Hepatocyte ApoB-Containing Lipoprotein Secretion Is Decreased by the Grapefruit Flavonoid, Naringenin, via Inhibition of MTP-Mediated Microsomal Triglyceride Accumulation†

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ABSTRACT: Naringenin, the principal flavonoid in grapefruit, reduces plasma lipids in vivo and inhibits apoB secretion, cholesterol esterification, and MTP activity in HepG2 human hepatoma cells. Although naringenin inhibits ACAT, we recently demonstrated that CE availability in the microsomal lumen does not regulate apoB secretion in HepG2 cells. We therefore hypothesized that inhibition of TG accumulation in the ER lumen, secondary to MTP inhibition, is the primary mechanism whereby naringenin blocks lipidation and subsequent secretion of apoB. Multicompartmental modeling of pulse-chase studies was used to compare cellular apoB kinetics in the presence of either naringenin or the specific MTP inhibitor, BMS-197636. At concentrations that reduced apoB secretion by 50%, both compounds selectively enhanced degradation via a kinetically defined, rapid, proteasomal pathway to the same extent. Subcellular fractionation experiments revealed that naringenin and BMS-197636 reduced accumulation of newly synthesized TG in the microsomal lumen by 48% and 54%, respectively. Newly synthesized CE accumulation in the lumen was reduced by 80% and 33% with naringenin and BMS-197636, respectively, demonstrating for the first time that MTP is involved in CE accumulation in the microsomal lumen. Reduced TG availability at this initial site of lipoprotein assembly was associated with significant reductions in the secretion of apoB-containing lipoproteins. Both naringenin and BMS-197636 were most effective in reducing secretion of IDL and LDL, but also inhibited secretion of apoB-containing HDL-sized particles. Furthermore, in McA-RH7777-derived cell lines, naringenin reduced secretion of *h*apoB72 and *h*apoB100, which require significant assembly with lipid to be secreted, but did not reduce secretion of *h*apoB17, *h*apoB23, and *h*apoB48, which require only minimal lipidation. Taken together, our results indicate that naringenin inhibits the lipidation and subsequent secretion of apoB-containing lipoproteins primarily by limiting the accumulation of TG in the ER lumen, secondary to MTP inhibition.

Apolipoprotein B100 (apoB)[1] is the 550kDa hydrophobic molecule that provides the structural framework for the assembly of very low-density lipoproteins (VLDL). The synthesis and assembly of apoB-containing lipoproteins (apoB-Lp) in the liver is a complex and highly regulated process. ApoB has a pentapartite structure comprising three α-helical domains (α_1, α_2, and α_3) that alternate with two amphipathic β-sheet domains (β_1 and β_2) (reviewed in ref *1*). For secretion to occur, apoB must cotranslationally associate with lipids in the endoplasmic reticulum (ER) (*1*−*3*). Initially, the globular α_1 domain acquires a small amount of phospholipid, triglyceride (TG), and cholesteryl ester (CE). As the remaining α-helical and β-strand domains are translated/translocated into the ER, additional lipid is recruited. The primordial lipoprotein later acquires the majority of its neutral lipid core in the smooth ER, post-ER, and Golgi compartments (*3*−*7*). The sequential lipidation of apoB requires enzymes to synthesize lipids (reviewed in ref *2*) and a chaperone to mediate both the accumulation of these lipids in the ER lumen (*8*), as well as their transfer to apoB (reviewed in ref *9*). CE and TG are synthesized by acyl CoA: cholesterol acyltransferase (ACAT) and diacylglycerol acyltranserfase (DGAT), respectively (*10*, *11*). Recent studies have described two forms of ACAT, ACAT1 and ACAT2 (reviewed in refs *10* and *12*−*16*), which are predicted to esterify cholesterol for cytosolic storage and lipoprotein assembly, respectively (*15*, *17*, *18*). Knockout studies in mice have suggested that there may also be more than one form of DGAT (reviewed in ref *11*). Lipids are transferred to apoB by the microsomal triglyceride transfer protein (MTP), an

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[1] Abbreviations: apo, apolipoprotein; apoB-Lp, apoB-containing lipoprotein; MTP, microsomal triglyceride transfer protein; ER, endoplasmic reticulum; CE, cholesteryl ester; TG, triglyceride; DGAT, diacylglycerol acyltransferase; ALLN, *N*-acetyl-leucinyl-leucinyl-norleucinal; PDI, protein disulfide isomerase; MEM, minimum essential medium; DMEM, Dulbecco's modified Eagle's medium; LPDS, lipoprotein deficient serum.

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ER- and Golgi-localized (*19*, *20*) heterodimer consisting of protein disulfide isomerase (PDI) and a unique 97 kDa subunit. MTP activity is an absolute requirement for lipoprotein assembly and secretion (reviewed in refs *9* and *21*−*23*). According to studies in McArdle-RH7777 (McA-RH7777) rat hepatoma cells transfected with human cDNAs encoding various lengths of apoB, secretion of apoB molecules corresponding to at least 53% of the full-length protein is highly sensitive to inhibition of MTP activity, while secretion of smaller forms of apoB is unaffected (*24*). Recently, Wang et al. (*8*) demonstrated that MTP activity is also required for the accumulation of TG, but not phosphatidylcholine, within the ER lumen. Whether MTP is required for the accumulation of CE within the ER has not been determined.

In the liver, insufficiently lipidated or misfolded apoB is targeted for degradation (reviewed in ref *25*). Several reports have demonstrated that, in HepG2 cells, only 20% of newly synthesized apoB is secreted (reviewed in refs *1* and *26*). The majority of apoB degradation is believed to occur cotranslationally via the ubiquitin-26S cytosolic proteasome pathway (*27*−*31*); however, degradation also occurs in the ER lumen and post-ER compartment (*25*, *32*). We have previously shown, using pulse-chase studies combined with a mulitcompartmental modeling approach, that in HepG2 cells, greater than 90% of newly synthesized apoB is degraded and that the vast majority of this degradation occurs via a kinetically defined rapid pathway (*33*−*35*). Furthermore, we demonstrated that this rapid pathway is ALLN and lactacystin-sensitive and thus represents mainly proteasomal degradation (*35*).

Naringenin and hesperetin, found predominantly in grapefruit and oranges, have been shown to reduce plasma lipids in rodent models (reviewed in ref *36*) and, more recently, to reduce atherosclerosis (*37*). These hypocholesterolemic effects have been associated with reduced hepatic HMG-CoA reductase and ACAT activities (*38*). We recently reported that the citrus flavonoids, naringenin and hesperetin, reduce basal apoB secretion (*39*, *40*) and that naringenin inhibits newly synthesized apoB secretion in oleate-stimulated HepG2 cells with a selective increase in degradation via the rapid kinetic pathway described above (*35*). We also demonstrated that, in HepG2 cells, the flavonoid-induced reduction in basal apoB secretion was associated with reduced ACAT and MTP activities, as well as decreased expression of ACAT2 and MTP (*40*). Furthermore, naringenin inhibits accumulation of both TG and CE in the microsomal lumen (*35*). However, by comparing naringenin to specific inhibitors of ACAT and HMG CoA reductase, we concluded that newly synthesized CE within the microsomal lumen does not regulate apoB secretion in HepG2 cells (*35*).

Therefore, in this report, we assessed the contribution of MTP inhibition to the naringenin-induced decrease in apoB secretion by comparing the impact of naringenin with the specific MTP inhibitor BMS-197636 on (i) the kinetics of apoB secretion and degradation using multicompartmental modeling, (ii) the availability of newly synthesized lipids within the ER, and (iii) the density distribution of secreted apoB100-Lp by HepG2 cells. Furthermore, in McA-RH7777 cells, we assessed the ability of naringenin to inhibit the secretion of human apoB C-terminal truncations that require significant lipidation for secretion (*h*apoB72 and *h*apoB100) compared to those constructs which require only minimal lipidation for secretion (*h*apoB17, *h*apoB23, and *h*apoB48). Our results demonstrate that naringenin inhibits MTP activity, leading to reduced apoB secretion and enhanced intracellular degradation via a rapid, kinetically defined, proteasomal pathway. Changes in cellular apoB kinetics were entirely consistent with reduced MTP-mediated accumulation of newly synthesized TG within the microsomal lumen, leading to reduced apoB-Lp assembly and secretion.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture. HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown as described previously (*41*). For experiments, HepG2 cells were plated in either 100 mm or in 6-well (35 mm) culture plates from Falcon Scientific (VWR, Missisauga, ON) and maintained in minimal essential medium (MEM) containing 5% human lipoprotein deficient serum (LPDS). The appropriate concentrations of naringenin (Sigma, St. Lois, MO) and BMS-197636, solubilized in DMSO (concentration in cell cultures did not exceed 0.5%), were added to the dishes. BMS-197636 was a gift from Dr. J. Wetterau (Bristol-Myers Squibb, Princeton, NJ).

Stable McArdle-RH7777 (McA-RH7777) cells expressing various C-terminal truncations of human apoB were a generous gift from Dr. Z. Yao (University of Ottawa Heart Institute, Ottawa, ON, Canada). Cell lines expressing apoB proteins corresponding to 17% (*h*apoB17), 23% (*h*apoB23), 48% (*h*apoB48), 72% (*h*apoB72), and 100% (*h*apoB100) of the full-length molecule were grown and maintained as previously described (*8*). For experiments, cells were plated in 6-well (35 mm) culture plates (Falcon Scientific) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% human LPDS.

MTP Activity. MTP activity was assessed using an isotopic transfer assay with modifications, as previously described (*40*). For in situ assays, HepG2 cells were preincubated for 24 h in the absence or presence of either naringenin or BMS-197636, harvested, and sonicated. Cell homogenates were then used as the MTP source. For in vitro assays, porcine hepatic microsomes were prepared as previously described (*42*), and naringenin or BMS-197636 was added exogenously to the assay mixture. HDL_3 ($d > 1.13$ g/mL) and LDL (1.019 g/mL $< d <$ 1.063 g/mL) were isolated from human plasma. HDL_3 was labeled with [1α,2α(n)-^{3}H]cholesterol (Amersham, Oakville, ON, Canada) and was subsequently reisolated by ultracentrifugation. The MTP reaction mixture contained donor lipoproteins ([^{3}H] CE-containing HDL) and acceptor lipoproteins (LDL) in a ratio of 1:10 (based on protein concentration), 50 μg of HepG2 cell homogenate or porcine hepatic microsomes, and dialysis buffer in a final volume of 50 μL. The ratio of donor to acceptor lipoproteins and the quantity of MTP source added to the reaction mixture were optimized such that results obtained were within the linear range of lipid transfer activity for this assay. MTP-mediated [^{3}H]CE transfer was allowed to proceed for 6 h at 37 °C. Donor and acceptor lipoproteins were then separated by ultracentrifugation, radioactivity in the acceptor particles was counted, and percent transfer of CE was calculated.

Pulse Chase, Immunoprecipitation, and Multicompartmental Modeling. Secreted and cellular apoB were measured

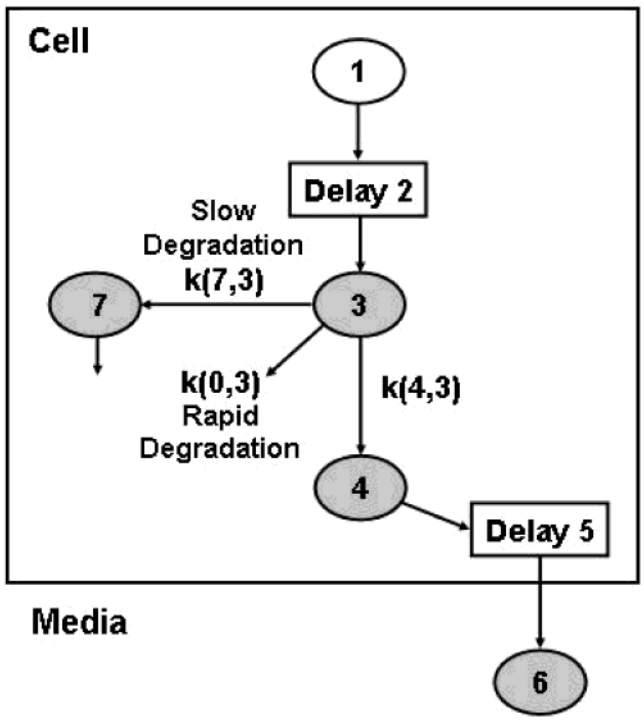

FIGURE 1: Kinetics of apoB100 metabolism in HepG2 cells. Compartments 1−5 and 7 are within the cell. Compartment 6 represents apoB in the cell culture media. Shaded compartments represent apoB radioactivity measured experimentally (3, 4, and 7 in the cell lysates and 6 in the media). Compartments 1 and 2 represent an intracellular pool of tracer and a delay to allow for apoB synthesis, respectively. Compartment 3 represents newly synthesized apoB. From compartment 3, apoB destined for secretion (rate constant $k(4,3)$) proceeds through compartment 4, a delay, and is then secreted into the media (compartment 6). Alternatively, apoB can be degraded directly from compartment 3 by a rapid degradation pathway (rate constant $k(0,3)$), or it can move through compartment 7 and be degraded more slowly (rate constant $k(7,3)$).

following preincubation of HepG2 cells for 24h in the absence or presence of naringenin or BMS-197636. Cells were pulsed for 10 min with 100 μCi/ml Tran [^{35}S]label (1000 Ci/mmol, [^{35}S]L-methionine, and [^{35}S]L-cysteine, ICN, Costa Mesa, CA) and chased for either 60 or 0−120 min (*33*). Media and cellular apoB100 was immunoprecipitated using a polyclonal anti-human apoB antibody (Boehringer Mannheim, Montreal, PQ), resolved, and quantitated as described previously (*33*).

Data obtained from pulse chase experiments, which included time points from 0 to 130 min post-pulse, were analyzed by multicompartmental modeling using the SAAM II program (SAAM Inst., Seattle, WA). We previously described a compartmental model of apoB synthesis, secretion, and degradation, which we developed using full-length apoB (apoB100) radioactivity data obtained from pulse-chase experiments (*33*). Figure 1 shows the compartments and pathways between compartments included in the model. In short, the model includes a number of intracellular compartments and a single extracellular compartment. The shaded compartments within the cell comprise the apoB100 radioactivity measured in cell lysates. The distribution of apoB100 among these compartments (3, 4, and 7) was determined by the model. The media compartment (compartment 6) represents apoB100 radioactivity measured in media samples. Compartment 1 is a dosing compartment for the [^{35}S]label. The transport of tracer into cells from the media was assumed to be essentially instantaneous. The second compartment (Delay 2) was added to account for the time from the initial pulse until cellular radioactivity in immunoprecipitable apoB100 was detected. Compartments 3, 4, and 7 describe the kinetics of intracellular apoB100 radioactivity. Rate constants calculated by the model allow quantitation of the movement of apoB100 radioactivity between compartments, as well as the loss (secretion and degradation) of newly synthesized apoB from the cell.

Cellular Lipid Synthesis and Mass. Changes in cellular lipid synthesis and mass were measured in HepG2 cells preincubated for 24 h with or without either naringenin or BMS-197636. For lipid synthesis experiments, cells were incubated for an additional 2.5 h with either [^{14}C]oleic acid (0.08 μCi, 50 mCi/mmol, Amersham) complexed with fatty acid-free BSA or [^{14}C]acetic acid (0.5 μCi, 57 mCi/mmol, Amersham). Radioactivity incorporated into TG, CE, and cholesterol was determined after separation of the lipid species by thin-layer chromatography, as previously described (*33*). Cellular TG, total cholesterol, and free cholesterol mass were quantitated by a modification of the method of Carr et al. (*43*) using enzymatic reagents from Boehringer Mannheim, as previously described (*33*). CE mass was calculated as the difference between cellular total cholesterol and free cholesterol masses.

Subcellular Fractionation. HepG2 cells (100 mm dishes) were preincubated for 24 h as described in the paragraph above, followed by 2.5 h incubation with [^{14}C]oleic acid complexed with fatty acid-free BSA (0.2 μCi/ml cell culture media) to label cellular TG and CE. Cytosolic, microsomal membrane, and lumenal fractions were prepared as previously described (*35*). The purity of each subcellular fraction was assessed by immunoblotting with antibodies for the 97kDa MTP subunit and protein disulfide isomerase (PDI). Strong signals for both MTP and PDI were detectable in the lumenal fraction only. Lipids in each subcellular fraction were extracted (*44*), and lipid species were separated by thin-layer chromatography as previously described (*33*). Radioactivity associated with TG and CE was counted and normalized to cell protein.

Density Distribution of Secreted apoB-Lp. HepG2 cells (100 mm dishes) were incubated for 24 h with or without either naringenin or BMS-197636, and 100 μCi/ml Tran [^{35}S] label (ICN). Media were collected and concentrated using Macrosep centrifugal concentrators (Pall Filtron, Northborough, MA). VLDL (d < 1.006 g/mL), IDL (1.006 g/mL < d < 1.019 g/mL), LDL (1.019 g/mL < d < 1.063 g/mL), and HDL (1.063 g/mL < d < 1.21 g/mL) were separated by sequential ultracentrifugation as previously described (*45*). ApoB in each lipoprotein fraction was immunoprecipitated using a polyclonal anti-human apoB antibody (Boerhinger Manheim), resolved, and quantitated as described for pulse-chase experiments. Data were normalized to cell protein and expressed as percent of total apoB in the media of control cultures.

ApoB Western Blotting. McA-RH7777 cells expressing *h*apoB17, *h*apoB23, *h*apoB48, *h*apoB72, or *h*apoB100 were incubated with naringenin for 24 h. Media were collected, and aliquots of samples, based on cell protein, were resolved by 4.5% SDS−PAGE. Proteins were electrophoretically transferred to PVDF membranes and blocked overnight at 4

Table 1: Effect of Naringenin and BMS-197636 on ApoB Secretion and MTP Activity[a]

treatment	ApoB secretion % of control	in situ activity % of control	in vitro activity % of control
control	100.00 ± 7.02	100.00 ± 11.35	100.00 ± 10.00
naringenin 50 μM	ND	ND	83.00 ± 13.00
naringenin 75 μM	48.75 ± 10.70	67.92 ± 8.33	ND
naringenin 100 μM	ND	ND	68.00 ± 3.00[b]
naringenin 200 μM	29.40 ± 5.80	65.28 ± 2.55[b]	ND
BMS-197636 10 nM	44.24	60.17 ± 6.67[b]	55.77 ± 7.35[b]
BMS-197636 50 nM	26.15	23.69 ± 5.36[c]	25.03 ± 10.26[c]

[a] ApoB secretion was determined by pulse chase. HepG2 cells incubated for 24 h with either naringenin or BMS-197636 were pulsed for 10 min with Tran [^{35}S] label and chased for 60 min. ApoB was then immunoprecipitated from the media, quantitated and expressed as percent of control. MTP activity was determined by [^{3}H]CE transfer from donor to acceptor lipoproteins. For in situ activity measurements, HepG2 crude cell homogenates prepared from cells incubated for 24 h with either naringenin or BMS-197636 were used as the MTP source and no additional naringenin or BMS-197636 was added exogenously. For in vitro measurements, untreated porcine hepatic microsomes were used as the MTP source, and either naringenin or BMS-197636 were added exogenously at the time of the assay. Values are reported as % of control and are the means ± SEM for 4 separate experiments. Control values were 0.44% ± 0.05% and 0.76% ± 0.08% CE transfer h^{-1} (mg protein)$^{-1}$ for in situ and in vitro assays, respectively. [b] $p < 0.05$, [c] $p < 0.001$ compared to control. ND, not determined.

°C with 5% nonfat dry milk in phosphate-buffered saline, 0.1% Tween 20. Membranes were then incubated with a mouse monoclonal antibody against human apoB (1D1) (Lipoprotein and Atherosclerosis Group, Heart Institute Research Corporation, Ottawa, ON, Canada), followed by incubation with anti-mouse IgG peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA). ApoB was detected using Chemiluminescence Blotting Substrate (Roche Diagnostics, Laval, QC, Canada). Quantitative analysis on the developed films was performed using an Imaging Densitometer (GS-700, Biorad, Mississauga, ON, Canada). The optical densities of bands corresponding to apoB were linear over the range of protein concentrations loaded.

Statistics. All values are presented as means ± SEM. Means were compared by either T-tests or ANOVA followed by T-tests to determine statistical significance. $P < 0.05$ was considered significant.

RESULTS

Naringenin Inhibits MTP Activity in Situ and in Vitro. We previously demonstrated that the citrus flavonoids, naringenin and hesperetin, inhibited MTP activity in HepG2 cells by up to 40% (*40*). Here, we compared the abilities of naringenin and the specific inhibitor, BMS-197636, to inhibit MTP activity in situ and in vitro (Table 1). The concentrations of each compound used were matched for their ability to inhibit apoB secretion in HepG2 cells. Naringenin (75 μM) and BMS 197636 (10 nM) reduced apoB secretion by ~50% (IC_{50}), while naringenin (200 μM) and BMS 197636 (50 nM) inhibited apoB secretion by ~70% (Table 1). In the presence of IC_{50} concentrations for apoB secretion, naringenin and BMS-197636 inhibited in situ MTP activity by 32% ($p = 0.06$) and 40% ($p < 0.05$), respectively. Concentrations that reduced apoB secretion by ~70% inhibited MTP activity by 35% for naringenin ($p < 0.05$) and by 76% for BMS-197636 ($p < 0.001$). In vitro activity, measured using porcine hepatic microsomes as an MTP source, was inhibited by up to 32% ($p < 0.05$, 100 μM) and 75% ($p < 0.001$, 50 nM) by naringenin and BMS-197636, respectively. Naringenin at concentrations greater than 100 μM interfered with the in vitro assay.

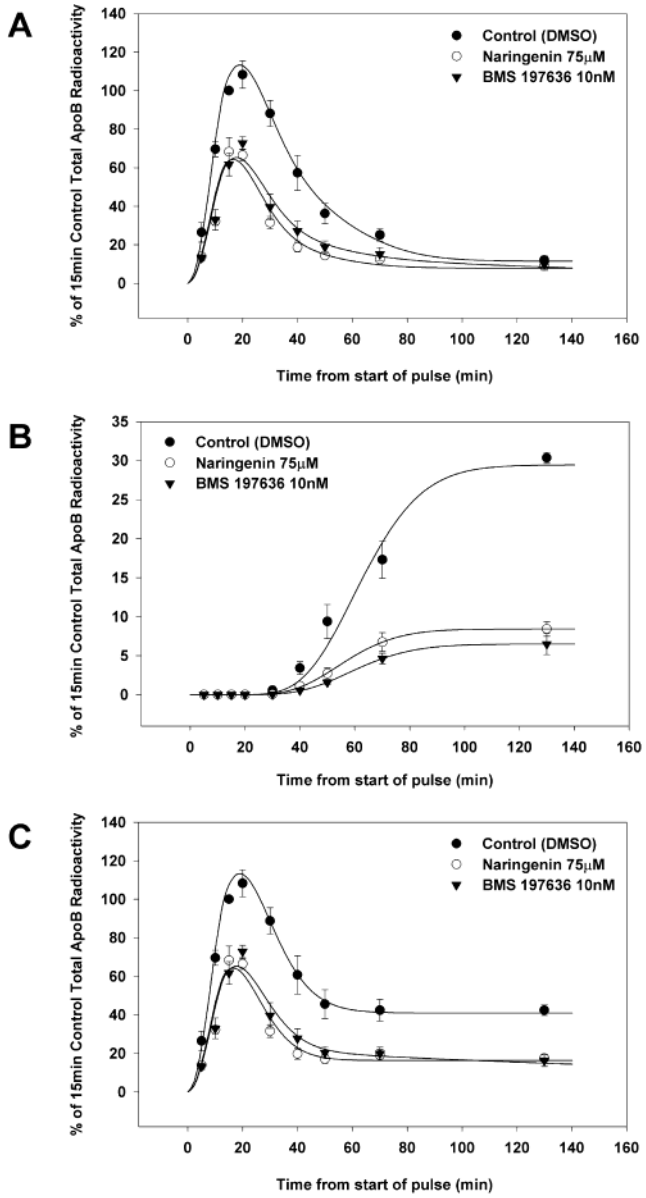

FIGURE 2: Effect of naringenin and BMS-197636 on the secretion and intracellular degradation of apoB100. HepG2 cells were preincubated for 24 h with DMSO (●), naringenin (○), or BMS-197636 (▼). Cells were pulsed for 10 min and chased for 0−120 min. Naringenin and BMS-197636 were present throughout the pulse and the chase as indicated. Cell lysate apoB100 radioactivity is shown in panel A. ApoB100 radioactivity secreted into the media is shown in panel B. Total apoB100 radioactivity, determined as the sum of apoB in the media plus in the cell, is shown in panel C. Data points represent the observed values and are means ± SEM for 4 experiments. The lines are the best fit generated by the kinetic model shown in Figure 1.

MTP Inhibition Enhances Degradation of ApoB via a Kinetically Defined, Rapid Pathway. Pulse-chase experiments were conducted to compare the effects of naringenin (75 μM) on the kinetics of apoB secretion and intracellular degradation to those of BMS-197636 (10 nM). Data points in Figure 2A-C represent apoB radioactivity measured experimentally. The curves in each graph are fits to the data obtained from

Table 2: Kinetic Parameters of apoB Secretion and Intracellular Degradation in the Presence of Either Naringenin or BMS-197636[a]

parameter	control	naringenin 75 μM	BMS-197636 10 nM
total secreted (%)[d]	6.97 ± 0.56[b]	3.53 ± 0.59[c]	2.22 ± 0.25[c]
total degraded (%)[e]	93.03 ± 0.56[b]	96.47 ± 0.59[c]	97.78 ± 0.25[c]
k(4,3) (pools/min)[f]	0.012 ± 0.002[b]	0.006 ± 0.001[c]	0.004 ± 0.001[c]
k(0,3) (pools/min)[f]	0.143 ± 0.010[b]	0.162 ± 0.004[c]	0.173 ± 0.006[c]
k(7,3) (pools/min)[f]	0.010 ± 0.003[b]	0.007 ± 0.001[b]	0.008 ± 0.002[b]
proportion degraded via rapid pathway (%)[g]	87.36 ± 0.02[b]	92.70 ± 0.01[c]	93.50 ± 0.01[c]

[a] ApoB pulse-chase data were analyzed by multicompartmental modeling using SAAMII. The percent of newly synthesized apoB secreted and degraded were determined using the kinetic model. Values are means ± SEM for four separate experiments. [b,c] Means not sharing a lower case superscript are significantly different, $p < 0.05$. [d] Calculated using the formula [k(4,3)/k(4,3)+k(0,3)+k(7,3)] × 100. [e] Calculated using the formula [k(0,3) + k(7,3)/k(4,3) + k(0,3) + k(7,3)] × 100. [f] k(4,3) is the rate constant for apoB transfer from compartment 3 to compartment 4, or the rate constant for secretion; k(0,3) is the rate constant of apoB degradation directly from compartment 3 (rapid degradation), and k(7,3) is the rate constant for apoB transfer from compartment 3 to compartment 7. [g] The percent of apoB degraded directly from compartment 3 is calculated using the formula [k(0,3)/k(4,3) + k(0,3) + k(7,3)] × 100.

analyses using the multicompartmental model shown in Figure 1. Inspection of the curves in Figure 2B showed that naringenin and BMS-197636 dramatically reduced the secretion of newly synthesized apoB to the same extent. Both compounds simultaneously increased intracellular degradation to the same extent (Figure 2C). The inclusion of time points during the pulse revealed that both naringenin and BMS-197636 substantially decreased peak apoB radioactivity (Figure 2C, 20 min). Although differences in labeling observed at the start of the chase are traditionally interpreted as effects on apoB synthesis, neither naringenin (*40*) nor MTP blockade with a specific inhibitor (*46*, *47*) alter apoB message abundance. Blunted peak heights are therefore interpreted as increased co-translational degradation and/or decreased rates of chain elongation.

The pathways for secretion and intracellular degradation of apoB were defined and quantitated by multicompartmental modeling. The key kinetic parameters determined by the model (Figure 1) are shown in Table 2. Naringenin and BMS-197636 significantly decreased the percents of apoB secreted due to 50% and 67% decreases ($p < 0.05$), respectively, in the rate constants for the pathway through which apoB is destined for secretion, k(4,3). Consequently, the total percents degraded were significantly increased due to 13% and 20% increases ($p < 0.05$) in the rate constants for the rapid degradation pathway, k(0,3), while the slow degradation pathway, k(7,3), was unaffected. Due to these selective changes in degradation rate constants, the proportions of apoB degraded via the rapid pathway were significantly increased.

MTP Inhibition Does Not Affect Cellular Lipid Synthesis or Mass. We previously showed that naringenin inhibits CE synthesis and increases TG synthesis and that these effects are accompanied by corresponding changes in celllular lipid content (*40*). MTP inhibition has been shown to increase hepatic TG in rodents (*48*). Therefore, we hypothesized that the increased TG synthesis and mass observed with naringenin could be due to reduced TG secretion, secondary to MTP inhibition. To test this, we compared the effects of naringenin and BMS-197636 on cellular lipid synthesis and mass at concentrations that equally inhibited apoB secretion. Naringenin increased TG synthesis 32% at 200 μM ($p < 0.05$), increased fatty acid synthesis to a similar extent (data not shown), and concentration-dependently reduced CE synthesis by up to 63% ($p < 0.01$) (Table 3). On the other hand, BMS-197636 had no effect on either TG or CE synthesis. The changes in lipid synthesis were accompanied by corresponding changes in cellular lipid mass. Naringenin tended to increase cellular TG content (23%, $p = 0.11$, 200 μM) and concentration-dependently reduced CE mass (−31%, $p < 0.05$, 200 μM). In contrast, BMS-197636 did not affect TG mass, but tended to increase cellular CE content (19%, $p = 0.06$, 50 nM). Neither free cholesterol synthesis nor mass was altered with naringenin or BMS-197636 treatment.

MTP Inhibition Limits the Accumulation of Newly Synthesized TG and CE in the Microsomal Lumen. Using a subcellular fractionation protocol, we recently demonstrated that naringenin, specific ACAT inhibitors, and specific HMG-CoA reductase inhibitors reduce the content of newly synthesized CE in the cytosol, microsomal membranes, and microsomal lumen of HepG2 cells (*35*). In addition, naringenin inhibited TG accumulation in the microsomal lumen (*35*). We postulated that reduced microsomal accumulation of TG was due to the ability of naringenin to inhibit MTP, whereas reduced microsomal CE accumulation was due to both ACAT inhibition and limited transfer of CE to the ER lumen via MTP. To test this, we measured the accumulation of newly synthesized TG and CE in subcellular fractions of HepG2 cells treated with either naringenin or BMS-197636.

Table 3: Effect of Naringenin and BMS-197636 on Cellular Lipid Biosynthesis and Mass[a]

	lipid synthesis			lipid mass		
treatment	triglyceride (nmol [^{14}C]oleate/mg cell protein)	cholesteryl ester (pmol [^{14}C]oleate/g cell protein)	cholesterol (nmol [^{14}C]acetate/mg cell protein)	triglyceride (μg/mg cell protein)	cholesteryl ester (μg/mg cell protein)	cholesterol (μg/mg cell protein)
control	0.37 ± 0.03	9.14 ± 1.03	0.32 ± 0.04	66.55 ± 2.97	6.98 ± 0.38	18.15 ± 0.62
naringenin 75 μM	0.46 ± 0.04	5.74 ± 0.64	0.26 ± 0.04	71.93 ± 10.79	5.92 ± 0.65	17.23 ± 0.18
naringenin 200 μM	0.49 ± 0.01[b]	3.35 ± 0.90[c]	0.23 ± 0.05	81.83 ± 7.54	4.85 ± 0.38[b]	17.39 ± 0.43
BMS-197636 10 nM	0.38 ± 0.02	8.83 ± 1.48	0.30 ± 0.04	62.43 ± 5.49	7.03 ± 0.69	18.04 ± 0.69
BMS-197636 50 nM	0.38 ± 0.01	8.82 ± 1.35	0.29 ± 0.05	65.50 ± 7.64	8.31 ± 0.45	17.47 ± 0.77

[a] HepG2 cells were incubated in MEM containing 5% LPDS with naringenin or BMS-197636 at the concentrations indicated for 24 h. For lipid synthesis experiments, cells were incubated for a further 2.5 h with either [^{14}C]acetate or [^{14}C]oleate. Lipids were extracted and separated by thin-layer chromatography and label incorporated into each lipid species was quantitated by liquid scintillation. For cellular lipid mass determinations, extracted lipids were quantitated by spectrophotometric assays. TG, CE, and cholesterol synthesis and mass are reported as mean ± SEM for a minimum of four experiments with duplicate samples. [b] $p < 0.05$, [c] $p < 0.01$ compared to control.

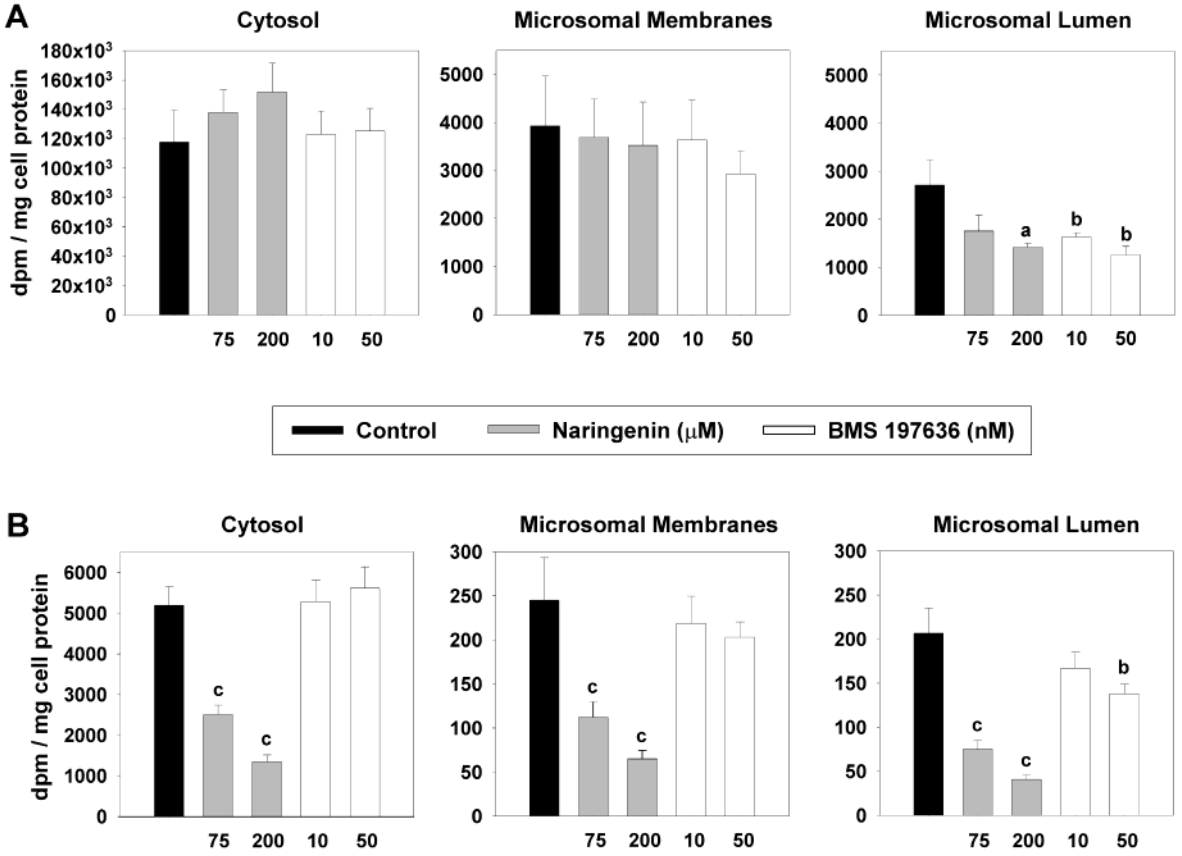

FIGURE 3: Effect of naringenin and BMS-197636 on the cellular distribution of newly synthesized TG and CE. HepG2 cells were preincubated for 24 h with or without the indicated concentrations of naringenin or BMS-197636. Cells were continuously labeled for 2.5 h with [^{14}C]-oleic acid. Naringenin and BMS-197636 were present during labeling at the concentrations indicated. Cells were then homogenized, and subcellular fractions were isolated (cytosol, microsomal membranes, and microsomal lumen). Lipids in each fraction were extracted, and TG (A) and CE (B) were separated by thin-layer chromatography. Label incorporated into each lipid species was quantified by liquid scintillation. Values are means ± SEM for at least 4 experiments. $^{a}p < 0.005$, $^{b}p < 0.05$ and $^{c}p < 0.001$ compared to control.

Naringenin tended to increase newly synthesized TG in the cytosol (29%, $p = 0.29$, 200 μM) and concentration-dependently reduced TG accumulation in the microsomal lumen by up to 48% ($p < 0.05$, 200 μM) (Figure 3A), while BMS-197636 significantly reduced TG accumulation only in the microsomal lumen, by up to 54% ($p < 0.05$, 50 nM). In the case of CE, naringenin concentration-dependently reduced accumulation by up to 74%, 73%, and 80% in the cytosol, microsomal membranes, and microsomal lumen, respectively ($p < 0.001$, 200 μM) (Figure 3B), while BMS-197636 inhibited CE accumulation only in the microsomal lumen, by 19% at 10 nM ($p = 0.28$, not significant) and 33% at 50 nM ($p < 0.05$).

MTP Inhibition Reduces Secretion of Large ApoB-Lp More Effectively than Small, Dense Particles. The effect of reduced availability of ER lumenal lipids on the density distribution of secreted apoB-Lp was determined in HepG2 cells (Figure 4). In control cells, 5% of apoB was secreted as IDL, 76% as LDL, and 17% as apoB-containing HDL-sized particles. Since less than 2% of apoB was secreted as VLDL, data was obtained from IDL, LDL, and HDL fractions only. Both naringenin and BMS-197636 concentration-dependently reduced the secretion of IDL, LDL, and apoB-containing HDL-sized particles to a similar extent. Naringenin (75 μM) and BMS-197636 (10 nM) reduced IDL secretion by 57% ($p < 0.05$) and 62% ($p < 0.05$), respectively. The same concentrations of each compound were less effective in reducing the

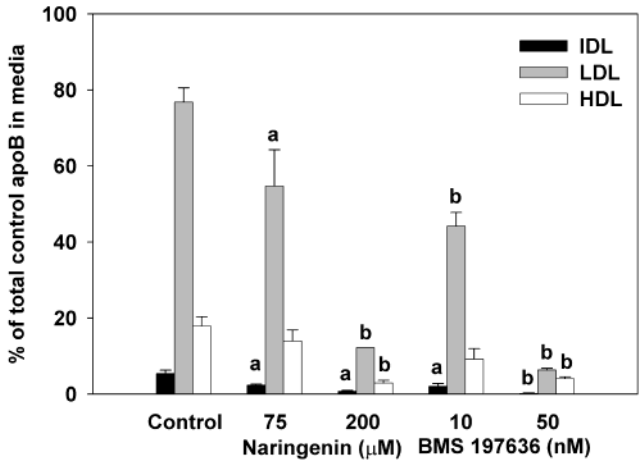

FIGURE 4: Effect of MTP inhibition on the density distribution of secreted of apoB-Lp. HepG2 cells were continuously labeled for 24 h with Tran [^{35}S] label in the presence of increasing concentrations of either naringenin or BMS-197636. Media were collected and concentrated, and lipoproteins were isolated by sequential ultracentrifuagation. ApoB in each lipoprotein fraction was immunoprecipitated, resolved by SDS−PAGE, and quantitated. Total apoB secreted (IDL + LDL + HDL) from control cells was set at 100%. Percentages of total apoB secreted from cells treated with naringenin were 70.93% ± 12.78% and 15.80% ± 0.98% for 75 and 200 μM, respectively, and from cells treated with BMS-197636 were 55.37% ± 7.11% and 10.55% ± 1.03% for 10 and 50 nM, respectively, compared to control cells. Values are means ± SEM for three experiments. $^{a}p < 0.05$ and $^{b}p < 0.01$.

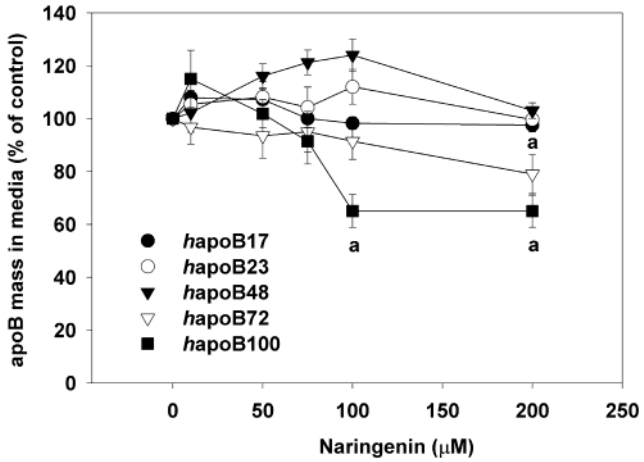

FIGURE 5: Effect of naringenin on the secretion of human C-terminal truncations of apoB from McA- RH7777 rat hepatoma cells. Cell lines stably transfected with cDNAs corresponding to 17% (*h*apoB17), 23% (*h*apoB23), 48% (*h*apoB48), 72% (*h*apoB72), and 100% (*h*apoB100) of the human protein were incubated for 24 h with increasing concentrations of naringenin. ApoB in the media was detected by immunoblotting and bands were quantitated by densitometry. Values are means ± SEM for 4 experiments. [a] $p < 0.05$ compared to the control for each cell line.

secretion of smaller, denser particles. Naringenin reduced LDL and apoB-containing HDL-sized particle secretion by 29% ($p < 0.05$) and 22% ($p = 0.34$), respectively. BMS-197636 reduced LDL and HDL secretion by 42% ($p < 0.01$) and 49% ($p = 0.06$), respectively. The high concentrations of naringenin and BMS-197636 inhibited secretion of IDL, LDL and apoB-containing HDL-sized particles by 84% to 87% ($p < 0.05$) and 80% to 96% ($p < 0.01$), respectively.

Naringenin Inhibits Secretion of Hapob72 and Hapob100, but not Shorter C-Terminal Truncations. Since naringenin inhibited MTP activity (Table 1) and consequently reduced accumulation of newly synthesized TG in the microsomal lumen (Figure 3), we hypothesized that the secretion of apoB molecules which require significant assembly with lipid to be secreted would be inhibited, while secretion of shorter proteins, which can be secreted with minimal lipidation, would be unaffected. To test this, stable McA-RH7777 cell lines expressing the human C-terminal apoB truncations, *h*apoB17, *h*apoB23, *h*apoB48, *h*apoB72, or full-length *h*apoB100 were incubated for 24 h with increasing concentrations of naringenin (Figure 5). Naringenin inhibited apoB72 secretion by 21% (200 μM, $p < 0.05$) and inhibited full-length apoB secretion by 35% (100 μM and 200 μM, $p < 0.05$). In contrast, naringenin did not alter *h*apoB17 and *h*apoB23 secretion, while *h*apoB48 secretion tended to increase with 75 μM and 100 μM concentrations.

DISCUSSION

Since the discovery of MTP and its involvement in abetalipoproteinemia, this protein has been a target for the pharmacological reduction of apoB secretion. MTP activity is an absolute requirement for the assembly of apoB-Lp in the intestine and liver, and numerous studies, in cell culture and in vivo, have demonstrated the effectiveness of MTP inhibition in reducing apoB secretion (reviewed in ref *9*). Flavonoids, which are small polyphenolic molecules found in all plants and plant products, have recently been identified as lipid-lowering agents (reviewed in ref *36*). We previously demonstrated that, in HepG2 cells, the citrus flavonoids, naringenin and hesperetin, reduce apoB secretion, inhibit ACAT actvity, selectively decrease ACAT2 expression, and reduce MTP activity and expression (*40*). Further studies with naringenin revealed that reduced accumulation of newly synthesized CE in the ER lumen, secondary to ACAT inhibition, was not the primary mechanism responsible for reducing apoB secretion (*35*). In the present study, we clearly demonstrate that the ability of naringenin to inhibit MTP activity, and thereby reduce accumulation of newly synthesized TG in the ER lumen, represents a primary mechanism responsible for the dramatic reduction in apoB lipidation and secretion.

Multicompartmental modeling analyses of pulse-chase data allowed us to identify, quantitate, and compare changes in the kinetics of intracellular pools of apoB resulting from naringenin or BMS-197636 treatment. Although previous pulse-chase studies have demonstrated that MTP inhibtion blocks apoB secretion and increases degradation in HepG2 cells (*46*, *47*, *49*, *50*), the impact on intracellular apoB kinetics has not been reported. The curves generated by the model to fit data points collected in pulse-chase experiments show that peak radioactivity in full length apoB100 is consistently reached 20 min post-pulse, or after 10 min of chase (Figure 2C). This indicates that, due to the size of the apoB molecule, there is a 10 min lag between the end of the pulse and completion of synthesis of labeled apoB. Decreases in peak height of apoB radioactivity observed in the presence of either naringenin or BMS-197636 likely reflect increased cotranslational degradation and/or decreased rates of chain elongation during translation (*35*). Although neither naringenin (*40*) nor MTP blockade with a specific inhibitor (*46*, *47*) affect apoB mRNA levels, MTP inhibition has been associated with increased cotranslational degradation (*50*) and reduced rates of elongation of nascent apoB (*47*). Both naringenin and BMS-197636 decreased apoB secretion (Figure 2), and the reduced percentages of apoB secreted were reflected by decreases in the rate constants for secretion with each treatment (Table 2). The large proportion of apoB degraded in these cells (93% in control cells, Table 2) agrees well with previously reported estimates of greater than 80% degradation of newly synthesized apoB, obtained from pulse-chase studies not subjected to multicompartmental kinetic analysis (reviewed in refs *1* and *26*). Multicompartmental analyses revealed that apoB not targetted for secretion is degraded by two kinetically distinct pathways in HepG2 cells (Figure 1). The majority of newly synthesized apoB is degraded via a rapid pathway through which 14% of the total pool of newly synthesized apoB in control cells is degraded each minute. By contrast, approximately 1% per min of the apoB pool is catabolized by the slow degradation pathway. Naringenin and BMS-197636 selectively increased degradation via the rapid pathway, while the slow pathway was unaffected. The changes observed are best reflected by the increases in the proportion of apoB degraded via the rapid pathway in the presence of either naringenin or BMS-197636 (Table 2). The similarity between the effects of naringenin

and BMS-197636 on cellular apoB kinetics suggests similar mechanisms of action.

We previously observed selective changes in degradation via the rapid pathway in HepG2 cells following alterations in cellular cholesterol, CE, or TG concentrations. The HMG-CoA reductase inhibitor, atorvastatin, the ACAT inhibitor, CI-1011 (avasimibe), and naringenin (200 μM) (*33*−*35*) increased only rapid degradation, whereas oleic acid selectively decreases rapid degradation (*35*). Collectively, these data suggest that treatments which alter the availability of cellular lipids selectively affect degradation via a kinetically defined rapid pathway without affecting the slow pathway. Using the proteasome inhibitors, ALLN and lactacystin, we identified this pathway as primarily proteasomal (*35*), consistent with previous reports demonstrating that the majority of newly synthesized apoB is degraded via the cytosolic ubiquitin-proteosome pathway (*27*−*31*). The slow pathway was insensitive to either ALLN or lactacystin and is, therefore, nonproteasomal and may represent either ER lumenal or lysosomal degradation.

In HepG2 cells cultured under either basal conditions or in the presence of oleic acid, naringenin decreases CE synthesis and increases TG synthesis, with corresponding changes in cellular lipid content (*35*, *39*, *40*). Since MTP inhibition has been shown to increase hepatic TG in rodents (*48*), we hypothesized that the increased TG synthesized and mass observed with naringenin was due to reduced TG secretion secondary to inhibiton of MTP. In the present study, BMS-197636 at apoB-lowering concentrations equivalent to those of naringenin did not significantly alter CE or TG synthesis. Thus, the changes in cellular TG synthesis and mass observed with naringenin are unrelated to MTP inhibition. Although the mechanism for this effect remains unexplained, we have previously demonstrated that specific ACAT inhibitors also increase TG synthesis in HepG2 cells (*33*, *35*).

Although naringenin effectively inhibited MTP activity in both in situ and in vitro assays, it was less effective than BMS-197636. MTP activity was reduced by up to 38% in cells preincubated with flavonoid for 24 h (in situ) and up to 32% in isolated porcine hepatic microsomes (in vitro), while concentrations of BMS-197636 which reduced apoB secretion to a similar extent inhibited MTP activity by up to 75% in both assays (Table 1). However, this assay quantitates only the transfer of lipids from isolated donor particles to isolated acceptor particles and cannot assess the ability of MTP to accumulate newly synthesized lipid in a given cellular compartment. For this reason, we conducted subcellular fractionation experiments. We recently showed that naringenin reduced the accumulation of newly synthesized CE in cytosol, microsomal membranes, and microsomal lumen, whereas TG accumulation was only inhibited in the microsomal lumen (*35*). Since MTP activity is required for TG accumulation within the microsomal lumen of McA-RH7777 cells (*8*) and primary mouse hepatocytes (*51*), we hypothesized that naringenin reduced lumenal accumulation of newly synthesized TG via MTP inhibition. Furthermore, we hypothesized that naringenin reduced lumenal CE accumulation due to the combination of limited cellular CE, secondary to ACAT inhibition, and limited CE transfer to the ER lumen, secondary to MTP inhibition. Despite increased TG synthesis with naringenin, leading to increased newly synthesized cytosolic TG, the accumulation of newly synthesized TG in the ER lumen was concentration-dependently reduced to approximately the same extent as in cells treated with BMS-197636 (Figure 3A). In contrast, newly synthesized CE accumulation was concentration-dependently reduced in all three subcellular fractions with naringenin, while BMS-197636 reduced accumulation in the lumen only (Figure 3B). This significant but incomplete inhibition of lumenal CE accumulation in the presence of BMS-197636 demonstrates, for the first time, that MTP activity is involved in, but not required for CE accumulation within the microsomal lumen. Furthermore, since we recently reported that CE availability in the lumen does not regulate apoB secretion in HepG2 cells (*35*), these results suggest that naringenin blocks apoB secretion by limiting TG availability in the ER lumen, secondary to inhibition of MTP. However, the possibility exists that another mechanism is also involved in naringenin-induced inhibition of apoB secretion. Recently we demonstrated that naringenin substantially increased the expression and activity of the LDL receptor (*40*). Primary hepatocytes from LDL receptor null mice secrete twice as much newly synthesized apoB as wild-type hepatocytes (*51*, *52*). It has been proposed that the LDL receptor reduces net apoB accumulation in the media both by binding the nascent protein within the ER, thereby preventing its secretion and shunting it into a degradation pathway, and by binding and endocytosing the nascent protein at the cell surface for eventual lysomal degradation (*52*). However, recent studies in vivo showed no difference in VLDL production rate between LDL receptor null and wild-type mice (*53*). Thus, the contribution of increased LDL receptor expression in the presence of naringenin remains to be clarified.

Both naringenin and BMS-197636 concentration-dependently reduced the secretion of IDL, LDL, and apoB-containing HDL-sized particles. Although the shift toward secretion of more dense lipoproteins was not significant, both compounds were most effective in reducing the secretion of IDL and LDL, particularly at lower concentrations. These data supports the concept that the assembly of larger, lipid-rich apoB-Lp is more sensitive to MTP inhibition than that of small, dense particles (*8*). Further support was provided by experiments using stable McA-RH7777 cell lines expressing human C-terminal apoB truncations (Figure 5). Secretion of proteins corresponding to 17%, 23%, and 48% of full-length apoB (*h*apoB17, *h*apoB23, and *h*apoB48) was not inhibited by naringenin. However, secretion of *h*apoB72 and *h*apoB100, which contain the β_2 domain, believed to require extensive MTP-mediated lipid transfer for secretion (*24*), was reduced by as much as 35%. This is consistent with studies in McA-RH7777-derived cell lines and primary mouse hepatocytes demonstrating that MTP inhibitors preferentially reduced the secretion of proteins larger than apoB48 (*8*, *24*, *51*). These observations, combined with our subcellular fractionation data, suggest that the ability of naringenin to inhibit MTP activity, and thereby limit TG accumulation in the ER lumen, is the primary mechanism responsible for reducing apoB-Lp secretion.

In summary, naringenin directly and effectively inhibits MTP activity. Thus, apoB secretion is reduced and intracellular degradation is enhanced via a rapid, kinetically defined, proteasomal pathway—a pathway also modulated

by a variety of compounds which alter cellular cholesterol, CE or TG concentrations (*33*−*35*). We conclude that these changes in cellular apoB kinetics result primarily from reduced MTP-mediated accumulation of newly synthesized TG within the ER lumen, the initial site of apoB-Lp assembly. Consequently, apoB lipidation and subsequent lipoprotein secretion are inhibited. This mechanism is likely responsible for the significant reductions in plasma cholesterol and TG observed in rodent models given naringenin-supplemented diets (reviewed in ref *36*).

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