

Secretion of hepatocyte apoB is inhibited by the flavonoids, naringenin and hesperetin, via reduced activity and expression of ACAT2 and MTP

Lisa J. Wilcox,¹ Nica M. Borradaile,¹ Linda E. de Dreu, and Murray W. Huff²

Departments of Medicine and Biochemistry and The John P. Robarts Research Institute, 4-16, University of Western Ontario, 100 Perth Drive, London, Ontario N6A 5K8, Canada

Abstract The citrus flavonoids, naringenin and hesperetin, lower plasma cholesterol *in vivo*. However, the underlying mechanisms are not fully understood. The ability of these flavonoids to modulate apolipoprotein B (apoB) secretion and cellular cholesterol homeostasis was determined in the human hepatoma cell line, HepG2. apoB accumulation in the media decreased in a dose-dependent manner following 24-h incubations with naringenin (up to 82%, $P < 0.00001$) or hesperetin (up to 74%, $P < 0.002$). Decreased apoB secretion was associated with reduced cellular cholesteryl ester mass. Cholesterol esterification was decreased, dose-dependently, up to 84% ($P < 0.0001$) at flavonoid concentrations of 200 μ M. Neither flavonoid demonstrated selective inhibition of either form of acyl CoA:cholesterol acyltransferase (ACAT) as determined using CHO cells stably transfected with either ACAT1 or ACAT2. However, in HepG2 cells, ACAT2 mRNA was selectively decreased (-50% , $P < 0.001$) by both flavonoids, whereas ACAT1 mRNA was unaffected. In addition, naringenin and hesperetin decreased both the activity (-20% to -40% , $P < 0.00004$) and expression (-30% to -40% , $P < 0.02$) of microsomal triglyceride transfer protein (MTP). Both flavonoids caused a 5- to 7-fold increase ($P < 0.02$) in low density lipoprotein (LDL) receptor mRNA, which resulted in a 1.5- to 2-fold increase in uptake and degradation of 125 I-LDL. We conclude that both naringenin and hesperetin decrease the availability of lipids for assembly of apoB-containing lipoproteins, an effect mediated by 1) reduced activities of ACAT1 and ACAT2, 2) a selective decrease in ACAT2 expression, and 3) reduced MTP activity. Together with an enhanced expression of the LDL receptor, these mechanisms may explain the hypocholesterolemic properties of the citrus flavonoids.—Wilcox, L. J., N. M. Borradaile, L. E. de Dreu, and M. W. Huff. Secretion of hepatocyte apoB is inhibited by the flavonoids, naringenin and hesperetin, via reduced activity and expression of ACAT2 and MTP. *J. Lipid Res.* 2001. 42: 725–734.

Supplementary key words flavonoids • naringenin • hesperetin • apoB • microsomal triglyceride transfer protein • HepG2 cells • cholesterol esterification

Flavonoids are naturally occurring molecules abundant in fruit, vegetables, nuts, seeds, and beverages such as tea and wine. The roles of naringenin and the related citrus flavonoid, hesperetin, in prevention and treatment of

disease have recently received considerable attention (1), with particular interest in the use of these flavonoids as anti-cancer (2) and anti-atherogenic (3, 4) compounds. Naringenin and hesperetin belong to the class of flavonoids called flavanones and are found largely as the glycosides naringin and hesperidin in grapefruit and oranges, respectively. These glycosides are hydrolyzed to their active forms, naringenin and hesperetin, by intestinal bacteria (4).

Studies in a number of animal models have shown that diets supplemented with grapefruit juice, orange juice (5), or glycosylated forms of naringenin (6–8) or hesperetin (6, 8, 9) result in reduced plasma cholesterol levels. In a recent study by Kurowska et al. (5), replacing the drinking water of rabbits with either grapefruit juice or orange juice attenuated the elevation of serum low density lipoprotein (LDL) cholesterol and hepatic cholesteryl ester (CE) levels induced by feeding a cholesterol-free, casein diet. It was hypothesized that the hypocholesterolemic effects of the juices were due to their flavonoid components. Studies by Choi et al. have shown that administration of the glycosylated forms of naringenin and hesperetin results in significant reductions in plasma triglyceride (TG) and total cholesterol (TC) concentrations in hyperlipidemic (6) and streptozotocin-diabetic (7) rats. More recently, Bok et al. (8) observed significant decreases in plasma and hepatic cholesterol levels in rats fed a mixture of glycosylated naringenin and hesperetin. These results suggest that citrus flavonoids may regulate the synthesis, secretion, and/or catabolism of apolipoprotein B (apoB)-containing lipoproteins.

Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; apo, apolipoprotein; CE, cholesteryl ester; MTP, microsomal triglyceride transfer protein; DMSO, dimethylsulfoxide; ELISA, enzyme linked immunosorbent assay; ER, endoplasmic reticulum; FC, free cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; TC, total cholesterol; TG, triglyceride; PL, phospholipid.

¹ These two authors contributed equally to this work.

² To whom all correspondence should be addressed.

e-mail: mhuff@julian.uwo.ca

The hepatic production of apoB-containing lipoproteins is regulated largely at posttranscriptional levels, with nascent apoB molecules being secreted or degraded intracellularly (reviewed in 10). The assembly of apoB with lipid to form a secretion-competent particle is a complex process (10, 11). It is widely accepted that hepatic lipid availability is obligatory for apoB-containing lipoprotein assembly within the liver, a finding supported by studies demonstrating the necessity of triglyceride (11, 12) and phospholipid (13). The availabilities of cholesterol and CEs, synthesized by the HMG-CoA reductase and acyl-CoA: cholesterol acyltransferase (ACAT) pathways, respectively, have also been shown to be important determinants of the rate of hepatic apoB secretion (14–16). Recently, two distinct ACAT activities, ACAT1 and ACAT2, have been described. It has been postulated that ACAT2, which is predominant in the liver and intestine, is responsible for the synthesis of CEs for lipoprotein assembly (17). The microsomal triglyceride transfer protein (MTP) also plays a key role in apoB secretion by catalyzing the transfer of lipids to the nascent apoB molecule as it is cotranslationally translocated across the endoplasmic reticulum (ER) membrane (18, 19).

A recent study by Borradaile, Carroll, and Kurowska (20) demonstrated that naringenin and hesperetin dose-dependently reduced apoB accumulation in the media of HepG2 cells. This was associated with reduced CE synthesis. Reduced activities of HMG-CoA reductase and ACAT have also been observed in livers of rats fed naringenin (21) or a mixture of naringin and hesperidin (8) along with a high cholesterol diet. These studies suggest a link between flavonoid-induced modulation of cholesterol biosynthesis and esterification and apoB secretion. The present study was undertaken to define the mechanisms whereby naringenin and hesperetin regulate hepatic apoB metabolism. Specifically, we investigated the effects of these flavonoids on the expression and activity of ACAT1, ACAT2, HMG-CoA reductase, MTP, and the LDL receptor in HepG2 cells. Our results demonstrate that naringenin and hesperetin inhibit apoB secretion in HepG2 cells by two mechanisms: 1) reduced activity of ACAT1 and ACAT2 and a selective decrease in ACAT2 expression and 2) reduced MTP activity. Furthermore, increased LDL-receptor activity contributed to the reduced accumulation of apoB in the media.

MATERIALS AND METHODS

Cell culture

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown as described previously (22). For experiments, HepG2 cells were plated in either 100-mm or in 6-well (35-mm) culture plates from Falcon Scientific (VWR, Mississauga, ON) and maintained in minimal essential medium (MEM) containing 5% human lipoprotein-deficient serum (LPDS). The appropriate concentrations of flavonoids, solubilized in dimethyl sulfoxide (DMSO, concentration did not exceed 0.5%), were added to the dishes. Naringenin and hesperetin were obtained from Sigma (St. Louis, MO).

AC29 cells, a Chinese hamster ovary cell line lacking endogenous ACAT activity (23), stably transfected with African green monkey ACAT1 or ACAT2 cDNA (17), were kindly provided by Dr. L. Rudel (Wake Forest University School of Medicine, Winston-Salem, NC). Stable transfections were done using pIRESneo vectors from Clontech Labs (Palo Alto, CA), containing ACAT1 or ACAT2 cDNAs, with a FuGENE 6 (Boehringer Mannheim, Roche Diagnostics, Laval, PQ, Canada) to DNA ratio of 6 μ l:1 μ g, and selection was done in the presence of geneticin (G418) (24). The cells were maintained in Ham's F-12 Nutrient Mixture medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 1% MEM vitamin solution, and 800 μ g/ml geneticin (G418). All cell culture reagents were obtained from Life Technologies (Burlington, ON, Canada). For experiments, cells were plated as described above and maintained in F-12 medium containing 5% LPDS. Flavonoids, solubilized in DMSO, were added to the dishes as described above.

Secreted apoB and cellular lipid mass

ApoB concentrations in media were measured by an enzyme-linked immunosorbent assay (ELISA) described previously (15). Media samples were diluted 20-fold for analyses. ApoB mass results are reported as μ g/mg cell protein. Secreted and cellular apoB, synthesized in the absence or presence of 0.1 mM oleic acid, was also measured following preincubation of cells for 24 h with or without flavonoid. Cells were pulsed for 10 min with [35 S] methionine and chased for 60 min (15). Media and cellular apoB was immunoprecipitated using a polyclonal anti-human apoB (Boehringer Mannheim), resolved and quantitated as described previously (15). Newly synthesized apoAI was similarly immunoprecipitated and analyzed using a polyclonal anti-human apoAI antibody (Boehringer Mannheim). Cellular TG, free cholesterol (FC), and TC were quantitated by a modification of the method of Carr, Andresen, and Rudel (25) using enzymatic reagents from Boehringer Mannheim, as previously described (15). Cellular lipid results are reported as μ g of cellular lipid (CE, TG, or FC) per mg cell protein.

Lipid synthesis and CE hydrolysis

The incorporation of [1- 14 C]oleic acid (Amersham, Oakville, ON, Canada) or [1- 14 C]acetic acid (Amersham) into cellular lipids was determined as described previously (15). Radioactivity incorporated into CE, TG, and phospholipids (PL) was determined after separation of the lipid species by thin layer chromatography. Incorporation of [14 C]oleic acid into CE was used as a measure of whole cell ACAT activity. CE hydrolysis was determined following a 24-h preincubation with [14 C]oleic acid (in the absence of the flavonoids) to label an intracellular pool of CE. This was followed by incubations of 2–24 h in the presence of the flavonoids. The latter incubations were carried out in the presence of the specific ACAT inhibitor, DuP 128 (10 μ M, generously provided by the DuPont Merck Pharmaceutical Co., Wilmington, DE), to inhibit cholesterol esterification. [14 C]-oleic acid incorporation into cellular CE was determined as described above.

ACAT1 and ACAT2 activities

The direct effect of naringenin and hesperetin on ACAT1 and ACAT2 activities was determined in AC29 cells expressing either enzyme. In whole cells, the incorporation of [14 C]oleic acid into cellular CE was measured in the presence or absence of naringenin or hesperetin over 5 h, essentially as described above. In further experiments, microsomes were isolated from AC29 cells expressing either ACAT. Cells were scraped from the plates and disrupted by sonication in ice-cold buffer containing 0.1 M phos-

phate, 0.25 M sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 $\mu\text{g}/\text{ml}$ leupeptin at pH 7.4. Microsomes were isolated as in the method of Carr, Parks, and Rudel (26), and frozen at -80°C until analysis. ACAT activity was determined in the presence of exogenous cholesterol, according to the methods of Billheimer, Tavani, and Nes (27), using 25–75 μg of microsomes as the source of ACAT. Naringenin and hesperetin were dissolved in DMSO (0.5% per assay) and added along with a cholesterol/triton WR-1339 buffer for 15 min at 37°C prior to a 10-min incubation with the radiolabeled substrate. Results were compared with those obtained using 10 μM of the ACAT inhibitors DuP 128 and CI-1011 (generously provided by Dr. B. Krause, Parke-Davis, Ann Arbor, MI). Previously, we established in HepG2 cells that 10 μM of DuP 128 or CI-1011 inhibited cellular esterification by 85% and 61%, respectively (15). Furthermore, Cases et al. (28) reported that, in membranes from ACAT1 and ACAT2 baculovirus-infected insect cells, CI-1011 inhibited ACAT2 with an IC_{50} of 2.5 μM and ACAT1 with an IC_{50} of 10 μM .

MTP activity

MTP activity was assessed using an isotopic transfer assay (29), with modifications. Briefly, HepG2 cells were preincubated for 24 h in the absence or presence of naringenin or hesperetin at the indicated concentrations, harvested and sonicated as described above. Cell homogenates were then used as the MTP source. High density lipoprotein-3 (HDL_3) ($d > 1.13 \text{ g}/\text{ml}$) and LDL ($1.019 < d < 1.063 \text{ g}/\text{ml}$) were isolated from human plasma and dialyzed against 10 mM Tris, 150 mM NaCl, 5 mM Na_2EDTA , 3 mM NaN_3 , pH 7.4 (dialysis buffer). [$1\alpha, 2\alpha(n)\text{-}^3\text{H}$]-cholesterol (Amersham) in toluene was evaporated under nitrogen, resuspended in absolute ethanol, and incubated with HDL_3 for 24 h at 37°C in a shaking water bath. Subsequently, [^3H]CE-containing HDL ($1.13 < d < 1.21 \text{ g}/\text{ml}$) was reisolated by ultracentrifugation. The MTP reaction mixture contained donor lipoproteins ([^3H]CE-containing HDL) and acceptor lipoproteins (LDL) in a ratio of 1:10 (based on protein concentration), 50 μg of HepG2 cell homogenate, and dialysis buffer in a final volume of 50 μl . MTP-mediated [^3H]CE transfer was allowed to proceed for 6 h at 37°C in a shaking water bath, after which the reaction was stopped by placing the samples on ice for 5 min. Radioactivity in an aliquot of the reaction mixture was measured in a liquid scintillation counter to determine total dpm. The density of the mixture was adjusted to 1.07 g/ml, and donor and acceptor lipoproteins were separated by ultracentrifugation. Radioactivity in the acceptor particles was counted, and percent transfer of CE was determined as dpm of the $d < 1.07 \text{ g}/\text{ml}$ fraction divided by total dpm added to the reaction.

MTP Western blotting

HepG2 cells were incubated with naringenin for 24 h or 5 days. Following the incubations, HepG2 cells were harvested in 100 μl per well (35 mm) of 1 \times RIPA buffer (1% v/v Triton X-100, 0.1% w/v sodium dodecyl sulfate, 0.5% w/v sodium deoxycholate, 10 mM sodium phosphate, pH 7.5, 5 mM EDTA, 5 mM EGTA, 100 mM sodium chloride, 1 mM PMSF, 20 μM leupeptin, 20 mM methionine, and 1 mM cysteine) (30). The samples were resolved on a 5% stacking, 8% running gel; proteins were transferred to a nitrocellulose membrane. The membranes were blocked overnight at 4°C with 5% nonfat dry milk in phosphate buffered saline, 0.1% Tween 20. The membranes were then incubated with goat anti-bovine MTP primary antibody (provided by Dr. J. R. Wetterau, Bristol-Myers Squibb, Princeton, NJ) followed by incubation with rabbit anti-goat IgG peroxidase conjugate (The Binding Site, Intermedico, Markham, ON, Canada).

MTP was detected using BM Chemiluminescence Blotting Substrate (Roche Diagnostics, Laval, QC, Canada).

^{125}I -LDL binding, uptake and degradation studies

LDL (Sf 0-12) was isolated from human plasma and radiolabeled with ^{125}I using the iodine monochloride technique (31). ^{125}I -LDL binding, uptake and degradation studies were carried out as described by Goldstein, Basu, and Brown (32), with minor modifications described previously (33).

RNase protection analysis of HepG2 mRNA

A HindIII/PstI fragment of human apoB, cloned into psp72 (Promega, provided by Dr. N. Azrolan, Rockefeller University, NY), a Hind III/Xba I fragment from glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ATCC, Rockville, MD), subcloned into pGEM-7Zf (Promega), a Pst I/Pst I fragment from the human LDL receptor (ATCC), subcloned into pbluescript SK+ (Stratagene), a Hind III/Pst I fragment of the human HMG-CoA reductase (ATCC), subcloned into pbluescript SK+, a Pst I/Pst I fragment of human MTP (provided by Dr. J. R. Wetterau, Bristol-Myers Squibb) in pbluescript SK+, a plasmid encoding human ACAT1 (full-length ACAT1 cDNA in PRC/CMV) (provided by Dr. S. L. Sturley, Columbia University, New York, NY), and a plasmid-encoding African green monkey ACAT2 (full-length ACAT2 cDNA in pcDNA3⁻) (provided by Dr. L. Rudel, Wake Forest University School of Medicine) served as templates to synthesize antisense RNA probes. These riboprobes were used to measure mRNA concentrations in a modification of the RNase protection/solution hybridization assay of Azrolan and Breslow (34), as described previously (35).

Statistics

All values are presented as mean \pm SEM (standard error of mean). Means were compared by *t*-tests to determine statistical significance. A *P* value of < 0.05 was considered significant.

RESULTS

ApoB secretion

HepG2 cells were incubated with naringenin (10–200 μM) for 24 h, after which the accumulation of apoB in the media was measured using an ELISA. As shown in Fig. 1A, naringenin dose-dependently decreased the secretion of apoB into the media by 27% (not significant, NS), 56% ($P < 0.002$), and 82% ($P < 0.00001$) at 50, 100, and 200 μM , respectively. At concentrations of 10 μM or less, naringenin had little effect on apoB secretion. The reduction in apoB secretion following incubation with naringenin was also observed for the structurally related citrus flavonoid, hesperetin. Hesperetin decreased apoB secretion by 40% (NS) and 74% ($P < 0.002$) (Fig. 1B) at concentrations of 50 and 200 μM , respectively. In cells incubated with 0.1 mM oleate, immunoprecipitation of radiolabeled apoB from the media and cell lysates revealed that naringenin decreased the secretion of newly synthesized apoB by up to 69% ($P < 0.05$) compared with cells treated with oleate alone (Fig. 2). In contrast, apoAI secretion was unaffected (data not shown). Cellular labeled apoB decreased concomitantly with the decrease in secretion into the media (Fig. 2), demonstrating that apoB is degraded and does not accumulate in the cell. At the concentrations of flavonoids used in this study, there were no significant effects on total

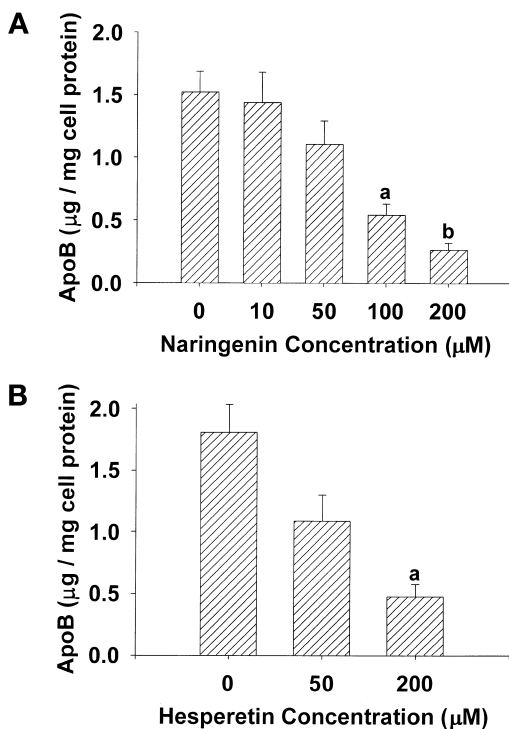


Fig. 1. Effect of naringenin (A) and hesperetin (B) on apoB accumulation in the media. HepG2 cells were incubated with naringenin and hesperetin, at the concentrations indicated, for 24 h. Following the incubation, apoB in the media was measured by ELISA. Values are reported as $\mu\text{g apoB/mg cell protein}$, and are the mean \pm SEM for four to eight experiments with duplicate samples. a, $P < 0.002$; b, $P < 0.00001$.

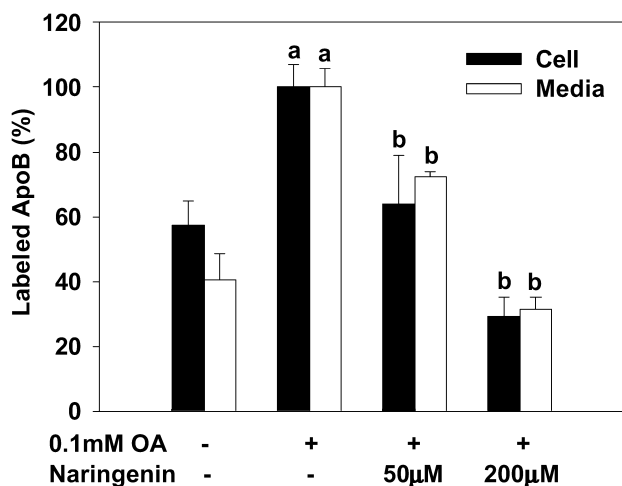


Fig. 2. Effect of naringenin on cellular and secreted, newly synthesized apoB. HepG2 cells were preincubated for 24 h in the presence or absence of naringenin (50 or 200 μM), followed by a 20-min incubation with or without 0.1 mM oleate (OA). The cells were then pulsed for 10 min with [^{35}S]methionine and chased for 1 h. Oleate and naringenin were present throughout the pulse and the chase as indicated. Following the chase, media and cell lysates were collected and labeled; apoB was immunoprecipitated and quantified as described in Materials and Methods. The results are the mean \pm SEM for four experiments. a, $P < 0.05$ as compared to the untreated control; b, $P < 0.05$ as compared with 0.1 mM oleate alone.

cell protein or viability as assessed by an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Thiazolyl blue) assay (data not shown). Kim et al. (36) have reported the IC_{50} for cell growth was greater than 1 mM for both naringenin and hesperetin.

Cellular lipid mass

The mass of cellular CE, FC, and TG was determined after 24-h incubations with either naringenin or hesperetin at doses of 50 and 200 μM . As shown in **Table 1**, FC mass was not significantly altered by flavonoid treatment. CE mass was significantly reduced by 200 μM naringenin (26% decrease, $P < 0.01$) and hesperetin (21% decrease, $P < 0.02$). Cellular TG mass tended to increase (30–50%) following incubation with either flavonoid at concentrations of 200 μM .

Cellular lipid biosynthesis, ACAT activities, and CE hydrolysis

The incorporation of [^{14}C]oleic acid or [^{14}C]acetic acid into cellular lipids was carried out over 5 h, either immediately after flavonoid addition or after a 19-h preincubation with the flavonoids. As shown in **Table 2**, without a 19-h preincubation, the incorporation of [^{14}C]oleic acid into CE was reduced by 37% ($P < 0.05$) and 70% ($P < 0.001$) at 50 and 200 μM naringenin, respectively. Hesperetin reduced cholesterol esterification by 22% (50 μM , NS) and 57% (200 μM , $P < 0.01$). Following a 19-h preincubation, naringenin reduced the incorporation of [^{14}C]oleic acid into CE by up to 84% (200 μM , $P < 0.0001$), whereas hesperetin inhibited cholesterol esterification by up to 76% (200 μM , $P < 0.01$). The incorporation of [^{14}C]oleic acid into cellular TG was increased by naringenin (200 μM) without a preincubation (29% increase, $P < 0.01$) and with a 19-h preincubation (27% increase, $P < 0.05$). The incorporation of [^{14}C]oleic acid into phospholipid was not altered by flavonoid treatment, with or without the 19-h preincubation. The incorporation of [^{14}C]acetate into cholesterol was affected by flavonoid treatment only following the 19-h preincubation. Cholesterol synthe-

TABLE 1. Effect of naringenin and hesperetin on cellular lipid mass

Treatment	Cholesteryl Ester	Free Cholesterol	Triglyceride
	<i>μg/mg cell protein</i>		
Control	5.75 ± 0.28	16.04 ± 0.64	38.80 ± 3.58
Naringenin 50 μM	5.30 ± 0.37	16.71 ± 0.67	44.24 ± 5.16
Naringenin 200 μM	4.23 ± 0.33 ^a	17.21 ± 1.03	51.95 ± 4.58
Control	5.52 ± 0.49	16.82 ± 0.90	38.67 ± 5.25
Hesperetin 50 μM	4.62 ± 1.13	17.34 ± 0.98	39.80 ± 7.64
Hesperetin 200 μM	4.37 ± 0.55 ^b	17.28 ± 1.26	58.15 ± 8.78

HepG2 cells were incubated in minimal essential medium (MEM) containing 5% lipoprotein-deficient serum with naringenin or hesperetin at concentrations of 50 and 200 μM , for 24 h. Following the incubation, lipids were extracted from cell monolayers and quantitated by spectrophotometric assays. CE, FC, and TG mass are reported as the mean \pm SEM for seven experiments with duplicate samples for naringenin and four experiments with duplicate samples for hesperetin. CE mass is calculated as the difference between total and FC mass values.

^a $P < 0.01$ (vs. control).

^b $P < 0.02$ (vs. control).

TABLE 2. Effect of naringenin and hesperetin on cellular lipid biosynthesis

Treatment	Cholesteryl Ester	Triglyceride	Phospholipid	Cholesterol
	<i>nmol oleic acid incorporated/mg cell protein</i>			<i>pmol acetic acid incorporated/mg cell protein</i>
Preincubation, 0 h				
Control	3.21 ± 0.33	104.32 ± 5.95	52.88 ± 5.15	878.5 ± 32.0
Naringenin 50 μM	2.02 ± 0.37 ^a	117.97 ± 11.41	54.99 ± 9.96	826.1 ± 85.8
Naringenin 200 μM	0.97 ± 0.11 ^b	134.15 ± 6.02 ^c	53.68 ± 6.46	850.3 ± 99.8
Hesperetin 50 μM	2.50 ± 0.34	126.57 ± 11.38	63.56 ± 8.14	809.8 ± 110.4
Hesperetin 200 μM	1.37 ± 0.21 ^c	141.12 ± 15.40	61.21 ± 6.90	886.6 ± 147.7
Preincubation, 19 h				
Control	4.32 ± 0.52	128.98 ± 8.28	56.33 ± 4.99	1565.9 ± 123.6
Naringenin 50 μM	1.73 ± 0.32 ^c	133.56 ± 7.43	55.87 ± 5.86	1323.3 ± 72.4
Naringenin 200 μM	0.68 ± 0.10 ^d	164.20 ± 7.23 ^a	56.90 ± 2.82	909.3 ± 129.3 ^a
Hesperetin 50 μM	2.96 ± 0.47	127.01 ± 11.64	52.50 ± 5.95	1369.2 ± 82.3
Hesperetin 200 μM	1.03 ± 0.18 ^c	140.23 ± 8.21	49.29 ± 4.60	977.3 ± 143.9 ^a

HepG2 cells treated with naringenin or hesperetin at concentrations of 50 and 200 μM were incubated with [¹⁴C]oleic acid or [¹⁴C]acetic acid for 5 h. The 5-h incubation was initiated immediately after addition of the flavonoids (0–5 h) or following a 19-h incubation (19–24 h). At the end of the incubation, the cell lipids were extracted and analyzed by thin-layer chromatography as outlined in Materials and Methods. Values are given as mean ± SEM from at least four separate experiments with duplicate samples. 0–5 h and 19–24 h incubations were determined in separate experiments.

^a $P < 0.05$ (vs. control).

^b $P < 0.001$ (vs. control).

^c $P < 0.01$ (vs. control).

^d $P < 0.0001$ (vs. control).

sis was decreased by naringenin (42% at 200 μM, $P < 0.05$) and hesperetin (38% at 200 μM, $P < 0.05$).

The discovery and cloning of a second ACAT enzyme, ACAT2 (17, 28, 37), and the concept that ACAT2 may preferentially provide CEs for lipoprotein assembly (17, 38) raised the possibility that the citrus flavonoids selectively inhibited ACAT2 activity. AC29 cells (a Chinese hamster ovary cell line lacking endogenous ACAT activity) stably transfected with either ACAT1 or ACAT2 cDNA were treated with either naringenin or hesperetin (50–200 μM). Naringenin and hesperetin reduced cholesterol esterification in cells expressing either ACAT1 or ACAT2 to the same extent, as determined by assays using intact cells or isolated microsomes (Table 3).

The observation that cellular ACAT activity was decreased to a greater extent than cellular CE mass suggested that CE hydrolysis may be modulated by naringenin and hesperetin. As shown in Fig. 3, the rate of CE hydrolysis in HepG2 cells was reduced by 34% and 36% after 24 h by naringenin (200 μM, $P < 0.002$) and hesperetin (200 μM, $P < 0.001$), respectively.

MTP activity and expression

The MTP activity in homogenates of HepG2 cells preincubated for 24 h with either flavonoid was determined as the transfer of [³H]CE from donor to acceptor lipoproteins. As shown in Table 4, transfer of [³H]CE was reduced by up to 40% by naringenin (200 μM, $P < 0.00004$) and up to 33% by hesperetin (100 μM, $P < 0.00004$).

MTP is a heterodimer consisting of a 97-kDa large subunit and protein disulfide isomerase (PDI) (39). Following a 24-h incubation with naringenin (200 μM), the amount of the MTP large subunit, as determined by Western blotting, was not significantly altered (Fig. 4). MTP

has a reported half-life of approximately 4.4 days (40). Therefore, the flavonoid incubations were extended to 5 days to determine whether the reduced MTP mRNA concentrations observed in flavonoid-treated cells (see below)

TABLE 3. Effect of naringenin and hesperetin on ACAT activity

Treatment	ACAT1	ACAT2
	<i>[¹⁴C]oleic Acid Incorporation in Intact Cells, % of control</i>	
Control	100	100
Naringenin 50 μM	88.7 ± 1.4 ^a	90.3 ± 2.0 ^c
Naringenin 100 μM	79.5 ± 2.1 ^b	80.2 ± 2.8 ^b
Naringenin 200 μM	65.9 ± 0.8 ^b	64.7 ± 1.9 ^b
Hesperetin 50 μM	86.2 ± 2.8 ^c	89.1 ± 2.6 ^c
Hesperetin 100 μM	79.0 ± 3.1 ^c	80.1 ± 4.0 ^c
Hesperetin 200 μM	58.2 ± 2.9 ^b	63.1 ± 4.2 ^b
	<i>Microsomal ACAT Activity, % of control</i>	
Control	100	100
Naringenin 50 μM	90.0 ± 3.2 ^d	93.8 ± 1.6 ^d
Naringenin 100 μM	88.0 ± 2.3 ^d	88.3 ± 3.8 ^d
Naringenin 200 μM	72.5 ± 1.8 ^a	72.0 ± 2.2 ^a
Hesperetin 50 μM	97.4 ± 5.7	100.8 ± 6.9
Hesperetin 100 μM	90.8 ± 1.9 ^d	100.4 ± 8.5
Hesperetin 200 μM	80.5 ± 5.6 ^d	87.5 ± 6.1
Dup 128 10 μM	0.0	0.0
CI-1011 10 μM	2.3	0.4

ACAT1 and ACAT2 activities were determined using AC29 cells stably transfected with African green monkey ACAT1 or ACAT2 cDNA. For microsomal assays, naringenin and hesperetin were added in the presence of exogenous cholesterol. The values are reported as percent of control and are the mean ± SEM for at least three separate experiments.

^a $P < 0.0002$ (vs. control).

^b $P < 0.00006$ (vs. control).

^c $P < 0.003$ (vs. control).

^d $P < 0.04$ (vs. control).

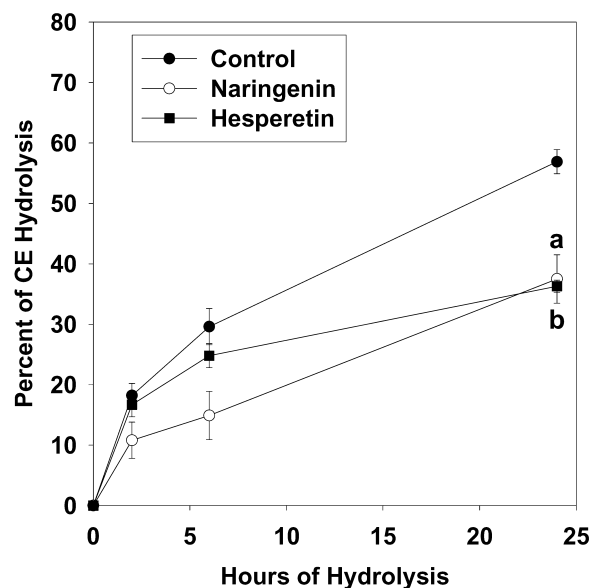


Fig. 3. Effect of naringenin and hesperetin on cholesteryl ester hydrolysis. HepG2 cells were preincubated with [^{14}C]oleic acid for 24 h to label an intracellular pool of CE. DMSO (control) (●), naringenin (200 μM) (○), or hesperetin (200 μM) (■) were then added in the presence of the ACAT inhibitor, DuP 128 (10 μM). The rate of CE hydrolysis was then measured over 24 h. The results are expressed as the percent of the labeled CE pool hydrolyzed and are the mean \pm SEM for three experiments. a, $P < 0.002$; b, $P < 0.001$.

were associated with reduced MTP protein levels. The amount of immunodetectable MTP large subunit was nearly completely depleted in flavonoid-treated cells following a 5-day incubation (Fig. 4).

Binding, uptake, and degradation of ^{125}I -LDL

HepG2 cells were preincubated with naringenin and hesperetin (200 μM) for 24 h prior to determining the cellular binding, uptake, and degradation of ^{125}I -LDL at 37°C. As shown in Fig. 5, naringenin and hesperetin increased the cellular binding of ^{125}I -LDL by 24% (NS) and 137% ($P < 0.02$), respectively. Cellular uptake of ^{125}I -LDL was increased by naringenin (66%, $P < 0.05$) and hesperetin (149%, $P < 0.05$). ^{125}I -LDL degradation was in-

TABLE 4. Effect of naringenin and hesperetin on MTP activity

Concentration μM	Naringenin	Hesperetin
	% of Control	
0	100 \pm 0	100 \pm 0
50	81.3 \pm 5.8 ^a	92.4 \pm 3.8
100	67.6 \pm 8.4 ^b	66.9 \pm 2.9 ^b
200	60.2 \pm 4.1 ^b	78.2 \pm 4.2 ^b

MTP activity was determined by [^3H]CE transfer from donor to acceptor lipoproteins, as described in Materials and Methods. HepG2 crude cell homogenates prepared from cells incubated for 24 h with either naringenin or hesperetin were used as the MTP source. No additional naringenin or hesperetin was added exogenously at the time of the assay. The values are reported as % of control and are the mean \pm SEM for eight separate experiments.

^a $P < 0.001$ (vs. control).

^b $P < 0.00004$ (vs. control).

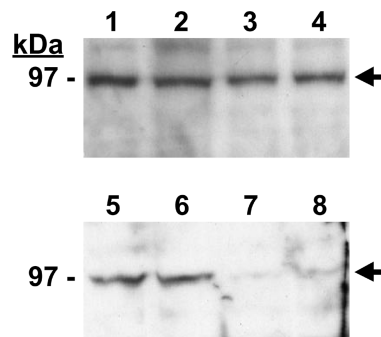


Fig. 4. Effect of naringenin on MTP expression. HepG2 cells were incubated with 200 μM naringenin for 24 h or 5 days. Following the incubations, MTP large subunit was detected by Western blotting as described in Materials and Methods. Lanes 1 and 2 are control cells incubated for 24 h. Lanes 3 and 4 are cells incubated with naringenin for 24 h. Lanes 5 and 6 are control cells incubated for 5 days. Lanes 7 and 8 are cells incubated with naringenin for 5 days. The arrow indicates the position of MTP large subunit (97 kDa). The gels shown are representative of the results obtained in at least three separate experiments.

creased by naringenin and hesperetin by 11% (NS) and 118% ($P < 0.05$), respectively.

HepG2 mRNA content

The abundances of specific mRNAs were determined in HepG2 cells following 24-h incubations with the flavonoids. As shown in Table 5, ACAT2 mRNA was significantly reduced by 49% ($P < 0.0001$) with naringenin (200 μM) and by 53% ($P < 0.001$) with hesperetin (200 μM). ACAT1 mRNA was unaffected. MTP mRNA levels were also significantly reduced by naringenin (−31% at 200 μM , $P < 0.02$) and hesperetin (−47% at 200 μM , $P < 0.001$). In contrast,

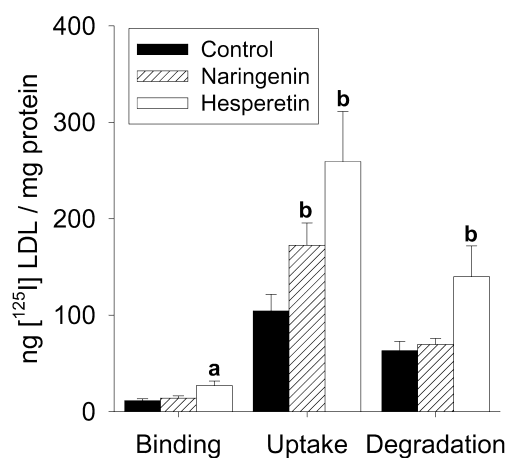


Fig. 5. Effect of naringenin and hesperetin on LDL receptor activity. HepG2 cells were incubated with DMSO (control, solid bars), naringenin (striped bars), and hesperetin (open bars) at concentrations of 200 μM for 24 h. ^{125}I -LDL cell binding, uptake, and degradation were determined following 24-h incubations with the flavonoids. Naringenin and hesperetin were present throughout the ^{125}I -LDL binding, uptake, and degradation studies. Values are reported as ng ^{125}I -LDL associated or degraded per mg cell protein and are the mean \pm SEM for five experiments. a, $P < 0.02$; b, $P < 0.05$ (compared with control).

TABLE 5. Effect of naringenin and hesperetin on mRNA levels

mRNA	Control	Naringenin 50 μ M	Naringenin 200 μ M	Hesperetin 50 μ M	Hesperetin 200 μ M
<i>pg mRNA/μg total RNA</i>					
ApoB	109.2 \pm 11.1	94.5 \pm 17.0	104.6 \pm 21.7	107.8 \pm 17.6	93.6 \pm 8.0
ACAT1	0.55 \pm 0.03	0.53 \pm 0.05	0.50 \pm 0.04	0.57 \pm 0.04	0.48 \pm 0.07
ACAT2	0.47 \pm 0.02	0.51 \pm 0.06	0.24 \pm 0.04 ^c	0.41 \pm 0.08	0.22 \pm 0.04 ^b
MTP	1.31 \pm 0.07	1.42 \pm 0.11	0.90 \pm 0.13 ^a	1.52 \pm 0.15	0.69 \pm 0.09 ^b
LDL receptor	0.63 \pm 0.11	0.89 \pm 0.08	3.07 \pm 0.87 ^a	0.73 \pm 0.07	4.13 \pm 1.11 ^a
HMG-CoA reductase	4.27 \pm 0.26	3.68 \pm 0.56	4.28 \pm 0.79	3.84 \pm 1.39	5.09 \pm 0.82
GAPDH	35.5 \pm 4.0	46.3 \pm 1.6	30.3 \pm 1.9	43.0 \pm 7.5	37.7 \pm 2.3

HepG2 cells were incubated with naringenin or hesperetin at concentrations of 50 and 200 μ M for 24 h. Subsequently, total RNA was extracted from the cells and quantitated as described in Materials and Methods. ApoB, ACAT1, ACAT2, MTP, LDL receptor, HMG-CoA reductase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were analyzed by RNase protection/solution hybridization assay. The results are reported as pg mRNA/ μ g of total RNA. Values are given as the mean \pm SEM from at least three separate experiments.

^a $P < 0.02$.

^b $P < 0.001$.

^c $P < 0.0001$.

LDL receptor mRNA levels were significantly increased approximately 5-fold at 200 μ M naringenin ($P < 0.02$) and 7-fold at 200 μ M of hesperetin ($P < 0.02$). ApoB, HMG-CoA reductase, and GAPDH mRNA levels were not significantly affected by either flavonoid (50 or 200 μ M) when compared with control cells.

DISCUSSION

In vivo studies in a number of animal models have shown that diets supplemented with grapefruit juice or orange juice (5), or glycosylated forms of naringenin (6–8) or hesperetin (6, 8, 9), reduce plasma cholesterol levels. We demonstrate here that the citrus flavonoids, naringenin and hesperetin, dose-dependently reduce the accumulation of apoB in the media of HepG2 cells. Our results indicate that this is due to a decrease in the assembly and secretion of apoB-containing lipoproteins, as well as enhanced re-uptake of these lipoproteins via the LDL receptor.

In this study, we examined the mechanisms whereby naringenin and hesperetin altered hepatic apoB metabolism. ApoB mRNA levels were not affected by flavonoid treatment, consistent with the concept that, under most conditions of altered apoB secretion from HepG2 cells, apoB mRNA levels remain unchanged (12, 15, 41). The inhibition of apoB secretion in the presence of these flavonoids may result in posttranscriptionally enhanced intracellular degradation of apoB, as shown for other inhibitors of apoB secretion (15, 41). Our data, showing parallel decreases in radiolabel incorporation into both newly synthesized cellular and secreted apoB in the presence of naringenin, support this possibility.

Both flavonoids caused dose-dependent inhibition of cholesterol esterification, consistent with earlier observations in HepG2 cells (20). Inhibition of newly synthesized CEs catalyzed by ACAT has been shown to decrease apoB secretion in HepG2 cells (15, 16, 42, 43), although this has not been a universal finding (15). Furthermore, we have previously shown, using in vivo kinetic studies in pigs (14, 44, 45), that inhibition of hepatic cholesterol esterifi-

cation decreases hepatic apoB secretion. In the present study, the flavonoids decreased ACAT activity in HepG2 cells by 75–85%, which was associated with a 74–82% reduction in apoB secretion. Therefore, reduced cholesterol esterification upon incubation with either naringenin or hesperetin may contribute to the observed reduction in apoB secretion from HepG2 cells. Inhibition of hepatic ACAT has also been demonstrated for a flavonoid structurally different from these flavanones, called baicalein (IC₅₀ for ACAT inhibition: 100 μ M) (46). Whether inhibition of ACAT and apoB secretion are general features of flavonoids remains to be determined. It is of particular interest that both flavonoids also reduce CE hydrolysis. This observation explains the modest reduction in HepG2 cellular CE mass, since the reduction in cholesterol esterification is offset by a concomitant decrease in CE hydrolysis.

The mechanism by which flavonoids reduce hepatic ACAT activity and apoB secretion may be mediated through interaction with the plasma membrane transporter, multidrug resistance p-glycoprotein. Recently, Conseil et al. (47) have shown that flavonoids can bind mouse p-glycoprotein and modulate its activity. Inhibition of p-glycoprotein activity by a number of drugs has been shown to inhibit cholesterol esterification in HepG2 cells (48) and in the intestinal cell line, Caco-2 (48, 49). The secretion of apoB in Caco-2 cells was also reduced following inhibition of p-glycoprotein (49). Thus, inhibition of cholesterol esterification and apoB secretion by naringenin and hesperetin in HepG2 cells may be mediated through inhibition of p-glycoprotein.

A novel finding in this study is the selective decrease in ACAT2 mRNA in HepG2 cells incubated with either flavonoid. Although the regulation of ACAT2 is largely unknown (38), this is the first report demonstrating that regulation of ACAT2 occurs at the level of mRNA abundance. The specificity of the flavonoids for ACAT2 is of interest in light of the tissue-specific expression and predicted topology of this enzyme (17). It has been postulated that ACAT2 may preferentially provide CE for lipoprotein assembly within the endoplasmic reticulum lumen of the liver and intestine (17). Our results show

that the message abundance for ACAT1 and ACAT2, in HepG2 cells grown under control conditions, were quantitatively similar. This would suggest that the enzyme activities for each ACAT would also be similar. Chang et al. (50), using an immunodepletion strategy, found that the activity of ACAT2 in HepG2 cells was approximately 15% of the total activity. However, mRNA levels were not reported.

We have established that naringenin and hesperetin are direct inhibitors of total ACAT activity; however, specificity for either enzyme was not observed. Addition of either flavonoid to microsomes from AC29 cells expressing either ACAT1 or ACAT2 produced similar reductions in cholesterol esterification. However, it is not known whether the subcellular distribution (51) or topology (38) of ACAT1 and ACAT2 in AC29 cells resembles hepatocytes. Nevertheless, selective inhibition of ACAT2 expression, together with the inhibition of its activity, would result in a preferential reduction in CE available for association with apoB.

In the present study, naringenin and hesperetin caused an increase in the synthesis of TG, but the reason for this is not readily apparent. In previous studies, an increase in TG synthesis was observed in HepG2 cells incubated with the ACAT inhibitors DuP128 (15) or Sandoz 58-035 (52). Therefore, it is possible that the increased TG synthesis is a consequence of inhibition of cholesterol esterification. The flavonoids did not significantly increase TG mass, although a trend was observed. It is important to note that, despite the increased TG synthesis, apoB secretion was inhibited, demonstrating discordance between these two processes.

The observed decrease in cholesterol synthesis by flavonoid treatment is likely secondary to the inhibition of cholesterol esterification. We speculate that this would lead to increased FC levels within specific regulatory pools not revealed by whole cell FC mass measurements. Cholesterol synthesis was not decreased by flavonoid treatment without preincubation, suggesting that the flavonoids do not have a direct effect on HMG-CoA reductase. Previously (15), we observed a reduction in cholesterol synthesis following incubation of HepG2 cells with the ACAT inhibitors DuP 128 and CI-1011. This may explain the reduced hepatic HMG-CoA reductase activities observed in rats consuming glycosylated forms of naringenin and hesperetin (8).

A second novel finding of this study was the flavonoid-induced reduction in microsomal triglyceride transfer protein (MTP) activity and expression. MTP has been shown to be essential for the hepatic secretion of apoB-containing lipoproteins (19) by mediating the transfer of TG, CE, and PL to nascent apoB (18). Naringenin and hesperetin (200 μ M) inhibited cellular MTP activity by approximately 30–40%. This is consistent with the findings of Wang, Tran, and Yao (53), who showed that, in McArdle RH7777 rat hepatoma cells, inhibiting MTP activity by 40% with a specific MTP inhibitor resulted in a 60% decrease in apoB secretion. Thus, the reduction in MTP activity we observed following incubation with naringenin or hesperetin likely contributes to the 74–82% reduction in apoB secretion in HepG2 cells. The flavonoids also reduced MTP mRNA by 30–50%,

following a 24-h incubation. Note that, because MTP has a half-life of approximately 4.4 days (40), it is unlikely that the reduction in MTP mRNA observed in this study would result in decreased apoB secretion during a 24-h incubation. This altered expression may, however, be important in vivo. Nevertheless, direct inhibition of MTP by these flavonoids likely contributes to the reduced secretion of apoB.


The inhibition of both ACAT and MTP by the flavonoids suggests that reduced availability and transfer of lipids to apoB is the primary mechanism of action in HepG2 cells. This concept is consistent with our observation that naringenin not only decreased apoB secretion in cells grown under basal conditions, but was equally effective under conditions in which apoB secretion was increased by oleate. Preincubation of cells for 24 h with 200 μ M naringenin inhibited oleate-stimulated apoB secretion by 69%, a value below that observed in control cells. Recently, Borradaile, Carroll, and Kurowska (20) reported that acute exposure to either naringenin or hesperetin for 4 h did not alter apoB secretion by HepG2 cells preincubated with oleate. Thus, incubations longer than 4 h are required to observe any effect of flavonoids on apoB secretion in oleate-treated HepG2 cells.

Naringenin and hesperetin increase the cell association and degradation of 125 I-LDL in HepG2 cells, indicating increased LDL receptor expression. This is consistent with the observed increases in LDL receptor mRNA. Interestingly, LDL receptor mRNA increased approximately 5-fold, whereas cellular uptake of labeled LDL increased only 1.6- to 2.5-fold, suggesting a disconnection between gene expression and receptor activity. The reason for this is not known. Although increased re-uptake of apoB-containing lipoproteins may partially explain the diminished apoB accumulation in the media of flavonoid-treated HepG2 cells, it is unlikely to be responsible for the observed 80% decrease in apoB accumulation. Recently, Twisk et al. (54) provided evidence that hepatocyte LDL receptor expression modulates net apoB secretion. Using primary hepatocytes from wild-type and LDL receptor knockout mice, these investigators showed that a complete absence of hepatic LDL receptors increased apoB secretion by only 44%.

Our observed increase in LDL receptor expression may be related to the estrogenic activity of these flavonoids (55, 56). Naringenin has been shown to elicit an estrogenic response through interaction with estrogen receptors (55) and can bind both estrogen receptors α and β (56). The ability of hesperetin to act as an estrogen has not been investigated; however, its structural similarity to naringenin suggests that it may exert a similar effect. Estrogen has been shown to increase hepatic LDL receptor expression in a number of animal models (57–60) and to increase LDL receptor transcription in HepG2 cells (61). The significant increase in LDL receptor mRNA without a change in HMG-CoA reductase mRNA was somewhat unexpected, as LDL receptor and HMG-CoA reductase are generally regulated coordinately (62). However, discordance has been observed previously in estrogen-treated

frogs (60) in which LDL receptor mRNA increased without any increase in HMG-CoA reductase mRNA.

It is unlikely that the estrogenic action of these flavonoids is directly responsible for their effect on apoB secretion and cholesterol esterification in HepG2 cells. Estrogen has been reported to have no effect (63, 64) or to increase (64) apoB secretion from HepG2 cells. Furthermore, estrogen treatment has been shown to result in no change (65) or increases (66) in hepatic ACAT activity. Whether the estrogenic action of these flavonoids is responsible for the reduced concentrations of ACAT2 mRNA, thus affecting cholesterol esterification and apoB secretion, is currently unknown.

In summary, the citrus flavonoids naringenin and hesperetin significantly reduce apoB accumulation in the media of HepG2 cells. We conclude that this effect is mediated via the following: 1) reduced activities of ACAT1 and ACAT2, 2) a selective decrease in ACAT2 expression, and 3) reduced MTP activity. Together, these mechanisms reduce the availability of lipids, in particular CE, for the assembly of apoB-containing lipoproteins. Furthermore, increased LDL receptor activity contributed to the reduced accumulation of apoB in the media. Future studies are required to determine whether citrus flavonoids modulate hepatic apoB secretion and clearance *in vivo*. 

This work was supported by a grant from the Heart and Stroke Foundation of Ontario (T-3371 to M.W.H.). L.J.W. is a recipient of a Medical Research Council of Canada Studentship. N.M.B. is a recipient of a Heart and Stroke Foundation of Canada Studentship. M.W.H. is a Career Investigator of the Heart and Stroke Foundation of Ontario. We thank Cindy Sawyez, Dawn Telford, and Jennifer Epstein for expert technical assistance. We are grateful to Dr. Lawrence Rudel (Wake Forest University, Winston-Salem, NC) for providing plasmids encoding African green monkey ACAT1 and ACAT2 and AC29 cells stably transfected with monkey ACAT1 and ACAT2.

Manuscript received 29 August 2000 and in revised form 5 December 2000.

REFERENCES

- Montanari, A., J. Chen, and W. Widmer. 1998. Citrus flavonoids: a review of past biological activity against disease. In *Flavonoids in the living system*. Manthey and Buslig, editors. Plenum Press, New York. 103–113.
- Guthrie, N., and K. K. Carroll. 1998. Inhibition of mammary cancer by citrus flavonoids. In *Flavonoids in the living system*. J. A. Manthey and B. S. Buslig, editors. Plenum Press, New York. 227–236.
- Samman, S., P. M. L. Wall, and N. C. Cook. 1999. Flavonoids and coronary heart disease: dietary perspectives. In *Flavonoids in the living system*. J. A. Manthey and B. S. Buslig, editors. Plenum Press, New York. 469–481.
- Elias, N., B. W. Patterson, and G. Schonfeld. 1999. Decreased production rates of VLDL triglycerides and apoB-100 in subjects heterozygous for familial hypobetalipoproteinemia. *Arterioscler. Thromb. Vasc. Biol.* **19**: 2714–2721.
- Kurowska, E., N. Borradaile, J. D. Spence, and K. K. Carroll. 2000. Hypocholesterolemic effects of dietary citrus juices in rabbits. *Nutr. Res.* **20**: 121–129.
- Choi, J. S., T. Yokozawa, and H. Oura. 1991. Antihyperlipidemic effect of flavonoids from *Prunus davidiana*. *J. Nat. Prod.* **54**: 218–224.
- Choi, J. S., T. Yokozawa, and H. Oura. 1991. Improvement of hyperglycemia and hyperlipemia in streptozotocin-diabetic rats by a methanolic extract of *Prunus davidiana* stems and its main component, prunin. *Planta Med.* **57**: 208–211.
- Bok, S.-H., S.-H. Lee, Y.-B. Park, K.-H. Bae, K.-H. Son, T.-S. Jeong, and M.-S. Choi. 1999. Plasma and hepatic cholesterol and hepatic activities for 3-hydroxy-3-methyl-glutaryl-CoA reductase and acyl CoA: cholesterol transferase are lower in rats fed citrus peel extract or a mixture of citrus bioflavonoids. *J. Nutr.* **129**: 1182–1185.
- Monforte, M. T., A. Trovato, S. Kirjavainen, A. M. Forestieri, E. M. Galati, and R. B. Lo Curto. 1995. Biological effects of hesperidin, a citrus flavonoid (note II): hypolipidemic activity on experimental hypercholesterolemia in rat. *Farmacol.* **50**: 595–599.
- Yao, Z., K. Tran, and R. S. McLeod. 1997. Intracellular degradation of newly synthesized apolipoprotein B. *J. Lipid Res.* **38**: 1937–1953.
- Ginsberg, H. N. 1997. Role of lipid synthesis, chaperone proteins and proteasomes in the assembly and secretion of apoprotein B-containing lipoproteins from cultured liver cells. *Clin. Exp. Pharmacol. Physiol.* **24**: A29–A32.
- Pullinger, C. R., J. D. North, B.-B. Teng, V. A. Rifci, A. E. Ronhild de Brito, and J. Scott. 1989. The apolipoprotein B gene is constitutively expressed in HepG2 cells: regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life. *J. Lipid Res.* **30**: 1065–1077.
- Yao, Z., and D. E. Vance. 1988. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. *J. Biol. Chem.* **263**: 2998–3004.
- Burnett, J. R., L. J. Wilcox, D. E. Telford, S. J. Kleinstiver, P. H. R. Barrett, R. S. Newton, and M. W. Huff. 1999. Inhibition of ACAT by avasimibe decreases both VLDL and LDL apolipoprotein B production in miniature pigs. *J. Lipid Res.* **40**: 1317–1327.
- Wilcox, L. J., P. H. R. Barrett, R. S. Newton, and M. W. Huff. 1999. ApoB100 secretion from HepG2 cells is decreased by the ACAT inhibitor CI-1011: an effect associated with enhanced intracellular degradation of apoB. *Arterioscler. Thromb. Vasc. Biol.* **19**: 939–949.
- Cianflone, K., Z. Yasruel, M. A. Rodriguez, D. Vas, and A. D. Sniderman. 1990. Regulation of apoB secretion from HepG2 cells: evidence for a critical role for cholesterol ester synthesis in the response to a fatty acid challenge. *J. Lipid Res.* **31**: 2045–2055.
- Anderson, R. A., C. Joyce, M. Davis, J. W. Reagan, M. Clark, G. S. Shelness, and L. L. Rudel. 1998. Identification of a form of acyl-CoA:cholesterol acyltransferase specific to liver and intestine in nonhuman primates. *J. Biol. Chem.* **273**: 26747–26754.
- Jamil, H., J. K. Dickson, Jr., C. Chu, M. W. Lago, J. K. Rinehart, S. A. Biller, R. E. Gregg, and J. R. Wetterau. 1995. Microsomal triglyceride transfer protein: specificity of lipid binding and transport. *J. Biol. Chem.* **270**: 6549–6554.
- Wetterau, J. R., R. E. Gregg, T. W. Harrity, C. Arbeeny, M. Cap, F. Connolly, C.-H. Chu, R. J. George, D. A. Gordon, H. Jamil, K. G. Jolibois, L. K. Kunselman, S.-J. Lan, T. J. Maccagnan, B. Ricci, M. Yan, D. Young, Y. Chen, O. M. Fryszman, J. V. M. Logan, C. L. Musial, M. A. Poss, J. A. Robl, L. M. Simpkins, W. A. Slusarchyk, R. Sulsky, P. Taunk, D. A. Magnin, J. A. Tino, R. M. Lawrence, J. K. Dickson, Jr., and S. A. Biller. 1998. An MTP inhibitor that normalizes atherogenic lipoprotein levels in WHHL rabbits. *Science*. **282**: 751–754.
- Borradaile, N. M., K. K. Carroll, and E. M. Kurowska. 1999. Regulation of HepG2 cell apolipoprotein B metabolism by the citrus flavanones hesperetin and naringenin. *Lipids*. **34**: 591–598.
- Brown, A., D. Wiggins, and G. F. Gibbons. 1999. Glucose phosphorylation is essential for the turnover of neutral lipid and the second stage of triacylglycerol-rich apoB-containing lipoproteins in primary hepatocyte cultures. *Arterioscler. Thromb. Vasc. Biol.* **19**: 321–329.
- Evans, A. J., C. G. Sawyez, B. M. Wolfe, and M. W. Huff. 1992. Lipolysis is a prerequisite for lipid accumulation in HepG2 cells induced by large hypertriglyceridemic very low density lipoproteins. *J. Biol. Chem.* **267**: 10743–10751.
- Chang, C. C. Y., J. Chen, M. Thomas, D. Cheng, V. A. Del Priore, R. S. Newton, M. E. Pape, and T. Y. Chang. 1995. Regulation and immunolocalization of acyl-coenzyme A: cholesterol acyltransferase in mammalian cells as studied with specific antibodies. *J. Biol. Chem.* **270**: 29532–29540.
- Lee, R. G., M. L. Willingham, M. A. Davis, K. A. Skinner, and L. L. Rudel. 2000. Differential expression of ACAT1 and ACAT2 among cells within liver, intestine, kidney and adrenals of nonhuman primates. *J. Lipid Res.* **41**: 1991–2001.
- Carr, T. P., C. J. Andresen, and L. L. Rudel. 1993. Enzymatic determination of triglyceride, free cholesterol, and total cholesterol in tissue lipid extracts. *Clin. Biochem.* **26**: 39–42.

26. Carr, T. P., J. S. Parks, and L. L. Rudel. 1992. Hepatic ACAT activity in African green monkeys is highly correlated to plasma LDL cholesterol ester enrichment and coronary artery atherosclerosis. *Arterioscler. Thromb.* **12**: 1274–1283.
27. Billheimer, J. T., D. Tavani, and W. R. Nes. 1981. Effect of a dispersion of cholesterol in Triton WR-1339 on acyl CoA:cholesterol acyltransferase in rat liver microsomes. *Anal. Biochem.* **111**: 331–335.
28. Cases, S., S. Novak, Y. Zheng, H. M. Myers, S. R. Lear, E. R. Sande, C. B. Welch, A. J. Lusis, T. A. Spencer, B. R. Krause, S. K. Erickson, and R. V. Farese, Jr. 1998. ACAT-2, a second mammalian acyl CoA:cholesterol acyltransferase: its cloning, expression, and characterization. *J. Biol. Chem.* **273**: 26755–26764.
29. Lagrost, L. 1998. Determination of the mass concentration and the activity of the plasma cholesteryl ester transfer protein (CETP). In *Lipoprotein Protocols*. J. M. Ordovas, editor. Humana Press, Totowa, NJ.
30. Mathur, S. N., E. Born, S. Murthy, and F. J. Field. 1997. Microsomal triglyceride transfer protein in CaCo-2 cells: characterization and regulation. *J. Lipid Res.* **38**: 61–67.
31. Huff, M. W., and D. E. Telford. 1985. Direct synthesis of low-density lipoprotein apolipoprotein B in the miniature pig. *Metabolism*. **34**: 36–42.
32. Goldstein, J. L., S. K. Basu, and M. S. Brown. 1983. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Meth. Enzymol.* **98**: 241–260.
33. Whitman, S. C., C. A. Argmann, C. G. Sawyez, D. B. Miller, R. A. Hegele, and M. W. Huff. 1999. Uptake of type IV hypertriglyceridemic VLDL by cultured macrophages is enhanced by interferon-gamma. *J. Lipid Res.* **40**: 1017–1028.
34. Azrolan, N., and J. L. Breslow. 1990. A solution hybridization/RNase protection assay with riboprobes to determine absolute levels of apoB, A-I, and E mRNA in human hepatoma cell lines. *J. Lipid Res.* **31**: 1141–1146.
35. Burnett, J. R., L. J. Wilcox, D. E. Telford, S. J. Kleinstiver, P. H. R. Barrett, R. S. Newton, and M. W. Huff. 1997. Inhibition of HMG-CoA reductase by atorvastatin decreases both VLDL and LDL apolipoprotein B production in miniature pigs. *Arterioscler. Thromb. Vasc. Biol.* **17**: 2589–2600.
36. Kim, D-H., E-A. Jung, I-S. Sohng, J-A. Han, T-H. Kim, and M. J. Han. 1998. Intestinal bacterial metabolism of flavonoids and its relation to some biological activities. *Arch. Pharm. Res.* **21**: 17–23.
37. Oelkers, P., A. Behari, D. Cromley, J. T. Billheimer, and S. L. Sturley. 1998. Characterization of two human genes encoding acyl coenzyme A:cholesterol acyltransferase related enzymes. *J. Biol. Chem.* **273**: 26765–26771.
38. Joyce, C., K. Skinner, R. A. Anderson, and L. L. Rudel. 1999. Acyl-coenzyme A:cholesteryl acyltransferase 2. *Curr. Opin. Lipidol.* **10**: 89–95.
39. Wetterau, J. R., M. C. M. Lin, and H. Jamil. 1997. Microsomal triglyceride transfer protein. *Biochim. Biophys. Acta.* **1345**: 136–150.
40. Lin, M. C. M., D. Gordon, and J. R. Wetterau. 1995. Microsomal triglyceride transfer protein (MTP) regulation in HepG2 cells: insulin negatively regulates MTP gene expression. *J. Lipid Res.* **36**: 1073–1081.
41. Wilcox, L. J., P. H. R. Barrett, and M. W. Huff. 1999. Differential regulation of apolipoprotein B secretion from HepG2 cells by two HMG-CoA reductase inhibitors: atorvastatin and simvastatin. *J. Lipid Res.* **40**: 1078–1089.
42. Musanti, R., L. Giorgini, P. P. Lovisolo, A. Pirillo, A. Chiari, and G. Ghiselli. 1996. Inhibition of acyl-CoA:cholesterol acyltransferase decreases apolipoprotein B-100-containing lipoprotein secretion from HepG2 cells. *J. Lipid Res.* **37**: 1–14.
43. Kohen Avramoglu, R., K. Cianflone, and A. D. Sniderman. 1995. Role of the neutral lipid accessible pool in the regulation of secretion of apoB-100 lipoprotein particles by HepG2 cells. *J. Lipid Res.* **36**: 2513–2528.
44. Burnett, J. R., L. J. Wilcox, D. E. Telford, S. J. Kleinstiver, P. H. R. Barrett, and M. W. Huff. 1998. Inhibition of cholesterol esterification by DuP 128 decreases hepatic apolipoprotein B secretion in vivo: effect of dietary fat and cholesterol. *Biochim. Biophys. Acta.* **1393**: 63–79.
45. Huff, M. W., D. E. Telford, P. H. Barrett, D. W. Billheimer, and P. J. Gillies. 1994. Inhibition of hepatic ACAT decreases apoB secretion in miniature pigs fed a cholesterol-free diet. *Arterioscler. Thromb.* **14**: 1498–1508.
46. Yotsumoto, H., T. Yanagita, K. Yamamoto, Y. Ogawa, J. Y. Cha, and Y. Mori. 1997. Inhibitory effects of oren-gedoku-to and its components on cholesterol ester synthesis in cultured human hepatocyte HepG2 cells: evidence from the cultured HepG2 cells and in vitro assay of ACAT. *Planta Med.* **63**: 141–145.
47. Conseil, G., H. Baubichon-Cortay, G. Dayan, J-M. Jault, D. Barron, and A. Di Pietro. 1998. Flavonoids: A class of modulators with bifunctional interactions at vicinal ATP- and steroid-binding sites on mouse P-glycoprotein. *Proc. Natl. Acad. Sci. USA.* **95**: 9831–9836.
48. Debry, P., E. A. Nash, D. W. Neklason, and J. E. Metherall. 1997. Role of multidrug resistance P-glycoprotein in cholesterol esterification. *J. Biol. Chem.* **272**: 1026–1031.
49. Field, F. J., E. Born, H. Chen, S. Murthy, and S. Mathur. 1995. Esterification of plasma membrane cholesterol and triacylglycerol-rich lipoprotein secretion in CaCo-2 cells: possible role of p-glycoprotein. *J. Lipid Res.* **36**: 1533–1543.
50. Chang, C. C., N. Sakashita, K. Ornvold, O. Lee, E. T. Chang, R. Dong, S. Lin, C. Y. Lee, S. Strom, R. Kashyap, J. Fung, J. Farese, J. F. Patoiseau, A. Delhon, and T. Y. Chang. 2000. Immunological quantitation and localization of ACAT-1 and ACAT-2 in human liver and small intestine. *J. Biol. Chem.* **275**: 28083–28092.
51. Vance, J. E. 1998. Eukaryotic lipid-biosynthetic enzymes: the same but not the same. *Trends Biochem. Sci.* **23**: 423–428.
52. Ooyen, C., A. Zecca, T. Zanelli, and A. L. Catapano. 1997. Decreased intracellular degradation and increased secretion of apoB-100 in HepG2 cells after inhibition of cholesteryl ester synthesis. *Atherosclerosis*. **130**: 143–152.
53. Wang, Y., K. Tran, and Z. Yao. 1999. The activity of microsomal triglyceride transfer protein is essential for accumulation of triglyceride within microsomes in McA-RH7777 cells. *J. Biol. Chem.* **274**: 27793–27800.
54. Twisk, J., D. L. Gillian-Daniel, A. Tebon, L. Wang, P. H. R. Barrett, and A. D. Attie. 2000. The role of the LDL receptor in apolipoprotein B secretion. *J. Clin. Invest.* **105**: 1–12.
55. Balaguer, P., A. Joyeux, M. S. Denison, R. Vincent, B. E. Gillesby, and T. Zacharewski. 1996. Assessing the estrogenic and dioxin-like activities of chemicals and complex mixtures using in vitro recombinant receptor-reporter gene assays. *Can. J. Physiol. Pharmacol.* **74**: 216–222.
56. Kuiper, G. G. J. M., J. G. Lemmen, B. Carlsson, J. C. Corton, S. H. Safe, P. T. van der Saag, B. van der Burg, and J-A. Gustafsson. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β . *Endocrinology*. **139**: 4252–4263.
57. Kovanen, P. T., M. S. Brown, and J. L. Goldstein. 1979. Increased binding of low density lipoprotein to liver membranes from rats treated with 17 α -ethinyl estradiol. *J. Biol. Chem.* **254**: 11367–11373.
58. Srivastava, R. A., D. Baumann, and G. Schonfeld. 1993. In vivo regulation of low-density lipoprotein receptors by estrogen differs at the post-transcriptional level in rats and mouse. *Eur. J. Biochem.* **216**: 527–538.
59. Parini, P., B. Angelin, and M. Rudling. 1997. Importance of estrogen receptors in hepatic LDL receptor regulation. *Arterioscler. Thromb. Vasc. Biol.* **17**: 1800–1805.
60. Di Croce, L., G. Bruscalupi, and A. Trentalance. 1997. Independent responsiveness of frog liver low-density lipoprotein receptor and HMGCoA reductase to estrogen treatment. *Pflugers Archiv.* **435**: 107–111.
61. Croston, G. E., L. B. Milan, K. B. Marschke, M. Reichman, and M. R. Briggs. 1997. Androgen receptor-mediated antagonism of estrogen-dependent low density lipoprotein receptor transcription in cultured hepatocytes. *Endocrinology*. **138**: 3779–3786.
62. Brown, M. S., and J. L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science*. **232**: 34–47.
63. Adeli, K., and C. Sinkevitch. 1990. Secretion of apolipoprotein B in serum-free cultures of human hepatoma cell line, HepG2. *FEBS Lett.* **263**: 345–348.
64. Tam, S-P., T. K. Archer, and R. G. Deeley. 1986. Biphasic effects of estrogen on apolipoprotein synthesis in human hepatoma cells: Mechanism of antagonism by testosterone. *Proc. Natl. Acad. Sci. USA.* **83**: 3111–3115.
65. Angelin, B., H. Olivecrona, E. Reihner, M. Rudling, D. Stahlberg, M. Eriksson, S. Ewerth, P. Henriksson, and K. Einarsson. 1992. Hepatic cholesterol metabolism in estrogen-treated men. *Gastroenterology*. **103**: 1657–1663.
66. Erickson, S. K., S. Jaecle, S. R. Lear, S. M. Brady, and R. J. Havel. 1989. Regulation of hepatic cholesterol and lipoprotein metabolism in ethinyl estradiol-treated rats. *J. Lipid Res.* **30**: 1763–1771.