Overexpression of human diacylglycerol acyltransferase 1, acyl-CoA:cholesterol acyltransferase 1 or acyl-CoA:cholesterol acyltransferase 2 stimulates secretion of apolipoprotein B-containing lipoproteins in McA-RH7777 cells

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Abstract:
The relative importance of each core lipid in the assembly and secretion of very low density lipoproteins (VLDL) has been of interest over the past decade. The isolation of genes encoding diacylglycerol acyltransferase (DGAT) and acyl-CoA:cholesterol acyltransferases (ACAT1 and 2) provided the opportunity to investigate the effects of isolated increases in triglycerides (TG) or cholesteryl esters (CE) on apolipoprotein B (apoB) lipoprotein biogenesis. Overexpression of human DGAT1 in rat hepatoma McA RH7777 cells resulted in increased synthesis, cellular accumulation, and secretion of TG. These effects were associated with decreased intracellular degradation and increased secretion of newly synthesized apoB as VLDL. Similarly, overexpression of human ACAT1 or ACAT2 in McA RH7777 cells resulted in increased synthesis, cellular accumulation, and secretion of CE. This led to decreased intracellular degradation and increased secretion of VLDL apoB. Overexpression of ACAT2 had a significantly greater impact upon assembly and secretion of VLDL from liver cells than did overexpression of ACAT1. Addition of oleic acid (OA) to media resulted in a further increase in VLDL secretion from cells expressing DGAT1, ACAT1, or ACAT2. VLDL secreted from DGAT1-expressing cells incubated in OA had a higher TG:CE ratio than VLDL secreted from ACAT1- and ACAT2-expressing cells treated with OA. These studies indicate that increasing DGAT1, ACAT1 or ACAT2 expression in McA RH7777 cells stimulates the assembly and secretion of VLDL from liver cells and that the core composition of the secreted VLDL reflects the enzymatic activity that is elevated.
Apolipoprotein B100 (apoB) is the major protein of atherogenic very low density (VLDL) and low density lipoproteins (LDL). There is a wide range of apoB secretion from the liver in humans studied on average American diets, and overproduction of VLDL and LDL is a common feature of human dyslipidemias (1-3). The secretion of apoB in cultured primary hepatocytes and hepatoma cells is regulated primarily at the post-translational level by degradation of newly synthesized apoB (4). This pre-secretory degradation is, in turn, regulated mainly by the availability of newly synthesized core lipids (triglyceride (TG) and cholesterol esters (CE)) (5-7). In the presence of active microsomal triglyceride transfer protein (MTP) and adequate phospholipids, these core lipids facilitate the translocation of nascent apoB across the endoplasmic reticulum, targeting it away from proteasomal degradation and towards secretion (8,9).

The relative importance of each core lipid in the assembly of VLDL (10) is a fundamental issue that has been a topic of interest in several laboratories over the past decade. The central role of TG in assembly and secretion of apoB-lipoproteins has been demonstrated repeatedly in cultured liver cells (6,11-13). Stimulation of the secretion of apoB-lipoproteins by oleic acid (OA) is closely associated with increased TG synthesis and secretion, while decreased apoB-lipoprotein secretion in the presence of triacin D (a potent inhibitor of fatty acyl CoA synthetase) is associated with decreased TG synthesis (11). Diacylglycerol acyltransferase (DGAT) is a microsomal enzyme activity that catalyzes the final committed step in TG synthesis by using DAG and fatty acyl CoA as its substrates (14). DGAT activity is present in the liver and, therefore, is likely to be involved in apoB-lipoprotein assembly, and in the regulation of plasma TG concentrations (10,15). In contrast to TG, the role of CE in the assembly and secretion of apoB-lipoproteins has been less clearly defined (16). Because acyl-CoA:cholesterol acyltransferases (ACATs) catalyze the formation of CE from cholesterol and long-chain fatty acyl-coenzyme A (14,17), their role in regulating apoB-lipoprotein secretion has been studied extensively in cultured cells and in vivo (11,18-30). However, the results of those studies have been variable and often contradictory.
Recently, several genes encoding DGATs have been identified (31-33). Although targeted deletion of murine DGAT1 has indicated a role for the enzyme in obesity and insulin sensitivity (34,35), the activity of this enzyme is not required for chylomicron formation (36). Similarly, obviation of the major alternate pathway of TG synthesis encoded by the DGAT2 gene significantly reduced but did not eliminate lipoprotein secretion by the liver or intestine (37). Thus the role of DGAT in the assembly and secretion of apoB-containing lipoproteins remain unclear. Two genes encoding human ACAT (ACAT1, ACAT2) have also been identified (38-41) with different tissue locations and predicted membrane orientations. This has promoted the hypothesis that CE formed by ACAT1 and ACAT2 have different functions (41-44). Targeted deletion of the murine ACAT1 gene has no apparent consequences with regard to serum lipids, while deletion of the murine ACAT2 gene results in resistance to hyperlipidemia induced by a diet high in fat and cholesterol, suggesting a role for ACAT2 in the incorporation of dietary cholesterol and fat into intestinal lipoproteins (45). Conversely, on the background of an apolipoprotein E deficient animal, ACAT2 mutant mice secreted increased numbers of triglyceride enriched VLDL that were severely depleted in CE (46). This suggests a major role of ACAT2 in loading lipoprotein particles with steryl esters, but no direct role in VLDL biogenesis.

In the present studies, we overexpressed human DGAT1, ACAT1 and ACAT2 individually in McA RH7777 rat hepatoma cells to investigate the effects of isolated increases in TG or CE synthesis on the assembly and secretion of apoB-lipoproteins. We found that not only did overexpression of each of the three cDNAs increase secretion of apoB in VLDL, but that the core-lipid content of VLDL reflected the enzyme that was overexpressed. We also observed that the ACAT2 enzyme was more efficient in its promotion of lipoprotein biogenesis than was ACAT1, despite similar elevations in CE biosynthesis.
Materials and Methods:

Materials: N-Acetyl-leu-leu-norleucinal (ALLN), dimethyl sulfoxide, leupeptin, pepstatin A, and bovine serum albumin (fatty acid-free) were purchased from Sigma. Sheep anti-human apoB100 polyclonal antibodies were from Roche or CalBiochem. They cross-react with mouse and rat apoB. [3H]glycerol and [35S]methionine/cysteine were from PerkinElmer Life Sciences. Protein A-Sepharose CL-4B was from Amersham Biosciences, Inc. Minimum essential medium (MEM) was purchased from Invitrogen. Bovine serum albumin (fatty acid-free) and oleic acid (OA) were purchased from Sigma.

O-acyltransferase Expression Constructs: Human DGAT1, and ACAT2 cDNAs were expressed from the CMV promoter of pCR3.1 (Invitrogen) with approximately 25 bp of 5’ and 3’ untranslated sequences (38). ACAT1 was isolated by RT-PCR from mRNA extracted from the human macrophage cell line THP1, using oligos ACAT4 and ACAT5 (TGTGGAGAAGCCTATTAGCTGGG and ATCTGTAAAGCCTGGAGTGGTCC, respectively) to include 250 bp and 642 bp of the 5’ and 3’ untranslated regions. This was performed to maximize levels of sterol esterification from ACAT1, which was demonstrated in pilot cell culture experiments to require more upstream sequences than for the other genes.

Stable Transfected Cells: McA RH7777 rat hepatoma cells, obtained from ATCC, were grown in six-well plates coated with collagen and maintained in DMEM containing penicillin (100 units/ml), streptomycin (100 µg/ml), 10% fetal bovine, and 10% horse serum (Invitrogen). Stably transfected McA RH7777 cells expressing human DGAT1, ACAT1, and ACAT2 were generated by co-transfection of DGAT1 or ACAT plasmids with a neomycin vector and Lipofectamine 2000: Positive clones were isolated using G418 selection media. A total of 6 DGAT1 clones, 4 ACAT1 clones, 4 ACAT2 clones, and 5 neomycin vector control clones were isolated. These clones were maintained in culture medium containing 400 µg/ml G418, which was removed prior to experiments. Expression of human ACAT1, ACAT2 or DGAT1 in the individual clones was assessed by gene specific RT-PCR assays from oligo-dT primed cDNA as described previously (38,47). In vitro microsomal and cell culture O-acyltransferase enzymatic activities were determined as described (38,47).
**Immunoprecipitation:** For steady state studies, cells were pre-incubated in methionine free medium for 2 hours and then labeled with \[^{35}\text{S}]\text{Met}\) for 2 hours. Studies of intracellular degradation of apoB in McA RH7777 cells transfected with DGAT1, ACAT1, ACAT2 or vector control (Neo) involved pre-incubation in serum free, methionine free medium for 2 hours, pulse labeling with \[^{35}\text{S}]\text{Met}\) for 30 mins, and a 90 minute chase protocol in medium supplemented with cold methionine. Samples were taken every 15 minutes and processed for intracellular apoB by immunoprecipitation and SDS-PAGE as below. After labeling, media were transferred to tubes containing a mixture of protease inhibitors (PI) (30 µl/ml, PI: 1 mM benzamidine, 5 mM EDTA, 100 units/ml aprotinin, 50 µg/ml leupeptin, 50 µg/ml pepstatin A, and 10 mM Hepes, pH 8.0) and 0.86 mM freshly added phenylmethylsulfonyl fluoride (PMSF). Cells were harvested with homogenization buffer (62.5 mM sucrose, 0.5% sodium deoxycholate, 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and PI). Immunoprecipitation of apoB100 in medium or cellular lysate was carried out according to the method of Dixon et al. (9). Protein A-Sepharose CL4B was added to the reaction solution to precipitate apoB100, which was released with sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% β-mercaptoethanol) by boiling for 5 min. Samples were resolved with SDS-polyacrylamide gel (SDS-PAGE). The gel was treated with Autofluor (National Diagnostic, Atlanta, GA) and, after drying, exposed to x-ray film (Kodak X-Omat AR) at -70 °C and quantified using laser scanning densitometry.

**Metabolic labeling, Lipid Extraction and Thin Layer Chromatography.** McA RH 7777 cells were labeled with \[^{3}\text{H}]\text{glycerol}\) (20 µCi/ml) or \[^{3}\text{H}]\text{OA}\) (15 µCi/ml) for 4 hours. Labeled cells were washed with cold PBS and then extracted with hexane/isopropanol (3/2, v/v) (1.5-2 ml/well) at room temperature for 2 h (48). The extraction solution was collected, and the remaining cellular proteins were lysed with 0.1 N NaOH. The media were extracted with 20 volumes of chloroform/methanol (2/1, v/v) at room temperature for 2 h. Then five volumes of H₂O were added to the mixture, which was spun for 5 min at 2,000 rpm. The lower organic phase was carefully transferred to a new tube and dried under a stream of N₂ gas. The lipid content of the cells and/or the media was re-suspended in a small volume of hexane and applied to a plastic thin layer chromatography plate, which was developed in hexane-ethyl ether-glacial acetic acid (70/30/1, v/v/v). The lipid spots were visualized with iodine vapor and analyzed by liquid scintillation counting. Statistical significance was assessed by students two tailed t-test or Analysis of Variance.
Sucrose Gradient Ultracentrifugation: Sucrose gradient ultracentrifugation of apoB-containing lipoproteins were conducted as described by Boren et al. (49). Briefly, the sucrose gradient was formed by layering from the bottom of the tube: 2 ml of 47% sucrose, 2 ml of 25% sucrose, 5 ml of sample in 12.5% sucrose, and 3 ml of PBS. All solutions contained 0.1 mM leupeptin, 1 µM pepstatin A, 0.86 mM PMSF, 100 units/ml aprotinin, 5 µM ALLN, 5 µM EDTA, 150 mM NaCl, and 50 mM PBS, pH 7.4. The gradients were spun at 35,000 rpm in a Beckman SW40 rotor for 65 h at 12 °C, and unloaded into 12 fractions from the top of the tube. Each fraction was subjected to immunoprecipitation of apoB, SDS-PAGE and autoradiography as described above.

Cellular TG, CE, and protein mass determination: TG mass in cell extracts was determined using a triglyceride determination kit from Boehringer Mannheim. CE mass in cell extracts was determined using an enzymatic assay after separation of free and esterified cholesterol by gas chromatography. Cellular protein levels were determined with a BioRad protein assay kit.

Medium lipoprotein lipid composition: Serum free culture medium was collected after 6 hour incubations of cells at 37°C, 5% CO₂. The culture medium was adjusted with solid NaCl to d:1.006 and centrifuged for 22 hours at 39,000 rpm in a 50.3 rotor. VLDL (d<1.006 lipoproteins) were isolated from the top 1 ml and material from several wells were pooled and analyzed by HPLC (50). Fraction densities were determined by conventional assessment of refractive index using a refractometer. The VLDL fraction (3 ml) was extracted (51), and after addition of internal standard (4 µg, 1,2-di-O-hexadecyl-rac-glycerol), and 2 ml acetone (with 0.01 % butylated hydroxytoluene), was heated in a culture tube with Teflon lined cap at 90° for 30 min. Ethyl acetate (6 ml) was added and the mixture was heated again at 90° for 30 min. After centrifugation at 4° C at 2000 rpm for 15 min, the upper layer was removed, dried under a nitrogen stream, transferred to a fresh tube with 2 X 500 µl hexane (hexane wash), dried again, and dissolved in 200 µl isoctane:tetrahydrofuran (9:1). The sample (20 µl) was injected onto a 10 cm x 4.6 mm silica gel column (Spherisorb S5W, Waters, Milford, MA) and monitored using a SEDEX 55 evaporative light scattering detector (Richard Scientific, Inc., Novato, CA). The chromatograms were stored and evaluated using Star Chromatography software (version
4.0) (Varian, Walnut Creek, CA). A standard curve utilizing triolein (Gnu-Check),
cholesteryl oleate (Gnu-Check), cholesterol (Sigma) and 1,2-di-O-hexadecyl-rac-glycerol
(Sigma) as internal standards was run every day.
Results:

Overexpression of DGAT1 increases the synthesis and secretion of triglycerides in McA RH7777 cells: We first attempted to determine whether overexpression of human DGAT1 increased TG synthesis in McA RH7777 rat hepatoma cells. Overexpression of DGAT1 in McA-RH7777 cells increased TG synthesis, as measured by the incorporation of \[^{3}H\]glycerol, by 5 fold \((102384 \pm 3815 \text{ cpm/mg cell protein})\) compared to Neo-vector control cells \((20275 \pm 2718 \text{ cpm/mg cell protein, p<0.01})\). Secretion of newly synthesized TG was also increased almost 3 fold in DGAT1 cells \((14876 \pm 3785 \text{ cpm/mg cell protein})\) compared to Neo-vector control cells \((5326 \pm 1786 \text{ cpm/mg cell protein, p<0.01})\) (Fig. 1A). Consistent with the radioactivity data, total cellular TG mass in DGAT1 cells was also significantly increased compared to neomycin-transfected cells \((18.25 \pm 3.10 \mu g \text{ vs. } 12.75 \pm 2.87 \mu g \text{ TG/mg cell protein: } p<0.05)\) (Fig. 1B). By contrast, overexpression of DGAT1 had no significant effect on either phospholipid \((4002 \pm 2432 \times 10^3 \text{ cpm/mg cell protein compared to control 4080.7} \pm 2818 \times 10^3 \text{ cpm/mg cell protein; n=4})\) or CE synthesis \((565 \pm 29.9 \times 10^3 \text{ cpm/mg cell protein compared to control 562 } \pm 43.7 \times 10^3 \text{ cpm/mg cell protein})\) as measured by incorporation of \(^{3}H\)-OA into these lipids in cells. These results are the means of three separate experiments using one clone of stably transfected McA RH7777 cells. Similar results were obtained with 3 different clones.

Overexpression of DGAT1 increases the assembly and secretion of VLDL from McA RH7777 cells: Since TG synthesis and availability plays a critical role in the assembly and secretion of apoB-lipoproteins in cultured liver cells \((6,11-13)\), we next determined whether overexpression of DGAT1 in McA RH7777 cells affected apoB secretion. As expected, concomitant with increased synthesis and secretion of TG, secretion of apoB was increased by more than 2 fold \((2.54 \pm 0.65 \text{ normalized to neomycin transfected cells: } p<0.05)\) in cells overexpressing DGAT1, as measured by the incorporation of \(^{35}S\)methionine (Fig. 1C). Increased secretion of apoB was associated with decreased intracellular disappearance of newly synthesized \(^{35}S\)methionine labeled apoB over a 90 minute pulse chase protocol, relative to the Neo control cells (Fig. 1D). This is consistent with rescue of apoB from intracellular degradation as a result of DGAT1 overexpression and targeting of nascent apoB for secretion on lipoproteins. Simultaneous stimulation of TG and apoB secretion suggested that more VLDL particles were being assembled and secreted. Indeed, experiments using sucrose gradient ultracentrifugation demonstrated that the
increased numbers of apoB-containing lipoproteins secreted by McA RH7777 cells over-expressing DGAT1 had buoyant densities characteristic of VLDL particles (Fig. 2).

**Overexpression of either ACAT1 or ACAT2 stimulates the synthesis and secretion of CE in McA RH7777 cells:** We next evaluated the role of CE synthesis and availability in the assembly and secretion of apoB-lipoproteins. Recent studies have suggested that the enzymes for CE formation, ACAT1 and ACAT2, have different functions based on different tissue locations and membrane orientations (41-44). However, the roles of these enzymes in the assembly and secretion of apoB-lipoproteins remain unclear. Hence, we examined the effects of overexpressing either ACAT1 or ACAT2 on the synthesis and secretion of cholesterol ester in McA RH7777 cells. Importantly, we observed similar increases in CE synthesis regardless of which ACAT enzyme was expressed. As measured by incorporation of [3H]OA, overexpression of either ACAT1 or ACAT2 significantly increased CE synthesis (ACAT1: 14,272 ± 2,176 cpm/mg cell protein; ACAT2: 12,681 ± 1,978 cpm/mg cell protein; p<0.01 both compared to control: 5,247 ± 1,567 cpm/mg cell protein; n=3) (Fig. 3A). Secretion of newly synthesized CE was also increased in these cells (ACAT1: 3,265 ± 716 cpm/mg cell protein; ACAT2: 2,864 ± 565 cpm/mg cell protein; p<0.01 both compared to control: 1,726 ± 312 cpm/mg cell protein; n=3) (Fig. 3B). Consistent with these results, total cellular CE mass was also significantly increased by overexpression of either ACAT1 or ACAT2 (19.0 ± 2.4 and 18.0 ± 3.2 ug CE/mg cell protein, respectively, both p<0.05 compared to control: 13.0 ± 1.7, n=3 in each group (Fig. 3C). By contrast, overexpression of either ACAT1 or ACAT2 had no significant effect on the synthesis of PL or TG, or secretion of newly synthesized PL and TG (Figures 3A, 3B).

**Overexpression of either ACAT1 or ACAT2 increases the secretion of VLDL from McA RH7777 cells:** We next examined the effects of increased CE synthesis and secretion in cells overexpressing ACAT1 or ACAT2 on the assembly and secretion of apoB-lipoproteins. Overexpression of either enzyme resulted in significant increases in the secretion of apoB-lipoproteins relative to the Neo-transfected controls, as measured by the incorporation of [35S]methionine (Fig 3D). Increased secretion with overexpression of either enzyme was associated with decreased intracellular disappearance rates of newly synthesized apoB relative to the Neo control cells over a 90 minute chase following [35S]methionine pulse labeling (Fig. 3E). These data indicated that increased secretion was associated with
reduced intracellular degradation of nascent apoB in the ACAT1 and ACAT2 expressing cells. In a series of several secretion experiments, we determined there was a marked difference in total apoB secreted by ACAT2 vs. ACAT1 transfected cells. Thus, despite similar elevations in CE synthesis in the transfected cells, overexpression of ACAT2 resulted in a 1.8-fold increase in apoB secretion relative to cells overexpressing ACAT1 (P<0.001, Fig. 3D). Sucrose gradient ultracentrifugation indicated that the increased numbers of apoB-containing lipoproteins secreted from cells overexpressing either ACAT1 or ACAT2 were in the form of VLDL particles (Fig. 4). These data indicate that elevation of either ACAT1 or ACAT2 promotes CE synthesis and coordinately increases secretion of apoB-lipoproteins in McA RH7777 cells. Furthermore these results suggest ACAT2 to be more effective than ACAT1 in promoting apoB recruitment into lipoprotein particles.

Effect of Oleic Acid on the stimulation of apoB secretion by DGAT1, ACAT1 or ACAT2: Overexpression of each enzyme may have resulted in a non-physiological state, making it difficult to extrapolate results to non-transfected cells. In an attempt to address this issue, we determined the effects of incubating the cells with OA, which is known to stimulate apoB secretion (4). In these experiments, cells were pre-incubated in 4mM OA (OA:BSA ratio of 2:1) for 30 min and then radiolabeled in the continued presence of 4mM OA for two hrs. The media were then subjected to sucrose gradient ultracentrifugation and apoB was immunoprecipitated from each of 12 fractions. SDS-PAGE gels of each fraction were scanned and fractions 1 and 2, with average densities of 1.005 and 1.008 g/L were defined as VLDL. All cell lines responded to the addition of OA to the medium by assembling and secreting more VLDL. Thus, in the presence of OA, Neo-expressing cells increased secretion of VLDL apoB (density gradient fractions 1 and 2) to 67.7% of total apoB secreted compared to secretion of 49.6% apoB as VLDL in the absence of OA (n=4 separate experiments). The effects of OA on the proportion of apoB secreted as VLDL in cells overexpressing DGAT1 (61.9% with OA vs. 48.9% without OA; n=4), ACAT1 (67.7% with OA vs. 44.8% without OA; n=3), and ACAT2 (77.3% with OA vs. 47.5% without OA; n=3) were similar to the effects of OA in Neo-transfected cells. These results indicated that all of the cell lines were able to respond to increased core lipid synthesis by incorporating these lipids into apoB-lipoproteins.
Overexpression of DGAT1 results in the secretion of a TG-rich VLDL. Our results to this point indicated that increased synthesis of either TG or CE could stimulate the secretion of lipid-rich, buoyant apoB-lipoproteins. Several recent studies have supported a model in which the assembly of apoB-containing VLDL particles occurs in two discrete steps (52,53), with the merging of a lipid–poor nascent apoB-lipoprotein and a TG-rich droplet comprising the second step. Our present findings raised the question whether the lipid-rich VLDL secreted would be specifically TG-enriched, regardless of the enzyme that was overexpressed, or, alternatively, would have a lipid core reflecting the enzyme that was specifically increased. To address this question, we carried out analyses of the lipid composition of apoB-lipoproteins secreted from cells overexpressing DGAT1, ACAT1, or ACAT2. Because the mass of lipids associated with secreted d<1.006 lipoproteins was too low to detect when cells were incubated with BSA containing media, we carried out these measurements on media from the experiments where 0.4 mM OA was added to the incubation media. Media from cells expressing DGAT1 secreted VLDL with a marked enrichment in TG over CE (fold change, 6.93 ± 3.13; n=6). By contrast, ACAT1 or ACAT2 transfected cells secreted VLDL with similar composition in which TG on average was only ~70% higher than CE (1.71 ± 0.51; n=8), similar to Neo-transfected controls. These data, together with the sucrose gradient ultracentrifugation results (Figs. 2 & 4), suggest that DGAT1 overexpression stimulated the assembly and secretion of increased numbers of buoyant VLDL that were TG-enriched, while expression of either ACAT1 or ACAT2 stimulated the assembly and secretion of more VLDL particles.
Discussion

A large body of literature, including several reviews (4-7) has focused on factors regulating the assembly and secretion of apoB-lipoproteins from the liver. Although much has been learned about this complex process, some aspects, including how the bulk of the core lipids are added, remain to be defined. One issue that continues to be debated is whether TG or CE is dominant in determining the formation of VLDL. Although there is consensus that increased availability of cellular TG can stimulate apoB secretion, there has been continuing controversy regarding the role of cellular CE availability. The role of CE has been further complicated by questions regarding the functions of the two ACAT genes products, ACAT1 and ACAT2. The cloning of genes for the DGAT and ACAT reactions provided us with the opportunity to address this issue.

To date, two distinct genes for the DGAT reaction have been isolated from mammals (14,31-33). Lipoprotein secretion was diminished in animals lacking DGAT1 (34,35), particularly after a high fat challenge, but clearly the remaining TG synthesis or ACAT mediated synthesis of CE was sufficient to maintain particle biogenesis. Similarly, DGAT2 expression is not essential for TG secretion, although this enzyme appears to play a predominant role in lipoprotein production (32); plasma TG in DGAT2 deficient mice at birth was ~70 –90 % reduced relative to littermate controls. Our results indicate that overexpression of DGAT1 in McA RH7777 cells stimulates a selective increase in the synthesis and secretion of TG, and the secretion of VLDL particles. We further found that the VLDL particles being secreted were particularly enriched in TG relative to CE. Our studies support, therefore, a significant role for DGAT1 in the assembly and secretion of apoB-lipoproteins from the liver.

As noted earlier, the role of CE in apoB-lipoprotein assembly and secretion has been controversial. Some studies in HepG2 suggested that CE availability was critical (and dominant over TG availability) for apoB-lipoprotein secretion (26-28). We, however, found no relationship between the availability of CE and apoB secretion in the same cultured liver cells (11,18). Studies of ACAT inhibitors in cultured cell lines have generated variable results; some have found a reduction in apoB secretion in the presence of an ACAT inhibitor
(22,24,54) while others have not (18,25). Two groups have shown that different ACAT inhibitors, with equal ability to inhibit ACAT, had very different effects on apoB secretion from HepG2 cells, suggesting other mechanisms were involved in inhibiting apoB secretion (19,55). On the other hand, inhibition of ACAT has been associated with reduced apoB secretion from primary rabbit hepatocytes (20) and perfused monkey livers (21). Orally administered ACAT inhibitors have also inhibited apoB secretion, as determined by in vivo tracer kinetic methods in miniature swine (56,57).

Our studies indicate that any neutral lipid core is sufficient for lipoprotein assembly; DGAT1, ACAT1 and ACAT2 all promote lipoprotein secretion. Overexpression of either ACAT1 or ACAT2 selectively stimulated the synthesis and secretion of CE and secretion of VLDL from McA RH7777 cells. Thus, despite the conflicting data derived from studies of ACAT inhibitors, increased ACAT activity and the concomitant increase in hepatic CE content are important stimuli for the assembly and secretion of lipid enriched apoB-lipoproteins. Our data are consistent with the in vivo studies of Spady et al. (58) in which increased apoB-lipoprotein secretion resulted from adenoviral-mediated increases in hepatic ACAT1 activity. However consistent with the ACAT2 mutant mouse model, ACAT2 was more efficient in promoting the secretion of VLDL from McA RH7777 cells. Clearly, either ACAT1 or ACAT2 activity is sufficient for lipoprotein assembly, however we demonstrate that with approximately equivalent levels of sterol esterification, more apoB is secreted from ACAT2 compared to ACAT1 expressing cells.

Studies in non-human primates have shown the presence of ACAT1 primarily in the Kupffer cells of the liver, in non-mucosal cell types in the intestine, and in kidney and adrenal cortical cells, whereas ACAT2 is present only in hepatocytes and in intestinal mucosal cells (41). By contrast, in studies of human tissues obtained at autopsy, ACAT1 predominated in adult liver, adrenal gland, macrophages and kidney, whereas ACAT2 was the major enzyme in adult small intestine and fetal liver (42,59). The former data, however are supported by the findings from studies of targeted deletions of either ACAT1 or ACAT2 in mice which indicate that the physiologic role of ACAT1 is mostly confined to macrophages (60,61), while ACAT2 plays the major role in CE formation in the liver and small intestine (45). Chang et al. (44) speculated that both ACAT enzymes carry out their catalytic activities within the lipid bilayer of the ER, with the resulting CE capable of
moving either into the lumen of the ER or the cytosol. Our results suggest that the ability of newly synthesized CE to stimulate secretion of apoB is preferentially modulated by a topological difference in an active site or some other unique characteristic of the ACAT2 enzyme. The proposed differences in orientation of these enzymes in the ER, with ACAT1 having a serine (Ser\textsubscript{269}) essential for activity on the cytoplasmic side of the endoplasmic reticulum (ER) membrane and ACAT2 having the analogous serine (Ser\textsubscript{249}) on the luminal side of the ER (43) may thus have physiologic relevance. Although overexpression of ACAT enzymes resulting from transfection may not represent the physiologic state of endogenous ACAT1 or ACAT2, the fact that these cells did respond to OA stimulation by shifting the density of secreted apoB-lipoproteins into the VLDL range provides support for the extrapolation of our results to the native state.

Overexpression of DGAT1 and the ACATs in McA RH7777 cells offered us the potential to speculate on the mechanism whereby bulk addition of core lipids are added to nascent apoB-lipoproteins (52,53,62). Interestingly, we found that when cells transfected with DGAT were stimulated with OA, they secreted VLDL with high TG:CE ratios; when cells were transfected with either ACAT1 or ACAT2, they secreted VLDL with closer to equivalent amounts of TG and CE. We believe these results suggest that when VLDL is being assembled in hepatocytes, the core lipid that is most available will be added to the core. When DGAT expression is high, TG is added to nascent apoB both early, targeting it away from degradation and toward lipoprotein formation, and late, forming TG-enriched VLDL. When ACAT expression is high, CE could be added either early, creating a CE-rich lipid-poor apoB-lipoprotein that then merges with a TG droplet in the ER, or later, with CE droplets being present in the ER. Our data do not allow us to choose between these possibilities. Our results do, however, indicate that the lipid composition of VLDL can vary, depending on the availability of core lipids in the hepatocyte, thus allowing apoB to maintain neutral lipid homeostasis regardless of the particular neutral lipid status of the liver.

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Figure legends.

Figure 1: Effect of overexpression of DGAT1 on synthesis and secretion of TG, cellular TG mass and secretion of apoB-lipoproteins in McA RH 7777 cells. McA RH 7777 cells transfected with Diacylglycerol acyltransferase (hDGAT1) or Neomycin vector control (Neo), were pre-incubated in serum free medium for 2 hours and then labeled with [3H]glycerol for 4 hours for lipid analysis (A, B), or with [35S]Met for 2 hours for protein synthesis, without (C) or with (D) cold chase (90 minutes) analysis. A: Overexpression of DGAT1 in McARH7777 cells increased TG synthesis (102384 ± 3815 cpm/mg protein, p<0.01 compared to control 20275 ± 2718) and TG secretion (14876 ± 3785 cpm/mg protein, *p<0.01 compared to control 5326 ± 1786), as measured by the incorporation of [3H]glycerol. DGAT overexpression had no effect on either PL or CE synthesis (see text). Mean (±SD) results from three separate experiments are presented. B: Overexpression of DGAT1 increased total cellular TG mass (18.25 ± 3.10 µg TG/mg cell protein, *p<0.05 compared to control: 12.75 ± 2.87). Mean (±SD) results from three separate experiments are presented. C: Overexpression of DGAT1 increased secretion of apoB by more than 2 fold (relative densitometric unit: 2.54 ± 0.65 VS control, *p<0.05). Results of a representative experiment (of three total) are shown. D: Overexpression of DGAT1 promoted subcellular stabilization of apoB. McARH7777 cells transfected with DGAT1 or vector control (Neo) were analyzed by a [35S]Met pulse/chase. Samples were taken every 15 minutes and processed for intracellular apoB by immunoprecipitation and SDS-PAGE. The results are means of three experiments.

Figure 2. Effect of overexpression of DGAT1 on the density of secreted apoB-lipoproteins. McA RH 7777 cells were pre-incubated in serum free medium for 2 hours and then labeled with [35S]Met for 2 hours for protein synthesis. After labeling, medium was collected and subjected to sucrose gradient ultracentrifugation as described in Methods. Fraction 1 has an average density of 1.005 and fraction 2 has an average density of 1.008. Density of relevant fractions are shown. Overexpression of DGAT stimulated increased secretion of buoyant (VLDL) apoB-lipoprotein particles. The results depicted are representative of two separate experiments.
Figure 3. Effect of overexpression of ACAT1 and ACAT2 on synthesis and secretion of CE, CE mass and secretion of apoB-lipoproteins in McA RH 7777 cells. McA RH 7777 cells were pre-incubated in serum free medium for 2 hours and then labeled with [3H]-OA (4 µCi/ml) for 4 hours for lipid analysis (panels A, B, C), or with [35S]Met for 2 hours for protein synthesis without (D) or with (E) cold chase analysis. A: Overexpression of ACAT1 and ACAT2 in McARH7777 cells significantly increased CE synthesis (ACAT1: 14272 ± 2176 cpm/mg. cell protein; ACAT2: 12681 ± 1978 cpm/mg. cell protein; *p<0.01 compared to control: 5247 ± 1567). There was no effect of ACAT overexpression on either PL or TG synthesis. Mean (±SD) results from three separate experiments are presented. B: Overexpression of ACAT1 and ACAT2 in McARH7777 cells significantly increased CE secretion (ACAT1: 3265 ± 716 cpm/mg.cell protein; ACAT2: 2864 ± 565 cpm/mg. cell protein; *p<0.01 compared to control: 1726 ± 312), but had no significant effect on either PL or TG synthesis and secretion. Mean (±SD) results from three separate experiments are presented. C: Total cellular CE mass was significantly increased by either overexpression of ACAT1 (19.0 ± 2.4 µg CE/mg. cell protein, *p<0.05 compared to control: 13.0 ± 1.7) or ACAT2 (18.0 ± 3.2 µg CE/mg. cell protein, *p<0.05 compared to control: 13.0 ± 1.7). Mean (±SD) results from three separate experiments are presented. D: Secretion of apoB-lipoproteins is increased by overexpression of either ACAT1 or ACAT2. Pooled data (3 experiments) are shown graphically with a representative experiment depicted in the inset. E: Overexpression of ACAT1 or ACAT2 promoted subcellular stabilization of apoB. McARH7777 cells transfected with ACAT1, ACAT2, or vector control (Neo) were pulse labeled with [35S]Met for 2 hours and chased for 90 minutes. Samples were taken every 15 minutes and processed for intracellular apoB by immunoprecipitation and SDS-PAGE. The results are representative of three experiments.

Figure 4. Effect of overexpression of ACATs on the density of secreted apoB-lipoproteins. RH 7777 cells transfected with ACAT1, ACAT2 or Neomycin control (Neo) were labeled with [35S]Met and subjected to sucrose gradient ultracentrifugation and SDS-PAGE analysis as described in Fig. 2. Fraction 1 has an average density of 1.005 and fraction 2 has an average density of 1.008. Density of relevant fractions are shown. The data depicted are representative of two separate experiments. The apoB-containing lipoproteins secreted from cells overexpressing either ACAT1 or ACAT2 were mainly in the form of VLDL particles.
Figure 1
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Figure 1
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Figure 2
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ApoB Secretion levels (relative densitometric units)

D.

Neo control

AC AT1

AC AT2

0 50 100 150 200 250

Figure 3
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E.

Figure 3
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Figure 4
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Overexpression of human diacylglycerol acyltransferase 1, acyl-CoA:cholesterol acyltransferase 1 or acyl-CoA:cholesterol acyltransferase 2 stimulates secretion of apolipoprotein B-containing lipoproteins in McA-RH7777 cells

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