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HIV protease inhibitors protect apolipoprotein B from degradation by the proteasome: A potential mechanism for protease inhibitor-induced hyperlipidemia

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Highly active anti-retroviral therapies, which incorporate HIV protease inhibitors, resolve many AIDS-defining illnesses. However, patients receiving protease inhibitors develop a marked lipodystrophy and hyperlipidemia. Using cultured human and rat hepatoma cells and primary hepatocytes from transgenic mice, we demonstrate that protease inhibitor treatment inhibits proteasomal degradation of nascent apolipoprotein B, the principal protein component of triglyceride and cholesterol-rich plasma lipoproteins. Unexpectedly, protease inhibitors also inhibited the secretion of apolipoprotein B. This was associated with inhibition of cholesteryl-ester synthesis and microsomal triglyceride transfer-protein activity. However, in the presence of oleic acid, which stimulates neutral-lipid biosynthesis, protease-inhibitor treatment increased secretion of apolipoprotein B-lipoproteins above controls. These findings suggest a molecular basis for protease-inhibitor–associated hyperlipidemia, a serious adverse effect of an otherwise efficacious treatment for HIV infection.

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HIV protease inhibitors (PIs) are peptidomimetic active-site competitors of the aspartyl protease required for production of infectious viral particles. Highly active anti-retroviral therapies (HAART), which incorporate PIs such as ritonavir (RTV) or saquinavir (SQV), allow immunological recovery, resolution of AIDS defining illnesses, and increased survival[1–3]. However, patients receiving PIs develop a marked lipodystrophy and hyperlipidemia[4–6]. The direct association of PIs with hyperlipidemia, especially hypertriglyceridemia, has been demonstrated in normal healthy subjects taking RTV (ref. 7) and is often associated with findings of the insulin resistance syndrome[4]. Thus patients on HAART are at elevated risk for premature atherosclerosis[8].

Lipoproteins that contain apolipoprotein B (ApoB) are the major determinants of human plasma lipid levels[9]. Extensive studies of cultured primary cells have established that secretion of these lipoproteins is regulated primarily by proteasomal degradation of newly synthesized ApoB (refs. 10–13). The HIV PI, RTV, has been demonstrated to inhibit the chymotryptic activity of the 20S proteasome required for the generation of antigenic peptides[14]. We reasoned, therefore, that PI-inhibition of pre-secretory ApoB degradation at the proteasome might increase the assembly and secretion of very low density (VLDL) and low-density lipoproteins (LDL). In our study of three established models of lipoprotein assembly we show that newly synthesized cellular ApoB is stabilized by PI-treatment, but unexpectedly accumulates within the cell due to a specific block in ApoB secretion. We further demonstrate that the inhibitory effects of PIs on ApoB secretion are reversed by oleic acid, such that ApoB is ultimately secreted at higher levels than in untreated controls.

HIV-PI treatment inhibits ApoB degradation and secretion

We first studied the effects of PIs on ApoB metabolism in human hepatoma (HepG2) cells that are used extensively to characterize the assembly and secretion of lipoproteins. Treatment of these cells with therapeutically relevant concentrations of RTV (5–100 μM)[15] or SQV resulted in significant intracellular accumulation of ApoB (Fig. 1*a*). These effects were comparable to those seen in cells treated with proteasome inhibitors such as acetyl-leucyl-leucyl-norleucinal (ALLN). Furthermore, PI treatment significantly protected nascent ApoB from intracellular degradation (data not shown) and concomitantly increased levels of ubiquitinated ApoB (Fig. 1*b*); a clear indication that the proteasome was inhibited.

We anticipated that protection of nascent ApoB from proteasomal degradation by RTV or SQV would be associated with increased secretion of ApoB-lipoproteins as observed with other inhibitors of the proteasome[16] such as ALLN (Fig. 1*c*). We were surprised therefore, that PI treatment resulted in marked impairment of ApoB secretion (Fig. 1*c*). This treatment effect was specific for ApoB as no change in the secretion of either apolipoprotein AI or albumin was observed (data not shown).

HIV-PI compromises neutral-lipid biosynthesis and transfer

ApoB fails to be translocated into the lumen of the endoplasmic reticulum when neutral-lipid (triglyceride and cholesteryl ester)

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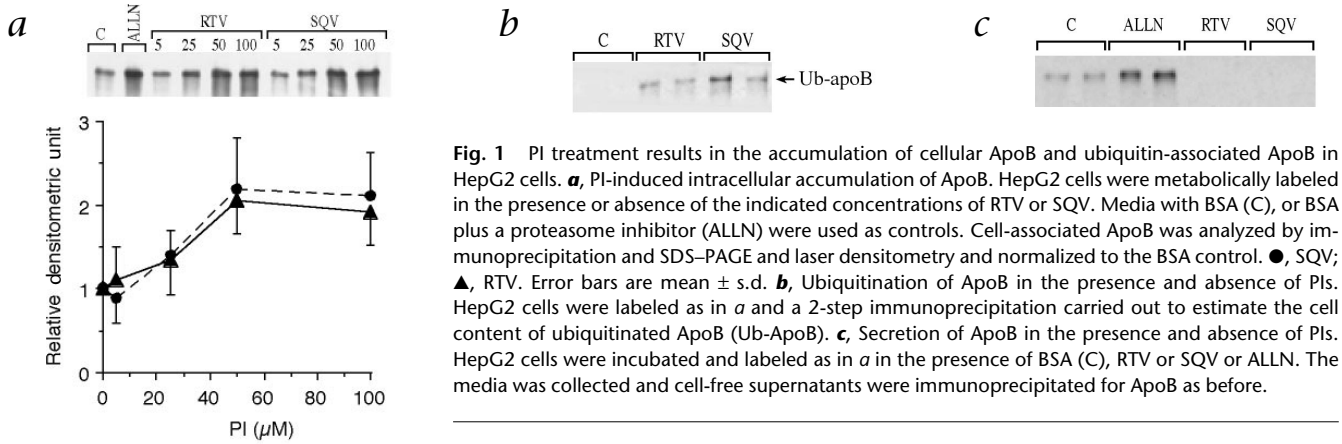

Fig. 1 PI treatment results in the accumulation of cellular ApoB and ubiquitin-associated ApoB in HepG2 cells. ***a***, PI-induced intracellular accumulation of ApoB. HepG2 cells were metabolically labeled in the presence or absence of the indicated concentrations of RTV or SQV. Media with BSA (C), or BSA plus a proteasome inhibitor (ALLN) were used as controls. Cell-associated ApoB was analyzed by immunoprecipitation and SDS–PAGE and laser densitometry and normalized to the BSA control. ●, SQV; ▲, RTV. Error bars are mean ± s.d. ***b***, Ubiquitination of ApoB in the presence and absence of PIs. HepG2 cells were labeled as in *a* and a 2-step immunoprecipitation carried out to estimate the cell content of ubiquitinated ApoB (Ub-ApoB). ***c***, Secretion of ApoB in the presence and absence of PIs. HepG2 cells were incubated and labeled as in *a* in the presence of BSA (C), RTV or SQV or ALLN. The media was collected and cell-free supernatants were immunoprecipitated for ApoB as before.

availability and/or the activity of microsomal triglyceride transfer protein (MTP) are limiting. Consequently, it becomes ubiquitinated and is degraded by the proteasome[10–13]. To assess whether the PI-induced block in ApoB secretion could be due to impaired neutral-lipid biosynthesis, we treated HepG2 cells with RTV and SQV in the presence of radiolabeled lipid precursors. PI treatment was consistently associated with a dose-dependant decrease in cholesteryl ester (CE) synthesis that did not resolve over the course of 48 hours (Fig. 2*a*). The 50% inhibitory concentration (IC_{50}) meaurements for CE synthesis in HepG2 cells were approximately 7 μM and 15 μM for RTV and SQV, respectively. 50 μM RTV produced an approximately 95% decrease in CE synthesis ($P < 0.001$). By contrast, although the acute effect of PI treatment for two hours, to modestly reduce triglyceride (TG) synthesis, was significant, a net increase in TG synthesis was observed with prolonged treatment, consistent with the work of Lenhard and colleagues[17]. Similar effects were observed with indinavir, another PI associated with hyperlipidemia (data not shown). PI treatment also inhibited CE synthesis in Chinese hamster ovary cells expressing either human acyl-coenzyme A (CoA): cholesterol *O*-acyltransferase (ACAT) 1 or 2 (data not shown) indicating that the effects are not isoform specific. ACAT inhibition is unlikely to be a sequela of proteasome inhibition, as under the same culture conditions, ALLN had no effect on neutral-lipid biosynthesis (data not shown). To further assess the mechanism by which neutral-lipid biosynthesis is compromised by PI treatment, we tested the direct inhibition of the acyltransferase reactions catalyzed by ACAT or diacylglycerol acyltransferase (DGAT) using *in vitro* assays in rat liver microsomes (Fig. 2*b*). The ACAT reaction was markedly inhibited (~40%) by 5 μM RTV or 50 μM SQV, whereas the DGAT reaction was only modestly affected, consistent with the cell-culture experiments. Furthermore, this demonstrates that the effects of PIs on neutral-lipid biosynthesis are direct rather than secondary to processes such as transcriptional regulation or substrate provision.

Sterol esterification plays a role in lipoprotein assembly *in vivo*[18,19]; however, the inhibition of ACAT by PIs is unlikely to account for the complete block in ApoB secretion observed with PI treatment. An established ACAT inhibitor (DuP 128)[20], did not affect ApoB secretion in our studies, despite a 90% reduction in sterol ester synthesis (data not shown). By contrast, pharmacological inhibition of MTP-mediated transfer of neutral lipids onto ApoB particles severely reduces ApoB secretion[21]. We therefore tested the effect of PIs on MTP in an *in vitro* TG transfer assay and found that RTV and SQV inhibited the activity of bovine MTP with IC_{50} meaurements of 6.6 and 7.2 μM, respectively (Fig. 2*c*).

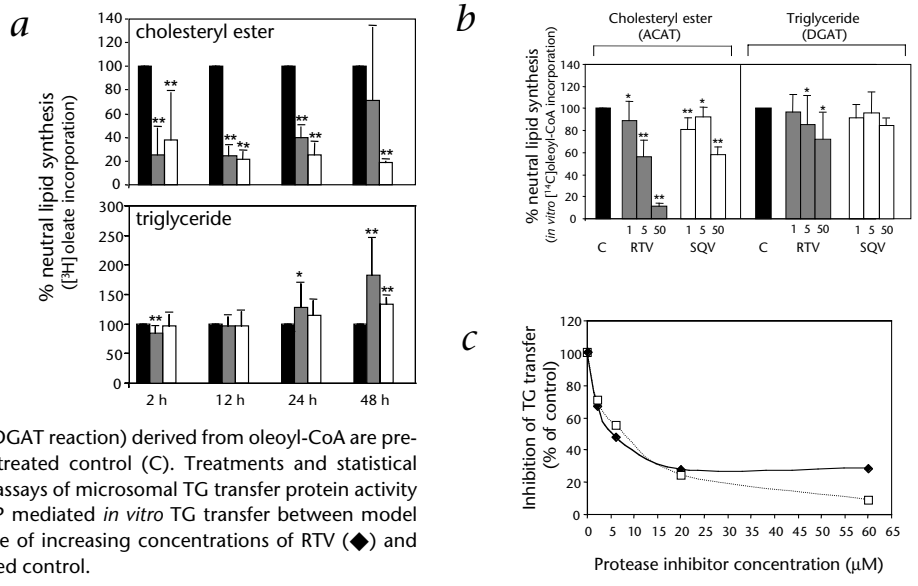

Fig. 2 PI treatment results in marked alterations in neutral-lipid synthesis and transfer. ***a***, Neutral-lipid biosynthesis in the presence and absence of PIs. HepG2 cells were incubated with RTV (■) or SQV (□) and [9,10-^{3}H(N)]oleic acid. Cell-associated lipids (TG and CE) were extracted and resolved by TLC at the times indicated. Data represents percent of untreated control (■). Statistical significance relative to untreated controls; *, $P < 0.05$; **, $P < 0.01$. ***b***, *In vitro* inhibition of *O*-acyltransferase activities by PIs. Rat liver microsomes were incubated with [^{14}C]oleoyl-CoA and increasing concentrations of RTV or SQV followed by organic extraction and TLC analysis. CE(an acyl-coenzyme A (CoA): cholesterol *O*-acyl transferase, ACAT reaction) and TG (a diacylglycerol acyltransferase, DGAT reaction) derived from oleoyl-CoA are presented as percent incorporation relative to untreated control (C). Treatments and statistical significance are denoted as in panel *a*. ***c***, *In vitro* assays of microsomal TG transfer protein activity in the presence and absence of PIs. Bovine MTP mediated *in vitro* TG transfer between model membrane systems was assessed in the presence of increasing concentrations of RTV (◆) and SQV (□). Data is presented as percent of untreated control.

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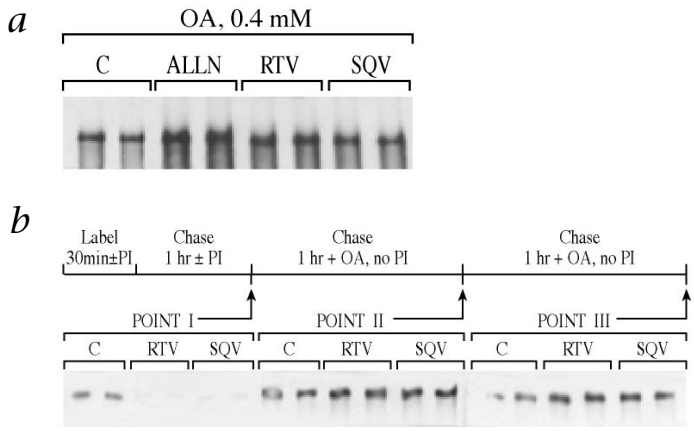

Fig. 3 PI treatment increases net secretion of ApoB-containing lipoproteins in the presence of added fatty acids. ***a***, Oleic-acid–induced secretion of ApoB in the presence and absence of PIs. HepG2 cells were labeled with L-[4,5-^{3}H]leucine in the presence of oleic acid (OA) plus BSA (C), RTV, SQV or ALLN. In the absence of OA, secretion of ApoB-lipoproteins was markedly inhibited during PI treatment (Fig. 1*c*). In the presence of OA, secretion of ApoB-lipoproteins was significantly increased by RTV (1.64 ± 0.43 relative densitometric units; $n = 3$) or SQV (1.68 ± 0.49; $n = 3$) compared with cells incubated with BSA only. ***b***, Enhanced induction of ApoB secretion by oleic acid following PI-removal. HepG2 cells were pulse-labeled and chased in the presence or absence of RTV or SQV. The cells were chased in BSA medium containing PIs in new media and then medium containing OA (no PIs) and assayed at the points shown. Relative to control cells, total secretion of ApoB in pooled media collected at point I + point II + point III was significantly increased by RTV (1.47 ± 0.33-fold; $n = 3$) or SQV (1.42 ± 0.29-fold; $n = 3$).

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Neutral-lipid biosynthesis reverses the effects of HIV-PI

Stimulation of neutral-lipid synthesis by oleic acid is associated with increased translocation, reduced ubiquitination and increased secretion of ApoB (refs. 16,22). We therefore treated HepG2 cells with 0.4 mM oleic acid and 50 μM RTV or SQV to determine the effects of stimulating core-lipid synthesis on PI-associated inhibition of ApoB secretion. In PI-treated cells, the presence of oleic acid resulted in approximately 65% greater secretion of ApoB-lipoproteins compared with cells treated with OA in the absence of PIs (Fig. 3*a*). Furthermore, the results of pulse-chase experiments (Fig. 3*b*) indicated that the PI-protected cellular ApoB could be assembled with core lipids and secreted into medium when sufficient lipids were provided soon after PI treatment. Addition of oleic acid after removal of PI from the media induced an approximately 45% increase in ApoB secretion compared with cells treated only with oleic acid. This relief from PI inhibition of ApoB secretion was associated with increased TG biosynthesis (data not shown). This data implies that the PI-mediated impediments to ApoB secretion can be overcome by elevating the supply of core lipids.

Effects of HIV-PIs on lipoprotein metabolism are universal

Although HepG2 cells represent an excellent model for the early events in lipoprotein biogenesis, the secreted lipoproteins from these cells are unusually TG-poor due to a defective 'second step' in lipoprotein assembly. Moreover, human hepatocytes do not produce ApoB48, the major component of dietary lipid containing chylomicrons, which in humans arises from intestine-specific ApoB100 mRNA editing. By contrast, cultured rodent McA RH7777 hepatoma cells secrete both ApoB100- and ApoB48-containing particles that closely resemble very low density lipoproteins (VLDL) in terms of lipid composition[23,24]. The effects of PIs on lipoprotein assembly observed in HepG2 cells were also found in McA RH7777 cells (Fig. 4). Both ApoB100 and ApoB48 were stabilized and retained following incubation with RTV. Moreover, RTV treatment and co-incubation with oleic acid elevated the secretion of more buoyant ApoB100 and ApoB48 lipoprotein particles. Densitometric analysis of total levels of ApoB100 over all fractions indicated a 69% net increase in ApoB100-lipoprotein secretion in oleic acid plus RTV-treated cells relative to untreated control and a 43% increase relative to cells treated with oleic acid alone. Secretion of ApoB48-containing particles was similarly elevated (37%) by the combined application of RTV and oleic acid relative to cells treated with oleic acid alone.

Numerous animal models for the receptor-mediated and enzymatic events that regulate plasma lipoprotein levels have been described[25]. However, the mouse carries the majority of cholesterol in apolipoprotein A1-containing high-density lipoproteins. Thus murine models of apolipoprotein B biogenesis are currently inadequate to fully address production of these lipoproteins *in vivo*, particularly with regard to the subcellular events that lead to degradation of ApoB in response to lipid status. We

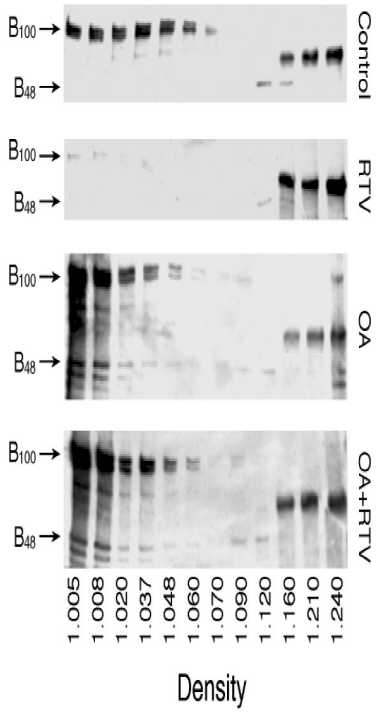

Fig. 4 RTV increases the secretion and buoyant density of ApoB-containing lipoproteins in McA RH7777 cells. McA RH7777 cells were labeled with [^{35}S]methionine in medium containing BSA (control) or RTV in the presence or absence of oleic acid (OA). Media samples were subjected to sucrose density-gradient ultracentrifugation and fractions with the indicated densities were analyzed for ApoB100 and ApoB48 by immunoprecipitation, polyacrylamide gel electrophoresis and fluorography.

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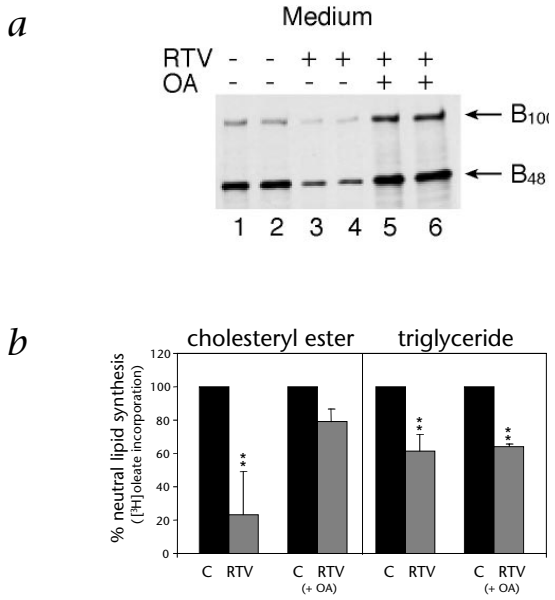

Fig. 5 RTV-inhibition of apoB secretion and neutral lipid synthesis is reversed by oleic acid in cultured primary hepatocytes from ApoB transgenic mice. ***a***, Effect of RTV on the secretion of ApoB in mouse hepatocytes in the presence or absence of OA. Fasted mouse hepatocytes were isolated from overnight-fasted human ApoB-transgenic mice, and labeled with [^{35}S]methionine in BSA (control, lanes 1–2), or RTV (lanes 3–6), in the absence or presence of oleic acid (OA, lanes 1–4, and 5–6, respectively). After labeling, ApoB100 and ApoB48 in medium were analyzed as described above. ***b***, RTV-mediated alterations in neutral-lipid synthesis in mouse hepatocytes in the presence or absence of OA. Primary mouse hepatocytes were isolated and cultured as above and labeled with [9,10-^{3}H(N)]oleic acid in the presence of BSA (control; ■) or RTV (■) in the absence or presence of oleic acid. Cell-associated lipids (TG and CE) were extracted and resolved by TLC. Data represents percent of untreated control. Statistical significance relative to untreated controls; *, $P < 0.05$; **, $P < 0.01$.

chose an *ex vivo* approach of culturing primary hepatocytes from human ApoB transgenic mice that had been maintained on a lipid-free diet and fasted overnight before killing. The primary hepatocytes were then further depleted of lipid in serum-free media to reveal the ApoB degradation pathways. Under these circumstances, we demonstrate essentially the same effects of PIs and oleic acid on ApoB100/48 secretion (Fig. 5*a*) and neutral-lipid synthesis (Fig. 5*b*) to those observed in the *in vitro* models. ApoB100 and ApoB48 secretion and sterol esterification were significantly impaired by treatment with RTV (59, 52 and 77% respectively). The inclusion of oleic acid with RTV significantly elevated ApoB100 and ApoB48 secretion (107 and 40%, respectively) relative to untreated cells.

Discussion

These findings further confirm previous studies that proteasomal degradation of nascent ApoB has a significant role in the regulation of assembly and secretion of ApoB-containing lipoproteins in cultured cells. The current studies, conducted in three distinct and established model systems for lipoprotein production, implicate inhibition of proteasomal degradation as a physiologically relevant molecular mechanism by which PIs may cause hyperlipidemia. The intracellular stabilization of ApoB due to proteasomal inhibition in conjunction with a specific block in lipoprotein secretion due to inhibition of neutral-lipid biosynthesis and transfer results in 'stockpiling' of ApoB within the cell. We hypothesize that this represents a population of nascent particles primed for secretion when core lipid availability is increased, such as after delivery of remnant lipoproteins or when fatty-acid flux to the liver is increased by the concomitant presence of peripheral insulin resistance[26].

The widespread applications of HAART regimens that include nucleoside analogues and protease inhibitors have been associated with a dramatic reversal of the AIDS epidemic in the western world. However the adverse effects of PI-based therapies, particularly hyperlipidemia and findings of the insulin resistance syndrome[4] have led to suggestions that the benefits of the therapy may not outweigh the long term outcome for some patients[27]. Indeed, anecdotal reports of accelerated atherosclerosis and coronary heart disease[8,28] suggest that this is a significant public health issue. Furthermore, it is conceivable that any pharmaceuticals that inhibit the proteasome could induce similar syndromes. Our data suggest that the development of PIs that do not affect proteasomal activities might avoid one of the major adverse outcomes associated with the present therapies and thus have a significant impact on the long-term survival of these patients.

Methods

Cell culture and metabolic labeling of ApoB. HepG2 or McA RH7777 cells (ATCC, Rockville, Maryland) were grown for 3 d and subjected to metabolic labeling (200 μCi/ml L-[4,5-^{3}H]leucine, or [^{35}S]methionine, Amersham) for 2 h in the presence or absence of 5–100 μM RTV, SQV or 40 μg/ml acetyl-leucyl-leucyl-norleucinal (ALLN). Primary hepatocytes were isolated from overnight-fasted human ApoB transgenic mice[29,30]. After 2 h incubation in 10% FBS MEM culture medium, hepatocytes were washed twice with PBS and cultured for another 20 h in 1.5% BSA MEM. Finally, hepatocytes were labeled with [^{35}S]methionine (100 μCi/ml) for 2 h with BSA (control), 50 μM RTV or 40 μg/ml ALLN in the absence or presence of oleic acid (OA, 0.4 mM). Immunoprecipitations of cell extracts or cell-free supernatants were performed with excess anti-human ApoB antibody and quantified by laser densitometry following SDS–PAGE (4%) and fluorography as described[31]. For fatty-acid treatment, cells were stably-labeled for 2 h (Fig. 3*a*) or pulse labeled for 30 min and chased for 2 separate 1 h periods (Fig. 3*b*) in the presence of 0.4 mM oleic acid with 1.5% BSA alone, BSA plus ALLN, or BSA plus PI (50 μM RTV or SQV). The cell content of ubiquitinated ApoB was estimated in a two-step immunoprecipitation of labeled HepG2 cell lysates with rabbit anti-ubiquitin antibody (StressGen, Victoria, Canada). This was followed by solubilization in 2% SDS, re-immunoprecipitation with anti-human ApoB antibody and SDS–PAGE analysis[32]. Lipoproteins secreted by McA RH7777 cells in 5 ml culture medium were further characterized by sucrose gradient density centrifugation (twelve fractions of 1 ml with the indicated densities, Fig. 4), immunoprecipitation, SDS–PAGE, and fluorography as before.

***In vivo* and *in vitro* assays of lipid metabolism.** HepG2, CHO-ACAT1 or CHO-ACAT2, McA RH7777 cells and primary hepatocytes were grown in the presence of 5 or 50 μM RTV or SQV and 2 μCi/ml [9,10-^{3}H(N)]oleic acid as indicated and then extracted with hexane/isopropanol at room temperature for 2 h and dried under a stream of N_2 gas. An ACAT inhibitor (10 μg/ml DuP 128) was included as a control for sterol esterification (data not shown). Lipids were analyzed by thin layer chromatography (TLC) in hexane-ethyl ether-glacial acetic acid (70:30:1) and normalized to total cell protein following visualization with iodine vapor and analysis by liquid scintillation counting. Qualitatively similar results were obtained using alternate metabolic precursors such as [1-^{14}C]acetate, [5-^{3}H]mevalonolactone or [1,2-^{3}H(N)]cholesterol in phospholipid liposomes (data not shown).

Rat liver microsomes were isolated by homogenization and successive centrifugation[33]. Neutral-lipid biosynthetic enzyme activity in the presence or absence of RTV was determined by incorporation of [^{14}C]oleoyl-CoA into CE or TG for 2.5 min after preincubation for 15 min with or without RTV or SQV in dimethylsulfoxide.

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Lipid-transfer activity from donor to acceptor membranes by the microsomal TG transfer protein was measured using bovine MTP in an *in vitro* assay similar to that described[34].

Acknowledgments

We thank N. Buss for the PIs used in this study; J. Billheimer for rat liver microsomes; and J. D. Rich and S. Santos for encouragement at the early stages of this work. This study was supported by Glaxo–Wellcome, Roche Products (Australia) and Merck and Company (to D.C.), NIH HL55638 (to H.G.), HL40404 (to R.J.D.) and the Columbia-Rockefeller Center for AIDS Research (NIH P30 AI42848 pilot award to S.L.S., R.J.D. and J.L.). R.J.D. and S.L.S. were supported in part by NIH HL65954 to N. S. Shachter. NCHECR is supported by the Research Advisory Committee of the Australian National Council on AIDS, Hepatitis C and Related Diseases through the Commonwealth Department of Health and Aged Care.

RECEIVED 7 SEPTEMBER; ACCEPTED 2 NOVEMBER, 2001

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