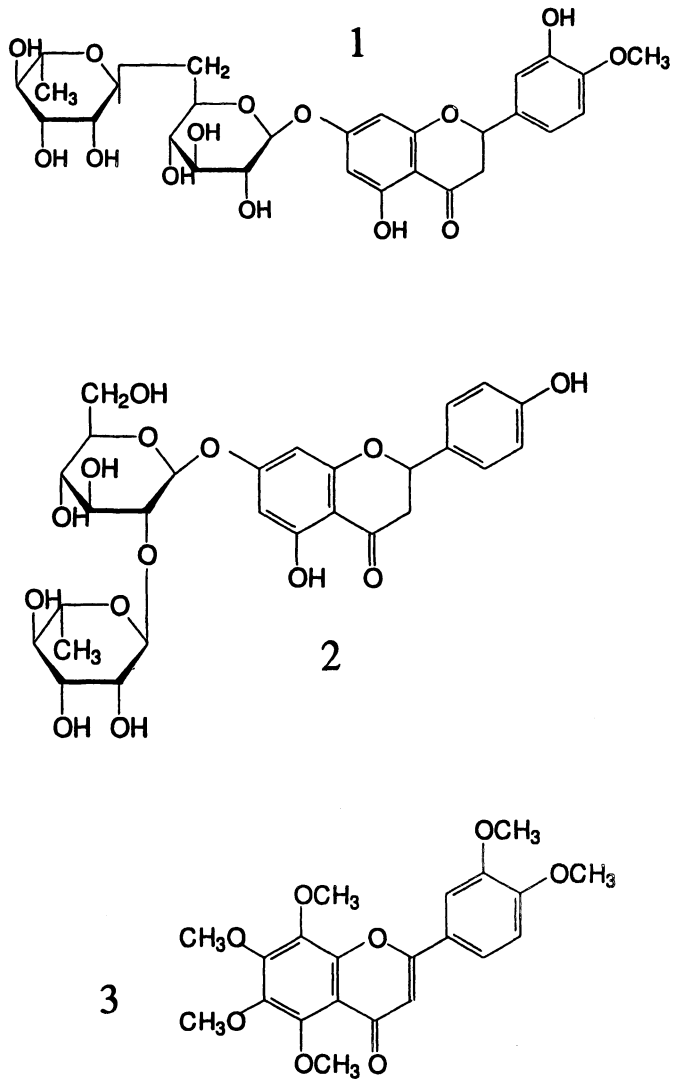

Figure 1. Structures of citrus PMFs and flavanone glycosides. 1, hesperidin;, 2, naringin; 3, nobiletin; 4, tangeretin; 5, heptamethoxyflavone; 6, sinensetin; 7, tetramethylscutellarein. Continued on next page.

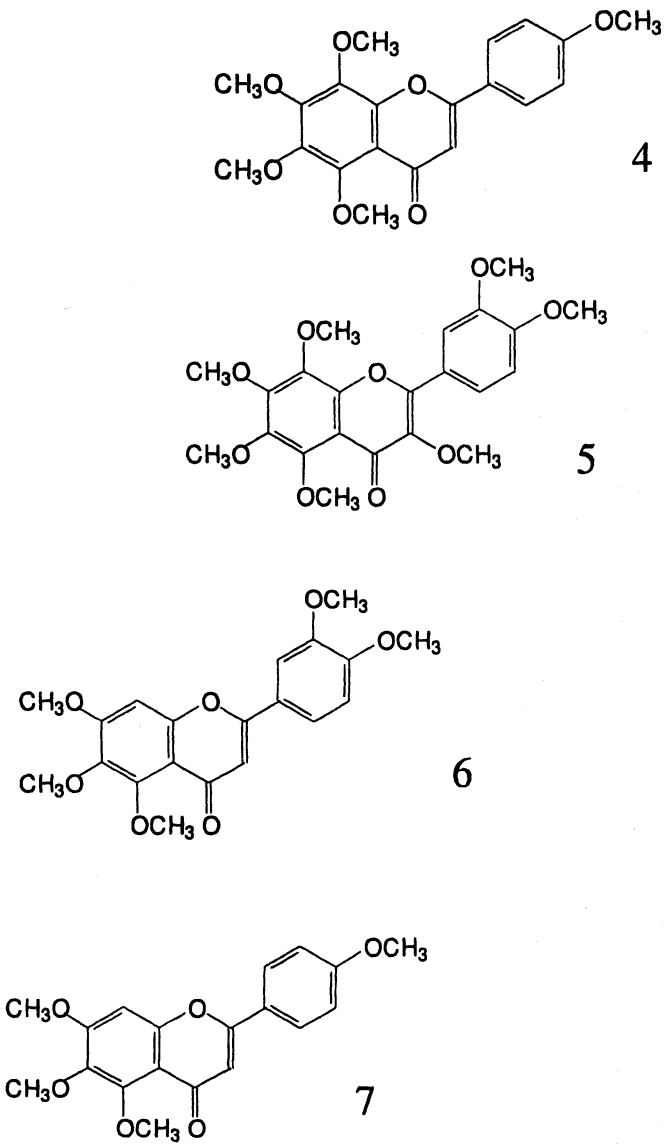

Figure 1. Continued.

subintimal foam cell infiltration on microscopic morphometry (7). Naringin inhibited the expression of the intercellular adhesion molecule -1 (ICAM-1) on endothelial cells of these treated animals. Attenuation of ICAM-1 similarly occurred in ischemic tissue treated with structurally related diosmin and hesperidin (*8*). In hypercholesterolemic rabbits, a diet containing 0.5% naringin produced dramatically lowered plasma total and LDL cholesterol and hepatic lipids. Male rabbits fed naringin exhibited lowered hepatic ACAT activities and exhibited sharply decreased levels of morphological damage in the vasculature induced by the high cholesterol diet (*9*). Similar decreases in hepatic ACAT activity and plasma cholesterol were detected in hesperidin- and naringin-fed rats maintained on a high cholesterol diet (*10,11*). An important distinction was observed between hesperidin and naringin in these animal studies. Significant decreases occurred in the levels of the plasma TGs in the naringin-fed rats, but not in the hesperidin-fed rats. Finally, male rats fed a high cholesterol diet along with 0.02% hesperetin, and alternatively a mixture of hesperetin metabolites (*m*-hydroxycinnamic acid, 3,4-dihydroxyphenylpropionic acid, and ferulic acid) had significantly reduced plasma TG and total cholesterol compared to the untreated controls (*12*). As part of a recent feeding trial, hypercholesterolemic hamsters were given a diet of a 3.0% hesperidin/naringin mixture. This diet produced a 28% decrease in serum total cholesterol, a 38% decrease in the serum VLDL and LDL cholesterol, an 18% decrease in the serum high density lipoprotein (HDL) cholesterol, and a 57% decrease in serum TGs (*13*). In contrast, this diet produced substantial increases in hepatic lipids, including, a 75% increase in total cholesterol, a 77% increase in free cholesterol, a 72% increase in cholesterol esters, but a 23% decrease in TGs. This latter effect on the TGs is in sharp contrast to the concurrent changes that occurred in the hepatic cholesterol and cholesterol conjugates.

In a recent human trial, a modified, water-soluble glucosyl hesperidin significantly lowered serum TGs in hypertriglyceridemic subjects, while no significant decreases occurred in total serum cholesterol (*14*). In contrast, administration of naringin to hypercholesterolemic humans produced only modest decreases in serum cholesterol and no decreases in plasma TGs (*15*). It would be interesting to determine what property of the soluble glucosyl hesperidin imparts the TG-lowering activity. Future trials with hesperidin would help determine if the activity of the modified glucosyl hesperidin is linked to the chemical constituents of the flavonoid aglycone, or to the increased solubility, and possibly to the increased bioavailability, of the modified, water-soluble glucosyl hesperidin.

In a similar manner, citrus PMFs exhibited lipid-lowering properties in cell culture studies as well as in small animal trials. Kurowska and Manthey (*5*) reported that PMFs isolated from orange oil inhibited apoB secretion by *HepG2* cells. IC_{50} values for these compounds were significantly lower than those of the flavanone aglycones, hesperetin (43 ppm) and naringenin (48 ppm). The three most active inhibitors were tangeretin (2.5 ppm), nobiletin (4.9 ppm), and 3,5,6,7,8,3',4'-heptamethoxyflavone (7.8 ppm).

Formulations containing PMFs were subsequently tested for lipid-lowering properties in hypercholesterolemic hamsters (*13*). Diets containing either 1.0% tangeretin or 1.0% of a 1/1 mixture of tangeretin and nobiletin had similar effects in lowering serum lipids. The 1.0% tangeretin diet produced a 25% decrease in serum total cholesterol, a 39% decrease in VLDL + LDL cholesterol, and a 48% decrease in serum TGs, while no change occurred in the high-density lipoprotein (HDL) cholesterol. In contrast, significant increases in liver lipids were observed, *i.e.* a 46% increase in total cholesterol, a 48% increase in free cholesterol, a 47% increase in cholesterol esters, but a 29% decrease in TG. A diet containing 0.25% of a 1/1 mixture of tangeretin and nobiletin showed the same trends, but produced smaller changes in the serum and liver lipids, with the important exception of serum TGs, which decreased by nearly the same levels associated with the higher percent PMF diets. A second hamster trial using a diet of a 1.0% PMF mixture produced similar changes in the serum lipids that occurred with the 1.0% tangeretin and 1.0% tangeretin/nobiletin diets.

A study was also conducted to identify and quantify tangeretin and nobiletin metabolites in blood serum, urine, and liver of hypercholesterolemic hamsters. HPLC-MS analyses of the metabolites in the blood serum showed the conversion of tangeretin into a series of dihydroxytrimethoxyflavone and monohydroxytetramethoxyflavone glucuronides. Only trace levels were detected of the aglycones and of unmodified tangeretin. The estimated total concentrations of all metabolites in the serum solids were 110 ppm, or an equivalent of 21 μM intact tangeretin. The occurrence of glucuronides in the liver and urine was similarly detected. Free aglycones ranged from 38 to 65% of the total metabolites in the liver, with the dihydroxytrimethoxyflavones constituting the major portions of these compounds. The total concentrations of all hepatic tangeretin metabolites were within the range of 25-106 ppm, an equivalent of 16-67 μM tangeretin in the fresh tissue. The total concentrations of PMF metabolites in the urine from hamsters fed 1.0% tangeretin, or 1.0 tangeretin/nobiletin diets were in the range from 11 to 21 mM. Nearly all of the metabolites in the urine were glucuronidated (*13*).

Ensuing biochemical studies of the lipid-lowering properties of the PMFs focused on the roles of tangeretin, for which the reduction of apoB secretion by *HepG2* cells was both rapid, and apoB specific (*16*). Figure 2 shows the inhibition time course resulting from exposure of the liver cells to 72 μM tangeretin for 0, 2, 4, and 8 hours. By 2 hours there was a nearly 50% reduction of apoB secretion, and no further medium accumulation of apoB occurred beyond 4 hours. The inhibition was not due to decreased viability of the tangeretin-treated cells, nor was it due to increased proteosomal degradation of extracellular apoB. *HepG2* cells incubated with 72 μM tangeretin for 24 h, then postincubated in tangeretin-free media, secreted apoB within 70 to 80% of the levels of the untreated cells, suggesting a partial reversibility of the apoB-lowering effect. Another important finding was that when the liver cells were incubated with ^{3}H-leucine, with and without tangeretin, there was no difference

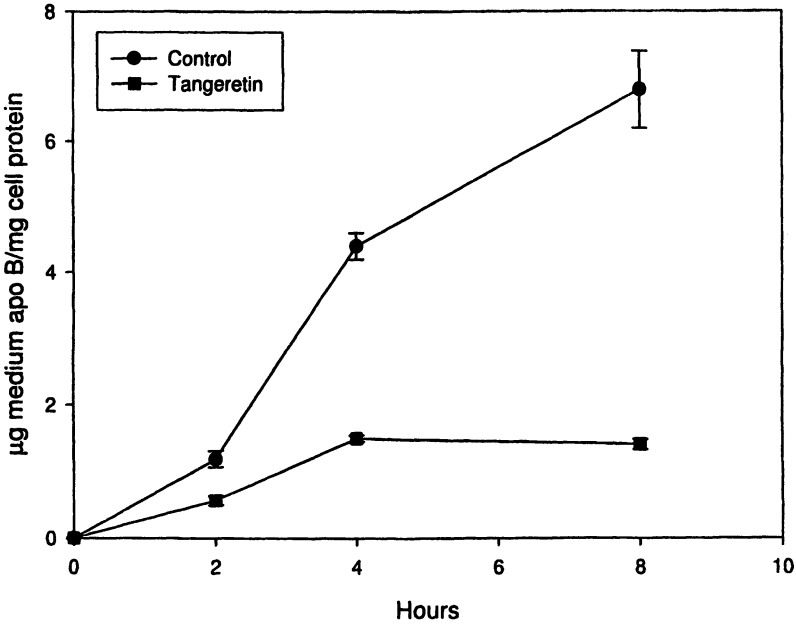

Figure 2. Inhibition time course resulting from exposure of HepG2 *liver cells to 72 µM tangeretin for 0, 2, 4, 8 hours. (Reproduced with permission from reference 16. Copyright 2004 AOCS.)*

in total trichloroacetic acid precipitable radioactivity, whereas, the sharp decrease that occurred for the apoB following this treatment indicated that the effects produced by tangeretin were specific for apoB.

One possible mechanism of blocking apoB-Lp secretion by liver cells is the inhibition of the synthesis of lipids necessary for its assembly and export. To test this mechanism, tangeretin was evaluated for its ability to inhibit lipid synthesis and accumulation in *HepG2* cells. This was done by measuring the effects of tangeretin on the cellular synthesis of cholesterol, cholesterol esters and TGs (*16*). Cells incubated with [1- ^{14}C]acetate or [1- ^{14}C]glycerol were treated for 24 h in the presence vs. absence of 72 □M tangeretin. Tangeretin reduced the rates of incorporation of radiolabeled precursors into cellular cholesterol, cholesterol esters and TGs by 45, 82, and 64%, respectively.

The inhibition of TG synthesis by naringenin, similar to that for tangeretin, was previously shown to strongly reduce the rate of early assembly and secretion of lipids into apoB-Lp (*3,4*). One of the critical control points in the assembly of lipids into apoB is the rate of TG synthesis, catalyzed by diacylglycerol acyltransferase (DGAT). By controlling the rate determining step between diacylglycerol and fatty acid CoA, DGAT controls the availability of TGs for lipid assembly into apoB and subsequent transport of lipoproteins out of

the cell (*17*). To test whether tangeretin influences the activity of DGAT in liver cells, DGAT activity was measured after exposure of *HepG2* cells to tangeretin for 24 h (*16*). Tangeretin at 29 μM and 72 :M decreased the rates of incorporation of ^{14}C-palmitoyl-CoA into cellular TGs by DGAT by 35% and 39%, respectively. These results failed to show a strong dose dependency at these tangeretin concentrations, but did provide an indication that tangeretin is able to influence TG synthesis at this DGAT-catalyzed rate determining step.

A second important control point in the early assembly of lipids into lipoproteins involves the MTP, where the inhibition of this transfer protein has been shown to influence the secretion of apoB-Lp (*18*). Using the same basic protocol as before, tangeretin was tested for its effect on the MTP at 29 □M and 72 □M tangeretin. On incubation at these concentrations, the cellular MTP activity was inhibited by 22% and 35%, respectively (*16*).

A final mechanism investigated for tangeretin pertained to the activation of the peroxisome proliferator-activated receptor (PPAR), which in the liver, reduces the quantity of fatty acids available for the synthesis of TGs by enhancing beta-oxidation of fatty acids via the activation of acyl CoA oxidase and carnitine palmitoyl-CoA transferase (*19,20*). To evaluate this, liver cells were treated with tangeretin at 29 □M and 72 □M for 24 h and PPAR activity in homogenates of these treated cells was assessed by electophoretic mobility shift assays and autoradiography (*16*). The results showed that in the presence of tangeretin PPAR activation increased by 25% at 29 □M and by 36% at 72 □M. These results suggest that tangeretin-induced increases in PPAR activity might contribute to decreases in cellular accumulation of TGs *via* beta-oxidation, and represent a DGAT-independent mechanism of inhibiting TG synthesis. The mechanistic findings on the actions of tangeretin suggest that PMFs influence lipid synthesis, lipid assembly into apoB-Lp, and secretion of lipoproteins out of liver cells by multiple mechanisms. These mechanisms are summarized in Figure 3. The complex actions of PMFs are consistent with their effects on the plasma lipids observed in hypercholesterolemic hamsters (*13*). Clinical trials are in progress to determine if similar effects occur in humans following dietary intake of PMFs.

Other important cardioprotective actions of the citrus PMFs pertain to their effects on macrophages and vascular inflammation. Macrophages are intimately linked to many different chronic diseases including atherosclerotic plaque formation (*21*). In large part, the involvement of macrophages in atherosclerosis is their uptake of LDL and oxidized LDL, and their subsequent transformation into foam cells. Uptake of lipoproteins by macrophages is initiated by their binding to cell surface receptors followed by their internalization and catabolism (*22-24*). There are specificities in the receptors involved in the uptake of lipoprotein by macrophages, including the LDL receptor (*23*), and the class A scavenger receptors (SR-A), which selectively bind chemically modified LDL (*i.e.* oxidized LDL). Generation of oxidized LDL is largely driven by the action of lipoxygenases and the generation of superoxide anion (*25,26*). While the uptake of native, unmodified LDL by macrophage receptors is a highly

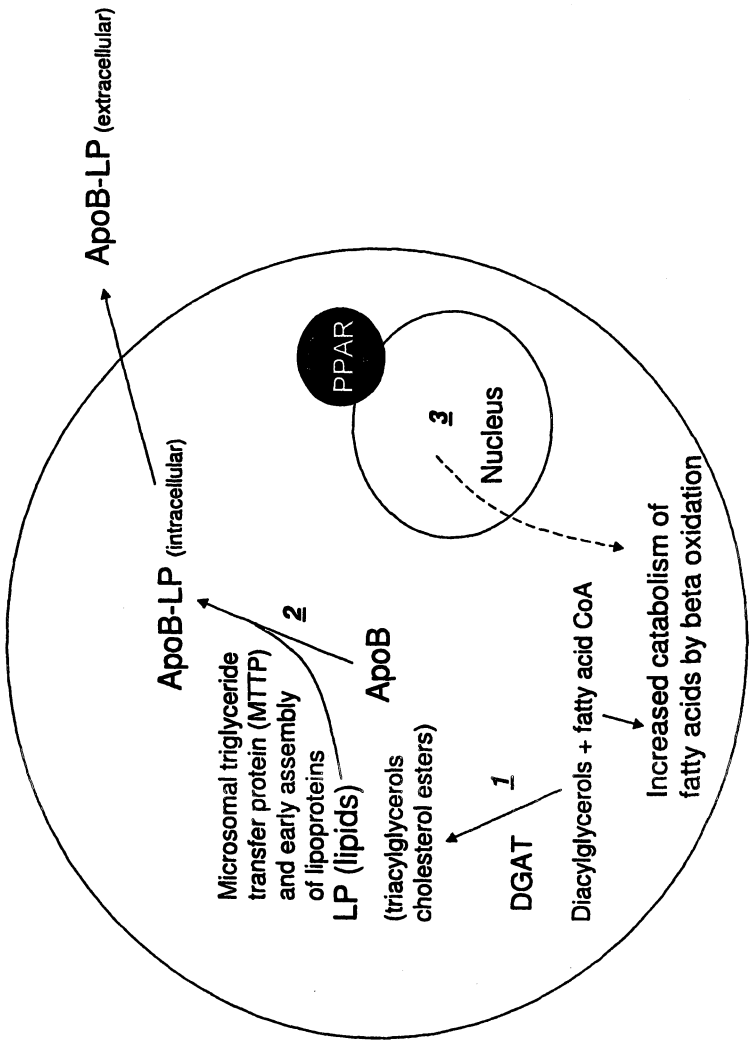

Figure 3. Summary of sites of activity of the PMFs in decreasing apoB secretion and TG synthesis. 1: Inhibition of diacylglycerol acyltransferase (DGAT). 2: Inhibition of MTP. 3: Activation of PPAR, and increased catabolism of fatty acids.

regulated event (*27*), the uptake of modified cholesterol of LDL by the SR-A receptors is unregulated, and is a primary event that drives atherosclerotic plaque formation (*28*).

The abilities of naringenin, hesperetin, tangeretin, and nobiletin, to mediate SR-A expression and metabolism were examined in cultured mouse J774A.1 macrophages (*29*). A known ligand of the SR-A receptor, acetylated LDL (acLDL), was used in these studies as a marker of the SR-A receptor activity (*30*). In macrophages, all four flavonoids inhibited cellular uptake of beta-VLDL by the LDL receptor, while only nobiletin inhibited macrophage-mediated metabolism of acLDL. This inhibition occurred without changes in the total levels of SR-A protein or in the expression of the receptor on the plasma membrane. Sites of action were speculated by Whitman *et al.* (*29*) to possibly include second-messenger signaling pathways involving phosphatidylinositol-3-OH kinase (*31*), the inhibitory/other subclass of guanine nucleotide binding proteins ($G_{i/o}$-proteins) (*32*), or similar pathways. Many flavonoids have a propensity to interact with nucleotide binding sites (discussed below), and a similar type of receptor/protein binding may be a mode of action of nobiletin in this particular receptor system. These results suggest, therefore, that nobiletin may prevent atherosclerosis *via* a number of mechanisms, the first by reducing plasma VLDL, LDL, and TGs, and by directly inhibiting macrophage-derived foam cell formation at the site of injury in vascular tissue.

Relevant to this discussion of the anti-atherosclerotic properties of PMFs are the findings that PMFs exhibit yet other biological actions that may prevent cardiovascular disease, particularly their inhibition of chronic inflammation. Again, many of these actions are attributable to the effects of PMFs on macrophages and on proinflammatory responses of activated monocytes. Manthey *et al.* (*33*) reported that PMFs suppress production of the tumor necrosis factor-α by lipopolysaccharide (LPS)-activated human monocytes. Also inhibited was the formation of the macrophage inflammatory protein-lα (MIP-lα) and interleukin-l0 (IL-10). IL-10 plays complex roles in lipid uptake by macrophage-derived foam cells and atherosclerotic plaque stabilization (*34*). The most active compound, 3,5,6,7,8,3',4'-heptamethoxyflavone (HMF), induced a substantial elevation of cAMP in activated monocytes. Similarities in the profiles of cytokine inhibition by PMFs to the profile of the known phosphodiesterase-4 inhibitor, 3-isobutyl-1-methylxanthine, suggest that PMFs inhibit cytokine production by suppression of phosphodiesterase-4. Further actions of PMFs on macrophages during inflammation were subsequently shown by nobiletin's inhibition of LPS-induced production of prostaglandin E2 in J774A.1 macrophages (*35*). Nobiletin inhibited gene expression of the pro-inflammatory IL-1α, IL-β, IL-6 and tumor necrosis factor-α in these LPS-activated macrophages. The inhibitory effects of nobiletin on the expression of the cycbooxygenase-2 and inducible NO synthase proteins, and of prostaglandin E2 release in mouse macrophage RAW 264.7 cells have also been reported (*35*,*36*). Inhibitory actions similar to these are likely to contribute to the anti

inflammatory properties of PMFs, and possibly play roles in the prevention of cardiovascular disease.

While many of the biological activities of flavonoids, such as the PMFs, can be linked to their inhibition of enzymes involved in chronic diseases (*37*), the mechanisms of these inhibitions at the molecular level are unclear. Yet, the evidence of the binding of flavonoids suggests that these compounds, or their metabolites, attenuate cellular events *via* receptor binding, and thus influence cell activation processes. It would be logical to suggest that allosteric binding may contribute to the inhibition of a number of enzymes involved in inflammation and atherosclerotic plaque formation. Yet, specificity has been widely reported for the binding of many flavonoids to the ATP binding sites of a number of regulatory enzymes (kinases, ATPases, phosphodiesterases, etc.) (*37,38*). In the inhibition of phosphodiesterases, quantum chemical calculations have suggested that the competition between the pyrone ring of the flavonoids and the pyrimidine ring of cAMP occurs *via* $\pi \rightarrow \pi$ interactions between these molecules and the nucleotide binding sites (*38*). Similarities were also detected in the charge distributions of the pyrone rings of the flavonoids and the pyrimidine ring of cAMP, suggesting that similar interactions occur for the binding of flavonoids and cAMP with the nucleotide binding pocket.

Similar types of interactions between PMFs and nucleotide binding sites have been further indicated by the tight binding of these compounds to the several classes of human adenosine receptors. These receptors are involved in the immune, cardiovascular, and central nervous systems (*39,40*); thus, these systems are potential targets of action by agonists/antagonists of adenosine. The A_3 receptors have already been implicated in vascular effects, inflammation, and cancer (*41*), three areas where flavonoids show considerable activity (*2*). Structure/activity relationships for the binding of plant flavonoids to the three subtypes of adenosine receptors (A_1, A_{2A}, and A_3) have been extensively analyzed (*42*), where methylations of hydroxyl constituents typically increased binding to the three subclasses of receptors (*43*). These findings led to the study of the binding of citrus PMFs to the three subtypes of adenosine receptors. Most of the PMFs analyzed in this study exhibited affinities to the A_1 and A_{2a} receptors in the micromolar range, but showed little selectivity (*41*). Three of the PMFs, 3-hydroxy-5,7,3',4'-tetramethoxyflavone, sinensetin, and 5,7,3',4'-tetramethylquercetin exhibited binding constants, K_i, below 1 μM for their binding to the A_3 receptor.

The understanding of the biological actions of PMFs in the prevention of chronic diseases is still incomplete, but the wide range of actions detected for these compounds suggests that there are numerous sites of action with far ranging effects on cell activation and cell signaling processes. Thus far, data suggest that PMFs influence a variety of events relating to atherosclerosis and inflammation and their inhibition of these underlying disease states may prove useful for their eventual commercialization as either nutraceuticals or food ingredients with targeted pharmacological endpoints.

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