Molecular Structures of Citrus Flavonoids Determine Their Effects on Lipid Metabolism in HepG2 Cells by Primarily Suppressing ApoB Secretion

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ABSTRACT: This study investigated the underlying mechanisms of action for blood lipid lowering effects of citrus flavonoids and their methoxylated analogues ($n = 19$; dose range: $0-100\ \mu M$) in HepG2 cells. Cholesterol (CH) and triglyceride (TG) syntheses were assessed by measuring the incorporation of ^{14}C-acetate and ^{14}C-glycerol, respectively, whereas apoB secretion was determined by ELISA. Results show that two polymethoxylated citrus flavonoids (PMFs), tangeretin and nobiletin, potently inhibited apoB secretion ($IC_{50} = 13$ and $29\ \mu M$, respectively) and modestly inhibited CH synthesis ($IC_{50} = 49$ and $68\ \mu M$) and TG synthesis ($IC_{50} = 14$ and $73\ \mu M$), without effecting LDL-receptor activity. Other PMFs (e.g., sinensetin) and non-PMFs (e.g., hesperetin and naringenin) had only weak effects on CH and TG syntheses and apoB secretion ($IC_{50} > 100\ \mu M$). The structure−activity analysis indicated that a fully methoxylated A-ring of the flavonoid structure was associated with a potent inhibitory activity on hepatic apoB secretion. In conclusion, this study using HepG2 cells indicates that citrus flavonoids with a fully methoxylated A-ring may lower blood CH and TG concentrations primarily by suppressing hepatic apoB secretion as a main underlying mode of action.

KEYWORDS: HepG2 cells, cholesterol, triglycerides, polymethoxylated flavonoids (PMFs), tangeretin, nobiletin

■ INTRODUCTION

Elevated blood cholesterol (CH) and especially low-density lipoprotein-cholesterol (LDL-C) are established risk factors of cardiovascular diseases (CVD). Despite existing CH-lowering intervention strategies, reducing both blood CH and triglycerides (TG) concentrations via naturally derived ingredients as part of a dietary approach is regarded of importance in the prevention of CVD in the population.

Citrus fruits, including orange, grapefruit, lemon, lime, mandarin, and tangerine, are rich in flavonoids. Interest in citrus flavonoids has increased in recent years because of accumulating evidence suggesting that they may lower blood CH and TG, as was demonstrated in a number of animal studies using rabbits,[1] rats,[2] mice,[3] and hamsters.[4] However, from human studies conflicting data exist concerning the lipid-lowering effect of citrus flavonoids. Some human intervention studies showed that citrus flavonoids significantly reduced plasma CH and TG concentrations,[5,6] whereas other studies failed to confirm such effects.[7,8] The reasons for these conflicting data are unclear. Possibly differences in the activity of the various citrus flavonoids tested in these human studies may at least partly explain the discrepancy in study outcomes.

Hesperidin, naringin, tangeretin, and nobiletin are the most common citrus flavonoids. Hesperidin and naringin are rich in citrus juices, whereas tangeretin and nobiletin mainly exist in citrus peels. The sugar moieties of hesperidin and naringin can be hydrolyzed by intestinal enzymes (e.g., β-glucosidase)[9] or by the colonic microflora[10] to release corresponding aglycones, which are called hesperetin and naringenin, respectively. Only the aglycone forms of citrus flavonoids can be absorbed from the gut lumen.[11] Therefore, hesperetin and naringenin can be deemed as the bioactive forms of hesperidin and naringin in

the body. Citrus flavonoids, such as tangeretin and nobiletin, with more than two methoxy moieties are called polymethoxylated flavonoids (PMFs). In contrast to hesperidin and naringin, tangeretin and nobiletin exist only as aglycones because the binding sites for sugar moieties are not occupied by hydroxyl moieties. PMFs can be directly absorbed from the gut.[12]

So far, the underlying mechanisms of action for a lipid-lowering effect of citrus flavonoids have not yet been fully established. Several hypotheses have been proposed on the basis of data obtained from both in vitro and animal studies, although inconsistent and conflicting data exist. These hypotheses include (1) inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase[2] and acyl-CoA:cholesterol acyltransferase (ACAT) activity;[13] (2) reduction in activity and expression of microsomal TG transfer protein (MTP);[13] (3) suppression of apoB[14] and/or VLDL secretion;[15] (4) activation of fatty acid β-oxidation;[15] (5) increase in LDL-receptor mRNA expression;[16] and (6) activation of the mitogen-activated protein kinase pathway in the liver.[17] All of these hypotheses are linked to hepatic lipid metabolism. However, (1) the primary effect on hepatic lipid metabolism, (2) the key molecular structure responsible for the activity, and (3) the most active individual citrus flavonoid have not yet been intensively investigated or determined.

Therefore, the present study aimed to systematically investigate the effect of individual citrus flavonoids and PMFs on hepatic lipid metabolism and subsequently to identify the structure−activity relationship among 19 flavonoids including citrus

flavonoids, reported metabolites of citrus flavonoids in the body, and synthetic PMF analogues in cultured HepG2 cells. HepG2 cells have been chosen as the model because they have been shown to maintain a number of human liver functions, including lipid synthesis and secretion of albumin, apoB, and lipoproteins in response to various nutritional or physiological stimulations.

■ MATERIALS AND METHODS

Materials. Natural citrus flavonoids and their metabolites as well as (bio)synthetic PMF analogues[18] were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), AnalytiCon Discovery GmbH (Potsdam, Germany), Extrasynthese (Genay, France), and Gaia Chemical Corp. (Gaylordsville, CT) or were kindly provided by Dr. D. Buisson,[18] University of Paris-Sud, Paris, France. A PMF extract from peels of *Citrus nobilis* L. was provided by KGK Synergize (London, ON, Canada) and was produced by Chengdu Wagott Pharmaceutical Co., Ltd. (Sichuan, China), from which 5-hydroxy-6,7,8,3′,4′-pentamethoxy-flavone was isolated and identified via HPLC-MS methods adapted from previous publications (e.g., see ref 19). Dulbecco's modified Eagle's medium (DMEM, 4.5 g of glucose/L), fetal bovine serum (FBS), ViaLight Plus kit, and phosphate-buffered saline (PBS) were supplied by Lonza Benelux BV (Breda, The Netherlands). Penicillin/streptomycin (P/S) was ordered from Invitrogen (Breda, The Netherlands). 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate buffer II and Substrate Perox buffer II were provided by bioMérieux (Boxtel, The Netherlands). Acetic acid, acetonitrile, Tween 20, bovine serum albumin (BSA), Triton X-100, sulfuric acid, 5-(tetradecyloxy)-2-furoic acid (TOFA), mevalonic acid, and formaldehyde were supplied by Sigma-Aldrich (Zwijndrecht, The Netherlands). An enzyme-linked immunosorbent assay (ELISA) kit for determining ApoB was from Mabtech (Nacka Strand, Sweden). The ELISA kit for albumin measurements was from Bethyl Laboratories (Montgomery, TX). [1,1-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) labeled LDL (DiI-LDL) was purchased from Biomedical Technologies Inc. (Stoughton, MA). LDL-receptor antibody IgG-C7 was provided by Progen Biotechnik GmbH (Heidelberg, Germany), and the secondary antibody anti-mouse IgG conjugated horseradish peroxidase (IgG—HRP) was purchased from Bio-Rad Laboratories BV (Veenendaal, The Netherlands). ^{14}C-Acetate (2.07 GBq/mmol) and ^{14}C-glycerol (5.73 GBq/mmol) were obtained from Amersham PLC (Amersham, U.K.). TLC silica gel 60 (20 × 20 cm) plates were obtained from Merck, Darmstadt, Germany.

Cell Culture and Incubation with Test Ingredients. *(a) Routine Cell Culture.* HepG2 cells were obtained from the American Type Culture Collection and routinely cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

(b) Experimental Media. Citrus flavonoids were dissolved in dimethyl sulfoxide (DMSO) and stored as 50 mM stock solutions at −20 °C. Stock solutions were diluted with routine medium to form experimental media, which contained various flavonoid concentrations (0, 5, 10, 20, 50, and 100 μM). The final concentration of DMSO in the cell culture media, either with added flavonoids or as vehicle control, was maximally 1% (v/v), as it was demonstrated in our study (based on the ATP assay) that incubation with 1% DMSO for at least 24 h did not affect cell viability. Similarly, control compounds [TOFA (a fatty acid synthesis inhibitor), lovastatin, or mevalonic acid] were added to medium. Their concentrations in media were 20 μM, 3 μM, and 10 mM, respectively. Each experiment consisted of two to three (wells) replicates for each flavonoid dose and for the untreated control (medium alone) and positive controls.

Monitoring Cellular Viability. HepG2 cells were seeded in 96-well plates and cultured overnight using routine culture media. The routine culture media were replaced by experimental media, and the cells

were cultured for the period indicated under Results. Cellular viability was measured using the Lonza ViaLight Plus kit, which measures cellular ATP contents. Cells were cultured in 96-well plates and exposed to the experimental media for 24 h. Cells were lysed by adding 50 μL of ATP lysis buffer and were shaken for 15 min at 650 rpm. Ten microliters of cell lysate was pipetted into a 96-well plate, followed by 40 μL of ATP lysis buffer and 100 μL of Vialight reagent for each well. To quantify ATP, luminescence was measured on a 1450 Microbeta LSC Analyzer (Perkin-Elmer, Groningen, The Netherlands).

Determination of Lipid Synthesis. HepG2 cells were seeded in 24-well cell culture plates and cultured overnight using routine culture media. The routine culture media were replaced by experimental media, and the cells were cultured for the period indicated under Results. At 4 h before the end of the experimental incubation, 7 kBq of ^{14}C-acetate or 11 kBq of ^{14}C-glycerol was added to each well to trace CH synthesis or TG synthesis, respectively. At the end of the experiment, media were discarded and cells were washed with cold PBS and harvested for lipid synthesis analysis as described below. Radiolabeled cellular lipids were extracted with a mixture of hexane/isopropanol (ratio 3:2). The hexane/isopropanol was evaporated under N_2, and the lipids were redissolved in ethanol. Individual lipids (CH and TG) were separated with silica thin layer chromatography (TLC) using a developing solvent of petroleum ether/diethyl ether/acetic acid for 2 × 45 min. The spots on the TLC plate were made visible using a phosphor imaging plate in a cassette. After 24 h of exposure, the phosphor imaging plate was scanned on a Typhoon Imager (Amersham, UK) to semiquantify the radiolabeled CH and TG spots.

Determination of ApoB Secretion. HepG2 cells were seeded in 24-well cell culture plates and cultured overnight using routine culture media. The routine culture media were replaced by experimental media, and the cells were cultured for the period indicated under Results. The conditioned media were collected for apoB analyses. Adequate amounts of conditioned media were diluted (10×) with PBS containing 0.1% BSA and 0.05% Tween 20 and were added to an ELISA plate coated with the monoclonal antibody LDL-20/17 (1:500, 2 μg/mL in PBS) and incubated for 2 h. After washing, the plate was incubated for 1 h at room temperature with a biotinylated monoclonal antibody LDL-11 (1:1000, 1 μg/mL). After washing, incubation was performed using peroxidase-linked streptavidin (1:1000, for 1 h), followed by detection using TMB/peroxidase substrate. Absorbance was measured using a SpectraMax plate reader (450 nm) (Molecular Devices, Sunnyvale, CA).

Determination of Albumin Secretion. HepG2 cells were seeded in 24-well cell culture plates and cultured overnight using routine culture media. The routine culture media were replaced by experimental media, and the cells were cultured for the period indicated under Results. The conditioned media were collected for albumin analyses. Adequate amounts of the conditioned media were diluted (100×) with PBS containing 1% BSA and 0.05% Tween 20 and were added to an ELISA plate coated with goat anti-human albumin (1:100, 10 μg/mL) and incubated for 1 h. The incubation with the second peroxidase-labeled antibody (goat anti-human albumin HRP 1:150000, 1 h) was performed after washing. Finally, peroxidase activity was determined using the TMB/peroxidase substrate and was measured by a SpectraMax plate reader (450 nm) (Molecular Devices).

Determination of LDL-Receptor Activity. HepG2 cells were seeded in 96-well plates and cultured overnight using routine culture media. The routine culture media were replaced by experimental media, and the cells were cultured for the period indicated under Results. LDL-receptor activity of HepG2 cells was determined by measuring the uptake of fluorescently labeled DiI-LDL according to previously published methods described by Teupser et al.[20] Briefly, 4 h before the termination of cellular incubations, fluorescent DiI-LDL was added to the conditioned media (final concentration = 10 μg/mL) to monitor LDL uptake by the cells. At the end of the experimental incubation,

media were removed and cells were washed three times with PBS (pH 7.4) to remove the unbound DiI-LDL. Both internalized and cellularly bound DiI-LDL were measured by using a fluorescence plate reader (ex, 540 nm; em, 575 nm) (Flexstation II 384, Molecular Devices).

Determination of LDL-Receptor Protein Abundance. HepG2 cells were seeded in 96-well plates and cultured overnight using routine culture media. The routine culture media were replaced by experimental media, and the cells were cultured for the period indicated under Results. LDL-receptor protein abundance on the cell surface was detected with a cell-enzyme-linked immunosorbent assay (cell-ELISA) by using antibody IgG-C7 against the human LDL-receptor protein.[21] After experimental incubation, cells were washed with PBS and fixed with formaldehyde (3.7% in PBS) for 15 min at room temperature. After washing, the fixed cells were blocked with 2% fat-free milk powder plus 1 mM $CaCl_2$. After blocking and washing, cells were incubated for 2.5 h at room temperature with the primary antibody 1:200 (IgG-C7, Progen 61087) in PBS/milk powder plus 1 mM $CaCl_2$. The incubation with the second peroxidase-labeled antibody (anti-mouse IgG—HRP, 1:5.000, room temperature, for 1.5 h) was performed after washing. Finally, the amount of peroxidase present with the second antibody was detected using a TMB/peroxidase substrate and measured on a dedicated plate reader at 450 nm (SpectraMax, Molecular Devices).

Statistical Analysis. IC_{50} values for apoB and albumin secretion and synthesis of CH and TG were calculated by using GraphPad Prism software. Data (Tables 1 and 2) are presented as the mean and range of independent replicated experiments. Neither parametric (such as ANOVA) nor nonparametric tests (e.g., Kruskal—Wallis test) generated credible p values for comparison of mean IC_{50} values between treatment groups for these reasons: the low number of independent observations ($n \leq 3$) for each treatment group allowed no reliable estimate of variance for a parametric test; furthermore, no exact IC_{50} values ($>100 \mu$M) were available for some groups for comparison, and applying correction for multiple comparisons among 19 different treatment groups (Table 2) would lead to less credible p values. Alternatively, differences of IC_{50} values between treatment groups were considered significant when their IC_{50} "ranges" did not overlap.

Data of LDL-receptor activity and abundance (Figure 1) are presented as the mean ± SD of independent replicated experiments. Data (Figure 1) were analyzed using ANOVA. Statistical differences between the treatment groups and control were calculated using a Dunnett multiple-comparison test. Differences from untreated control were considered significant at $p < 0.05$ (2-sided).

■ RESULTS

Viability of HepG2 Cells Exposed to Flavonoids. Cellular ATP is a sensitive parameter for cytotoxicity. In the present study, cell viability was monitored by measuring cellular ATP contents when cells were exposed to the tested flavonoids (up to 100 μM for 24 h). With all study conditions, cellular ATP contents were in the range of 95—128% compared to control cells (without flavonoids) (data not shown), indicating that cell viability was not affected by citrus flavonoids within the concentration range studied in these experiments.

Modulation of Hepatic Lipid Metabolism by Citrus Flavonoids. To validate the HepG2 cell model, the positive controls lovastatin (HMG-CoA reductase inhibitor) and TOFA (acetyl-coenzyme A carboxylase inhibitor) were used in these experiments, which are well established compounds to modulate lipid metabolism in vivo and/or in vitro.[22,23] The half-maximal inhibitory concentrations (IC_{50}) of lovastatin, TOFA, and the individual citrus flavonoids on CH and TG synthesis and apoB secretion are presented in Table 1. As expected, lovastatin potently inhibited CH synthesis ($IC_{50} = 1.4 \mu$M). However, lovastatin did not notably suppress apoB secretion [IC_{50} values could not be calculated up to the highest dose (3 μM) used in this
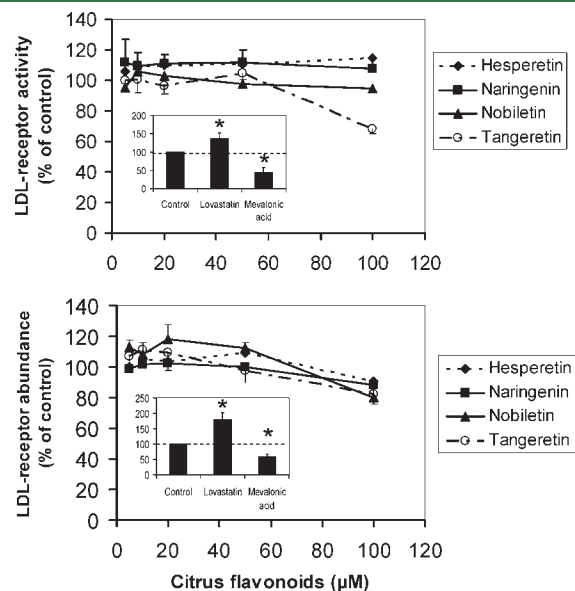

Figure 1. Effects of citrus flavonoids, lovastatin, and mevalonic acid (insets) on LDL-receptor activity (top) and LDL-receptor abundance (bottom). HepG2 cells were incubated using the indicated concentrations of flavonoids or lovastatin (3 μM) or mevalonic acid (10 mM) for 24 h. LDL-receptor activity of HepG2 cells was determined by measuring the uptake of fluorescently labeled DiI-LDL. LDL-receptor protein abundance on the cell surface was detected with a cellular ELISA. Data are presented as the mean ± SD of at least three independent experiments (n). In each experiment, there were two or three (wells) replicates for each treatment. For clarity purposes, SD values of hesperetin and tangeretin curves are not shown, which are within 10% of the corresponding mean values. In the inset bar charts, * indicates statistical significance ($p < 0.01$) compared to control treatment (100%).

Table 1. Inhibitory Effect (IC_{50}, μM) of the Tested Flavonoids and Positive Controls on Cellular Lipid Synthesis and ApoB Secretion[a]

	radiolabeled lipid synthesis		
	^{14}C-CH	^{14}C-TG	apoB secretion
tangeretin	49 (36—80)	14 (10—17)	13 (11—14)
nobiletin	68 (28—100)	73 (51—100)	29 (28—30)
hesperetin	>100	>100	>100
naringenin	>100	>100	>100
lovastatin	1.4 (0.5—2.2)	nd	>3
TOFA	nd	1.3 (0.7—1.9)	7.8 (7.3—8.2)

[a] Cells were cultured using different concentrations (0—100 μM) of individual citrus flavonoids for 24 h (for cholesterol synthesis and apoB secretion assays) or 4.5 h (triglyceride synthesis assay). ^{14}C-Acetate or ^{14}C-glycerol was added to each corresponding well at 4 h before the end of the experiments to monitor cholesterol (CH) and triglyceride (TG) synthesis, respectively. Data are presented as the mean and range (in parentheses) of three independent experiments ($n = 3$). In each experiment, there were two or three (wells) replicates for each treatment. IC_{50} values > 100 μM (the highest dose used in this study) are presented as >100. nd, not determined.

Table 2. Structures of Citrus Flavonoids, Their Metabolites, and Synthetic Analogues versus Their Inhibitory Effects (IC$_{50}$) on Secretion of ApoB and Albumin by HepG2 Cells[a]

no.	name	B-ring C3'	C-ring C4'	A-ring C3	C5	C6	C7	C8	apoB	albumin
Citrus flavonoids[19]										
1	sinensetin[1]	−OCH$_3$	−OCH$_3$		−OCH$_3$	−OCH$_3$	−OCH$_3$		>100	>100
2	tangeretin[2]		−OCH$_3$		−OCH$_3$	−OCH$_3$	−OCH$_3$	−OCH$_3$	13 (11−14)	73 (55−100)
3	nobiletin[2]	−OCH$_3$	−OCH$_3$		−OCH$_3$	−OCH$_3$	−OCH$_3$	−OCH$_3$	29 (28−30)	>100
4	naringenin[2]		−OH		−OH		−OH		>100	>100
5	hesperetin[2]	−OH	−OCH$_3$		−OH		−OH		>100	>100
6	3,5,6,7,8,3',4'-heptamethoxyflavone[3]	−OCH$_3$	−OCH$_3$	−OCH$_3$	−OCH$_3$	−OCH$_3$	−OCH$_3$	−OCH$_3$	29 (26−32)	51 (42−60)
7	5-hydroxy-6,7,8,3',4'-pentamethoxyflavone[4]	−OCH$_3$	−OCH$_3$		−OH	−OCH$_3$	−OCH$_3$	−OCH$_3$	>100	>100
Other PMFs (*non-citrus/synthetic*)										
8	5,8,4'-trihydroxy-6,7,3'-trimethoxyflavone[3]	−OCH$_3$	−OH		−OH	−OCH$_3$	−OCH$_3$	−OH	>100	>100
9	5,6,7,3'-tetramethoxyflavone[3]	−OCH$_3$			−OCH$_3$	−OCH$_3$	−OCH$_3$		62 (59−65)	>100
10	3'-hydroxy-5,6,7-trimethoxyflavanone[3]	−OH			−OCH$_3$	−OCH$_3$	−OCH$_3$		>100	>100
11	3,5,6,7,8,5'-hexamethoxy-3',4'-methylenedioxyflavone[3]	−CH$_2$−O−CH$_2$−		−OCH$_3$	−OCH$_3$	−OCH$_3$	−OCH$_3$	−OCH$_3$	20 (16−24)	>100
12	5,7-dihydroxy-3'-methoxyflavanone[3]	−OCH$_3$			−OH		−OH		>100	>100
13	5,7-dihydroxy-8-methoxyflavone[3]				−OH		−OH	−OCH$_3$	>100	>100
14	5,6,7,3'-tetramethoxyflavanone[3]	−OCH$_3$			−OCH$_3$	−OCH$_3$	−OCH$_3$		>100	>100
15	5,7-dihydroxy-6,8,3'-trimethoxyflavone[3]	−OCH$_3$			−OH	−OCH$_3$	−OH	−OCH$_3$	>100	>100
16	5-hydroxy-6,7,8,4'-tetramethoxyflav one[5]		−OCH$_3$		−OH	−OCH$_3$	−OCH$_3$	−OCH$_3$	>100	>100
17	3,4'-dihydroxy-5,6,7,8-tetramethoxyflavone[6]		−OH	−OH	−OCH$_3$	−OCH$_3$	−OCH$_3$	−OCH$_3$	39 (28−50)	59 (56−61)
PMF metabolite [12,39]										
18	4'-hydroxy-5,6,7,8,3'-pentamethoxyflavone[6]	−OCH$_3$	−OH		−OCH$_3$	−OCH$_3$	−OCH$_3$	−OCH$_3$	60 (53−66)	63 (54−69)
19	4'-hydroxy-5,6,7,8,-tetramethoxyflavone[6]		−OH		−OCH$_3$	−OCH$_3$	−OCH$_3$	−OCH$_3$	43 (36−49)	>100

[a] HepG2 cells were incubated in duplicate with the indicated flavonoids for 24 h at different concentrations (0−100 μM). ApoB and albumin contents of the conditioned media were determined by ELISA. Data are presented as the mean and range of at least two independent experiments. In each experiment, there were two or three (wells) replicates for each flavonoid dose. [b] The individual flavonoids were obtained from [1] Extrasynthese; [2] Sigma-Aldrich; [3] Analyticon; [4] in-house isolation as described under Materials; [5] Gaia Chemicals; and [6] University of Paris-Sud. [c] IC$_{50}$ values > 100 μM (the highest dose used in the study) are presented as >100.

study]. Similarly, TOFA potently inhibited TG synthesis (IC$_{50}$ = 1.3 μM). Nevertheless, the IC$_{50}$ value of TOFA for inhibition of apoB secretion was 7.8 μM, which was 6 times higher than the IC$_{50}$ (1.3 μM) for inhibiting TG synthesis. These data indicate that lovastatin and TOFA more potently inhibited cellular lipid synthesis than affected apoB secretion.

In contrast, tangeretin and nobiletin showed stronger effects on the inhibition of apoB secretion (IC$_{50}$ = 13 and 29 μM, respectively) than on inhibiting hepatic lipid synthesis, as indicated by the incorporation of ^{14}C-acetate into CH (IC$_{50}$ = 49 and 68 μM, respectively) and ^{14}C-glycerol into TG (C$_{50}$ = 14 and 73 μM, respectively). Hesperetin and naringenin had only weak effects on lipid synthesis and apoB secretion in HepG2 cells, with IC$_{50}$ values >100 μM (highest dose used in this study) for each parameter.

To gain more insights into the mechanisms of action of the four citrus flavonoids (tangeretin, nobiletin, hesperetin, and naringenin) on hepatic lipid metabolism, we further studied their effects on LDL-receptor abundance and activity (Figure 1). Lovastatin and mevalonate are well established to increase or decrease LDL-receptor activity/abundance, respectively.[22,24] As expected, in this study lovastatin increased LDL-receptor abundance and activity by 79 and 40% (both $p < 0.01$), whereas mevalonate reduced LDL-receptor abundance and activity by 55 and 40% (both $p < 0.01$), respectively, compared to control
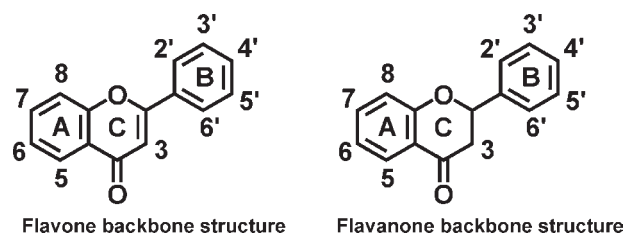

Figure 2. Backbone structures of citrus flavonoids (flavone and flavanone) and their synthetic analogues. The detailed substituent groups at the A-, B-, and C-rings of the tested individual flavonoids are indicated in Table 2.

treatment (inset panels in Figure 1), indicating that in the present study HepG2 cell LDL-receptor activity and abundance were modulated in response to cellular contents and synthesis of CH.

Both hesperetin and naringenin at the whole dose range of 5−100 μM slightly (7−12%) increased LDL-receptor activity, compared to control (0 μM) treatment ($p < 0.05$). However, such effects of hesperetin and naringenin were not in a dose-dependent manner. In addition, LDL-receptor abundance was not increased by either hesperetin or naringenin at any of the tested concentrations (5−100 μM). In contrast, nobiletin (5−100 μM) did not increase LDL-receptor activity, whereas

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it increased LDL-receptor abundance by 8–18% ($p < 0.05$) at the dose range of 5–50 μM. Tangeretin increased neither LDL-receptor activity nor LDL-receptor abundance. Furthermore, at high dose (100 μM) all four of these citrus flavonoids significantly ($p < 0.05$) lowered LDL-receptor abundance by 10–20%, whereas (except for tangeretin) such an effect was not associated with a parallel reduction of LDL-receptor activity. Therefore, taking these data together, (1) the lack of a dose-dependent increase in LDL-receptor activity and (2) the discrepancy in the effects of the four citrus flavonoids on LDL-receptor activity and abundance clearly indicate that they had virtually no effect on LDL-receptor activity/abundance.

Structure–Activity Relationship Analysis of Citrus Flavonoids and Their Analogues. As indicated by the data described in Table 1, the most notable effect of tangeretin and nobiletin was the suppression of apoB secretion by HepG2 cells, compared to their effects on CH and TG synthesis. To identify a structure–activity relationship for the apoB inhibitory activity, we compared in total 19 (natural and synthetic) flavonoids (Table 2) for their inhibitory activity on apoB secretion by HepG2 cells. These flavonoids differ in their number of substituting groups and their locations in the three rings of the flavone and flavanone basic structures as indicated in Figure 2. Except for naringenin and hesperetin, these flavonoids are all PMFs. Flavonoids with substituent groups other than hydrogen at C2′, C5′, or C6′ positions on the B-ring rarely exist in nature and are not commercially available. Therefore, these rare flavonoids were not included in the present study.

Substitutent Groups at the A-Ring. Seven flavonoids (tangeretin, nobiletin, and flavonoids **6**, **11**, and **17–19** in Table 2) with remarkable apoB inhibitory activities (IC$_{50}$ from 13 to 60 μM) have methoxy moieties ($-OCH_3$) at all C5, C6, C7, and C8 positions on the A-ring. Eliminating methoxy moieties ($-OCH_3$) at the C8 position [sinensetin and flavonoids **9**, **10**, **12**, and **14** or replacing C8 methoxy ($-OCH_3$) by hydroxyl ($-OH$) (flavonoid **8**)] significantly reduced their apoB inhibitory activity (IC$_{50}$> 62 μM). In addition, for naringenin, hesperetin, and flavonoids **12** and **13**, which have no methoxy moieties ($-OCH_3$) at C5, C6, C7, or C8 on the A-ring, IC$_{50}$ values of apoB inhibition were >100 μM. These data indicate that full methoxylation of the A-ring is required to exhibit a potent inhibitory effect on apoB secretion.

Substituent Groups at the B-Ring. Substituent groups at the B-ring of citrus flavonoids do not seem to play an important role in determining their apoB inhibitory activity because no consistent structure–activity relationship was observed concerning moieties at the C3′ and C4′ positions on the B-ring with regard to apoB secretion.

Substituent Groups at the C-Ring. The C-ring has only one position (C3) for a substituent group. Among flavonoids with full methoxylation of the A-ring, addition of a methoxy or hydroxy group on the C3 position (flavonoids **6**, **11**, or **17**, IC$_{50}$ - 20–39 μM) of the C-ring did not significantly increase or decrease the inhibitory activity compared to flavonoids possessing no methoxy or hydroxy group at this position (e.g., tangeretin, nobiletin, and flavonoids **18** and **19**, IC$_{50}$ = 13–60 μM).

Besides apoB, the liver also synthesizes and secretes most plasma proteins, including albumin.[25] The hepatic secretion of albumin is via a process that is different from apoB secretion. The latter can be secreted only when it is assembled as lipoproteins. Therefore, albumin was used as secretion control to study the specificity of the tested flavonoids on the inhibition of apoB secretion. The results (Table 2) revealed that the majority of the tested flavonoids do not affect albumin secretion (IC$_{50}$ > 100 μM), except tangeretin and flavonoids **6**, **17**, and **18**, which moderately reduced albumin secretion (IC$_{50}$ = 49–73 μM). Nobiletin inhibited apoB secretion without virtually inhibiting albumin secretion (IC$_{50}$ > 100 μM). Tangeretin had a much weaker effect on albumin secretion (IC$_{50}$ = 73 μM) compared to its apoB inhibitory activity (IC$_{50}$ = 13 μM). These data indicate that the inhibitory effect of the flavonoids on apoB secretion is (highly) selective.

■ DISCUSSION

ApoB Secretion Inhibitory Effect of Citrus Flavonoids and It Implications. Hepatic secretion of apoB-containing lipoproteins (i.e., VLDL) plays an important role in lipid homeostasis in the body. Overproduction of hepatic VLDL has been recognized to be linked to hyperlipidemia.[26] Because each VLDL particle contains only one apoB molecule, the secretion of hepatic apoB directly reflects the production of VLDL by the liver. Inhibiting hepatic apoB secretion has been demonstrated to lower blood CH and TG in humans.[27]

The present set of experiments demonstrated that the four citrus flavonoids tangeretin, nobiletin, hesperetin, and naringenin have different potencies in modulating hepatic lipid metabolism, especially in inhibiting hepatic apoB secretion. Tangeretin and nobiletin inhibited apoB secretion with IC$_{50}$ values of 13 and 29 μM, respectively. The IC$_{50}$ values for naringenin and hesperetin were >100 μM in the present study, which are in agreement with those reported by Kurowska et al.[28] showing IC$_{50}$ values for naringenin and hesperetin in HepG2 cells being 178 and 142 μM, respectively. However, some other studies, for example, by Wilcox et al.,[13] showed that the IC$_{50}$ values for naringenin and hesperetin on inhibiting apoB secretion by HepG2 cells were about 75 μM. The reasons for these discrepancies in the reported IC$_{50}$ values of naringenin and hesperetin in HepG2 cells are unclear, but are possibly due to differences in study conditions used among these reported studies.

Plasma peak concentrations of naringenin and hesperetin have been reported to be up to 6 and 2.2 μM, respectively, after ingestion of 400–720 mL of grapefruit juice (providing 199 mg of naringenin) or 400–760 mL of orange juice (providing 126 mg of hesperetin).[29] The IC$_{50}$ values for naringenin and hesperetin in the present study and other reported studies are in the range of 75–178 μM, which are much higher than human blood concentrations (2.2–6 μM) after the intake of 400–760 mL of citrus juices. Recently, a randomized, placebo-controlled, double-blind study, in which large doses of naringin (500 mg/day) or hesperidin (800 mg/day) were ingested, showed that the glycoside forms of naringenin and hesperetin did not (even had no trend to) change blood lipoprotein profiles compared to placebo control, indicating that dietary naringenin and hesperetin may have no blood lipid-lowering effects in humans.[7] In a clinical study that was not placebo controlled, administration of 500 mg/day for 6 weeks of glucosylhesperidin (a synthetic hesperidin derivative, which is water-soluble compared to conventional hesperidin) lowered plasma TG concentrations in hypertriglyceridemic (TG > 150 mg/dL) subjects without affecting blood concentrations of apoB and CH.[30] Glucosylhesperidin had, however, no TG-lowering effect in subjects with normal blood TG concentrations.[30] However, using a LDL receptor-null mouse model, Mulvihill et al.[15] showed that diets supplemented

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with 3% naringenin could reduce apoB100 production by 36%, indicating that this mouse model is more sensitive than human subjects in response to dietary intake of naringenin.

Plasma concentrations of tangeretin and nobiletin in humans have not been reported. We assume that similar plasma peak concentrations ($2.2-6 \mu M$ as for hesperetin and naringin) of tangeretin and nobiletin might be achieved after oral intake of corresponding amounts of these two PMFs in humans. Recently, a study in rats administered tangeretin or nobiletin by gavage at a high dose (50 mg/kg body weight, dissolved in corn oil) showed that plasma peak concentrations of these two flavonoids were 1.4 and $22 \mu M$, respectively.[31] Based on our in vitro data showing relatively low IC_{50} values ($13 \mu M$ and $29 \mu M$) for apoB secretion inhibition, this may suggest that tangeretin and nobiletin may have the potential to lower plasma CH and TG in humans. Accordingly, a reported clinical study showed that a mixture (270 mg/day) of tangeretin and nobiletin together with 30 mg/day of tocotrienols lowered plasma CH and TG concentrations.[6] Further clinical studies are, however, required to confirm the optimal dose range at which tangeretin and/or nobiletin alone (without tocotrienols) have blood lipid lowering activities in humans.

Mechanisms of Action of Inhibition of ApoB Secretion of Citrus Flavonoids. Suppressing apoB secretion is the most predominant lipid-related effect of tangeretin and nobiletin, as shown in this study. Although tangeretin and nobiletin moderately inhibited cellular TG and CH synthesis, the apoB secretion inhibitory effect cannot be attributed to such lipid synthesis inhibitory effects on the basis of a comparison of the mode of action of tangeretin and nobiletin with those of TOFA and lovastatin.

First, TOFA inhibited TG synthesis by 50% at a dose of 1.3 μM (Table 1). This dose had only a minor (5%, detailed data not shown) inhibitory effect on apoB secretion. These data indicate that inhibiting cellular TG synthesis by 50% had virtually no potent effect on apoB secretion. When this is taken into account, the observed apoB secretion inhibitory effects of tangeretin and nobiletin (IC_{50} = 13 and 29 μM, respectively) cannot be explained by their moderate or weak inhibitory effects on TG synthesis (IC_{50} = $14 \mu M$ and $73 \mu M$, respectively) (Table 1). In addition, the literature-reported apoB secretion inhibition of citrus flavonoids (naringenin and hesperetin) was even associated with an increased cellular TG synthesis.[13,32] Taken together, the apoB secretion inhibitory effect of citrus flavonoids cannot be or at least not only be attributed to their TG synthesis inhibitory effect.

Second, lovastatin more potently inhibited CH synthesis than apoB secretion (Table 1). At 3 μM, lovastatin inhibited CH synthesis by 60%, whereas apoB secretion was only modestly (by 17%) inhibited (detailed data not shown). These data are in agreement with literature reports showing that inhibiting HMG-CoA reductase activity was not associated with a decreased apoB secretion.[33] All of these data together indicate that hepatic apoB secretion seems to be less sensitive to the availability of hepatic CH content. Tangeretin and nobiletin inhibited CH synthesis to a much lesser than lovastatin (Table 1), indicating that the apoB secretion inhibitory effect of tangeretin and nobiletin cannot be or only partly be attributed to their modest inhibitory effect on CH synthesis.

Third, up-regulation of LDL-receptor activity and abundance was associated with a decreased cellular CH synthesis and content (mass) as demonstrated with lovastatin (Figure 1) in

this and a previously reported study.[22] Tangeretin and nobiletin did not affect LDL-receptor activity and abundance (Figure 1), indicating that they might not potently reduce cellular CH mass. This concept is supported by the observations of Wilcox et al. that the citrus flavonoids naringenin and hesperetin at concentrations up to 200 μM did not significantly change cellular total CH (free CH plus CH esters) mass, although apoB secretion was decreased.[13] Morin et al.[16] demonstrated in HepG2 cells that nobiletin (1−200 μM) did not significantly increase LDL-receptor mRNA compared to control treatment, whereas hesperetin increased LDL-receptor mRNA only at high doses (75−200 μM), although both nobiletin and hesperetin were shown to increase the transcriptional activity of the LDL-receptor gene. Similarly, naringenin was shown to increase LDL-receptor mRNA by approximately 70% in Hep G2 cells only at a very high dose (200 μM).[14] Taken together, no studies have so far demonstrated that any citrus flavonoids at doses of $\leq 30 \mu M$ (at which tangeretin and nobiletin actively inhibited apoB secretion in this study) could increase LDL-receptor abundance or its mRNA.

Inhibition of MTP leads to decreased apoB secretion both in vitro[34] and in vivo.[27] An accumulating number of in vitro studies have demonstrated that a number of flavonoids, such as taxifolin,[35] quercetin,[36] naringenin,[13] hesperetin,[13] and tangeretin,[37] as well as isoflavones (genistein and daidzein)[38] have MTP inhibitory activities. Interestingly, flavonoids have a molecular structure similar to that of a synthetic MTP inhibitor, CP-10447 (4-bromomethaqualone),[34] which suggests a relationship between the molecular structures of flavonoids and MTP inhibiting activity. When all of these data are taken together, tangeretin and nobiletin might lower apoB secretion by primarily inhibiting MTP.

Suppressed hepatic apoB secretion was commonly associated with an accumulation of CH and TG in the liver in both animals and humans.[27] Similarly, tangeretin and nobiletin have been shown to lead to hepatic accumulation of CH (esters) in hamsters.[4] Several studies have demonstrated that in HepG2 cells exposed to high doses of naringenin and hesperetin (75−200 μM), the decrease of apoB secretion was associated with an increase of cellular mass of both TG and CH.[13,32] The accumulation of hepatic CH and TG would inhibit the biosynthesis of CH and TG without affecting LDL-receptor activity and abundance, as observed in the present study, via the well-established negative feedback mechanisms in the liver.

Structure−Activity Relationship Analysis of Citrus Flavonoids. The present study compared the effects on apoB secretion by HepG2 cells among 19 flavonoids with different molecular structures, which allowed us to observe possible structure−activity relationships. Special attention was paid to the number and position of methoxy and hydroxy groups on the A-, B-, and C-rings of the citrus flavonoid basic structure. Previously, it was claimed that PMFs lowered blood lipids.[4] The present study demonstrated that only those PMFs with a fully methoxylated A-ring (i.e., at C5, C6, C7, and C8, see Figure 2) (e.g., tangeretin and nobiletin) had a potent apoB inhibitory effect. Substituting groups at the C-3′ and/or C-4′ position of the B- or C-ring does not have a strong impact on the inhibitory effect of apoB secretion. This concept generates insights to understand why other PMFs (e.g., sinensetin and flavonoids **1**, **12**, and **13**) and non-PMFs (naringenin and hesperetin) expressed only a weak inhibitory activity. In mammals, a proportion of the absorbed PMFs is subjected to metabolism mainly by demethylation at the C-3′ and/or C-4′ position on the B-ring of the flavonoid

molecules.[12,39] The present study indicates that metabolites of nobiletin and tangeretin (flavonoids **18** and **19**, respectively)[12,39] keep (to some extent) the inhibitory activity ($IC_{50} = 39-60 \mu M$) of their parent citrus flavonoids on apoB secretion (Table 2).

In conclusion, this study using HepG2 cells indicates that full methoxylation of the A-ring of citrus flavonoids appears to be the most optimal structure to express potent effects on modulating hepatic lipid metabolism via primarily suppressing apoB-containing lipoprotein secretion. Tangeretin and nobiletin, which have the most optimal molecule structure, may lower blood CH and TG concentrations, whereas other citrus flavonoids without a fully methoxylated A-ring may have virtually no or only weak lipid-lowering effects in humans.

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■ DISCLOSURE

Part of this study was presented as a poster at the Experimental Biology 2010 meeting in Anaheim, CA. The abstract can be found at http://www.fasebj.org/cgi/content/meeting_abstract/24/1_MeetingAbstracts/541.16.

■ ABBREVIATIONS USED

ACAT, acyl-CoA:cholesterol acyltransferase; CH, cholesterol; CVD, cardiovascular diseases; DiI, 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; IC_{50}, half-maximal inhibitory concentration; IgG—HRP, horseradish peroxidase conjugated IgG; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LDL, low-density lipoprotein; MTP, microsomal triglyceride transfer protein; PBS, phosphate-buffered saline; PMFs, polymethoxylated citrus flavonoids; TG, triglycerides; TLC, thin layer chromatography; TMB, 3,3′,5,5′-tetramethylbenzidine; TOFA, 5-(tetradecyloxy)-2-furoic acid; VLDL, very low density lipoprotein.

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