Transmembrane Lipid Transfer Is Crucial for Providing Neutral Lipids during Very Low Density Lipoprotein Assembly in Endoplasmic Reticulum*

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Very low density lipoprotein (VLDL), a large particle containing apolipoprotein B (apoB) and large amounts of neutral lipids, is formed in the luminal space within the endoplasmic reticulum (ER) of hepatic cells. The assembly mechanism of VLDL particles is a tightly regulated process where apoB, associated with an insufficient amount of lipids, is selectively degraded intracellularly. In this study we found that treatment of HuH-7 human hepatoma cells with verapamil inhibited secretion of apoB-containing lipoprotein particles through increasing degradation of apoB. Addition of N-acetylleucyl-leucyl-norleucinal, an inhibitor of proteasome and other cysteinyl proteases that are responsible for apoB degradation, restored apoB recovery from verapamil-treated cells. De novo synthesis of lipids from \[^{14}C\]acetate was increased in the presence of verapamil, suggesting that verapamil decreases lipid availability for apoB thus leading to the secretion of apoB-containing lipoprotein. We prepared cytosolic fractions from cells preincubated with \[^{14}C\]acetate and used as a donor of radioactive lipids. When this cytosolic fraction was incubated with microsomes isolated separately, radioactive triglyceride (TG) accumulated in the luminal space of the microsomes. The transfer of radioactive TG from the cytosolic fraction to the microsomal lumen was inhibited in the presence of verapamil, suggesting that there is a verapamil-sensitive mechanism for TG transfer across ER membranes that is involved in formation of apoB-containing lipoprotein particles in ER. Verapamil showed no inhibitory effect on microsomal TG transfer protein, a well known lipid transfer protein in ER. We propose from these results that there is novel machinery for transmembrane movement of neutral lipids, which is involved in providing TG for apoB during VLDL assembly in ER.

The liver plays a central role in lipoprotein metabolism through secretion of very low density lipoprotein (VLDL)\(^1\), which carries neutral lipids to all peripheral tissues. VLDL, a large particle containing apolipoprotein B (apoB) and various lipids, including triglyceride (TG), free cholesterol (FC), and cholesteryl ester (CE), is assembled in endoplasmic reticulum (ER) in hepatic cells. Mechanisms of VLDL particle formation are likely to be rigorously regulated, because association of lipids to apoB needs to occur cotranslationally, and poorly lipidated apoB-containing lipoprotein is degraded by proteasome or ER-resident proteases such as ER-60 prior to secretion (1–3). Thus it is thought that the association of lipids to apoB is a rate-limiting step for VLDL secretion, with regulation of this process controlled by as yet unknown mechanisms. One of the factors for this lipid transfer is microsomal triglyceride transfer protein (MTP), a lack of which was identified as the cause of abetalipoproteinemia, an autosomal recessive disease with total loss of apoB-containing lipoprotein in plasma (4, 5). MTP locates in the luminal space of ER by forming heterodimers with protein disulfide isomerase (6). MTP binds various lipid molecules, including TG and then presents those lipids to apoB (7). It was clearly shown that newly synthesized apoB in cells lacking MTP failed to associate with lipids and subsequently the apoB was degraded (8). It is also reported that MTP has a molecular chaperon-like activity (9) and contributes to TG transfer from cytosol to lumen of the endoplasmic reticulum (10, 11).

TG is synthesized from diglyceride and acyl-CoA by the action of diglyceride:acyl-CoA acyltransferase (DGAT). DGAT activity is detected in microsomes, and it is located on the cytosolic side of ER membranes (12, 13). Recently, Owen et al. (14) reported that the activity of DGAT was present also in the lumen of the ER; however, it is still unclear whether TG synthesis occurs physiologically within ER lumen. Enzymes involved in cholesterol synthesis are present in the cytosol and on the cytosolic side of ER membranes. Acyl-CoA:cholesterol acyltransferase (ACAT), which catalyzes esterification of cholesterol to form CE, is also located primarily on the cytosolic side of ER membrane (15). It is noteworthy that the active site of ACAT2, an isoform expressed primarily in liver and intestine, is predicted to be in the ER lumen (16). MTP can transfer these lipids from the ER membrane to apoB in the ER lumen. For proper assembly of VLDL particles to occur, however, these lipids must be moved from the site of synthesis into the luminal

apoAI, apolipoprotein AI; CE, cholesteryl ester; ER, endoplasmic reticulum; FC, free cholesterol; MTP, microsomal triglyceride transfer protein; PBS, phosphate-buffered saline; PC, phosphatidylcholine; TG, triglyceride; DGAT, diglyceride:acyl-CoA acyltransferase; ACAT, acyl-CoA:cholesterol acyltransferase; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.
space of the ER where MTP is present. Little is known about the precise mechanism of lipid transfer in hepatic cells especially on lipid movement across ER membranes.

There are many published reports that indicate that apoB secretion is dramatically affected by exogenous compounds (17–21). For example, addition of oleic acid to hepatic cells in culture leads to increased TG synthesis within the cells and enhanced apoB secretion (17), whereas in rat hepatocytes this is not the case (22). MTP inhibitors prevent association of lipids with apoB, which in turn affects apoB synthesis (18). It is thought that apoB-containing lipoproteins change the availability of neutral lipids necessary for lipoprotein formation not only by their amounts but also by decreasing the efficiency of intracellular movement of the lipids.

During the search for possible hypolipidemic compounds, we found that verapamil inhibited secretion of apoB-containing lipoproteins from human hepatoma cell line HuH-7 cells in culture. HuH-7 is well differentiated and is considered to be relevant to hepatic cells physiologically (23). Although HepG-2, one of the widely used hepatic cell lines, secretes only high density lipoprotein-sized, lipid-poor particles (24), low density lipoprotein-sized lipoprotein particles containing apoB-100 are secreted from HuH-7 cells (25). Thus we used the cell line as a good model to investigate human lipoprotein metabolism. Because apoB degradation within the HuH-7 cells was greatly enhanced with verapamil treatment, we speculate that verapamil reduces lipid availability for lipoprotein assembly. Verapamil did not inhibit either protein synthesis or lipid synthesis in the cells, but it inhibited lipid transfer from the cytosolic lipid pool to the luminal space in the ER across the ER membranes. Our present study provides the evidence for a novel transmembrane TG transfer system that is crucial for providing TG for VLDL assembly in ER.

MATERIALS AND METHODS

Cell Culture—HuH-7, a human hepatoma cell line, was obtained from Health Science Research Resources Bank (cell no. JCRB0043, Osaka, Japan). Cells were maintained in “medium A” (Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% 100× non-essential amino acids solution (ICN Biomedicals, Aurora, OH) and antibiotics (penicillin G potassium, 100 U/ml; streptomycin sulfate, 100 µg/ml; and fungizone, 2.5 µg/ml each of leupeptin, antipain, chymostatin, and pepstatin and 40 µg/ml of ALLN were added to the conditioned medium. The cells were lysed in cell-lysis buffer (20 mM Tris-HCl, pH 7.4, containing 0.2% Triton X-100, 0.2% SDS, 0.05% EDTA, and the following protease inhibitors: 20 µg/ml each of leupeptin, antipain, chymostatin, and pepstatin, 200 µg/ml phenylmethylsulfonyl fluoride and 80 µg/ml of N-acetyl-leucyl-leucyl-norleucine (ALLN)). Immuno precipitation of apoB was carried out as described previously (21). Briefly, conditioned medium and cell lysate were incubated with rabbit anti-human apoB antibody (Calbiochem-Novabiochem, San Diego, CA) at 4 °C, followed by incubation with protein A-Sepharose 4B at 4 °C. The autoradiogram after SDS-PAGE was visualized using a BAS-1500 bio-imaging analyzer (Fuji Photo). The relative intensity of radioactive spots was determined by BAS-1500 with photo-stimulating luminescence (PSL) as the unit of measure.

Pulse Labeling and Chasing of Intracellular apoB—HuH-7 cells were incubated with serum-free DMEM containing 40 µM verapamil for 4 h. After washing three times with PBS, the cells were incubated with Met-free medium containing 40 µM verapamil for 1 h in the presence or absence of 40 µg/ml ALLN. The cells were then pulsed with [35S]Met for 30 min and washed once with PBS and twice with serum-free DMEM containing 5 µM Met. After incubation with serum-free DMEM containing 5 µM Met for 60 min, cells and conditioned media were collected. Immediately, 10 µg/ml each of leupeptin, antipain, chymostatin, and pepstatin and 40 µg/ml of ALLN were added to the conditioned media. The cells were lysed in cell-lysis buffer. Radioactivity in the apoB band was quantified using a BAS-1500 bio-imaging analyzer after immunoprecipitation, and SDS-PAGE were carried out.

Analysis of the Lipids Secreted into Conditioned Medium—HuH-7 cells grown in 100-mm dishes were cultured for 24 h with serum-free DMEM containing 40 µM verapamil or vehicle and 37 kBq/ml [14C]acetate (Amersham Biosciences, Tokyo, Japan). Lipids were extracted from the conditioned medium with chloroform and methanol. The lipids were separated on thin-layer chromatography (TLC) as described previously (22). The radioactivities of the separated bands were quantified either using a BAS1500 bio-imaging analyzer or a liquid scintillation counter.

Measurement of [14C]Acetic Acid Incorporation into Lipids—Cells were cultured for 12 h with serum-free DMEM supplemented with 5 mM HEPES and 40 µM verapamil or vehicle. Following this, [14C]acetate was added to the culture, and the mixture was incubated for up to 4 h. Lipids, extracted from the cells with chloroform and methanol, were separated and quantified using TLC as described above. To determine [14C]acetate incorporation into microsomal lipids, cells were labeled with [14C]acetate in the presence of 40 µg/ml ALLN to prevent proteolytic degradation of apoB. After 4-h incubation with [14C]acetate, cells received trypsin treatment. The secreted lipids were collected and then incubated with ice-cold PBS. Field centrifugation was carried out as described previously (25). Briefly, cells were homogenized in cell-homogenization buffer (250 mM sucrose, 0.1% ethanol, and 20 mM Tricine, pH 7.8) using a Teflon homogenizer. After centrifugation at 1,300 × g for 10 min (to exclude large organelles and cell debris), the homogenates were centrifuged at 15,000 × g for 20 min, and the resulting supernatant was further centrifuged at 100,000 × g for 60 min to precipitate micromesomes. The microsomes suspended in cell-homogenization buffer were incubated with an equal volume of 200 mM Na2CO3 on ice for 60 min followed by centrifugation at 200,000 × g for 60 min. After this, microsomal membranes (precipitates) and luminal contents (supernatant) were separated and recovered. Lipids extracted from the precipitates and supernatants were analyzed by TLC. The radioactivities of the separated bands on TLC were quantified using a BAS1500 bio-imaging analyzer.

Measurement of MTP Activity—HuH-7 cells grown in DMEM supplemented with 10% FCS were washed three times with PBS and homogenized using a probe-type sonicator. MTP activity was determined using the MTP activity kit (Roche Biochemical, Tokyo, Japan) according to the manufacturer’s protocol. Briefly, 1 ml of cell homogenate (100 µg of protein), containing 10 µl each of suspensions of donor and acceptor particles, was incubated with either verapamil or vehicle in a water bath at 37 °C. The change of fluorescent intensity (excitation at 465 nm and emission at 535 nm) was subsequently monitored. The rate of MTP was defined by a linear plot analysis using a rabbit anti-human MTP polyclonal antibody (27).

Assay of Lipid Transfer Activity via a Microsomal Membrane—After culturing HuH-7 cells in serum-free DMEM with 37 kBq/ml [2-14C]acetate for 12 h, the cytosolic fraction was separated from the cells.
Lipids extracted from the cytosol with chloroform/methanol (2/1) were dried under an argon gas stream. The lipids were re-suspended in PBS using a sonicator, and aliquots of the suspension were used as the donor lipids for this lipid transfer assay. The microsomal fraction was prepared from another batch of HuH-7 cells as described above except for HEPES-buffered solution (250 mM sucrose, 25 mM HEPES-KOH, pH 7.4), which was used as the acceptor of the lipid transfer assay. An aliquot of the microsomes was suspended in assay buffer (100 mM potassium acetate, 5 mM magnesium acetate, 25 mM sucrose, 25 mM HEPES-KOH, pH 7.4, together with an ATP-regenerating system consisting of 1 mM ATP, 20 mM creatine phosphate, and 0.5 unit/ml creatine kinase), with the reaction started by addition of the lipid suspension. After an appropriate incubation time at 37 °C, the reaction mixture was diluted with the same volume of ice-cold HEPES-buffered solution and centrifuged at 100,000 × g for 60 min. Precipitated microsomes were re-suspended in ice-cold HEPES-buffered solution containing 3% bovine serum albumin (fatty acid-free) and then precipitated by centrifugation. Luminal contents of the microsomes were separated from membranes after treatment with Na2CO3 followed by centrifugation at 200,000 × g for 60 min (26). Lipids extracted from the luminal contents with chloroform/methanol were analyzed by TLC. The radioactivities of separated bands by TLC were quantified using a BAS1500 bio-imaging analyzer.

**Assay for Cholesterol Esterification—Plasma membrane FC was labeled by incubating HuH-7 cells with [14C]FC on ice for 90 min.** Then the cells were incubated with medium containing 40 μM verapamil or 4 μM reserpine at 37 °C for up to 4 h. Lipid was extracted from the cells and separated on TLC to measure the radioactivities in CE and FC. Amounts of apoB secreted into conditioned medium were determined using sandwich ELISA procedure.

**RESULTS**

**Verapamil Specifically Inhibits the Secretion of ApoB-containing Lipoprotein in HuH-7 Cells—**To study the regulatory mechanism of human apoB-100-containing lipoprotein formation, we tested various compounds on apoB secretion from the human hepatoma cell culture line HuH-7. HuH-7 cells secrete LDL-sized lipoprotein particles (density, ~1.04), which contain apoB-100 but not apoB-48 judging by Western blotting (data not shown). The amount of apoB secreted into culture media was measured using sandwich ELISA procedure. We found that verapamil inhibited apoB secretion from the cells in a dose-dependent manner (Fig. 1, circles). Because the secretion of albumin (Fig. 1, triangles) and apoAI (data not shown) was not decreased under the same conditions, it is very likely that verapamil has an inhibitory effect on assembly mechanisms of human apoB-100 but not on general protein secretory systems. This inhibitory effect of verapamil was readily detectable after 4 h of treatment and continued during 24 h of culture period (data not shown). When verapamil was washed out after 4 h of treatment, the apoB secretion was recovered to the control level (data not shown). During the treatment with verapamil, no significant change in amount of cellular protein was observed (ranging between 1.9 and 2.1 mg/dish). These results indicate that a short period of treatment with verapamil is enough to reduce apoB secretion and the effect of verapamil is reversible.

**Effects of Verapamil on the Synthesis and the Intracellular Degradation of ApoB—**Lipoprotein secretion can be reduced either when synthesis of apoB is inhibited or when intracellular degradation of apoB is accelerated. ApoB is degraded before secretion when it is poorly lipidated, and it is proposed that the rate of degradation is the major determinant of the rate of apoB secretion (1–3). The rate of apoB synthesis in HuH-7 cells was examined by counting [35S]Met incorporation into apoB. Treatment of the HuH-7 cells with verapamil showed no change in the levels of radioactivity incorporated in newly formed apoB (Fig. 2A). This suggests that verapamil does not change the rate of apoB protein synthesis. Next we examined the disappearance and secreting rate of apoB from the cell. When apoB synthesized de novo was pulse-labeled with [35S]Met and chased up to 60 min, verapamil treatment decreased the secretion of radiolaabeled apoB without affecting the rate of disappearance from the cells (Fig. 2B). Then we examined whether the decrease in rate of apoB secretion by verapamil was due to increased degradation within the cells. ApoB is known to be degraded by proteasomes in the cytosol and by proteases in the ER. A cysteiny protease inhibitor, ALLN, can block both of these protease activities (29). When the pulse-chase experiment was performed again in the presence of ALLN (Fig. 2C), ALLN completely prevented the degradation of apoB, as shown by the total recovery of apoB (sum of cellular fractions and media) being almost 100% in both the control and verapamil-treated cells. It is noteworthy that the cells treated with verapamil and ALLN still secreted lesser amounts of apoB than control cells, whereas the majority of apoB molecules prevented from being secreted remained within the cells (Fig. 2B, filled symbols). Addition of ALLN alone did not change the total recovery of apoB suggesting that apoB synthesis was not affected by ALLN. These results indicate that, with verapamil treatment, apoB was degraded by ALLN-sensitive protease activity, and hence secretion was prevented.

**Verapamil Affects Lipid Accumulation in ER—**It is well-known that lipid availability is an important factor in apoB secretion from hepatic cells; thus we tested the effects of verapamil on lipid availability for lipoprotein assembly in HuH-7 cells using [14C]acetate as a tracer. During 4 h of culture, incorporation of radioactivity into TG and FC was not reduced but, rather, was higher in the cells treated with verapamil than control cells (Fig. 3). Treatment with verapamil also increased incorporation of radioactivity into phospholipids (data not shown). These results indicate that de novo synthesis of lipids in the cells was not inhibited by verapamil.

After HuH-7 cells were incubated with [14C]acetate with or without verapamil for 24 h, incorporation of radioactivity in various lipids in the cells and medium was separately measured. As shown in Fig. 4A, relative amounts of radioactive lipids secreted in the medium, especially TG and CE, were decreased to about 40% of those of control cells following verapamil treatment; this is similar to the extent of inhibition as observed in apoB secretion (see Fig. 1). In the next experiments, HuH-7 cells were cultured for 4 h in serum-free medium containing [14C]acetate with or without verapamil. The micro-
**FIG. 2.** Effect of verapamil on synthesis and degradation of apoB in HuH-7 cells. A, effect of verapamil on apoB synthesis. After HuH-7 cells were cultured for 4 h in serum-free conditions, the medium was changed to Met-free medium containing $[^{35}S]$Met. During all these procedures, cells were treated with 40 μM verapamil (filled circles) or vehicle only (open circles). After the incubation periods indicated, cells were lysed and the lysates prepared from 5 x 10⁴ cells were subjected to immunoprecipitation. Radioactivity in apoB was then determined. Indicated values are the means ± S.E. from triplicates. PSB, photo-stimulating luminescence. B, effect of verapamil on disappearance of apoB from the cells. After cells were cultured for 4 h in serum-free conditions, the medium was changed to a Met-free DMEM containing $[^{35}S]$Met for 30 min. Then the cells were chased without $[^{35}S]$Met for up to 60 min. During all these procedures, cells were treated with 40 μM verapamil (filled symbols) or vehicle only (open symbols). The cell lysates (circles) and conditioned media (triangles) were immunoprecipitated with anti-apoB antibody, and radioactivity levels in apoB were determined. Each sample corresponds to 5 x 10⁴ cells. C, effect of the protease inhibitor ALLN on the disappearance of apoB from the cells. The cells were pulse-labeled with $[^{35}S]$Met and chased for up to 60 min using the same procedure as in B. During all these procedures, cells were treated with 40 μM verapamil (filled circles), 40 μM verapamil plus 40 μg/ml ALLN (filled squares), 40 μg/ml ALLN without verapamil (open squares), or vehicle only (open circles). Cell lysates and conditioned media were collected. Relative amounts of the total recovery of apoB (sum of immunoprecipitated apoB from both cell lysate and conditioned medium) are shown in comparison with the values for the chase-time “0.” Indicated values are the means ± S.E. from three independent experiments.
Verapamil Increases Lipid Transfer across ER Membranes

**Fig. 3. Effect of verapamil on lipid synthesis in HuH-7 cells.**
HuH-7 cells cultured in serum-free DMEM with 40 \( \mu \)g/ml verapamil for 12 h were further incubated with \( \left[{\text{14C}}\right] \text{acetate} for up to 4 h. During all these procedures, cells were treated with 40 \( \mu \)g verapamil (filled symbols with solid lines) or vehicle only (open symbols with dotted lines). Lipid extracted from the cells was separated on TLC, and the radioactivities for TG (circles), FC (triangles), and CE (squares) were determined. Indicated values are the means \( \pm \) S.E. from triplicates.

Our data (Figs. 1–3) show that verapamil decreases lipoprotein secretion without inhibiting synthesis of apoB protein as well as lipids. The observations shown in Fig. 4 suggest that the relative amounts of neutral lipids present in ER are reduced by verapamil and that this change in intracellular lipid distribution could lead to reduced secretion of lipoprotein. We then developed an *in vitro* assay system for examining lipid transfer into the microsomal luminal space. In this assay system, radiolabeled lipids and microsomes were mixed, followed by monitoring of radioactivity of lipids in microsomal lumen. Lipids were extracted from the cytosolic fraction of HuH-7 cells, which had already been cultured with \( \left[{\text{14C}}\right] \text{acetate} for 24 h. The extracts were re-suspended in buffer and used as a radio-labeled tracer. Microsomal fractions, prepared from HuH-7 cells separately and mixed with the radioactive lipids from the cytosolic fraction and an ATP-regenerating system, were incubated at 37 °C for up to 40 min. The washed microsomes were subsequently treated with \( \text{Na}_2\text{CO}_3 \) to separate luminal contents and membranes. We found that addition of verapamil to the assay mixture reduced the accumulation of TG radioactivity in the microsomal lumen (Fig. 5, TG). However, accumulation of radioactivity in CE or FC in microsomes was not altered by the addition of verapamil. Because 20 \( \mu \)g/ml ALLN was present throughout the incubation in this experiment, it is unlikely that apoB in the isolated microsomes is degraded. This observation strongly suggests that there is a novel regulated process of TG transfer across ER membrane and that the process is sensitive to verapamil.

Verapamil Does Not Affect Activity of MTP—MTP is a known factor involved in lipoprotein assembly that transfers lipids to apoB in ER (4–7). We therefore investigated whether verapamil affects MTP activity in HuH-7 cells. First, we measured MTP activity in homogenates of HuH-7 cells in the presence or absence of verapamil in the reaction mixture and found that MTP activity was not altered by verapamil *in vitro* (Fig. 6A). Second, we also tested possible changes in MTP activity in HuH-7 cells in culture. HuH-7 cells were cultured with or without verapamil for 4 h, then the MTP expression level and MTP activity in the cell homogenates were determined. There was no difference in MTP expression (Fig. 6B, inset) or lipid transfer activity between the verapamil-treated cells and the control cells (Fig. 6B). These results indicate that verapamil has no effect on MTP activity in HuH-7 cells.

Verapamil Inhibits ApoB Secretion Independently from Its Inhibitory Effect on P-glycoprotein—Verapamil is known to be a potent inhibitor of P-glycoprotein (30). It is reported that P-glycoprotein activity is involved in cholesterol traffic from plasma membrane to ER where ACAT locates (31, 32), and verapamil inhibits esterification of plasma membrane FC possibly by interfering with P-glycoprotein function in cholesterol traffic (32). We investigated whether verapamil decreases secretion of apoB-containing lipoprotein through its inhibitory effect on cholesterol traffic. HuH-7 cells in culture were incubated at 0 °C with \( \left[{\text{14C}}\right] \text{FC} to replace plasma membrane FC with radioactive FC. The culture was then continued at 37 °C, and the formation of \( \left[{\text{14C}}\right] \text{CE} was monitored. As shown in Fig. 7A, verapamil decreased \( \left[{\text{14C}}\right] \text{CE} formation during the 4 h of culture. Furthermore, another P-glycoprotein inhibitor, reserpine, decreased radioactive CE in HuH-7 cells (Fig. 7B), indicating that intracellular traffic of \( \left[{\text{14C}}\right] \text{FC} from the plasma membrane is impaired by treatment with these P-glycoprotein inhibitors. ApoB secretion from HuH-7 cells, however, was not reduced by reserpine, even at the concentration sufficient to inhibit FC esterification (Fig. 7C). These results strongly suggest that the inhibitory effects of verapamil on plasma FC esterification are not responsible for the decrease in apoB secretion upon treatment with verapamil.

Verapamil Inhibits ApoB Secretion Independently From Its Antagonistic Effect on Calcium Channels—Verapamil is also well known as an antagonist of the L-type calcium ion channel. We examined whether the inhibition of calcium influx into the cells is involved in the inhibition of apoB secretion by verapamil. Addition of 0.02 or 0.2 \( \mu \)mol of A23187, a calcium ionophore, together with verapamil, did not restore apoB secretion (Fig. 8A), suggesting that the inhibition of calcium influx is not responsible for the inhibition of apoB secretion by verapamil. Higher concentrations of A23187 up to 5 \( \mu \)mol did not restore the apoB secretion, whereas the cells were severely damaged and apoB secretion was further decreased when the concentration was more than 1 \( \mu \)mol (data not shown).

The verapamil used in this study is a racemic mixture. It is reported that the IC50 of the \((R)^{-}\)-isomer for inhibition of the channel is more than 10 times higher than that of the \((S)^{-}\)-isomer (33); thus the \((S)^{-}\)-verapamil is responsible for the calcium channel inhibition. Conversely, both isomers effectively inhibit P-glycoprotein (34). As shown in Fig. 8B, there was no significant difference between the two stereoisomers of verapamil or their inhibitory effects on apoB secretion at any concentration examined. These results suggest that the inhibitory effect of verapamil on apoB secretion is not related to the changes in intracellular calcium concentration.

**DISCUSSION**

VLDL particle formation is a well-controlled process, where, without proper association of lipids to apoB, the whole process cannot be completed and poorly lipidated apoB is degraded by proteasome or ER-residential proteases prior to secretion. Little is understood, however, about the precise mechanism of lipid transfer during the lipoprotein assembly process. In this study, using the human hepatic cell line HuH-7 in culture, we...
found that verapamil prevented apoB secretion from the cells without inhibiting de novo synthesis of apoB and lipids. The lipid composition in the luminal space of ER in HuH-7 cells in culture was greatly changed upon treatment with verapamil. Furthermore, we demonstrated that verapamil inhibited TG transfer of cytosolic lipids into the luminal space of ER across organellar membranes. We conclude that there is novel machinery for TG transfer into ER lumen across the ER membranes, which is crucial for proper lipid association with apoB during formation of lipoprotein particles. This is the first dem-
onstration, to our knowledge, of the importance of lipid transfer across the ER membranes in VLDL assembly. TG and CE, major lipid constituents in VLDL, are thought to be synthesized on the cytosolic side of ER membranes (12, 13, 15). A recent report pointed out that ACAT is present in small cytosolic vesicles in addition to ER membrane (35). Although some DGAT activity was found in the microsomal lumen, there was, however, no direct evidence for the synthesis of lipids on the luminal side of ER. MTP transfers TG and CE from the ER membrane to apoB, however, MTP is localized within the luminal space of ER. Therefore, these lipids must be moved from the site of synthesis to the luminal side of the ER prior to binding with MTP. To determine intracellular movement of lipids, we utilized a Na2CO3 treatment procedure, which separates luminal contents from ER membranes.

An in vitro assay was introduced in which isolated microsomal fractions were used as lipid donors. We found that verapamil inhibited time-dependent accumulation of TG in the microsomal luminal space in the in vitro assay. Verapamil has no effect on MTP activity. MTP activity in HuH-7 homogenates was assayed in the presence or absence of verapamil. 1 ml of assay mixture containing cell homogenate (100 μg of protein), 1 mM EDTA, 10 μl each of suspension of donor and acceptor particles, and either 40 μM verapamil (closed circles) or vehicle (open circles) was incubated at 37 °C for up to 120 min. During the incubation, change in fluorescent intensity was monitored. The expression of MTP in the cell homogenates was determined by Western blot analysis using a rabbit anti-human MTP polyclonal antibody.
formed TG present in the cytosolic lipid pool may be a good substrate for lipid transfer across ER membranes. Our present results suggest that there is verapamil-sensitive activity of TG transfer into ER lumen, which accounts for the decreased secretion of apoB by verapamil treatment. Previously, verapamil was reported to inhibit lipoprotein secretion from rat hepatocytes (36) and Caco-2 cells (31). Field et al. (31) found that verapamil inhibited both esterification of FB in plasma membrane and the secretion of apoB, apoAI, and lipids in Caco-2 cells, a human intestinal cell line. From these results, they pointed out that these inhibitory effects were dependent on normal vesicular traffic functioning within the cell. They postulated that verapamil disturbed the acidic environment of transport vesicles through inhibition of P-glycoprotein, and this disturbance led to impaired secretion of apoB. However, our observations using HuH-7 cells differ from these findings: first, verapamil did not inhibit secretion of albumin and apoAI in HuH-7 cells (Fig. 1A and data not shown), thus vesicular

**Fig. 7.** Verapamil and reserpine inhibited plasma membrane cholesterol esterification, but reserpine showed no effect on apoB secretion. Plasma membrane FC was labeled by incubating HuH-7 cells with [3H]FC on ice for 90 min. The cells were then incubated with medium containing 40 μM verapamil (A) or 4 μM reserpine (B) at 37 °C for up to 4 h. Lipid was extracted from the cells and separated on TLC to determine levels of radioactivity in CE and FC. Indicated are representative data from two independent experiments. C, HuH-7 cells were incubated with serum-free medium with either 40 μM verapamil, 4 μM reserpine, or vehicle for 4 h. Amounts of apoB secreted into medium were determined using a sandwich ELISA procedure. Indicated values are the means ± S.E. from four separate experiments.

**Fig. 8.** Calcium mobilization does not affect apoB secretion from HuH-7 cells. A, HuH-7 cells were incubated with serum-free medium either with or without verapamil (40 μM) for 4 h. In some of the cells, the calcium ionophore A23187 (0.02 or 0.2 μM) was added in addition to verapamil. Amounts of apoB secreted into conditioned medium were determined using the sandwich ELISA procedure. B, HuH-7 cells were incubated in serum-free medium containing either R(+) -verapamil (open triangles) or S(−)-verapamil (shaded circles) for 24 h. Amounts of apoB secreted into conditioned medium were determined using the sandwich ELISA procedure. Indicated values are the means ± S.E.
function should not be sensitive to verapamil. Second, reserine, another P-glycoprotein inhibitor, failed to decrease apoB secretion, although it reduced FC esterification probably through the inhibition of P-glycoprotein (Fig. 7).

Verapamil treatment certainly inhibited esterification of FC in the plasma membrane (Fig. 7) possibly through the inhibition of P-glycoprotein (32), and we observed that the content of CE in secreted lipoprotein in medium was decreased (data not shown). Impaired delivery of FC from plasma membranes to ER would reduce the availability of CE for lipoprotein assembly and would then cause apoB secretion to diminish. However, it is controversial whether availability of CE within hepatic cells is responsible for lipoprotein secretion. Some reports showed that hydroxymethylglutaryl-CoA reductase inhibitor (37) or an ACAT inhibitor (38) was effective in inhibiting the secretion of apoB-containing lipoprotein. But in other studies, including ours, inhibition of these enzymes showed no effect on apoB secretion (39, 40). There are some reports suggesting that the inhibitory effects of these compounds on apoB secretion are not due to their lipid lowering effects but rather to unknown mechanisms (19, 20, 21, 22).

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Transmembrane Lipid Transfer Is Crucial for Providing Neutral Lipids during Very Low Density Lipoprotein Assembly in Endoplasmic Reticulum

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