Differential regulation of apolipoprotein B secretion from HepG2 cells by two HMG-CoA reductase inhibitors, atorvastatin and simvastatin

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Abstract **The concept that hepatic cholesterol synthesis regulates hepatocyte assembly and secretion of apoB-containing lipoproteins remains controversial. The present study was carried out in HepG2 cells to examine the regulation of apoB secretion by the HMG-CoA reductase inhibitor atorvastatin. ApoB accumulation in the media was decreased by 24% and 36% at 10 μM ($P < 0.02$) and 20 μM ($P < 0.01$) of atorvastatin, respectively. Atorvastatin inhibited HepG2 cell cholesterol synthesis by up to 96% ($P < 0.001$) and cellular cholesteryl ester (CE) mass by 54% ($P < 0.001$). Another HMG-CoA reductase inhibitor, simvastatin, decreased cellular cholesterol synthesis and CE mass by up to 96% ($P < 0.001$) and 52% ($P < 0.001$), respectively. However, in contrast to atorvastatin, simvastatin had no effect on apoB secretion. To characterize the reduction in apoB secretion by atorvastatin (10 μM), pulse-chase experiments were performed and the kinetic data were analyzed by multicompartmental modeling using SAAM II. Atorvastatin had no affect on the synthesis of apoB, however, apoB secretion into the media was decreased by 44% ($P = 0.048$). Intracellular apoB degradation increased proportionately ($P = 0.048$). Simvastatin (10 μM) treatment did not significantly alter either the secretion or intracellular degradation of apoB, relative to control. The kinetics of apoB degradation were best described by a rapidly and a slowly turning over degradation compartment. The effect of atorvastatin on apoB degradation was largely confined to the rapid compartment. Neither inhibitor affected apoB mRNA concentrations, however, both significantly increased LDL receptor and HMG-CoA reductase mRNA levels. Atorvastatin treatment also decreased the mRNA for the microsomal triglyceride transfer protein (MTP) by 22% ($P < 0.02$). These results show that atorvastatin decreases apoB secretion, by a mechanism that results in an enhanced intracellular degradation of apoB.**—Wilcox, L. J., P. H. R. Barrett, and M. W. Huff. **Differential regulation of apolipoprotein B secretion from HepG2 cells by two HMG-CoA reductase inhibitors, atorvastatin and simvastatin.** ***J. Lipid Res.*** **1999.** 40: **1078–1089.**

Supplementary key words HMG-CoA reductase inhibitor • apoB • HepG2 cells • cholesterol synthesis • atorvastatin • simvastatin

Apolipoprotein B-100 (apoB), an essential structural component of very low density lipoproteins (VLDL), intermediate density (IDL) and low density lipoproteins (LDL) (1, 2), is required for the intracellular assembly and secretion of these lipoproteins, and serves as a ligand for receptor-mediated clearance. The regulation of apoB assembly into lipoproteins, and their secretion from the liver, is thought to occur primarily at post-transcriptional levels. These processes are complex and involve the translocation of apoB across the endoplasmic reticulum (ER) membrane and the intracellular degradation of apoB (3, 4). Several factors have been suggested to play key roles in regulating apoB secretion at these levels, including the availability of surface (phospholipid and free cholesterol) and core (triglyceride and cholesteryl ester) lipoprotein lipids.

The importance of hepatic triglyceride (5–9) and phospholipid (10) in regulating the secretion of apoB from cultured hepatocytes has been well documented. The role of hepatic free and esterified cholesterol in the assembly and secretion of apoB-containing lipoproteins, however, remains controversial. The regulation of apoB secretion by the rate of cholesterol synthesis, esterification, and/or the mass of cholesteryl ester (CE) has been documented in vitro (11–16) and in vivo (17–22). In contrast, several studies in HepG2 cells argue against the regulation of apoB by free cholesterol (FC) and/or CE availability (5, 7–9, 23). For example, Wu et al. (8) have shown that apoB

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; ALLN, N-acetyl-leucinyl-leucinyl-norleucinal; apo, apolipoprotein; CE, cholesteryl ester; DMSO, dimethylsulfoxide; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FC, free cholesterol; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; mRNA, messenger ribonucleic acid; MTP, microsomal triglyceride transfer protein; TC, total cholesterol; TG, triglyceride; PL, phospholipid; VLDL, very low density lipoprotein.

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secretion from HepG2 cells was unaffected by either long or short term changes in cellular CE content. Similar conclusions were reported by Furukawa and Hirano (7) and Sato, Imanaka, and Takano (23). Clearly, the role of free and esterified cholesterol in the assembly and secretion of apoB-containing lipoproteins is not fully understood.

The endoplasmic reticulum enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, catalyzes the reductive deacylation of HMG-CoA to mevalonate. This rate-limiting step in cholesterol synthesis can be inhibited by a group of compounds known as the HMG-CoA reductase inhibitors, or statins. These inhibitors have been widely used to lower plasma cholesterol levels; however, considerable debate exists over the mechanism by which these compounds lower plasma concentrations of apoB-containing lipoproteins. HMG-CoA reductase inhibitors have been shown to up-regulate LDL receptors, thereby enhancing LDL catabolism (24, 25). In addition, these inhibitors have been shown to inhibit the hepatic production of apoB-containing lipoproteins in vitro (12, 15) and in vivo (19–22, 26); however, the intracellular mechanisms involved have not been determined.

Atorvastatin and simvastatin are two HMG-CoA reductase inhibitors that have been shown to decrease the hepatic production of apoB-containing lipoproteins in vivo (20–22). Kinetic studies from our laboratory, performed in miniature pigs, have shown that atorvastatin decreases VLDL and LDL apoB production (20). In human studies, simvastatin decreased hepatic VLDL apoB secretion rates in both normolipidemic (22) and heterozygous familial hypercholesterolemic subjects (21). While both inhibitors have been shown to decrease hepatic apoB secretion, atorvastatin, in human and animal studies, has been found to cause greater reductions in LDL (27–29) or plasma (30, 31) cholesterol, triglyceride (27, 28, 30) and apoB (28) than milligram equivalent doses of other statins, including simvastatin. Therefore, we used atorvastatin and simvastatin as probes to investigate the effect of hepatic cholesterol synthesis inhibition on apoB secretion from HepG2 cells. Specifically, this study was conducted to determine any mechanistic differences between the two inhibitors by examining their effects on apoB secretion, lipid synthesis, and messenger RNA abundance for the LDL receptor, HMG-CoA reductase, apoB, and MTP. In addition, we examined whether the effect of HMG-CoA reductase inhibition on apoB secretion is associated with changes in intracellular apoB degradation. For the second objective, kinetics of intracellular apoB metabolism were determined using the novel approach of multicompartmental modelling, using SAAM II to analyze pulse-chase studies.

We demonstrate that atorvastatin is more effective than simvastatin in reducing apoB secretion from HepG2 cells, despite nearly identical inhibition of cholesterol synthesis, and induction of both LDL receptor and HMG-CoA reductase mRNA. Kinetic analysis revealed that the decreased secretion of apoB with atorvastatin treatment involves an enhanced intracellular degradation of apoB, primarily from a rapid degradation compartment. These results suggest that in HepG2 cells, atorvastatin, but not simvastatin, inhibits a pathway of apoB secretion involving a mechanism which cannot be explained simply by differences in the extent of cholesterol synthesis inhibition.

EXPERIMENTAL PROCEDURES

Cell Culture

HepG2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and grown as described previously (32). For experiments, HepG2 cells plated in either 100 mm or in 6-well (35 mm) culture plates (Falcon Scientific) were maintained in MEM containing 5% human lipoprotein-deficient serum (LPDS). The appropriate concentrations of HMG-CoA reductase inhibitors, solubilized in dimethyl sulfoxide (DMSO, concentration not to exceed 0.5%), were added to the dishes. Atorvastatin (sodium salt of the open acid form) was provided by Parke-Davis Pharmaceutical Research (Ann Arbor, MI) and simvastatin (sodium salt of the open acid form) was provided by Merck, Sharpe & Dohme (Rahway, NJ). In some experiments, 0.8 mm oleic acid complexed to fatty acid free bovine serum albumin (BSA, Sigma) in a molar ratio of 5:1 was added to the cells with the inhibitors.

After 24-h incubations, the media were collected and centrifuged at 2500 rpm (IEC Centra-8R centrifuge) for 10 min to remove detached cellular debris. The supernatant was immediately stored at −80°C until measurement of apoB concentrations. The cells were washed, cellular lipids were extracted, and the cell protein was analyzed, as described previously (8).

ApoB mass quantitation

ApoB concentrations in media were measured by enzyme-linked immunosorbent assay (ELISA) using a monoclonal human apoB antibody (Calbiochem, La Jolla, CA) as the capture antibody and an affinity-purified polyclonal apoB antibody conjugated to peroxidase (The Binding Site, Birmingham, UK) for detection. A purified human LDL standard (d 1.030–1.050 g/ml) was used to calibrate the assay, which was carried out as described previously (20). Media samples were diluted 20-fold for analysis. ApoB mass results are reported as μg per mg cell protein.

Lipid mass quantitation

Cellular triglyceride (TG), free cholesterol (FC), and total cholesterol (TC) were quantitated by a modification of the method of Carr, Andresen, and Rudel (33), using enzymatic reagents from Boehringer Mannheim (Indianapolis, IN) as described previously (34), with the following exception. For the determination of triglyceride mass, samples were diluted 2:5 (v:v) with a 2% Triton X-100 (in deionized H_2O, v/v) solution. Cellular lipid results are reported as μg of cellular lipid (CE, TG, or FC) per mg cell protein.

Lipid synthesis and secretion

The incorporation of [1-^{14}C]oleic acid (Amersham, Oakville, ON) or [1-^{14}C]acetic acid (Amersham, Oakville, ON) into cellular lipids was determined by a modification of the methods described previously (32). Each dish received 0.08 μCi of [1-^{14}C]oleic acid (50 mCi/mmol) complexed with fatty acid-free BSA or 0.5 μCi acetic acid (57 mCi/mmol). Incubations were carried out for 5 h. The cells were washed and the lipids were extracted in situ as described above. Extracted lipids from cells incubated with [1-^{14}C]acetic acid were divided into two equal fractions. One fraction was saponified as described previously (32) for the determination of TC after thin-layer chromatography (TLC). The other fraction and the lipid extract from [1-^{14}C]oleic

acid incorporation studies were not saponified. Radioactivity incorporated into lipid fractions was determined after TLC. The determination of radiolabeled CE, TG, and PL secreted into the media was performed as described above, but after 24-h incubations (with statins and [1-^{14}C]acetic acid or oleic acid). Lipids were recovered from the media by the method of Folch, Lees, and Sloane Stanley (35) and separated by TLC.

Analysis of lipoprotein fractions

The distribution of secreted apoB into lipoproteins with the density of VLDL (d < 1.006 g/ml), IDL (d 1.006–1.019 g/ml), and LDL (d 1.019–1.063 g/ml) was determined after the incubation of cells with 50 μCi (150 Ci/mmol) l-[4,5-^{3}H]leucine (Amersham) for 24 h. The media were removed after the incubation and mixed with 4 ml normal human plasma. VLDL, IDL, and LDL fractions were isolated by ultracentrifugation, as described previously (26). ApoB was isolated from each lipoprotein fraction by isopropanol precipitation (26) and analyzed by scintillation counting.

Pulse-chase experiments

Cells were preincubated with atorvastatin (10 μm), simvastatin (10 μm), or DMSO (control) for 24 h prior to metabolic labelling. The cells were washed twice with PBS (37°C) and incubated with methionine-free MEM (ICN, Aurora, OH) for 20 min. Cells were then 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 5–130 min in MEM containing 10 mm methionine and 5 mm cysteine. Statins or DMSO were present for each incubation. The media were collected and mixed with an equal volume of solubilization buffer (PBS containing 1% Nonidet P-40, 1% deoxycholate, 5 mm EDTA, 1 mm EGTA, 2 mm PMSF, 0.1 mm leupeptin, and 0.5 μm N-acetyl-leucinyl-leucinyl-norleucinal (Sigma)). The cells were washed with PBS and solubilized in 1 ml of solubilization buffer. Cell lysates were centrifuged at 14000 rpm for 5 min. The supernatants were used for immunoprecipitation.

To assess the uptake of newly secreted apoB-containing lipoproteins, untreated cells were pulsed with Tran [^{35}S] label for 10 min and chased for 60 min as outlined above. This media, containing [^{35}S] apoB-lipoproteins, was collected and placed on other cells that had been pre-treated for 24 h with atorvastatin (10 μm) or DMSO alone (control). The labeled media were collected after 60 min, and apoB was immunoprecipitated as outlined below.

Immunoprecipitation of cell lysates and media was carried out by a method similar to that described by Adeli (36), with some modification. Briefly, aliquots of cell extract and media were incubated with 10 μl preimmune rabbit serum for 1 h. Insoluble Protein A (*Staphylococcus aureus* suspension, Sigma) (30 μl) was added to each sample and incubated for a further 1 h. Each tube was centrifuged at 14,000 rpm for 5 min in a microcentrifuge and the supernatant was transferred to a tube containing 5 μl anti-apoB antiserum (Boehringer-Mannheim) or human anti-albumin antibody (The Binding Site, Birmingham, UK). The samples were incubated overnight at 4°C, with rocking. Insoluble Protein A suspension (75 μl) was added to each sample and incubated with rocking for 3 h at 4°C and then centrifuged at 14,000 rpm for 2 min. Immunoprecipitates were washed 3 times with wash buffer (PBS containing 1% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 5 mm EDTA). Immunoprecipitates were prepared for SDS-PAGE by suspending and boiling in 80 μl of electrophoresis buffer (10% SDS, 0.5 m Tris, pH 6.8, β-mercaptoethanol, 8% glycerol, and 0.1% bromophenol blue).

An aliquot of each sample, based on cell protein, was used for SDS-PAGE (3–15% gradient gel), as described by Laemmli (37). The gels were dried and the radioactivity in each band, corresponding to apoB or albumin, was determined using a phosphoimager system (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software (Molecular Dynamics). Radioactivity associated with apoB from cells after 5 min of chase was used as the maximal rate of incorporation into apoB. All time points were normalized to this value.

Development of a multicompartmental model

The pulse-chase data were analyzed by multicompartmental modeling using the SAAM II program (SAAM Inst., Seattle, WA). A compartmental model of apoB synthesis, secretion, and degradation was developed using apoB radioactivity data from HepG2 cells and media. **Figure 1** shows the compartments and the pathways that connect the compartments. Briefly, a simplified description of the model development is as follows. Initially, an intracellular compartment (compartment 1) was included to represent the dosing compartment (i.e., the [^{35}S]methionine). Although the tracer was added to the media, it was assumed that the transport of the tracer into the cells was essentially instantaneous. A second compartment, a delay compartment (compartment 2) was added to represent the time from the initial pulse of radioactivity until cellular radioactivity in immunoprecipitable apoB-100 was detected. Two compartments were then added to describe the kinetics of intracellular apoB radioactivity. ApoB radioactivity first enters a kinetically defined compartment

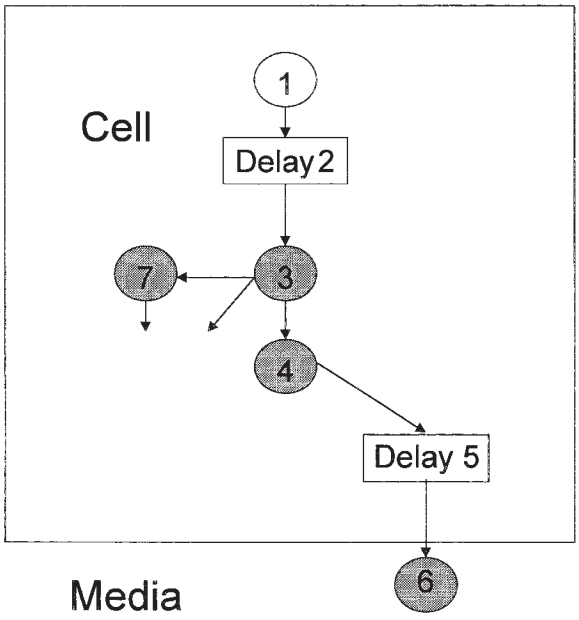

Fig. 1. Diagram of the multicompartmental kinetic model used for analysis of apoB secretion and intracellular degradation. Compartments 1–5 and 7 are within the HepG2 cell. Compartment 6 represents apoB in the culture media. Compartments 1 and 2 represent an intracellular pool of tracer and a delay compartment to allow for apoB synthesis after introduction of the tracer, respectively. Compartment 3 represents newly synthesized apoB that may be transferred to compartment 4 and subsequently secreted. From compartment 4, apoB passes through a delay compartment, compartment 5, prior to secretion into the media, compartment 6. ApoB may be degraded directly from compartment 3 by a rapid degradation pathway. A second, more slowly turning over pool of apoB destined for degradation is represented by compartment 7. The shaded compartments represent the compartments containing apoB radioactivity that is determined experimentally.

(compartment 3) and subsequently appears in a second, kinetically defined compartment (compartment 4). Intracellular apoB radioactivity was allowed to distribute between these two compartments (compartments 3 and 4) in such a manner as to best fit the experimental data. An extracellular compartment (compartment 6) was included to represent media apoB radioactivity and all of the apoB experimental data derived from media apoB radioactivity was assigned to this compartment. The compartmental model was fit to each data set (cell apoB and media apoB) by taking into account the pulse administration of the [^{35}S]methionine for 10 min. In order to optimize the fit, a delay compartment was introduced to represent the time it takes for apoB to appear in the media (compartment 5). Further, a second, more slowly turning over degradation compartment (compartment 7), containing apoB derived from compartment 3, was required to fit the rate of degradation defined by the experimental data. The addition of a pathway for apoB secretion directly from compartment 3 or for apoB degradation from compartment 4 was tested but neither of these pathways was required to fit the model to the experimental data.

RNase protection analysis of HepG2 mRNA

A HindIII/PstI fragment of human apoB, cloned into psp72 (Promega) (provided by Dr. N. Azrolan, Rockefeller University, NY), a Hind III/Xba I fragment from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ATCC, Rockville, MD) subcloned into pGEM-7Zf (Promega), a Pst I/Pst I fragment from the human LDL receptor (ATCC) subcloned into pbluescript SK+ (Stratagene), a Hind III/Pst I fragment of the human HMG-CoA reductase (ATCC) subcloned into pbluescript SK+, and a Pst I/Pst I fragment of human MTP (provided by Dr. J. R. Wetterau, Bristol-Myers Squibb) in pbluescript SK+, served as templates to synthesize antisense RNA probes. These riboprobes were used to measure mRNA concentrations in a modification of the RNase protection/solution hybridization assay of Azrolan and Breslow (38), as described previously (20). RNA was assayed in duplicate. Within assay coefficients of variation were <10%.

Statistical treatment of results

All values are presented as mean ± SEM (standard error of mean). Tests for statistical significance of differences were compared by unpaired *t* test for all experiments, with the exception of pulse-chase analysis which was compared by paired *t* test. A *P* value <0.05 was considered significant. The relationship between cellular CE mass and apoB secretion was determined by regression analysis.

RESULTS

Modulation of apoB secretion from HepG2 cells by HMG-CoA reductase inhibition

After 24-h incubations with HMG-CoA reductase inhibitors (10 nm to 20 μm), apoB in the media of HepG2 cells decreased by 24% ($P < 0.02$) and 36% ($P < 0.01$) at 10 μm and 20 μm of atorvastatin, respectively (**Table 1**). In contrast, apoB in the media was unchanged by simvastatin treatment at the same inhibitor concentrations. As shown in Table 1, oleate treatment (0.8 mm) induced a 2-fold increase in apoB secretion. Incubation of HepG2 cells with atorvastatin (10 μm) decreased apoB secretion by 22% ($P < 0.001$) compared to oleate-treated controls. In contrast, simvastatin (10 μm) did not significantly alter apoB in the media.

Effect on cell lipid mass

After a 24-h incubation, CE mass was decreased by 54% at 1 μm ($P < 0.001$), 54% at 10 μm ($P < 0.001$), and 62% at 20 μm ($P < 0.001$) of atorvastatin (**Fig. 2A**). Simvastatin decreased CE mass by up to 52% at 1 μm ($P < 0.001$), and was not further decreased by higher inhibitor concentrations (Fig. 2A). Atorvastatin (20 μm) caused a significantly greater decrease in CE mass compared to simvastatin at 20 μm ($P < 0.01$). The decrease in cellular CE mass observed with increasing concentrations of atorvastatin was positively correlated with the decrease in apoB secretion into the media ($r = 0.63$, $P < 0.003$), however, the cellular CE mass in simvastatin-treated cells did not ($r = 0.08$, $P = 0.74$). FC mass was decreased significantly by 13% at 10 μm of atorvastatin ($P < 0.05$) and by 19% ($P < 0.05$) and 12% ($P < 0.05$) at 10 μm and 20 μm of simvastatin, respectively (Fig. 2B). TG mass was not affected by atorvastatin treatment. However, simvastatin caused a significant increase in TG mass at a concentration of 20 μm (Fig. 2C). Incubation of HepG2 cells with 0.8 mm oleate resulted in a 3-fold increase in cellular TG mass and a 50% increase in CE mass after a 24-h incubation (data not shown). Atorvastatin decreased the cellular CE mass by 24% (NS), 43% ($P < 0.05$), and 44% ($P < 0.05$), compared to oleate-treated control cells, at 10 nm, 1 μm, and 10 μm, respectively (Fig. 2D). Simvastatin decreased cellular CE mass by 16% (NS),

TABLE 1. ApoB accumulation in media

	Treatment Concentration				
	0	10 nm	1 μm	10 μm	20 μm
	μg apoB/mg cell protein/24 h				
Atorvastatin	1.23 ± 0.08	1.21 ± 0.11	1.12 ± 0.11	0.93 ± 0.04[a]	0.79 ± 0.06[b]
Atorvastatin + oleate	2.50 ± 0.16	ND	ND	1.94 ± 0.24[c]	ND
Simvastatin	1.23 ± 0.08	1.20 ± 0.07	1.17 ± 0.07	1.20 ± 0.12	1.31 ± 0.11
Simvastatin + oleate	2.50 ± 0.16	ND	ND	2.53 ± 0.30	ND

HepG2 cells were incubated for 24 h in MEM containing 5% LPDS, with or without 0.8 mm oleate (complexed with BSA in a 5:1 molar ratio). Values are given as mean ± SEM from four experiments with duplicate samples; ND, not determined.

[a] $P < 0.02$ vs. control (no inhibitor, no oleate).

[b] $P < 0.01$ vs. control (no inhibitor, no oleate).

[c] $P < 0.001$ vs. oleate-stimulated control (no inhibitor).

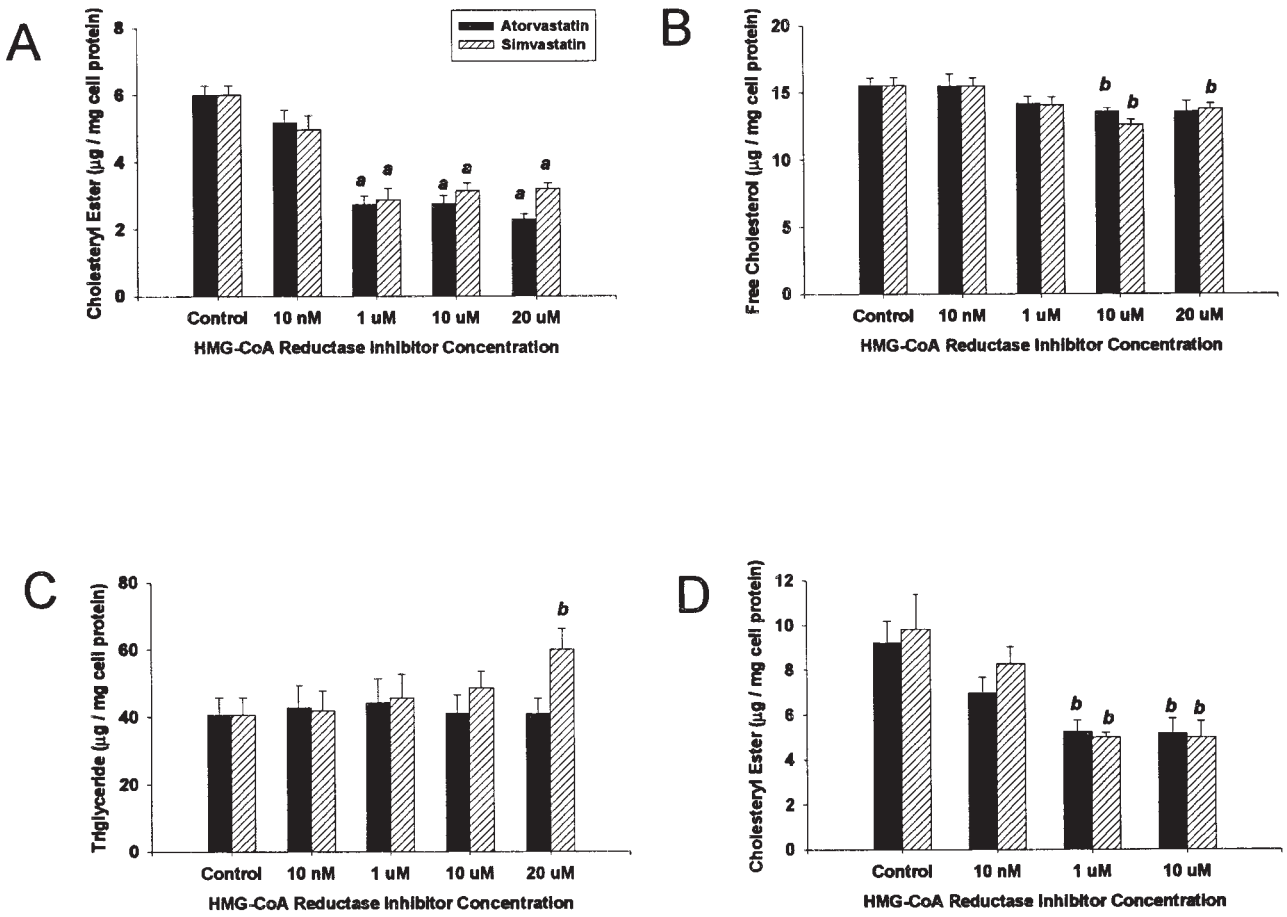

Fig. 2. Effect of atorvastatin and simvastatin on cellular lipid concentrations. HepG2 cells were incubated in MEM containing 5% LPDS with atorvastatin or simvastatin at concentrations of 10 nm, 1 μm, 10 μm, and 20 μm, for 24 h. After the incubation, the media were removed and the cells were washed. Lipids were extracted from cell monolayers and quantitated by spectrophotometric assays. CE mass (panel 2A), FC mass (panel 2B), and TG mass (panel 2C) are reported as the mean ± SEM for six experiments with duplicate samples for each condition. CE mass is calculated as the difference between total and free cholesterol mass values. The effect of atorvastatin and simvastatin on CE mass in oleate-loaded (0.8 mm) HepG2 cells (panel 2D) is reported as the mean ± SEM for four experiments. ***a*** indicates $P < 0.001$; ***b***, $P < 0.05$ compared to control.

49% ($P < 0.05$), and 49% ($P < 0.05$). In oleate-treated cells; the 3-fold increase in cellular TG was unaffected by either statin (data not shown). Free cholesterol mass, in oleate-treated cells, was reduced by 17% (NS) with atorvastatin (10 μm), and by 16% ($P < 0.05$) with simvastatin (10 μm) (data not shown).

Effect on cell lipid biosynthesis and secretion

Incorporation of [^{14}C]acetic acid or [^{14}C]oleic acid into cellular lipids was carried out from 0 h to 5 h or from 19 h to 24 h after the addition of atorvastatin (10 μm) or simvastatin (10 μm). This protocol allowed us to determine HMG-CoA reductase inhibition over time and to determine whether differences in apoB secretion are due to a difference in the metabolism or clearance of inhibitor from the hepatocyte, resulting in an attenuation of HMG-CoA reductase inhibition at later time points. As shown in **Table 2**, a reduction in the [^{14}C]oleic acid incorporation into CE was found for both inhibitors: 46% decrease for atorvastatin ($P < 0.01$) and 55% for simvastatin ($P < 0.01$) during the first 5-h incubation. This reduction was enhanced during the 19–24 h incubation period: 79% decrease for atorvastatin ($P < 0.001$) and 77% for simvastatin ($P < 0.001$). During the 0–5 h incubation, [^{14}C]oleic acid incorporation into TG was not significantly altered by either statin, but was increased by atorvastatin (11%, $P < 0.01$) and simvastatin (13%, $P < 0.01$) during the 19–24 h incubation.

The effects of atorvastatin (10 μm) and simvastatin (10 μm) on the incorporation of [^{14}C]acetic acid into CE, total cholesterol (TC), and PL are presented in Table 2. Incorporation into CE during the first 5 h and the 19–24 h period was significantly decreased by both statins, to an extent similar to that shown by [^{14}C]oleic acid incorporation. During the 0–5 h period, [^{14}C]acetic acid incorporation into TC was inhibited by 96% for both atorvastatin ($P < 0.001$) and simvastatin ($P < 0.001$), and inhibited to a similar extent during 19–24 h. TC synthesis was increased in the second incubation period (19–24 h) compared to the first (0–5 h), due to the longer incubation of

TABLE 2. Synthesis of intracellular lipids from [^{14}C]oleic acid or [^{14}C]acetic acid

	Cholesteryl Ester		Triglyceride		Phospholipid	
	0–5 h	19–24 h	0–5 h	19–24 h	0–5 h	19–24 h
	nmol oleic acid/mg cell protein/5 h					
Control	2.19 ± 0.3	1.69 ± 0.2	43.96 ± 6.3	46.48 ± 1.3	ND	ND
Atorvastatin	1.17 ± 0.4[a]	0.35 ± 0.1[b]	48.72 ± 7.3	51.70 ± 1.1[c]	ND	ND
Simvastatin	0.99 ± 0.4[a]	0.40 ± 0.1[b]	45.95 ± 12.3	52.34 ± 1.1[c]	ND	ND
	Cholesterol		Cholesteryl Ester		Phospholipid	
	pmol [^{14}C]acetic acid incorporated/mg cell protein/5 h					
Control	333 ± 31	683 ± 111	114 ± 10	128 ± 27	1592 ± 498	1055 ± 62
Atorvastatin	12.2 ± 4[d]	29.2 ± 5[b]	50.6 ± 9[d]	8.7 ± 2[b]	2089 ± 686	1280 ± 94
Simvastatin	12.3 ± 3[d]	21.8 ± 6[b]	59.3 ± 14[a]	4.0 ± 1[b]	2209 ± 697	1244 ± 65

HepG2 cells treated with 10 μm atorvastatin, or 10 μm simvastatin were incubated with 0.08 μCi [^{14}C]oleic acid or 0.5 μCi [^{14}C]acetic acid for 5 h. Values are given as mean ± SEM from three separate experiments with duplicate samples; 0–5 h and 19–24 h incubations were determined in separate experiments; ND, not determined.

[a] $P < 0.01$ vs. control (0–5 h).
[b] $P < 0.001$ vs. control (19–24 h).
[c] $P < 0.01$ vs. control (19–24 h).
[d] $P < 0.001$ vs. control (0–5 h).

HepG2 cells in the presence of LPDS-containing media. PL synthesis from acetic acid was not significantly altered by either statin.

Table 3 shows the effect of atorvastatin (10 μm) and simvastatin (10 μm) on the incorporation of oleic acid and acetic acid into secreted TG, CE, and PL. Simvastatin treatment significantly reduced the secretion of CE labeled with either [^{14}C]oleic acid (54% decrease; $P < 0.02$) or [^{14}C]acetic acid (50% decrease; $P < 0.01$), but had no effect on the secretion of ^{14}C-labeled TG or PL. Atorvastatin caused a reduction in the secretion of CE from [^{14}C]oleic acid (54% decrease; $P < 0.02$) and [^{14}C]acetic acid (58% decrease; $P < 0.01$). In contrast to simvastatin, atorvastatin resulted in a 32% ($P < 0.05$) reduction in the secretion of TG and a 17% (NS) decrease in the secretion of PL synthesized from [^{14}C]oleic acid. Atorvastatin decreased TG secretion despite an unchanged (0–5 h) or elevated (19–24 h) cellular TG synthesis and unchanged cellular TG mass.

TABLE 3. Incorporation of [^{14}C]oleic acid or [^{14}C]acetic acid into secreted lipids

	Cholesteryl Ester	Triglyceride	Phospholipid
	nmol oleic acid/mg cell protein/24 h		
Control	1.16 ± 1.15	11.98 ± 1.14	3.73 ± 0.22
Atorvastatin	0.53 ± 0.03[a]	8.20 ± 0.55[b]	3.07 ± 0.25
Simvastatin	0.53 ± 0.06[a]	10.85 ± 1.08	3.45 ± 0.34
	pmol [^{14}C]acetic acid/mg cell protein/24 h		
Control	119.2 ± 9.4	511.6 ± 35.1	327.3 ± 23.6
Atorvastatin	50.5 ± 4.7[c]	436.4 ± 32.9	294.1 ± 22.4
Simvastatin	59.9 ± 2.6[c]	544.7 ± 19.6	330.7 ± 7.85

HepG2 cells treated with 10 μm atorvastatin or 10 μm simvastatin were incubated with 0.08 μCi [^{14}C]oleic acid or 0.5 μCi [^{14}C]acetic acid for 24 h. Values are given as mean ± SEM from experiments with triplicate samples.

[a] $P < 0.02$ vs. control.
[b] $P < 0.05$ vs. control.
[c] $P < 0.01$ vs. control.

Effect on apoB distribution in secreted lipoprotein fractions

The distribution of apoB in media lipoprotein fractions was determined after 24-h incubations (**Fig. 3**). The lipoprotein distribution of apoB in control cells was similar to that reported previously in HepG2 cells cultured under similar conditions (39). Compared to control, simvastatin (10 μm) resulted in a 10% decrease in the recovery of apoB in the VLDL fraction but an increase in the recovery of apoB in the IDL (33% increase) and LDL (52% increase) fractions. The total recovery of apoB was unchanged. In contrast, atorvastatin (10 μm) resulted in a

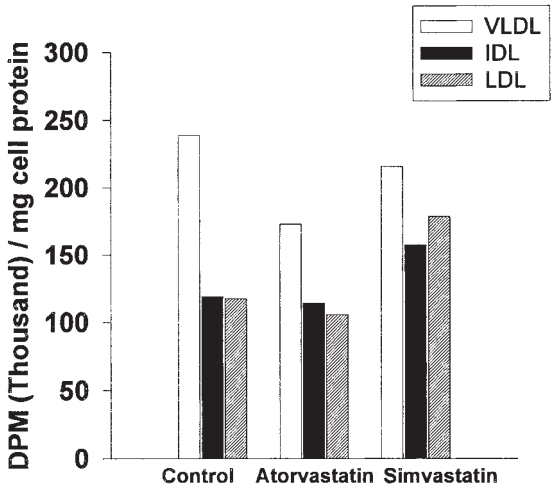

Fig. 3. Distribution of secreted apoB into lipoproteins. The distribution of secreted radiolabeled apoB into lipoproteins with the density of VLDL (d < 1.006 g/ml), IDL (d 1.006–1.019 g/ml), and LDL (d 1.019–1.063 g/ml) was determined after the incubation of cells with 50 μCi l-[^{3}H]leucine for 24 h. VLDL, IDL, and LDL fractions were isolated from the media by ultracentrifugation. Radiolabeled apoB was isolated from each lipoprotein fraction by isopropanol precipitation and analyzed by scintillation counting. The results are reported as the mean result from duplicate dishes of cells.

decreased recovery of apoB in the VLDL (28% decrease), IDL (4% decrease), and LDL (10% decrease) fractions. Total apoB recovery was decreased by 20%.

Effect of atorvastatin on apoB secretion and intracellular degradation

To better define the mechanism responsible for reducing the accumulation of apoB in the media of atorvastatin-treated HepG2 cells, pulse-chase studies were carried out. Total cellular immunoprecipitable apoB-100 radioactivity, after 5 min of chase, was not significantly different in atorvastatin- or simvastatin-treated (10 μm) cells compared to control ($P = 0.94$), indicating that apoB synthesis was not affected. **Figures 4A** and **4C** show the observed data points for apoB secreted into the media and the line of best fit generated by the kinetic model for two of eight separate experiments performed. Inspection of the curves shows that less apoB appears in the media of atorvastatin-treated cells (Fig. 4A), whereas simvastatin had little effect (Fig. 4C). The data for all experiments were analyzed by multicompartmental modeling using SAAM II, and the key kinetic parameters generated from the model are shown in **Table 4**. The rate constant k(4,3) represents the fraction of apoB synthesized that enters the pathway destined for secretion, whereas, k(0,3) + k(7,3) represents the fraction of apoB, defined kinetically, that enters the pathways destined for degradation. The percent of synthesized apoB that is secreted was determined from these rate constants and was found to be decreased by 44% ($P = 0.048$) in atorvastatin-treated cells (5.03% of apoB secreted) compared to control cells (8.95% of apoB secreted). The percent of apoB secreted was not altered by simvastatin treatment (6.72% vs. 8.15% for control; $P = 0.49$). The time from the addition of [^{35}S]methionine until radiolabeled apoB was initially detected in the media was approximately 24 min and was not affected by treatment. The re-

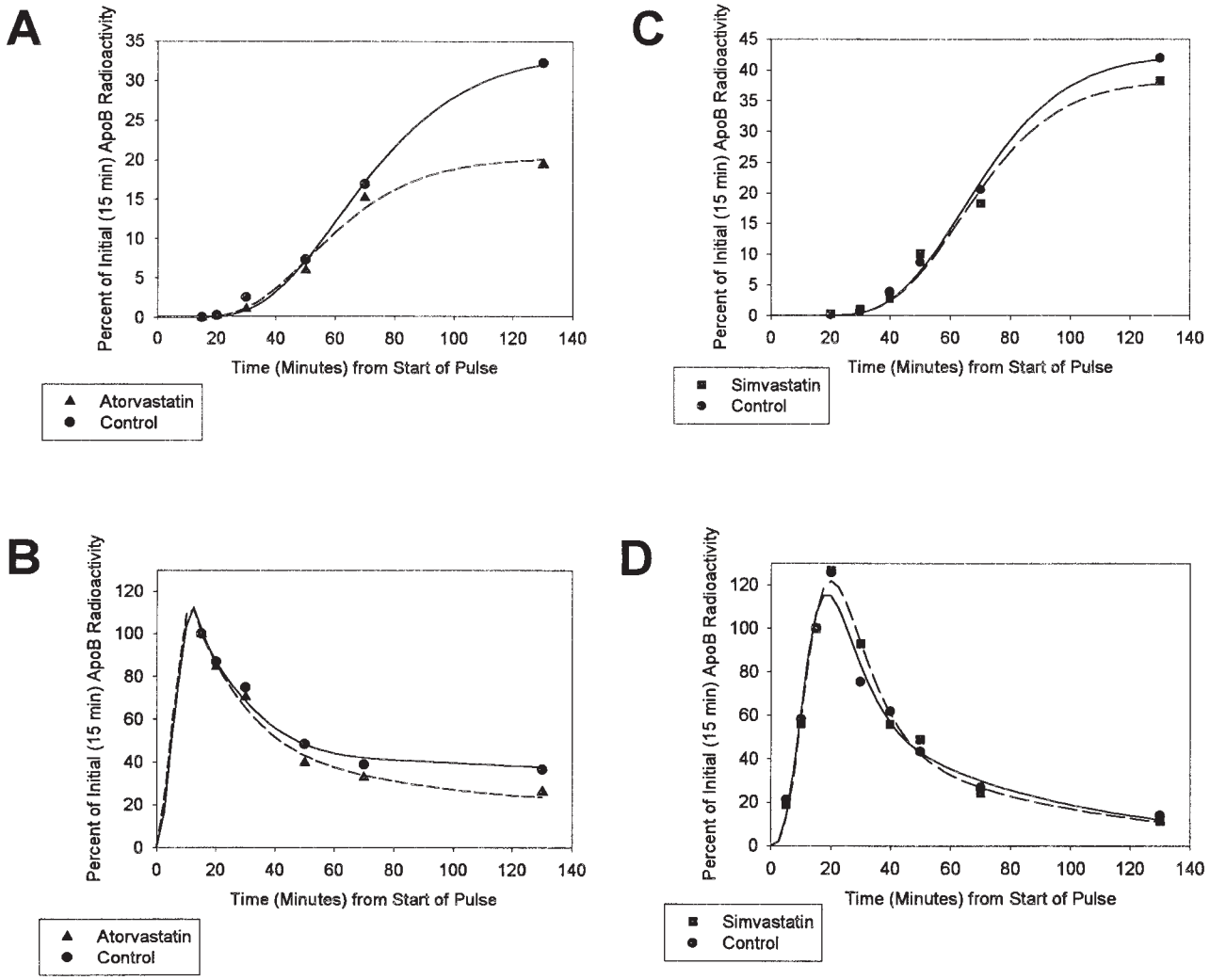

Fig. 4. Effect of atorvastatin on the secretion and intracellular degradation of apoB. HepG2 cells preincubated without statins (●) or with atorvastatin (▲) (10 μm) or simvastatin (■) (10 μm) for 24 h were pulse-labeled for 10 min (without oleate) and chased for various time periods (5–130 min). ApoB radioactivity secreted into the media is shown in Figs. 4A and 4C for two of eight experiments. Total apoB radioactivity, determined as the sum of apoB in the media plus the cell, is shown in Figs. 4B (corresponding to experiment in Fig. 4A) and 4D (experiment in 4C). The curves shown in Fig. 4 A, B corresponds to experiment #4 and Fig. 4 C, D to experiment #7 in Table 4. Data points represent the observed data (the mean of values from duplicate dishes) and the lines are the best fit generated by the kinetic model.

TABLE 4. Kinetic parameters of apoB secretion and intracellular degradation

	% Secreted[a]		% Degraded[b]		k[c](0,3)[d,e]		k(7,3)[d,e]	
Experiment	C	A	C	A	C	A	C	A
Atorvastatin								
1	9.46	3.01	90.54	96.99	0.143 (24.8)	0.327 (91.4)	0.435 (75.2)	0.031 (8.6)
2	8.33	6.08	91.67	93.92	0.141 (81.5)	0.795 (60.3)	0.032 (18.5)	0.524 (39.7)
3	5.72	4.22	94.28	95.78	0.142 (84.4)	0.141 (85.1)	0.026 (15.6)	0.025 (14.9)
4	12.31	6.8	87.69	93.2	0.386 (51.6)	0.623 (55.6)	0.362 (48.4)	0.498 (44.4)
Mean	8.95	5.03	91.05	94.97	0.203 (60.5)	0.472 (73.1)	0.214 (39.5)	0.270 (26.9)
±SEM	1.37	0.86	1.37	0.86	0.061 (14)	0.146 (8.9)	0.108 (14)	0.140 (8.9)
P vs. control	0.048		0.048		0.147		0.919	
	C	S	C	S	C	S	C	S
Simvastatin								
5	11.21	5.54	88.79	94.46	0.097 (15.0)	0.098 (32.7)	0.553 (85.0)	0.202 (67.3)
6	3.26	6.08	96.74	93.92	0.256 (88.9)	0.132 (15.1)	0.032 (11.1)	0.742 (84.9)
7	12.8	10.2	87.2	89.8	0.131 (78.4)	0.124 (84.9)	0.036 (21.6)	0.022 (15.1)
8	5.34	5.08	94.66	94.92	0.238 (87.2)	0.238 (87.9)	0.035 (12.8)	0.033 (12.1)
Mean	8.15	6.72	91.85	93.28	0.181 (67.4)	0.148 (55.1)	0.164 (32.6)	0.250 (44.9)
±SEM	2.29	1.18	2.29	1.18	0.039 (17.6)	0.031 (18.4)	0.130 (17.6)	0.169 (18.4)
P vs. control	0.486		0.486		0.362		0.727	

ApoB pulse-chase data from cells incubated with atorvastatin (A) or simvastatin (S), compared to control cells (C), were analyzed by multicompartmental modeling using SAAM II. The percent of synthesized apoB that is secreted and degraded were determined from the kinetic modeling. *P* value was determined by paired *t*-test.

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

[b] Calculated using the formula (k(0,3)+k(7,3))/(k(4,3) + k(0.3) +k(7,3)).

[c] k = pools/min.

[d] k(0,3) is the rate constant (pools/min) of apoB degradation directly from compartment 3 and k(7,3) is the rate constant for apoB transfer from compartment 3 to compartment 7.

[e] Numbers in parentheses represent the percent of degraded apoB that is degraded directly from compartment 3 calculated as [k(0,3)/(k(0,3)+k(7,3)] × 100, and the percent of degraded apoB that is degraded directly from compartment 7 (calculated as [k(7,3)/(k(0,3)+k(7,3)] × 100).

covery of radiolabeled albumin in cell lysates and media was not altered by atorvastatin (data not shown).

To determine whether the reduced apoB secretion was due to an enhanced intracellular degradation, the cellular apoB immunoprecipitates were analyzed. Figures 4B and 4D show the observed data points and the line of best fit generated by the kinetic model for total apoB (media plus intracellular) in two of eight experiments. These curves demonstrate that the intracellular degradation of apoB is enhanced by atorvastatin treatment (Fig. 4B), but not by simvastatin (Fig. 4D). As determined by the kinetic analysis (Table 4), simvastatin treatment did not significantly alter the percent of apoB degraded (93.3% vs. 91.9% for control; $P = 0.49$). The percent of apoB degraded was significantly greater in atorvastatin-treated cells (94.9% of total apoB was degraded) (Table 4) compared to control cells (91.1% of apoB degraded, $P < 0.05$). The rate of degradation directly from compartment 3 (Fig. 1) was approximately the same as the rate of conversion to the slow degradation compartment, compartment 7. Atorvastatin treatment did not significantly alter the rate constants for apoB degradation via the two degradation pathways, despite increasing the percent of total radiolabled apoB degraded. However, in 3 of 4 experiments, the rate constant describing degradation directly from compartment 3 [k(0,3)] was increased with atorvastatin treatment, such that the percent of apoB that was degraded via this pathway was nonsignificantly increased from 60.5% in control cells to 73.1% with atorvastatin. The percent transferred to compartment 7 [k(7,3)], prior to degradation, decreased from 39.5% (control) to 26.9% (atorvastatin, NS) (Table 4). For all experiments, once apoB is transferred to compartment 7, it turns over at approximately 10% of the rate of degradation from compartment 3.

Once secreted into the medium, apoB-containing lipoproteins may be rapidly taken up again by the cells, resulting in a net decrease in medium apoB. We determined whether atorvastatin (10 μm) treatment could affect the rate of this uptake. We found no difference in apoB radioactivity remaining in the media from control cells (87.4%) compared to atorvastatin treated cells (85.6%), indicating that the uptake of newly secreted apoB-containing lipoproteins was low and could not account for the reduced media apoB found with atorvastatin treatment.

Effect on apoB, LDL receptor, HMG-CoA reductase, and MTP mRNA content

The abundance of specific mRNAs was determined in HepG2 cells after 24-h incubations with the statins. As shown in **Table 5**, apoB and glyceraldehyde-3-phosphate dehydrogenase mRNA levels were not significantly changed by either statin, compared to control. The LDL receptor mRNA levels were significantly increased in cells treated with either atorvastatin (2.8-fold) or simvastatin (3.2-fold). HMG-CoA reductase mRNA was also significantly increased 2.2-fold with atorvastatin and 2.4-fold with simvastatin, compared to control. Atorvastatin caused a significant 22% ($P < 0.02$) decrease in MTP mRNA compared to

TABLE 5. ApoB, LDL receptor, HMG-CoA reductase, and MTP mRNA abundance

	Control	Atorvastatin 10 μm	Simvastatin 10 μm
	pg/μg total RNA		
Apolipoprotein B	72.34 ± 13.58	67.76 ± 12.71	81.04 ± 17.69
LDL receptor	0.52 ± 0.07	1.49 ± 0.20[a]	1.66 ± 0.17[a]
HMG-CoA reductase	4.86 ± 0.61	10.47 ± 1.09[a]	11.81 ± 0.93[a]
MTP	1.61 ± 0.08	1.26 ± 0.08[b]	1.39 ± 0.10
GAPDH	48.94 ± 3.76	46.09 ± 5.35	50.56 ± 8.97

HepG2 cells were incubated with 10 μm atorvastatin or 10 μm simvastatin for 24 h. ApoB, LDL receptor, HMG-CoA reductase, MTP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were analyzed by RNase protection/solution hybridization assay. The results are reported as pg mRNA per μg of total RNA. Values are given as the mean ± SEM from four experiments.

[a] $P < 0.002$ vs. control.

[b] $P < 0.02$ vs. control.

control, whereas, simvastatin reduced MTP mRNA by 14% (NS). There was no significant difference in MTP mRNA levels in cells treated with atorvastatin compared to simvastatin.

DISCUSSION

HMG-CoA reductase inhibitors have beneficial effects in the treatment of hyperlipidemia and atherosclerosis. These compounds have been shown to decrease the production and/or increase the clearance of apoB-containing lipoproteins in vivo (19–22, 24–26). However, the role of cholesterol synthesis and the mechanism by which HMG-CoA reductase inhibitors regulate hepatic apoB secretion remain controversial.

In HepG2 cells, we found that the HMG-CoA reductase inhibitor atorvastatin decreased apoB accumulation in the media. In contrast, simvastatin, at similar concentrations, did not affect the amount of apoB secreted. These results are consistent with a number of studies in humans (27–29) in which atorvastatin was found to cause greater reductions in LDL cholesterol (27–29), triglyceride (27, 28), and apoB (28) than milligram equivalent doses of simvastatin. Recent studies suggest that this may be due to a more pronounced (40) or prolonged (41) inhibition of HMG-CoA reductase, and thus, cholesterol biosynthesis. We examined this by determining the ability of these inhibitors to reduce the rate of cellular cholesterol and CE synthesis as well as cellular mass of FC and CE. Based on our finding that atorvastatin, but not simvastatin, reduced apoB accumulation in the media, we expected that atorvastatin would be more effective in reducing cellular cholesterol synthesis and CE availability. However, we found that the enhanced ability of atorvastatin to reduce apoB secretion was not related to any significant difference between atorvastatin and simvastatin with respect to inhibition of either the synthesis or mass of FC and CE. Although CE mass was lower in atorvastatin-treated cells (compared to simvastatin) at the 20 μm dose, the divergent response on apoB secretion was clearly established at concentrations of 10 μm, where cellular CE masses were similar. Furthermore, there was no significant difference between the two statins with respect to the mRNA levels of the LDL receptor and HMG-CoA reductase, suggesting similar effects on the regulation of cellular cholesterol homeostasis. Collectively, these results suggest that the greater efficacy of atorvastatin in reducing apoB secretion from HepG2 cells is unlikely to be explained by a greater efficacy in inhibiting cholesterol synthesis, in this model.

These results are consistent with the recent study by Bergstrom et al. (42), who show that atorvastatin and simvastatin caused similar inductions in HMG-CoA reductase mRNA and a nearly identical inhibition of HMG-CoA reductase in HepG2 cells. In contrast to our findings, however, they found that both statins reduced apoB secretion to the same extent, but only when cells were preincubated with oleate and butyrate (42). Consistent with our results, a recent study by Mohammadi et al. (43) found that atorvastatin (10 μm) reduced apoB secretion from HepG2 cells by an amount similar to that observed in the present study. However, it was not determined whether the reduced secretion of apoB was directly related to the inhibition of cholesterol synthesis or whether it was secondary to an increased LDL receptor expression leading to enhanced re-uptake of secreted lipoproteins.

Our observation that atorvastatin inhibits apoB secretion in HepG2 cells is also consistent with recent in vivo studies from this laboratory, in which miniature pigs receiving atorvastatin demonstrated a reduced hepatic secretion of VLDL apoB (20). These results are also similar to those found in atorvastatin-treated guinea pigs (44). Other, in vivo, human apoB kinetic studies have shown decreased apoB production by simvastatin (21, 22). The reason why both HMG-CoA reductase inhibitors decrease apoB secretion in vivo, but not in HepG2 cells, is not readily apparent. Although not measured in the present study, it is possible that atorvastatin produces greater reductions of FC and CE in specific regulatory pools within the ER. In addition, although the HepG2 cell line has been widely used as a model to study the regulation of apoB secretion (5–8, 13, 14, 45), these cells are thought to have a defect in TG mobilization (46, 47). Although not clearly understood, it is possible that this defect renders the HepG2 cell relatively insensitive to the regulation of apoB secretion by HMG-CoA reductase inhibition (5, 8, 9). Despite this, the HepG2 cell model has allowed us to determine, using the HMG-CoA reductase inhibitor atorvastatin, alternate pathway(s) regulating apoB secretion by mechanisms that may not be linked to an ability to alter cellular FC or CE concentrations.

Other potential mechanisms for the differential regulation of apoB secretion by atorvastatin and simvastatin were examined. Differences in apoB secretion might reflect an effect on the regulation of TG (5–9) and/or PL (10) availability. Neither inhibitor altered cellular TG or PL synthesis or mass at concentrations up to 10 μm. Simvastatin significantly increased cellular TG mass at a concentration of 20 μm, therefore, it is possible that the increased TG may compensate for reduced CE availability,

masking an effect on apoB secretion. However, this increase in TG mass is modest compared to that typically associated with enhanced apoB secretion (7, 46). It is possible that subtle changes in the concentration of TG or PL within specific membrane domains may regulate apoB secretion and may have escaped detection by our experimental techniques. However, our results suggest that inhibition of apoB secretion from atorvastatin-treated HepG2 cells is not caused by reductions in whole cell TG or PL.

To further characterize the regulation of apoB secretion by the statins, pulse-chase experiments were carried out. The analysis of apoB cellular pulse-chase data by multicompartmental modeling, in this study, is a novel approach. We have previously used compartmental modeling for in vivo apoB kinetic studies (18, 20, 48). We now extend this technique to the analysis of apoB pulse-chase data in HepG2 cells. This approach has allowed us to more precisely define the kinetics of apoB secretion and to identify kinetically distinct intracellular pools of apoB.

Atorvastatin exerted its effects post-translationally, as apoB synthesis and mRNA levels were unaltered. The time from the addition of the ^{35}S-labeled methionine to the first appearance of labeled apoB in the media was approximately 24 min, which was not affected by the HMG-CoA reductase inhibitor. This time is similar to the estimates previously reported by Bostrom et al. (49). The decreased apoB secretion was associated with an enhanced intracellular degradation of apoB. According to the model, apoB radioactivity initially appears in one, kinetically defined, intracellular compartment, designated compartment 3, and subsequently appears in a second kinetically defined compartment (compartment 4). We speculate that compartment 3 represents newly synthesized, partially lipidated apoB and that compartment 4 represents more lipidated or fully lipidated apoB. ApoB is either degraded directly from compartment 3 or after transfer to a more slowly turning over degradation compartment (compartment 7). Interestingly, the effect of atorvastatin on degradation is mainly on the rapid, direct degradation pathway, as evidenced by the trend towards an enhanced apoB degradation directly from compartment 3 and a decrease in conversion to compartment 7 with atorvastatin treatment. Although the intracellular pools of apoB in this study were defined kinetically, it is tempting to speculate that the degradation pathways represent different degradation processes as described by others (50–54). It is conceivable that the rapidly turning over degradation pool represents cytoplasmic degradation mediated by the proteasome (50–52) and that the slowly turning over pool represents apoB degradation within the ER lumen (53, 54).

Our pulse-chase results are similar to those in previous studies when analyzed by the conventional approach (i.e., assessing percent secretion and percent degradation from the relative amounts of media and total apoB radioactivity remaining at the end of the chase, 130 min) (3, 6, 8). Using the conventional approach, the percent secretion of apoB from HepG2 cells is generally reported to be higher than that determined by multicompartmental modeling. This is because multicompartmental modeling takes an integrated approach, simultaneously accounting for both data sets (media and cellular apoB) throughout the entire pulse-chase time course. Modeling the tracer data revealed that of the apoB that is synthesized, approximately 90% is destined for degradation and only 10% is destined for secretion. This does not imply that, during the course of the pulse-chase study, all of the apoB destined for degradation is actually degraded. Compared to synthesis and secretion, the degradation of apoB is a relatively slow process, particularly from compartment 7. Thus, at the end of the 130-min study, a significant proportion of the apoB destined for degradation is still present in the cell awaiting degradation.

There are a number of possible mechanisms that may play a role in the post-translational regulation of apoB secretion by atorvastatin. We have provided evidence that argues against an altered uptake of newly synthesized apoB-containing lipoproteins. Other possible mechanisms involve enhanced interaction of apoB with various proteases (36, 50–54) and chaperones (50) during the movement of apoB through the secretory pathway. A novel observation in this study is the significant decrease in MTP mRNA by atorvastatin. MTP has been shown to be essential for the secretion of apoB-containing lipoproteins (55), by mediating the transfer of TG, CE, and PL to nascent apoB (56). However, because the MTP protein has a half-life of approximately 4.4 days (57), it is uncertain whether the reduction in MTP mRNA, observed in this study, would result in decreased apoB secretion during 24 h. Whether atorvastatin decreases MTP activity in longer term, in vivo, studies remains to be determined.

In summary, the results of this study show that HMG-CoA reductase inhibitors, with differing properties, can exert different effects on the regulation of apoB secretion, which may not be due to their efficacy in inhibiting cholesterol synthesis. Specifically, treatment of HepG2 cells with atorvastatin has revealed potentially novel mechanisms that may contribute to the regulation of apoB secretion. The nature of these mechanisms, and whether they occur in primary hepatocytes or in vivo, requires further investigation.

This work was supported by a grant from the Heart and Stroke Foundation of Ontario (T-3371 to M. W. Huff), the National Institutes of Health (HL49110, NCRR12609, to P. H. R. Barrett), and Parke-Davis Canada (to M. W. Huff). L. J. Wilcox is a recipient of a Medical Research Council of Canada Studentship. P. H. R. Barrett is a recipient of a fellowship from the Fogarty Center (TW02272). M. W. Huff is a Career Investigator of the Heart and Stroke Foundation of Ontario. We thank Cindy Sawyez, Linda de Dreu, and Dawn Telford for expert technical assistance. We acknowledge Roger S. Newton, Parke-Davis Pharmaceutical Research (Warner Lambert Co., Ann Arbor, MI), for kindly providing atorvastatin and Merck, Sharpe & Dohme for providing simvastatin.

Manuscript received 29 September 1998 and in revised form 12 February 1999.

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