Inhibition of hepatocyte apoB secretion by naringenin: enhanced rapid intracellular degradation independent of reduced microsomal cholesteryl esters

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Abstract **The grapefruit flavonoid, naringenin, is hypocholesterolemic in vivo, and inhibits basal apolipoprotein B (apoB) secretion and the expression and activities of both ACAT and microsomal triglyceride transfer protein (MTP) in human hepatoma cells (HepG2). In this report, we examined the effects of naringenin on apoB kinetics in oleate-stimulated HepG2 cells and determined the contribution of microsomal lumen cholesteryl ester (CE) availability to apoB secretion. Pulse-chase studies of apoB secretion and intracellular degradation were analyzed by multicompartmental modeling. The model for apoB metabolism in HepG2 cells includes an intracellular compartment from which apoB can be either secreted or degraded by both rapid and slow pathways. In the presence of 0.1 mM oleic acid, naringenin (200 μM) reduced the secretion of newly synthesized apoB by 52%, due to a 56% reduction in the rate constant for secretion. Intracellular degradation was significantly increased due to a selective increase in rapid degradation, while slow degradation was unaffected. Incubation with either *N*-acetyl-leucinyl-leucinyl-norleucinal (ALLN) or lactacystin showed that degradation via the rapid pathway was largely proteasomal. Although these changes in apoB metabolism were accompanied by significant reductions in CE synthesis and mass, subcellular fractionation experiments comparing naringenin to specific ACAT and HMG-CoA reductase inhibitors revealed that reduced accumulation of newly synthesized CE in the microsomal lumen is not consistently associated with reduced apoB secretion. However, naringenin, unlike the ACAT and HMG-CoA reductase inhibitors, significantly reduced lumenal TG accumulation. We conclude that naringenin inhibits apoB secretion in oleate-stimulated HepG2 cells and selectively increases intracellular degradation via a largely proteasomal, rapid kinetic pathway. Although naringenin inhibits ACAT, CE availability in the endoplasmic reticulum (ER) lumen does not appear to regulate apoB secretion in HepG2 cells. Rather, inhibition of TG accumulation in the ER lumen via inhibition of MTP is the primary mechanism blocking apoB secretion.**—Borradaile, N. M., L. E. de Dreu, P. H. R. Barrett, and M. W. Huff. **Inhibition of hepatocyte apolipoprotein B secretion by naringenin: enhanced rapid intracellular degradation independent of reduced microsomal cholesteryl esters. *J. Lipid Res.* 2002.** 43: **1544–1554.**

Supplementary key words citrus flavonoid • ACAT • oleate • endoplasmic reticulum lumen • multicompartmental modeling • kinetics • degradation • triglycerides

The synthesis and assembly of apolipoprotein B-containing lipoproteins (apoB-Lp) in the liver is a complex process. Apolipoprotein B (apoB) is the 550 kDa hydrophobic molecule that provides the structural framework for the assembly of VLDL. For secretion to occur, apoB must cotranslationally associate with lipids in the endoplasmic reticulum (ER) (1–3). Initially, apoB acquires a small amount of phospholipid (PL), triglycerides (TG), and cholesteryl ester (CE), and as the protein is further translated/translocated into the rough ER, additional lipid is recruited. The primordial lipoprotein later acquires the majority of its neutral lipid core in the smooth ER (3–5).

The sequential lipidation of apoB requires enzymes to synthesize lipids [as reviewed in ref. (1)] and a chaperone to mediate both the accumulation of these lipids within the ER lumen (6), as well as their transfer to apoB [as reviewed in ref. (7)]. CE and TG are synthesized by ACAT and diacylglycerol acyltransferase (DGAT) respectively, and both lipids can be rate limiting for apoB secretion (8–

Abbreviations: ALLN, *N*-acetyl-leucinyl-leucinyl-norleucinal; apo, apolipoprotein; apoB-Lp, apoB-containing lipoprotein; CE, cholesteryl ester; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; LPDS, lipoprotein deficient serum; MTP, microsomal triglyceride transfer protein; PDI, protein disulfide isomerase; PL, phospholipid.

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10). Recent studies describing two forms of ACAT have been extensively reviewed (8, 9, 11–14). ACAT1 is ubiquitously expressed (15, 16) and is oriented in the ER membrane such that its active site faces the cytosol (17). ACAT2, however, is expressed exclusively in the liver and intestine, and its active site faces the ER lumen (17). These conformational differences have led to the hypothesis that ACAT1 esterifies cholesterol for cytosolic storage in all cells, whereas ACAT2 provides CE for lipoprotein assembly in the liver and intestine (13, 15, 17). However, the functional roles of ACAT1 and ACAT2 in lipoprotein assembly have not been completely elucidated [as reviewed in ref. (14)]. Knockout studies in mice have suggested that there may also be more than one form of DGAT [as reviewed in ref. (10)]. The lipids synthesized by ACAT and DGAT are transferred to apoB by the microsomal triglyceride transfer protein (MTP). MTP activity is an absolute requirement for apoB-Lp assembly and secretion [as reviewed in ref. (7, 18–20)]. Recently, Wang, Tran, and Yao (6) demonstrated that a critical component of MTP activity is the accumulation of newly synthesized TG within the ER lumen. Whether MTP is also required for the accumulation of CE within the ER is, as yet, undetermined.

In the liver, insufficiently lipidated or misfolded apoB is targeted for degradation. In HepG2 cells, it has been estimated that less than 20% of newly synthesized apoB is secreted [as reviewed in ref. (2, 21)]. The majority of apoB degradation is believed to occur cotranslationally via the ubiquitin-26S cytosolic proteasome pathway (22–26); however, degradation also occurs in the ER lumen and post-ER compartment (27, 28). Using mulitcompartmental modeling to quantitate the kinetics of cellular apoB metabolism, we reported 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 (29, 30).

Conditions that limit CE availability, such as treatment with inhibitors of HMG-CoA reductase and ACAT, can decrease apoB secretion in HepG2 cells and subsequently increase intracellular apoB degradation [as reviewed in ref. (4, 31)]. However, these effects are not observed for every HMG-CoA reductase or ACAT inhibitor (29, 30, 32–34). It is possible that to inhibit apoB secretion, the availability of newly synthesized CE within the ER lumen must be decreased. Thus, the role of CE availability in the secretion of apoB in cultured hepatocytes remains to be clarified. MTP inhibition, however, consistently inhibits apoB secretion in several hepatocyte cultures [as reviewed in ref. (7, 18, 19)].

We recently reported that the citrus flavonoids, naringenin and hesperetin, markedly reduce basal apoB secretion in HepG2 cells (35, 36). These flavonoids, found predominantly in grapefruit and oranges, reduce plasma lipids [as reviewed in ref. (37)] and atherosclerosis (38) in rodent models. The hypocholesterolemic effects observed in vivo were associated with reduced hepatic HMG-CoA reductase and ACAT activities (39). We demonstrated in HepG2 cells that the flavonoid-induced reduction in basal apoB secretion was associated with reduced ACAT and MTP activities, as well as reduced expression of ACAT2 and MTP (36). However, the relative contribution of each of these mechanisms has not been explored.

The objectives of the present study were: *i*) to define and quantitate the kinetic pathways of apoB secretion and degradation in HepG2 cells treated with oleate and/or naringenin using multicompartmental modeling analyses of pulse-chase experiments and *ii*) to determine if inhibition of ACAT by naringenin reduced the availability of newly synthesized CE within the ER lumen. Our results clearly show that naringenin decreased apoB secretion in HepG2 cells that were treated with oleate to stimulate apoB secretion. Intracellular apoB degradation increased significantly and was specific to a kinetically defined, rapid pathway, which was largely proteasomal. Furthermore, naringenin inhibited the appearance of CE within the ER lumen, but this was not the prime determinant for reduced secretion of apoB.

MATERIALS AND METHODS

Cell culture

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown as described previously (40). 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 MEM containing 5% human lipoprotein-deficient serum (LPDS), with or without the addition of oleic acid complexed to fatty acid-free BSA (Sigma, St. Louis, MO). The appropriate concentrations of naringenin, *N*-acetyl-leucinyl-leucinyl-norleucinal (ALLN), DuP 128, CI-1011 (avasimibe), simvastatin, and atorvastatin, solubilized in DMSO (concentration in cell cultures did not exceed 0.5%), were added to the dishes. Naringenin and ALLN were obtained from Sigma. Lactacystin was obtained from Calbiochem (La Jolla, CA). DuP 128 was provided by the Dupont Merck Pharmaceutical Co. CI-1011 and atorvastatin were provided by Parke-Davis Pharmaceutical Research (now Pfizer, Ann Arbor, MI). Simvastatin was provided by Merck (Rathway, NJ).

Pulse-chase and immunoprecipitation

Secreted and cellular apoB, synthesized in the absence or presence of either 0.1 mM oleic acid, 10 μM ALLN, or 10 μM lactacystin was measured following preincubation of cells for 24 h in the absence or presence of naringenin. Cells were pulsed for 10 min with 100 μCi/ml Tran ^{35}S-label (1000 Ci/mmol, L-[^{35}S]methionine and L-[^{35}S]cysteine, ICN, Costa Mesa, CA) and chased for 0–120 min (29). Media and cellular apoB-100 were immunoprecipitated using a polyclonal anti-human apoB antibody (Boehringer Mannheim, Montreal, Canada), resolved and quantitated as described previously (29). Newly synthesized apoAI and apoE were similarly immunoprecipitated and analyzed after 60 min of chase using polyclonal anti-human apoAI or apoE antibodies (Boehringer Mannheim).

Multicompartmental modeling

Data obtained from pulse-chase experiments, which included time points from 0 to 130 min postpulse, were analyzed by multicompartmental modeling using the SAAM II program (SAAM Institute, Seattle, WA). We previously described a compartmental model of apoB synthesis, secretion, and degradation, which we

developed using full-length apoB (apoB-100) radioactivity data obtained from pulse-chase experiments (29). **Figure 1** shows the compartments and pathways between compartments included in the model. Briefly, the model includes a number of intracellular compartments and a single extracellular compartment. The shaded compartments within the cell represent apoB-100 radioactivity measured in cell lysates. The media compartment (compartment 6) represents apoB-100 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 apoB-100 was detected. Compartments 3, 4, and 7 describe the kinetics of intracellular apoB-100 radioactivity. Rate constants calculated by the model allow quantitation of the movement of apoB-100 radioactivity between compartments, as well as the loss (secretion and degradation) of newly synthesized apoB from the cell.

Cellular lipid synthesis and mass

The incorporation of [1-^{14}C]oleic acid (Amersham, Oakville, ON, Canada) and [1-^{14}C]acetic acid (Amersham) into cellular lipids was determined as described previously (29). Cells were preincubated for 24 h with or without naringenin, followed by 2.5 h incubation with [^{14}C]oleic acid (0.08 μCi, 50 mCi/mmol) complexed with fatty acid-free BSA (molar ratio 5:1, oleic acid:BSA) or [^{14}C]acetic acid (0.5 μCi, 57 mCi/mmol). Radioactivity incorporated into CE, TG, and cholesterol 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.

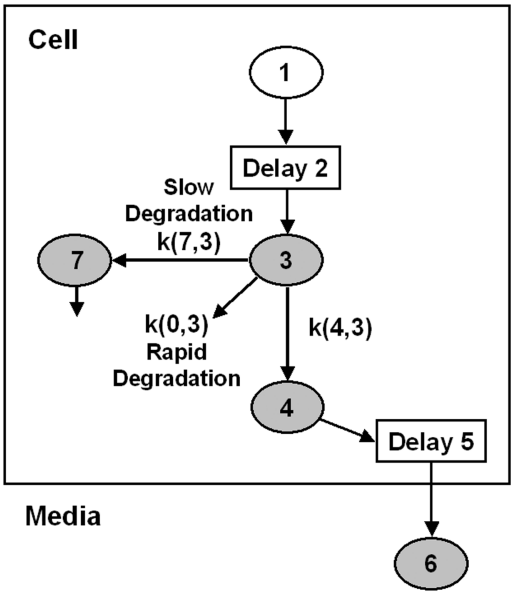

Fig. 1. Kinetics of apolipoprotein (apo)B-100 metabolism in HepG2 cells. Compartments 1–5 and 7 are within the cell. Compartment 6 represents apoB in the cell culture media. Shaded compartments represent apoB radioactivity measured experimentally (3, 4, and 7 in the cell lysates, and 6 in the media). 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 (compartment 5) 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)].

Cellular TG, free cholesterol, and total cholesterol were quantitated by a modification of the method of Carr, Andresen, and Rudel (41) using enzymatic reagents from Boehringer Mannheim, as previously described (29). Cellular lipid results are reported as μg of cellular lipid (CE, TG or cholesterol) per mg cell protein.

Subcellular fractionation

Cells (100 mm dishes) were preincubated as described above, followed by a 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 CE and TG. Cytosolic, microsomal membrane, and lumenal fractions were prepared as previously described (6) with minor modifications. Cells were scraped into 500 μl of Tris-sucrose buffer [10 mM Tris-HCl, 250 mM sucrose (pH 7.4)] containing 0.1 mM PMSF, 0.1 mM leupeptin, and 2 μg/ml ALLN and were homogenized by 30–35 strokes of a Dounce homogenizer. The postnuclear supernatant was obtained by centrifugation (2250 *g*, 4°C, 10 min). Microsomes and cytosol were isolated from the postnuclear supernatant by centrifugation using a Beckman TLA 120.2 rotor (100,000 *g*, 4°C, 1 h). Total microsomes were washed twice with Tris-sucrose buffer to minimize cytosolic contamination prior to extraction of lumenal contents by incubation with 0.1 M sodium carbonate (pH 11.5) (gentle rocking, room temperature, 30 min). Lumenal contents were separated from microsomal membranes by centrifugation (100,000 *g*, 4°C, 1 h). The purity of each subcellular fraction was assessed by immunoblotting with antibodies for MTP 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 (42), and lipid species were separated by thin layer chromatography as previously described (29). Radioactivity associated with CE and TG was counted and normalized to cell protein.

Statistics

All values are presented as mean ± 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 oleate-stimulated secretion of apoB, with no effect on apoAI or apoE

We previously established that naringenin dose-dependently reduced apoB secretion in HepG2 cells under basal conditions (36). Maximum inhibition of 82% was achieved with 200 μM naringenin. Here, we determined whether naringenin could also reduce the secretion of newly synthesized apoB under conditions in which apoB secretion was stimulated by exogenous oleate. Cells preincubated (24 h) with or without naringenin (200 μM) were exposed to increasing concentrations of oleic acid for 20 min prior to initiation of the pulse-chase protocol. Following 60 min of chase, naringenin significantly inhibited secretion of newly synthesized apoB in the presence of 0.1 mM (−84%), 0.4 mM (−55%), and 0.8 mM oleate (−55%) (**Fig. 2A**). For all subsequent experiments, 0.1 mM oleate was used because the increase in apoB secretion observed with this concentration lies on the linear portion of the control dose-response curve shown in Fig. 2A.

We next generated curves for the labeling of cellular and secreted apoB, apoAI, and apoE with increasing doses of naringenin in the presence of 0.1 mM oleate (Fig. 2B–D). Naringenin significantly reduced the secretion of newly synthesized apoB by up to 69% at 200 μM ($P < 0.001$) (Fig. 2B), with an IC_{50} of 75 μM. Reduced secretion was paralleled by reduced cellular apoB, indicating that protein not secreted in the presence of naringenin did not accumulate within the cell. The synthesis and secretion of apoAI (Fig. 2C) and apoE (Fig. 2D) were unaffected by naringenin over the range of doses tested.

Naringenin enhances degradation of apoB via a kinetically defined, rapid pathway

To further define the mechanisms responsible for reduced secretion of newly synthesized apoB in the presence of naringenin, extensive pulse-chase studies were done, essentially as described previously (29, 30). Prior to the start of each pulse-chase, HepG2 cells preincubated for 24 h with or without naringenin (200 μM) were incubated for 20 min with or without oleate (0.1 mM). Media and cell lysates were collected at eight time points throughout the pulse and the chase. Data points in **Figs. 3A**, **B** represent apoB radioactivity measured experimentally. The curves in each graph are fits to the data obtained from analyses using the multicompartmental model shown in Fig. 1. Inspection of the curves in Fig. 3A showed that oleate (0.1 mM) increased the secretion of newly synthesized apoB approximately 2-fold, while the rate of intracellular degradation was reduced, as indicated by the total apoB radioactivity curves (Fig. 3B). Naringenin, however, completely blocked the oleate-stimulated secretion of newly synthesized apoB to a level below that of the untreated control (Fig. 3A). A concomitant increase in intracellular degradation was observed (Fig. 3B).

It is important to note that the inclusion of time points during the pulse revealed substantial differences in peak apoB radioactivity (Fig. 3B, 20 min). Oleate increased peak apoB radioactivity, while naringenin had the opposite effect. Traditionally, differences in labeling observed at the start of the chase are interpreted as effects on apoB synthesis. As neither oleate (43–46) nor naringenin (36) alters apoB message levels, differences in peak height are interpreted as changes in cotranslational degradation and/or chain elongation.

Mathematical analysis of cellular apoB kinetics by multicompartmental modeling allowed us to define and quantitate the relative contributions of the pathways for secretion and intracellular degradation for each pulse-chase experiment. The key kinetic parameters determined by the model are shown in **Table 1**. Oleate (0.1 mM) increased the percent of apoB secreted by 1.4-fold ($P < 0.02$).

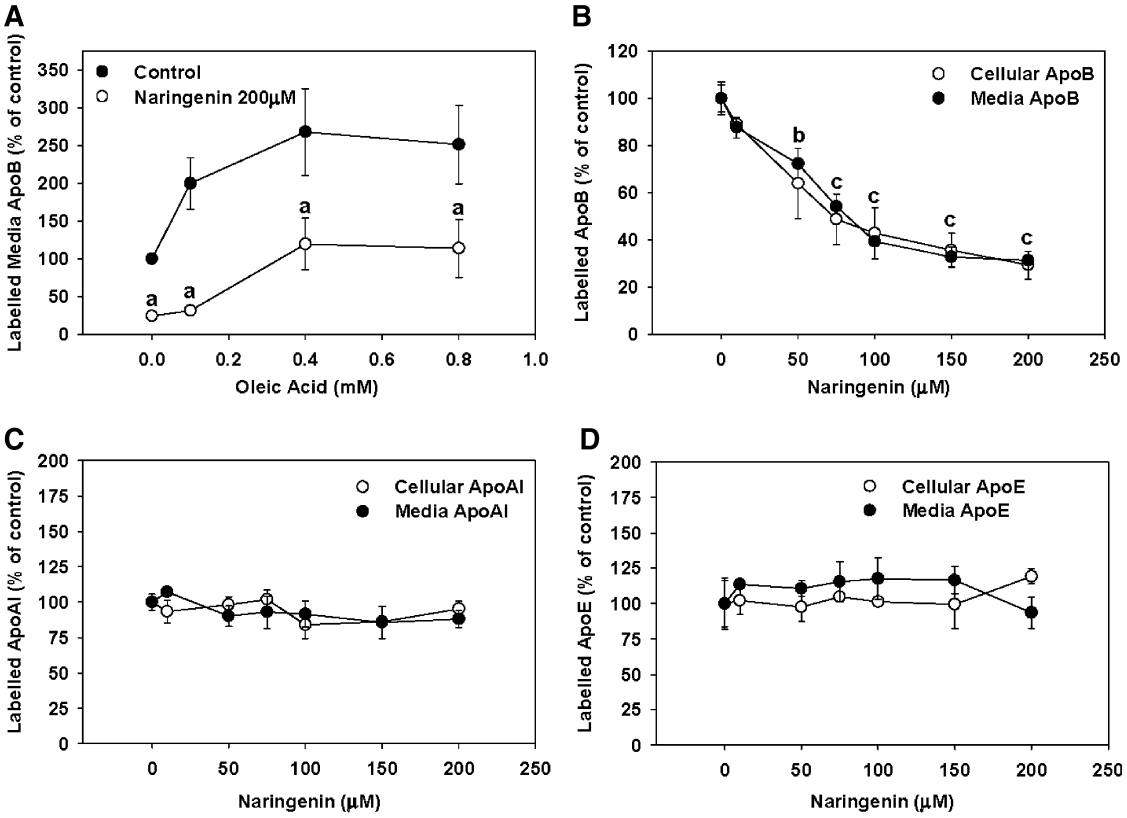

Fig. 2. Effect of naringenin on the secretion of newly synthesized apoB, apoAI, and apoE in the presence of oleic acid. HepG2 cells were preincubated for 24 h with various concentrations of naringenin. Cells were incubated for 20 min with either increasing doses of oleic acid (A) or 0.1 mM oleic acid (B, C, and D) prior to pulse-labeling for 10 min with [^{35}S]methionine. Cells were then chased for 60 min. Naringenin and oleic acid were present throughout the pulse and the chase. ApoB (A and B), apoA-I (C), and apoE (D) were immunoprecipitated from the media and cell lysates following 60 min of chase, as described in Materials and Methods. Values are means ± SEM for at least three experiments. a, $P < 0.05$; b, $P < 0.01$; and c, $P < 0.001$ compared with control.

Consequently, the percent degraded was significantly reduced. The increase in secretion was due to a 29% ($P < 0.02$) increase in the rate constant for the pathway through which apoB is destined for secretion, k(4,3). The resulting reduction in degradation was entirely due to a significant decrease in the rate constant for the rapid degradation pathway, k(0,3), while the slow degradation pathway, k(7,3), was unaffected. These selective changes in degradation rate constants resulted in a significant decrease in the proportion of apoB degraded via the rapid pathway.

In cells treated with oleate (0.1 mM) plus naringenin (200 μM), the kinetic parameters altered by treatment with oleate alone were changed in the opposite direction (Table 1). The percent of apoB secreted was significantly reduced compared with both oleate-treated (−58%) and control cells (−41%), while total degradation was significantly increased. The decreased secretion was due to significant reductions in the rate constant for secretion, k(4,3), compared with both oleate-treated (−56%) and control (−43%) cells. The resulting increase in degradation was again entirely due to a significant change in the rate constant for rapid degradation. This parameter, k(0,3), was increased by 11% ($P < 0.02$) compared with oleate-treated cells. Because the slow degradation pathway, k(7,3), was again unaffected, the proportion of apoB degraded via the rapid pathway was significantly increased compared with both oleate-treated and control cells.

The kinetically defined, rapid degradation pathway is ALLN- and lactacystin-sensitive

To determine the mechanism responsible for degradation via the rapid pathway, pulse-chase experiments were conducted, as described above, in the absence or presence of ALLN (10 μM). This compound effectively inhibits the proteasome in HepG2 cells, thereby reducing degradation of newly synthesized apoB (22, 23, 47). Prior to the start of each experiment, cells were incubated for 20 min with or without ALLN (10 μM). Media and cell lysates were collected at nine time points throughout the pulse and the chase. Data points in **Figs. 4A, B** represent apoB radioactivity measured experimentally, and the curves are fits to the data obtained from analyses using the multicompartmental model shown in Fig. 1. Inspection of the curves in Fig. 4A showed that ALLN markedly increased the secretion of newly synthesized apoB, while the rate of intracellular degradation was simultaneously reduced, as indicated by the total apoB radioactivity curve (Fig. 4B). The dramatic increase in peak apoB radioactivity in ALLN-treated cells suggests that cotranslational degradation was substantially inhibited. Qualitatively similar results were obtained with lactacystin (10 μM), a specific proteasome inhibitor (23, 28).

The key kinetic parameters determined by the multicompartmental model are shown in **Table 2**. ALLN (10 μM) significantly decreased the percent of apoB degraded, and this was entirely due to a 37% ($P < 0.05$) decrease in the rate constant for the rapid degradation pathway, k(0,3). The slow degradation pathway, k(7,3), was not significantly affected. These selective changes in degradation rate constants resulted in a 28% ($P < 0.05$) decrease in the proportion of apoB degraded via the rapid pathway. Consequent to this decreased degradation, the percent of apoB secreted was increased 3-fold ($P < 0.05$) due to a 3-fold increase in the rate constant for secretion, k(4,3). The specific proteasome inhibitor, lactacystin, also decreased total apoB degradation. This was, again, entirely due to a 24% decrease in rapid degradation, whereas slow degradation was unaffected.

Naringenin reduces CE synthesis and mass in oleate-treated cells

To determine whether naringenin could inhibit cholesterol esterification under conditions of increased fatty

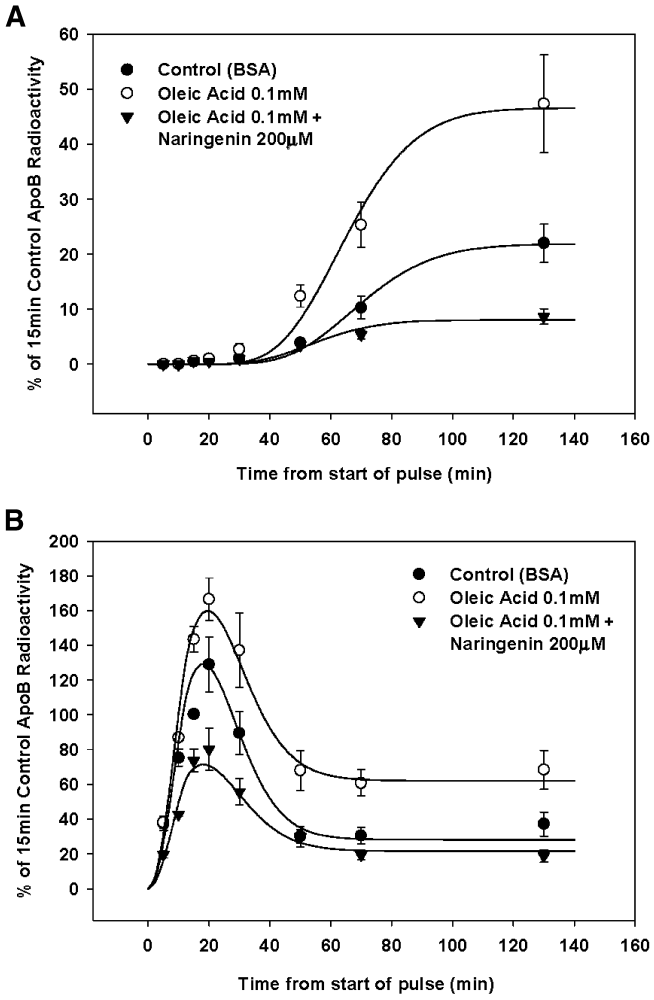

Fig. 3. Effect of oleic acid and naringenin on the secretion and intracellular degradation of apoB. HepG2 cells were preincubated for 24 h in the absence (closed circles, open circles) or presence (closed triangles) of naringenin. Cells were then incubated for a further 20 min in the absence (closed circles) or presence (open circles, closed triangles) of oleic acid prior to pulse labeling (10 min). Cells were then chased for 0–120 min. Naringenin and oleic acid were present throughout the pulse and the chase as indicated. ApoB-100 radioactivity secreted into the media is shown in A. Total apoB-100 radioactivity, determined as the sum of apoB in the media plus in the cell, is shown in B. Data points represent the observed values and are means ± SEM for four experiments. The lines are the best fit generated by the kinetic model shown in Fig. 1.

TABLE 1. Kinetic parameters of apoB secretion and intracellular degradation in the presence of oleic acid and naringenin

Parameter	Control (BSA)	Oleic Acid (0.1 mM)	Oleic Acid (0.1 mM) Naringenin (200 mM)
Total secreted (%)[d]	3.74 ± 0.37[a]	5.17 ± 0.59[b]	2.19 ± 0.29[c]
Total degraded (%)[e]	96.26 ± 0.37[a]	94.83 ± 0.59[b]	97.81 ± 0.29[c]
k(4,3) (pools/min)[f]	0.007 ± 0.001[a]	0.009 ± 0.002[b]	0.004 ± 0.001[c]
k(0,3) (pools/min)[f]	0.165 ± 0.020[a]	0.150 ± 0.020[b]	0.167 ± 0.014[a]
k(7,3) (pools/min)[f]	0.015 ± 0.004[a]	0.015 ± 0.005[a]	0.012 ± 0.005[a]
Proportion degraded via rapid pathway (%)[g]	88.24 ± 0.02[a]	86.21 ± 0.02[b]	91.26 ± 0.02[c]

Apolipoprotein B (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 experiments.

[a-c] Means not sharing a lowercase superscript are significantly different, $P < 0.02$.

[d] Calculated using the formula $[k(4,3)/k(4,3) + k(0,3) + k(7,3)] \times 100$.

[e] Calculated using the formula $[k(0,3) + k(7,3)/k(4,3) + k(0,3) + k(7,3)] \times 100$.

[f] k(4,3) is the rate constant for apoB transfer from compartment three to compartment four, or the rate constant for secretion. k(0,3) is the rate constant of apoB degradation directly from compartment three (rapid degradation). k(7,3) is the rate constant for apoB transfer from compartment three to compartment seven.

[g] The percent of apoB degraded directly from compartment three is calculated using the formula $[k(0,3)/k(4,3) + k(0,3) + k(7,3)] \times 100$.

acid availability, HepG2 cells were preincubated with naringenin (24 h) prior to labeling for 2.5 h with [^{14}C]oleic acid. This protocol allowed direct comparison with pulse-chase experiments, during which the total length of exposure to oleic acid was also 2.5 h. Naringenin dose-dependently inhibited CE synthesis in the presence 0.1 mM oleic acid (**Table 3**). Cholesterol esterification was reduced 37% ($P < 0.05$) by 75 μM naringenin (the IC_{50} dose for apoB secretion) and 63% ($P < 0.01$) by 200 μM naringenin. The synthesis of triglyceride, however, was significantly increased by 32% ($P < 0.01$) at the highest dose of naringenin (Table 3). Cholesterol synthesis, as determined by [^{14}C]acetate incorporation over the same period of time, was not significantly altered by naringenin (Table 3).

The changes in cellular lipid biosynthesis resulted in corresponding changes in cellular lipid mass (**Table 4**). Cellular CE mass was dose-dependently reduced by up to 30% ($P < 0.01$) with 200 μM naringenin in the presence of oleic acid. Triglyceride mass, however, increased 23% ($P < 0.05$) with 200 μM naringenin, while cellular free cholesterol mass was unaffected.

Naringenin inhibits accumulation of newly synthesized CE and TG in the ER lumen

We previously established that naringenin inhibited ACAT activity and the expression of ACAT2 and postulated that reduced availability of CE within an ER "regulatory pool" for lipoprotein assembly contributed to the reduction of apoB secretion in naringenin-treated cells (36). Although the effects of naringenin are consistent with studies in which ACAT inhibition decreased apoB secretion in HepG2 cells, this correlation is not observed for all ACAT inhibitors (29). Recently, Wang, Tran, and Yao (6) showed that decreased availability of newly synthesized TG within the microsomal lumen inhibited apoB secretion. We hypothesized that for ACAT inhibition to decrease apoB secretion, reduced availability of newly synthesized CE within the microsomal lumen was required. Therefore, HepG2 cells were preincubated with naringenin (24 h) and labeled with [^{14}C]oleic acid (2.5 h). Cell homogenates were separated into cytosolic, microsomal membrane, and microsomal lumen fractions, and radiolabeled lipids were quantitated. For comparison, cells were also treated with two ACAT inhibitors (CI-1011 and DuP 128) and two HMG-CoA reductase inhibitors (atorvastatin and simvastatin). Previously, we demonstrated that all four compounds decreased ACAT activity and CE concentrations in HepG2 cells. However, only CI-1011 and atorvastatin inhibited apoB secretion (29, 30).

Naringenin dramatically reduced the accumulation of newly synthesized CE in the cytosol (−74%, $P < 0.001$), microsomal membranes (−77%, $P < 0.001$), and microsomal lumen (−82%, $P < 0.001$) (**Fig. 5A**). Similar results were observed for DuP 128, simvastatin, and atorvastatin with average reductions in CE accumulation of approximately 89%, 82%, and 83% in the cytosol, microsomal membranes, and microsomal lumen, respectively. However, only atorvastatin decreases apoB secretion (−44%) (30), while DuP 128 and simvastatin have no effect (29, 30). CI-1011, on the other hand, decreased the accumulation of newly synthesized CE only in the cytosol (−32%, $P < 0.05$). Although CI-1011 decreases apoB secretion by 42% (29), CE accumulation in the microsomal membranes and lumen was unchanged.

Naringenin also significantly inhibited the accumulation of newly synthesized TG, but only in the lumenal fraction (−52%, $P < 0.01$). Accumulation in the other two fractions was not significantly affected (Fig. 5B), although a trend to increased TG in the cytosol was observed. Dup 128, CI-1011, simvastatin, and atorvastatin had no significant effect on TG accumulation in any of the three fractions. However, there were trends to decrease TG in the microsomal lumen for CI-1011 and atorvastatin (−9% and −10%, respectively), while small increases were observed for Dup 128 and simvastatin.

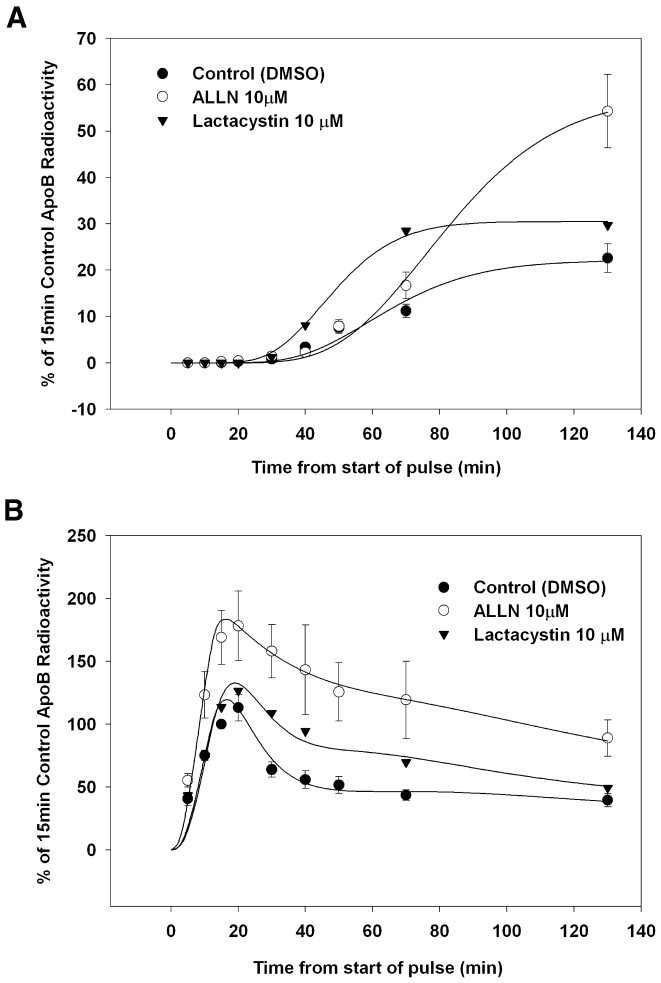

Fig. 4. Effect of *N*-acetyl-leucinyl-leucinyl-norleucinal (ALLN) and lactacystin on the secretion and intracellular degradation of apoB. HepG2 cells were incubated for 20 min with DMSO (closed circles), 10 μM ALLN (open circles), or 10 μM lactacystin (closed triangles) prior to pulse labeling (10 min). Cells were then chased for 0–120 min. ALLN and lactacystin were present throughout the pulse and the chase as indicated. ApoB-100 radioactivity secreted into the media is shown in A. Total apoB-100 radioactivity, determined as the sum of apoB in the media plus in the cell, is shown in B. Symbols represent the observed data points, and the lines are the best fit generated by the kinetic model shown in Fig. 1. Values for ALLN are means ± SEM for five experiments, and values for lactacystin are means for duplicate determinations.

TABLE 2. Kinetic parameters of apoB secretion and intracellular degradation in the presence of ALLN or lactacystin

Parameter	Control	ALLN (10 mM)	Lactacystin (10 mM)
Total secreted (%)[c]	6.14 ± 1.08[a]	18.34 ± 7.27[b]	8.64
Total degraded (%)[d]	93.86 ± 1.08[a]	81.66 ± 7.27[b]	91.36
k(4,3) (pools/min)[e]	0.014 ± 0.002[a]	0.042 ± 0.014[b]	0.017
k(0,3) (pools/min)[e]	0.172 ± 0.015[a]	0.109 ± 0.033[b]	0.130
k(7,3) (pools/min)[e]	0.042 ± 0.005[a]	0.078 ± 0.020[a]	0.053
Proportion degraded via rapid pathway (%)[f]	75.44 ± 0.06[a]	47.60 ± 0.13[b]	64.90

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. Control and *N*-acetyl-leucinyl-leucinyl-norleucinal (ALLN) values are means ± SEM for five experiments.

[a,b] Means not sharing a lowercase superscript are significantly different, $P < 0.05$. Lactacystin values are means for duplicate determinations.

[c] Calculated using the formula [k(4,3)/k(4,3) + k(0,3) + k(7,3)] × 100.

[d] Calculated using the formula [k(0,3) + k(7,3)/k(4,3) + k(0,3) + k(7,3)] × 100.

[e] k(4,3) is the rate constant for apoB transfer from compartment three to compartment four, or the rate constant for secretion. k(0,3) is the rate constant of apoB degradation directly from compartment three (rapid degradation). k(7,3) is the rate constant for apoB transfer from compartment three to compartment seven.

[f] 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.

DISCUSSION

Hepatic overproduction of apoB-Lp is associated with a number of hyperlipoproteinemias [as reviewed in ref. (48, 49)]. Consequently, over the past two decades, much research has focused on treatments that inhibit the assembly and secretion of apoB-Lp (49). More recently, a surge of research has revealed the lipid-lowering potential of neutraceutical compounds, including flavonoids [as reviewed in ref. (37, 50, 51)]. We recently reported that the hypocholesterolemic effects of the citrus flavonoids naringenin and hesperetin observed in rodents (52–56) were likely due to reduced assembly and secretion of hepatic apoB-Lp (36). Incubation of HepG2 hepatocytes with naringenin or hesperetin reduced apoB accumulation in the media. These effects were accompanied by reduced ACAT activity, a selective decrease in ACAT2 expression, and reduced MTP activity and expression. Consequently, we proposed that these mechanisms reduced the availability of lipids, particularly CE, for the assembly of hepatic apoB-Lp. In the present study, we clearly established that naringenin decreased the secretion of apoB in HepG2 cells in which secretion had been stimulated with oleic acid. In fact, secretion was decreased below the level observed for cells cultured under basal conditions. Analysis of intracellular apoB kinetic pathways by multicompartmental modeling revealed that naringenin selectively enhanced a rapid degradation pathway. Furthermore, naringenin markedly decreased the accumulation of newly synthesized CE within the microsomal lumen; however, this was not the primary mechanism whereby naringenin decreased apoB secretion.

It is well established that oleic acid increases the secretion of apoB from HepG2 cells by increasing cellular triglyceride synthesis, thereby providing more neutral lipid for lipoprotein assembly, resulting in the protection of nascent apoB from degradation [as reviewed in ref. (2, 57)]. However, naringenin, even under conditions in which apoB secretion is doubled, substantially inhibits apoB secretion in a dose-dependent manner (Figs. 2A, B). The effect of naringenin on apoB secretion is specific, because apoA-I and apoE secretion are unaffected (Figs. 2C, D).

To clearly identify and quantitate the pathways involved in the secretion and degradation of apoB in hepatocytes, we recently developed a novel multicompartmental model to describe the kinetics of apoB metabolism in HepG2 cells (Fig. 1) (29). We used this approach to determine which intracellular pathways of apoB secretion and/or

TABLE 3. Effect of naringenin on cellular lipid biosynthesis in the presence of oleic acid

Treatment	Triglyceride	Cholesteryl Ester	Cholesterol
	nmol [^{14}C]oleate/ mg cell protein	*pmol [^{14}C]oleate/ mg cell protein*	*nmol [^{14}C]acetate/ mg cell protein*
Oleic acid (OA) 0.1 mM	0.37 ± 0.03	9.14 ± 1.03	0.32 ± 0.04
Naringenin 75 μM + OA 0.1 mM	0.46 ± 0.04	5.74 ± 0.64[a]	0.26 ± 0.04
Naringenin 200 μM + OA 0.1 mM	0.49 ± 0.01[b]	3.35 ± 0.90[b]	0.23 ± 0.05

HepG2 cells were incubated in D MEM containing 5% LPDS and 0.1 mM oleic acid conjugated to BSA in the absence or presence of naringenin at the concentrations indicated for 24 h. Subsequently, cells were incubated for 2.5 h with either [^{14}C]acetic acid or [^{14}C]oleic acid. Lipids were then extracted from cell monolayers, separated by thin layer chromatography, and label incorporated into each lipid species was quantitated by liquid scintillation. Cholesteryl ester (CE), FC, and TG synthesis are reported as the mean ± SEM for a minimum of four experiments with duplicate samples.

[a] $P < 0.05$, [b] $P < 0.01$ (vs. oleic acid alone).

degradation were affected by naringenin. The curves generated by the model to fit data points collected in pulse-chase experiments show that peak radioactivity in full-length apoB-100 is consistently reached at 20 min postpulse, or after 10 min of chase (Fig. 3A). 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. Changes in peak height of apoB radioactivity observed in the presence of oleic acid and oleic acid plus naringenin likely reflect decreased and increased cotranslational degradation, respectively. These changes are unlikely due to altered apoB synthesis, because neither oleic acid (43–46) nor naringenin (36) affects apoB mRNA levels, but may also reflect altered rates of chain elongation during translation. Examination of the data points and fitted curves indicated that oleic acid dramatically increased the secretion of newly synthesized apoB and inhibited cellular apoB degradation (Fig. 3). Naringenin, however, had the opposite effect. Even in the presence of oleic acid, naringenin markedly decreased apoB secretion and enhanced cellular apoB degradation.

Multicompartmental modeling analyses allowed us to identify and quantitate changes in the kinetics of intracellular pools of apoB resulting from oleic acid or naringenin treatment. Although previous pulse-chase studies have demonstrated that oleic acid stimulates apoB secretion in HepG2 cells, the impact of oleate on intracellular apoB kinetics has not been reported. Oleic acid increased apoB secretion 1.4-fold, resulting in decreased cellular degradation (Table 1). In contrast, addition of naringenin to oleic acid-treated cells inhibited apoB secretion by 58% and increased degradation. The changes in the percentage of apoB secreted and degraded were reflected by changes in the rate constants for secretion with each treatment. Oleic acid increased the rate constant for secretion, while naringenin in the presence of oleic acid had the opposite effect. The large proportion of apoB degraded in these cells (96% in control cells, Table 1) agrees well with previously reported estimates [as reviewed in ref. (2, 21)]. The analyses revealed that apoB not targetted for secretion is degraded by two kinetically distinct pathways in HepG2 cells (Fig. 1). The majority of newly synthesized apoB is degraded via a rapid pathway with a basal turnover of 0.165 pools/min. Thus, in control cells (BSA only), 16.5% of the total pool of newly synthesized apoB is degraded via this pathway each minute. By contrast, less than 2% of the apoB pool is catabolized by the slow degradation pathway, with a basal rate of 0.015 pools/min. Both treatments in this study selectively altered the rapid pathway. Oleic acid reduced degradation, while naringenin, in the presence of oleic acid, significantly increased degradation via this pathway. The slow pathway was unaffected. The changes observed are best reflected in the calculated values for the proportion of apoB degraded via the rapid pathway: a decrease with oleic acid and an increase following addition of naringenin (Table 1).

We previously observed this selective increase in degradation via the rapid pathway in HepG2 cells treated with either the HMG-CoA reductase inhibitor atorvastatin or the ACAT inhibitor CI-1011 (avasimibe) (29, 30). This suggests that treatments that alter the availability of cholesterol, CE, or TG selectively affect degradation via a kinetically defined rapid pathway. Previous reports have demonstrated that the majority of newly synthesized apoB is degraded via the cytosolic ubiquitin-proteosome pathway (22–26). Based on the proportion of apoB degraded by the rapid pathway defined by our model (86–92%, Table 1), we hypothesized that this pathway was largely proteosomal. To test this hypothesis, we performed pulse-chase experiments using ALLN (Fig. 4), which inhibits the 26S cytosolic proteasome, lysosomal cathepsins B and L, and calcium-dependent calpains. Multicompartmental modeling analyses revealed that ALLN selectively decreased the rate constant for rapid degradation 37% without affecting the slow degradative pathway (Table 2). Consequently, the proportion of apoB degraded via the rapid pathway in the presence of ALLN was significantly reduced, whereas degradation via the slow pathway was unaffected and, if anything, was increased. Similarly, the specific proteasome inhibitor, lactacystin, decreased rapid degradation without affecting the slow degradation pathway. Collectively, these results suggest that the kinetically defined, rapid pathway described in our model represents proteasomal degradation. The slow degradation pathway is not sensitive to either ALLN or lactacystin and thus is nonproteasomal and involves mechanisms yet to be defined.

We previously demonstrated that in HepG2 cells cultured under basal conditions, naringenin inhibits choles-

TABLE 4. Effect of naringenin on cellular lipid mass in the presence of oleic acid

Treatment	Triglyceride	Cholesteryl Ester	Cholesterol
		μg/mg cell protein	
Control	56.38 ± 5.29	6.60 ± 0.60	17.85 ± 0.64
Oleic Acid (OA) 0.1 mM	66.55 ± 2.97	6.98 ± 0.38	18.15 ± 0.62
Naringenin 75 μM + OA 0.1 mM	71.93 ± 10.79	5.92 ± 0.65	17.23 ± 0.18
Naringenin 200 μM + OA 0.1 mM	81.83 ± 7.54[b]	4.85 ± 0.38[a]	17.39 ± 0.43

HepG2 cells were incubated in MEM containing 5% LPDS and 0.1 mM oleic acid conjugated to BSA with naringenin at the concentrations indicated for 24 h. Subsequently, lipids were extracted from cell monolayers and quantitated. CE, FC, and TG mass are reported as the mean ± SEM for a minimum of four experiments with duplicate samples. CE mass is calculated as the difference between total and free cholesterol mass values.

[a] $P < 0.01$, [b] $P < 0.05$ (vs. oleic acid alone).

terol ester synthesis and increases triglyceride synthesis, with corresponding changes in cellular lipid mass (35, 36). In this study, we demonstrated that this was also the case for cells exposed to exogenous oleic acid. Naringenin dose-dependently reduced CE synthesis by up to 63% and increased TG synthesis by 32% in the presence of oleic acid (Table 3), with corresponding changes in lipid mass (Table 4). All changes were comparable to those observed under basal conditions (35, 36). Collectively, these experiments demonstrate that the naringenin-induced inhibition of apoB secretion, even in the presence of oleic acid, was associated with decreased cellular CE synthesis and mass. Furthermore, apoB secretion was decreased despite increased TG synthesis. The mechanism for this increase in TG synthesis is unknown; however, we have previously demonstrated that specific ACAT inhibitors increase TG synthesis in HepG2 cells (29).

Although naringenin simultaneously inhibits cholesterol esterification and apoB secretion, ACAT inhibition is not universally associated with reduced apoB secretion (29, 32–34). Therefore, the second major objective of this study was to determine the extent to which ACAT inhibition by naringenin is responsible for the reduction in apoB secretion. We hypothesized that, for ACAT inhibition to limit the availability of CE for lipoprotein assembly and secretion, a decrease in newly synthesized CE within

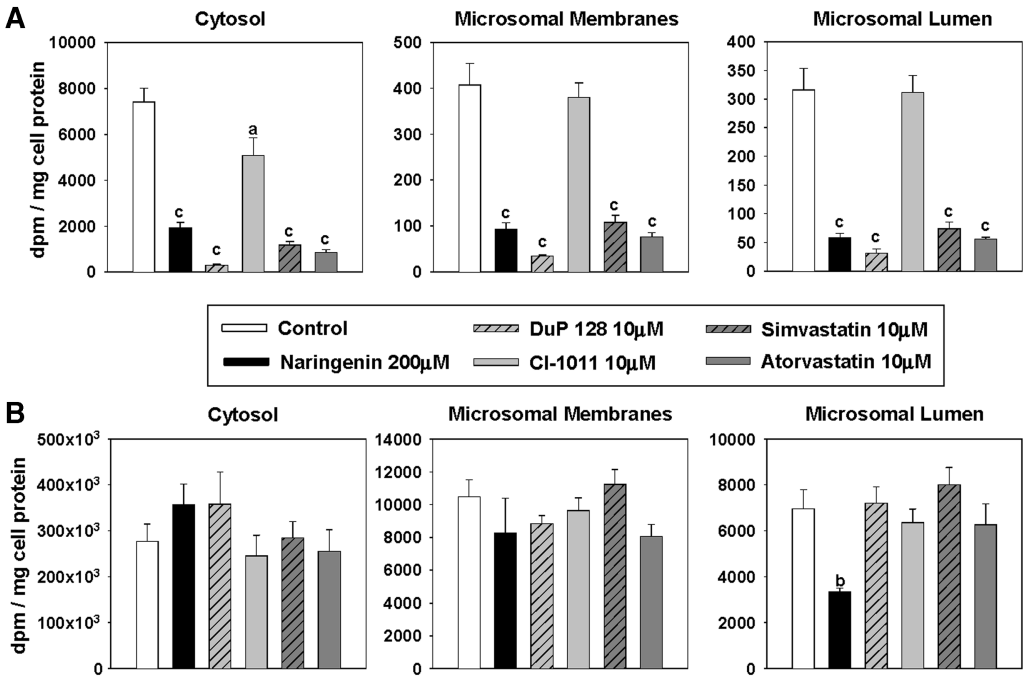

Fig. 5. Effect of naringenin and specific ACAT and HMG-CoA reductase inhibitors on the cellular distribution of newly synthesized cholesteryl ester (CE) and TG. HepG2 cells were preincubated for 24 h with or without naringenin, the ACAT inhibitors DuP 128 and CI-1011, or the HMG-CoA reductase inhibitors simvastatin and atorvastatin. Cells were continuously labeled for 2.5 h with [^{14}C]oleic acid such that the final concentration of oleic acid in the media was 0.1 mM. Naringenin, ACAT inhibitors, and HMG-CoA reductase inhibitors were present during labeling at the concentrations indicated. Cells were then homogenized and subcellular fractions were isolated (cytosol, microsomal membranes, and microsomal lumen), as described in Materials and Methods. Lipids in each fraction were extracted, and CE (A) and TG (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 four experiments. a = $P < 0.05$, b = $P < 0.01$, and c = $P < 0.001$ compared with control.

the microsomes would be required. We postulated that the "regulatory" pool of CE involved in apoB-Lp assembly would be found in the micrososmal (ER) lumen and that treatments that reduced this pool would also inhibit apoB secretion. For naringenin, this scenario is particularly appealing because we recently showed that this flavonoid selectively downregulates ACAT2 expression in HepG2 cells without affecting ACAT1 (36). Based on the discovery that the catalytic site of ACAT2 faces the ER lumen, it is believed that this isoform provides CE for association with apoB and that this step may be rate-limiting (17). To address this possibility, we measured newly synthesized CE in the cytosol, microsomal membranes, and microsomal lumen of HepG2 cells treated with naringenin and specific inhibitors of either ACAT or HMG-CoA reductase. Naringenin, DuP 128, simvastatin, and atorvastatin significantly decreased CE within the microsomal lumen to a similar extent (Fig. 5A). Of these, only naringenin [this report and ref. (35, 36)] and atorvastatin (30) decrease apoB secretion in HepG2 cells. CI-1011, on the other hand, had no effect on lumenal accumulation of newly synthesized CE, but does inhibit apoB secretion (29). This indicated that ACAT inhibition by naringenin, resulting in decreased lumenal CE, is not the primary mechanism whereby this flavonoid decreases apoB secretion. Furthermore, these results demonstrated that lumenal CE does not regulate apoB-Lp assembly and secretion in HepG2 cells.

The impact of naringenin on the accumulation of newly synthesized TG in subcellular fractions was also examined. Despite increased TG synthesis by naringenin, leading to increased cytosolic TG, the accumulation of newly synthesized TG in the ER lumen was reduced by 52% (Fig. 5B). This decrease in lumenal TG is likely due to the ability of naringenin to inhibit MTP, a property we recently reported (36). This conclusion is consistent with the findings of Wang, Tran, and Yao (6) who reported that in McA-RH7777 rat hepatocytes, inhibtion of MTP activity by 50% reduced TG accumulation in the lumen by approximately 60%, leading to a 75% reduction in apoB secretion. Interestingly, we observed slight reductions in TG accumulation (10%) in the lumenal fractions of cells treated with either CI-1011 or atorvastatin. Although these reductions were small, the reductions in apoB secretion with these compounds (30–40%) (29, 30) are less dramatic than that achieved with naringenin (80%). The compounds that do not affect apoB secretion, DuP 128 (29) and simvastatin (30), did not reduce lumenal TG accumulation. It is possible that HepG2 cell apoB secretion is very sensitive to changes in TG availability in the ER lumen, and this may contribute to the ability of both CI-1011 and atorvastatin to inhibit apoB secretion in these cells. However, detailed dose-response experiments comparing conditions which alter lumenal TG levels and apoB secretion are required to fully test this hypothesis.

We conclude that naringenin dramatically inhibits secretion of newly sythesized apoB from oleate-stimulated HepG2 cells with a selective increase in intracellular apoB degradation via a kinetically defined, rapid pathway–a pathway also activated in hepatocytes treated with compounds that reduce cellular CE content (29, 30). Although naringenin effectively inhibits ACAT, CE availability in the ER lumen, the site of initial apoB-Lp assembly, does not appear to regulate apoB secretion in HepG2 cells. Rather, the ability of naringenin to limit the accumulation of newly synthesized TG in the ER lumen via MTP inhibiton may be the primary mechanism blocking apoB secretion.

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