Myristic acid increases dense lipoprotein secretion by inhibiting apoB degradation and triglyceride recruitment

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Abstract Fatty acids of varying lengths and saturation differentially affect plasma apolipoprotein B-100 (apoB-100) levels. To identify mechanisms at the level of production, rat hepatoma cells, McA-RH7777, were incubated with [^{35}S]methionine and either fatty acid-BSA complexes or BSA alone. There were increases in labeled apoB-100 secretion with saturated fatty acids palmitic and myristic (MA) (153 ± 20% and 165 ± 11%, respectively, relative to BSA). Incubation with polyunsaturated docosahexaenoic acid (DHA) decreased secretion to 26 ± 2.0%, while monounsaturated oleic acid (OA) did not change it. In pulse-chase studies, MA treatment resulted in reduced apoB-100 degradation, in agreement with its promotion of secretion. In triglyceride (TG) studies, synthesis was stimulated equally by OA, MA, and DHA, but TG secretion was relatively decreased with MA and DHA. With OA, the majority of newly secreted apoB100-lipoproteins was d $\leq$ 1.006, but with MA, they were much denser (1.063 $<$ d). Furthermore, the relative recruitment of newly synthesized TG to lipoproteins was impaired with MA. We conclude that mechanisms for effects of specific dietary fatty acids on plasma lipoprotein levels may include changes in hepatic production. In turn, hepatic production may be regulated by specific fatty acids at the steps of apoB-100 degradation and the recruitment of nascent TG to lipoprotein particles.—Kummrow, E., M. M. Hussain, M. Pan, J. B. Marsh, and E. A. Fisher. **Myristic acid increases dense lipoprotein secretion by inhibiting apoB degradation and triglyceride recruitment.** ***J. Lipid Res.*** **2002.** 43: **2155–2163.**

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Elevated plasma levels of apolipoprotein B (apoB) and its associated cholesterol are well recognized risk factors for the development of atherosclerosis (1–4). There are two forms of apoB found in mammals. ApoB-100, a polypeptide of approximately 550 kDa, is produced by the liver and is required for the assembly and secretion of VLDL (5). Through a series of incompletely characterized steps, a portion of VLDL is converted to the highly atherogenic lipoprotein LDL. Many human and animal studies have shown that the amounts and type of fat in the diet can affect the plasma levels of apoB-100-containing lipoproteins and their associated cholesterol, and thereby influence atherosclerosis risk (6–12). General conclusions have been that diets rich in polyunsaturated fat tend to lower plasma cholesterol and apoB-100 levels, and that diets rich in saturated fats tend to elevate these parameters. Most of the mechanistic studies of these effects have been performed in intact animals and have focused on the ability of the types of dietary fat to affect LDL receptor (LDLR) activity and, thereby, plasma lipoprotein clearance (13, 14). In contrast, comparatively few studies have directly examined another way in which the type of dietary fat can influence plasma lipoprotein and lipid levels—namely, by the regulation of hepatic lipoprotein production. In addition, many previous animal studies have employed complex mixtures of fatty acids and other dietary lipids, thereby making it difficult to discern the effects of specific fatty acids.

To study the effects of specific fatty acids on hepatic apoB-100-lipoprotein production, we sought an in vitro system that would not only be convenient, but also be physiologically relevant. Particularly well suited for this purpose is the rat heptoma cell line McA-RH7777 (McA), which has been shown to express apoB-100 (15) and to re-

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Abbreviations: DHA, docosahexaenoic acid; MA, myristic acid; OA, oleic acid; PA, palmitic acid; SFA, saturated fatty acid(s); TG, triglyceride(s).

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spond to incubations with oleic acid (OA) and n-3 fatty acids as do rat primary hepatocytes (16, 17).

As summarized in this report, with this system we have determined that certain saturated fatty acids (SFA), relative to polyunsaturated fatty acids (PUFA), tend to increase apoB-100 lipoprotein secretion. Importantly, these results are consistent with the known effects on plasma lipoprotein levels in dietary studies in mammals, including humans. Using myristic acid (MA) as a representative SFA, the mechanisms for the relative effects of SFA appeared to be decreased intracellular degradation of apoB-100 and decreased recruitment of nascent triglycerides (TGs) to lipoprotein assembly, resulting in increased secretion of dense apoB-100-lipoprotein particles.

EXPERIMENTAL PROCEDURES

General

McArdle RH7777 (McA) cells were from the American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM) was from Mediatech (Herndon, VA), FBS and horse serum were from Gibco-BRL (Bethesda, MD). [^{35}S]methionine, [^{3}H]glycerol, [^{14}C]glycerol, [^{14}C]cholesterol, and Solvable were from Dupont/New England Nuclear (Boston, MA). Autofluor was from National Diagnostic (Manville, NJ). Anti-rat apoB antibodies were a kind gift from Drs. Janet and Charles Sparks (University of Rochester). All other chemicals were of the highest grade commercially available and purchased from the Sigma Chemical Co. (St. Louis, MO).

Cell culture

The McA cells were maintained in DMEM containing 10% FBS and 10% horse serum in 5% CO_2. The cells were plated on 60 mm culture dishes at a density of 2×10^5 cells/dish and then re-fed with 3 ml of medium on the second day. Experiments commenced on the third day after plating, by which time the cells had reached approximately 90% confluency.

Metabolic labeling and centrifugal analysis of secreted apoB-100

At the beginning of each experiment, cells were washed twice with warm (37°C) PBS and then incubated with experimental media consisting of DMEM, 0.8 mM fatty acid (the species are given in Results) complexed to BSA in a 5:1 molar ratio, and 70 μCi/ml [^{35}S]methionine. Control plates of cells were incubated with DMEM, 70 μCi/ml [^{35}S]methionine, and BSA alone (0.16 mM). Cells were incubated in the experimental media for 6 h at 37°C, 5% CO_2. In pilot studies, there was no evidence of cell toxicity after 6 h of incubation with any of the fatty acids at a concentration up to 0.8 mM. Cell toxicity was assessed by cell morphology, trypan blue exclusion, total protein mass, and protein synthetic activity.

At the conclusion of the incubation period, media were removed and spun in a Beckman tabletop centrifuge at 1,500 rpm for 10 min to remove any detached cells or cellular debris. Two and a half milliliters of the media were then placed in a Beckman polycarbonate ultracentrifuge tube with 0.5 ml of fresh rat plasma (to provide carrier lipoproteins), adjusted to density 1.25 g/ml, and overlaid with a 1.21 g/ml KBr solution (containing 0.2 mM PMSF and 2 mM EDTA). Tubes were centrifuged at 48,000 rpm for 40 h in a Beckman 50Ti rotor at 4°C. After centrifugation, the top 1 ml was carefully removed from each tube and dialyzed against normal saline for a minimum of 5 h at 4°C. After dialysis, volumes were carefully measured and aliquots were delipidated by adding 100 μl of buffer containing 62.5 mM Tris/HCI (pH 6.8), 2% (wt/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 10 mM dithiothreitol. After heating to 95°C for 5 min, apolipoprotein species were then separated by electrophoresis in a 3.5% polyacrylamide-18% glycerol gel (18). After staining, fixing, and enhancing in Autofluor, the gels were dried, exposed to Kodak XAR autoradiography film at −70°C for 3 to 5 days. The apoB-100 bands were excised from the gel and assayed by scintillation counting.

There were two variations in the density gradient analyses of secreted apolipoproteins. In the first, conditioned media from eight plates were combined and centrifuged as above. After centrifugation, the top 4 ml, containing total lipoproteins, were collected from each tube and placed in a fresh centrifuge tube. To fractionate the total lipoproteins, the 4 ml containing them were overlaid with 3 ml of d = 1.063 g/ml, 3 ml of d= 1.019 g/ml, and 2 ml of d = 1.006 g/ml solutions. After centrifugation in a SW40 rotor for 42 h, 40,000 rpm, 4°C, 1 ml fractions were collected from the top of the tube, dialyzed and processed as above.

In the second variation, 2.5 ml of conditioned media were placed in a Beckman polycarbonate ultracentrifuge tube with 0.5 ml fresh rat plasma and overlaid with d = 1.006 g/ml solution (containing 0.2 mM PMSF and 2 mM EDTA). Tubes were centrifuged for 18 h in a Beckman 50Ti rotor at 48,000 rpm, 4°C. The top 1 ml was carefully removed and the density of the tubes was readjusted to d = 1.21 g/ml by the addition of d = 1.35 g/ml solution and/or solid KBr. The tubes were then centrifuged at 48,000 rpm for 36 h, 4°C. The top 1 ml was removed and processed as above.

Pulse-chase studies of apoB-100 metabolism

McA cells were plated as above. At the beginning of each experiment, cells were washed twice in warm DMEM and then incubated for 4 h at 37°C, 5% CO_2 in media containing 0.8 mM of the indicated fatty acids complexed to BSA. Media were changed to methionone-free DMEM with 0.8 mM fatty acid-BSA complexes during the final 2 h of the initial incubation. Cells were then pulse-labeled for 10 min by adding methionine-free DMEM containing 0.8 mM fatty acid and 200 μCi/ml [^{35}S]methionine. After the 10 min pulse, media were removed and cells were chased for up to 120 min in DMEM containing 0.8 mM fatty acid and excess (10 mM) unlabeled methionine.

At the conclusion of each chase period, media were removed and the plates were immediately washed twice with ice-cold PBS. After the addition of protease inhibitors, media were centrifuged at 1,400 $g \times$ 10 min to remove detached cells and other debris and then placed in fresh tubes in 1 ml aliquots.

To cell monolayers were added 2 ml of lysis buffer (10 mM Tris-HCI, pH 7.4 containing 0.15 M NaCl, 1 mM EDTA, 0.1 mM PMSF, 1% nonidet P40, and 0.5% deoxycholate) containing freshly added protease inhibitors (2 mM benzamidine, 1 mM PMSF, 2 μg/ml aprotinin, 2.5 μg/ml leupeptin, 2.5 μg/ml antipain, and 1.25 μg/ml chymostatin), and the cells were scraped into culture tubes. After a 30 min incubation on ice, the tubes were vortexed vigorously and then clarified by centrifugation at top speed in a microcentrifuge for 15 min at 4°C. After clarification, supernatants were placed in fresh tubes in 1 ml aliquots for immunoprecipitation analysis.

Immunoprecipitation

To immunoprecipitate apoB-100, rabbit anti-rat apoB antiserum was added to either media or cell lysates prepared as above at a final dilution of 1:65. The tubes were rotated overnight at 4°C and the following morning, 50 μl of a 10% solution of protein A-Sepahrose were added, and the incubation continued for

2 h at 4°C. Protein A-anti-apoB complexes were collected by centrifugation at 6,000 rpm for 15 min. The pellets were then washed three times in buffer containing 0.142 M NaCl, 0.24 M KCl, 0.008 M Na_2PO_4, 0.5% sodium deoxycholate, and 1% Triton X-100. Antibody-antigen complexes were released from the pellets by adding 200 μl of buffer containing 10 mM Tris-glycine, pH 8.3, 8 M urea, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and then heating to 95°C for 5 min. After centrifugation to remove any insoluble material (6,000 rpm, 10 min), the supernatants were analyzed by electrophoresis in a 3.5% polyacrylamide-18% glycerol gel. After staining, fixing and enhancing in Autofluor, the gels were dried and exposed to XAR-5 film at −70°C. The radioactive bands corresponding to apoB-100 were excised and quantified by scintillation counting as described above.

Studies of TG metabolism

Synthesis and secretion: McA cells were incubated as above, except that instead of [^{35}S]methionine, the isotope was 10 μCi/ml [1,2,3-^{3}H]glycerol. At the conclusion of the incubation period, cell monolayers were washed with PBS and cellular lipids were extracted with 3 ml isopropanol at room temperature overnight. The extract was evaporated under N_2 at 60°C and the dried lipids dissolved in 0.5 ml isopropanol. An aliquot was then extracted with 4 ml Dole's solvent to isolate neutral lipids. The extract was dried as above, Scintiverse BD added, and radioactivity measured by liquid scintillation counting. Aliquots of conditioned media were also extracted with Dole's solvent as above. Previous work had confirmed that greater than 95% of the radioactive lipids extracted by Dole's solvent are TGs (19). In some experiments, the Dole's extract was placed on either TLC or "instant" TLC (ITLC) plates and developed with a toluene-ethyl acetate solvent system (97:3). Spots were then visualized by exposure to iodine vapor and the spots either scraped from the TLC plates or cut from the ITLC plates and quantified by scintillation counting.

Double-labeling of TGs: After reaching approximately 90% confluency, McA cells were incubated overnight in normal maintenance media (DMEM, 10% FBS, 10% horse serum) with 4.5 μCi/ml [1,2,3-^{3}H]glycerol. The following day cells were incubated in DMEM, 0.8 mM fatty acid-BSA complexes, and 0.5 μCi/ml [^{14}C(U)]glycerol. At the conclusion of the incubation, conditioned media were extracted with Dole's solvent and labeled TG quantified as above.

Other methods

Protein was measured by the method of Markwell (20) using BSA as the standard. Labeling of total cellular and medium protein was determined by TCA-phosphotungstic acid precipitation (21). All results were normalized to total cellular TCA-phosphotungstic acid-precipitable counts.

Statistical analysis

Data were analyzed by either Student's *t*-test or the ANOVA, (with multiple comparison testing as necessary), using the InStat program (Graphpad Software, San Diego, CA).

RESULTS

Effects of different fatty acids on the secretion of newly synthesized apoB

McA cells were incubated with 0.16 mM BSA alone (control condition) or 0.8 mM fatty acid-BSA complexes (0.8 mM fatty acid, 0.16 mM BSA) for 6 h in the presence of [^{35}S]methionine. Samples of conditioned media were subjected to density gradient ultracentrifugation to isolate the secreted lipoproteins. The amount of metabolically labeled apoB-100 that was secreted was assayed by delipidating the lipoproteins and separating the apoprotein species by SDS-PAGE. The apoB-100 and apoB48 bands were then excised and subjected to scintillation counting (Experimental Procedures). Secretion of labeled apoB48 was a small fraction (~20%) of the secretion of labeled apoB-100, as previously observed in McA cells (15). Therefore, only results for apoB-100 will be presented. It should be noted that the fatty acid treatments did not affect the synthetic rates of apoB48 or apoB-100 as assessed by the peak incorporation of [^{35}S]methionine (data not shown).

The results are summarized in **Fig. 1**. For any fatty acid, the result shown is the recovery of labeled apoB-100 in the presence of that fatty acid relative to the recovery in the presence of BSA alone. Note that relative to the BSA control group, the unchanged and decreased (−74%; $P < 0.001$) secretion of newly synthesized apoB-100 in the presence of OA (18:1) and the n-3 fatty acid docosahexaenoic acid (DHA) (22:6), respectively, are in agreement with our previous studies in rat primary hepatocytes and McA cells (17, 22, 23). The neutral effect of stearic acid (18:0) is consistent with the findings in animal and clinical studies (24). Incubation in the presence of the two other SFAs produced significant increases in the secretion of labeled apoB-100: palmitic (PA, 16:0) by 53% ($P < 0.02$) and MA (14:0) by 65% ($P < 0.001$).

These results were not due to toxic effects on general protein synthesis or secretion, as reflected by comparable TCA-precipitable labeled proteins in cell lysates or conditioned media samples from the different treatment groups (data not shown). In addition, the results could not be attributed to differences in the uptake of the fatty acid species, as judged by the measurement of cell-associated counts and disappearance of individual ^{14}C-labeled fatty acids that had been complexed to BSA and incubated as above (data not shown).

Overall, these initial experiments suggested that the secretion of newly synthesized apoB-100 in McA cells is sensitive to specific dietary fatty acids, with an almost 5-fold difference found between MA and DHA. We therefore focused our efforts on the possible reasons for these large relative differences.

Changes in apoB-100 secretion are independent of induction of TG synthesis by fatty acids

Because it is generally recognized that changes in TG synthesis may modulate apoB secretion from hepatic cells, the effects of MA, DHA, and OA on McA TG metabolism were explored. Cell cultures were incubated for 6 h with [1,2,3-^{3}H]glycerol and either 0.8 mM fatty acid-BSA complexes or BSA, after which TG were extracted from the cells and conditioned media and the incorporation of label determined by scintillation counting. **Figure 2A** displays the intracellular levels of newly synthesized TG at the end of the fatty acid incubation period relative to the results from incubation with BSA alone. While all three fatty acids increased the intracellular levels of labeled TG,

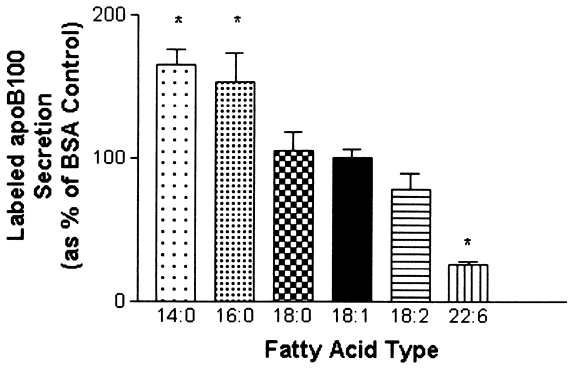

Fig. 1. Fatty acid effects on the secretion of newly synthesized apoB-100. After reaching approximately 90% confluency, McA cells were incubated with 0.16 mM BSA alone (control condition) or 0.8 mM of the indicated fatty acid-BSA complexes (0.8 mM fatty acid, 0.16 mM BSA) for 6 h in the presence of [^{35}S]methionine. Samples of conditioned media were subjected to density gradient ultracentrifugation to isolate the secreted lipoproteins. The amount of metabolically labeled apoB-100 that was secreted was assayed by delipidating the lipoproteins and separating the apolipoprotein species by SDS-PAGE. The apoB-100 bands were then excised and subjected to scintillation counting (Experimental Procedures). The dpm data were normalized to cell lysate TCA-precipitable labeled protein and the mean (±SEM, n ⩾ 9) values relative to the BSA-treated cells are displayed. Multiple comparisons were performed by ANOVA. *Significant differences from the BSA-treated group (see text for *P* values).

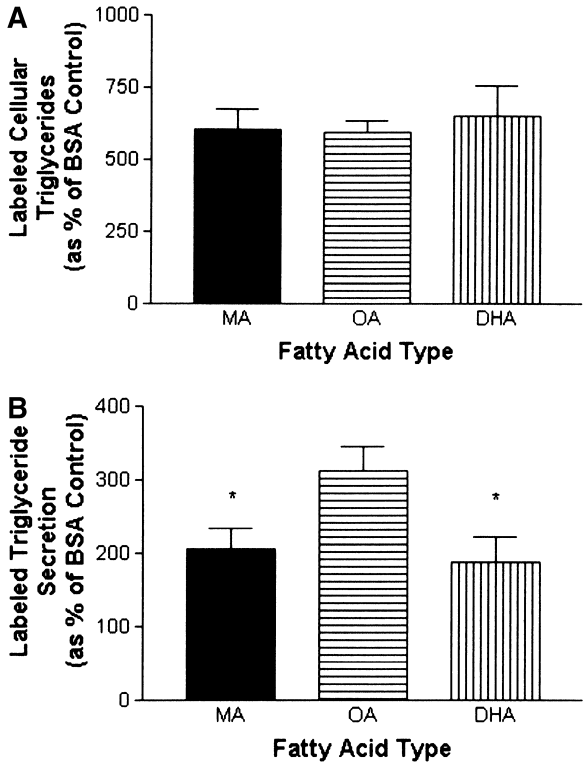

Fig. 2. Synthesis and secretion of newly synthesized triglycerides (TGs). McA cells were incubated with fatty acid-BSA complexes or BSA alone as in Fig. 1, but in the presence of 10 μCi/ml [^{3}H]glycerol. A: To measure stimulation of cellular TG synthesis, the cell monolayers were washed with PBS after incubation, and TG were extracted by Dole's solvent (Experimental Procedures) and then dissolved in Scintiverse BD. The amount of labeled glycerol incorporated into TG was determined by scintillation counting and normalized to milligrams of cell protein. Displayed are the mean (±SEM) values relative to the BSA-treated cells. B: To measure the secretion of newly synthesized TG, an aliquot of conditioned media was extracted and the labeled TG quantified and the data expressed as in A. Results in either panel were from three separate experiments, each one performed in triplicate. *Significantly less than oleic acid (OA) group ($P < 0.01$).

no significant differences were found among MA, OA, or DHA. In contrast, the accumulations of newly synthesized TG in conditioned media were clearly different after incubation with the various fatty acids. As shown in Fig. 2B, while all three fatty acids increased secretion of labeled TG (compared with the results from incubation with BSA alone), the greatest increase was seen with OA (∼3× greater, $P < 0.01$). As reported by us and others (17, 25), relative to the results with OA, incubation of McA cells with an n-3 fatty acid resulted in decreased secretion of labeled TG, but, surprisingly, so did incubation with MA ($P <$ 0.01 for either DHA or MA vs. OA). Because of this unexpected result, we wished to further explore the effects of MA that led to increased apoB-100 secretion and decreased TG secretion.

MA induces secretion of small, dense lipoproteins

Because McA cells incubated with MA secreted an increased amount of labeled apoB-100 but a decreased amount of labeled TG compared with incubation with OA, this implied that the apoB-100-lipoproteins secreted in the presence of MA were relatively dense. To directly determine this, cell cultures were incubated with [^{35}S]methionine and with 0.8 mM fatty acid-BSA complexes or BSA alone for 6 h, and conditioned media were subjected to ultracentrifugation to recover lipoproteins having a density of ⩽1.006 g/ml (i.e., VLDL). The top 1 ml containing these lipoproteins was collected from each tube and the infranatants were then raised to a density of 1.21 g/ml by addition of KBr and re-centrifuged to recover lipoproteins of $1.006 < d \leqslant 1.21$ g/ml. Labeled apoB-100 was recovered from these two density fractions and analyzed by SDS-PAGE and scintillation counting of excised gel bands (Experimental Procedures).

Figure 3 shows the fractional distribution of labeled apoB-100 between the two density fractions. As expected, most (>80%) of the newly secreted apoB-100 was found in the $d \leqslant 1.006$ g/ml fraction after incubation with OA, but the opposite pattern was seen after incubation with MA: approximately 76% of labeled apoB-100 was found in the $1.006 < d \leqslant 1.21$ g/ml fraction. In the absence of exogenous fatty acid, the majority (∼65%) of the labeled apoB-100 was found in the $d \leqslant 1.006$ g/ml fraction, similar to the results with DHA (∼60%).

It is possible that ultracentrifugation of conditioned media at 4°C could have influenced the results. If during incubation with MA the newly-formed VLDL TG were composed of tri-myristin, ultracentrifugation at 4°C could possibly lead to the formation of crystalline or liquid-crys-

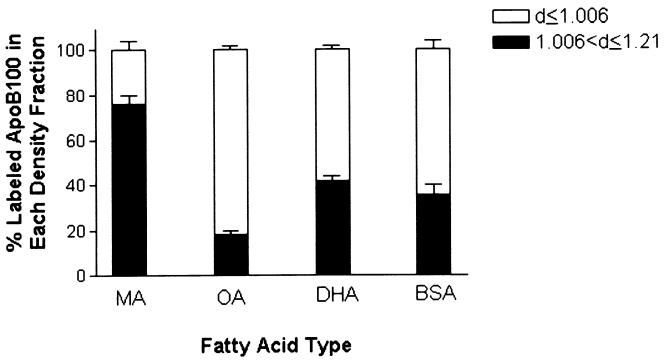

Fig. 3. Density distribution of newly synthesized apoB-100. McA cells were incubated as in Fig. 1 with BSA alone or the indicated fatty acids complexed to BSA. Conditioned media were placed in an ultracentrifuge tube and a saline solution of d = 1.006 g/ml was carefully layered on top. After centrifugation at 50,000 rpm for 16–18 h at 4°C, the top 1 ml was collected from each tube and reserved for SDS-PAGE analysis. Solid KBr was then added to the remaining solution in order to readjust the density to 1.21g/ml. After centrifugation at 50,000 rpm for 42 h at 4°C, the top 1 ml was again collected, dialyzed against a saline solution, and processed by SDS-PAGE. ApoB-100 bands were cut from the gels and the amount of incorporated label quantified by scintillation counting. Data are expressed as the percent in each fraction of the total apoB-100 counts in both fractions. Results are expressed as the means (±SEM) from four different experiments, each done in triplicate.

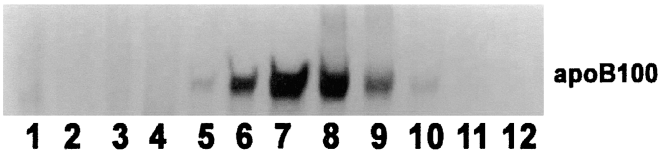

Fig. 4. Newly synthesized apoB-100 is secreted predominately as part of dense lipoprotein particles after incubation with myristic acid. McA cells were incubated as in Fig. 1. Conditioned media from eight plates were combined and the density adjusted to d = 1.265g/ml with KBr. A KBr solution of d = 1.25g/ml was then layered on top. After centrifugation at 50,000 rpm for 42 h at 4°C, the top 4 ml were collected and placed in another centrifuge tube. This was then overlaid with 3 ml d = 1.063g/ml, 3 ml of d = 1.019g/ml, and 2 ml of d = 1.006g/ml solutions. After centrifugation in a SW40 rotor for 42 h, 40,000 rpm, 4°C, 1 ml fractions were removed from the top of the tube, dialyzed against saline solution and processed by SDS-PAGE. After drying, the gels were exposed to XAR-5 film at −70°C for 18 h. A fluorogram representative of 2 independent experiments is shown. Fraction 1 is from the top of the gradient.

tal structures in the core of the lipoproteins, thus causing them to migrate to a lower density (26). In order to address this possibility, conditioned media from cells incubated with MA were subjected to sequential ultracentrifugation at 37°C, first at d ⩽ 1.006 g/ml and then again at 1.006 < d ⩽ 1.21 g/ml. As at 4°C, more labeled apoB-100 was recovered in the denser fraction (data not shown). Thus, the increased density of apoB-100-containing lipoproteins after MA incubation was unlikely to be an artifact caused by the temperature of isolation.

In order to better resolve the density distribution of the newly secreted apoB-100-containing lipoproteins in the conditioned medium of MA-treated cells, a discontinuous density gradient analysis was conducted (Experimental Procedures). **Figure 4** shows a representative fluorogram from this type of experiment. Clearly, after incubation with MA, the majority of labeled apoB-100 was secreted associated with lipoproteins in fractions 6–9, (densities of 1.078 to 1.117 g/ml). Thus, the results of Figs. 2B, 3, and 4 demonstrate that relative to OA, incubation with MA results in the secretion of denser, TG-poor lipoprotein particles.

Mechanism of the assembly of small, dense lipoproteins

Next, we turned our attention to the decreased secretion of TG by MA. Note that relative to OA, MA was as effective in stimulating the synthesis of TG, but it decreased the secretion of newly synthesized TG (Fig. 2). We have previously shown in Caco-2 cells that newly synthesized TG are recruited for the assembly of larger lipoproteins (27) and hypothesized that the decreased secretion of newly synthesized TG with MA was associated with a change in the recruitment from the cellular pool of pre-formed or nascent TGs. Therefore, cells were incubated overnight in maintenance medium and [1,2,3-^{3}H]glycerol in order to label intracellular TG to steady state. The next morning, the media were removed and cell monolayers washed and the medium changed to DMEM, 0.8 mM FA-BSA complexes, and [^{14}C(U)]glycerol to label the newly synthesized TG. After 6 h, TG were extracted from conditioned media and the incorporation of either the ^{3}H or ^{14}C label assessed by scintillation counting (Experimental Procedures).

Figure 5 shows the recoveries of the labeled TG from the conditioned media samples. As expected, the total label (^{14}C and ^{3}H) was greatest after incubation with OA. The relative percents of each label recovered were virtually identical for the incubations with MA and OA [e.g., for ^{3}H-TG, 33% (MA), 36% (OA)]. In those cultures incubated with BSA alone, the majority (60%) was ^{3}H-TG (i.e., from the pre-formed pool). In contrast, in those cultures incubated with MA or OA, the majority of the secreted TG was ^{14}C labeled, or derived from the newly synthesized pool. This indicated that recruitment of newly synthesized TG for lipoprotein formation occurs in the presence of MA or OA, but taken with the results in Figs. 2–4, there is a relative deficiency in the ability to fully lipid load lipoproteins in the presence of MA.

Mechanism of increased secretion of apoB

We have previously shown in McA cells that there is a degradation pathway for apoB-100 whose activity is directly proportional to the degree of lipidation of the associated lipoprotein (17, 23). Because the results above demonstrated that apoB-100 secreted in the presence of MA is associated with significantly denser (i.e., less lipidated) lipoproteins, we were interested in whether the increased (relative to OA) secretion of newly synthesized apoB-100 (Fig. 1) could be explained by decreased degradation. Therefore, pulse-chase studies were performed to directly determine whether incubation with MA affects intracellular synthesis or degradation of apoB-100, compared with incubation with OA.

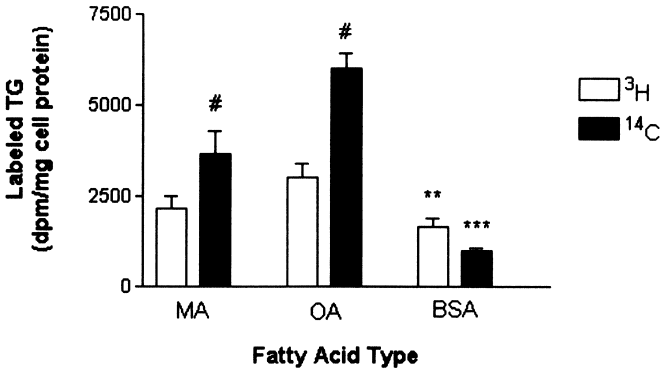

Fig. 5. Fatty acid effects on the secretion of pre-formed and nascent TGs. After reaching approximately 90% confluency, McA cells were labeled overnight in normal maintenance media (DMEM, 10% FBS, 10% horse serum) with 4.5 μCi/ml [1,2,3-^{3}H]glycerol. The following day, cells were incubated in DMEM, 0.8 mM fatty acid-BSA complexes, and 0.5 uCi/ml [^{14}C(U)]-glycerol. Aliquots of conditioned media were extracted with Dole's solvent as in Fig. 2. TG labeling was quantified by scintillation counting. Data are expressed as fractional distribution (±SEM) of each label. Results shown are from three separate experiments, each performed in triplicate. Multiple comparisons were performed by ANOVA: #Myristic acid (MA) vs. OA, $P < 0.02$; **BSA versus all other ^{3}H groups, $P < 0.02$; ***BSA versus all other ^{14}C groups, $P < 0.001$.

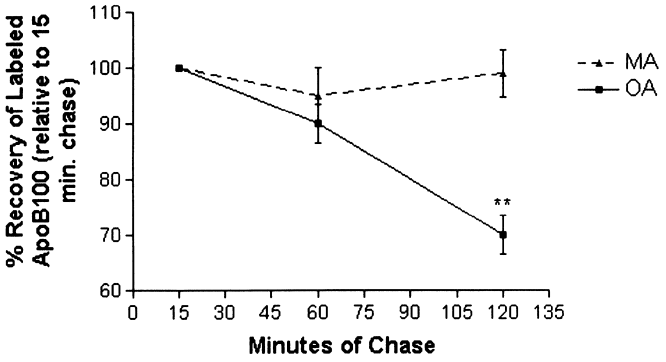

Fig. 6. Differential effects of MA and OA on apoB-100 degradation. After reaching approximately 90% confluency, McA cells were incubated for 4 h as in Fig. 1, then were pulse-labeled for 10 min with [^{35}S]methionine as described in Experimental Procedures. After pulse-labeling, media were changed to remove label, and incubations were continued for the indicated times in media containing an excess of unlabeled methionine. At the end of each chase period, labeled apoB-100 was isolated from cell lysates and conditioned media by immunoprecipitation. Quantification of labeled apoB-100 by SDS-PAGE and scintillation counting were performed as in Fig. 1. Each data point represents the recovery of total (cell + medium) labeled apoB-100 expressed as the mean percentage (±SEM) of the peak value of label incorporated into apoB-100 (which was at 15 min of chase), after normalization to TCA-precipitable counts of the cell lysates. Data presented are mean (±SEM) of three different experiments, each done in duplicate. **$P < 0.001$, total apoB-100 recovery from MA versus OA-treated cells.

Cell cultures were incubated for 4 h in the presence of 0.8 mM fatty acid-BSA complexes. Media were changed to methionine-free DMEM with 0.8 mM fatty acid-BSA complexes during the final 2 h of the incubation. Cells were then pulse-labeled for 10 min by adding 100 μCi/ml [^{35}S]methionine. Synthetic rates of apoB-100, estimated as peak incorporation of isotope at 15 min of chase, were similar for the two fatty acid treatments (MA: 10286.7 ± 4054 dpm, OA: 10334.3 ± 3917.4 dpm, n = 3). **Figure 6** shows the relative recovery of total labeled (cell+medium) apoB-100 during the120 min chase period. Consistent with the results shown in Fig. 1, there was increased recovery of total apoB-100 in the presence of MA: the recovery of total labeled apoB-100 after 120 min of chase was 98% after incubation with MA compared with 70% after incubation with OA, a statistically significant difference ($P < 0.001$). Thus, the approximately 1.6-fold increase in apoB-100 secretion with MA (vs. OA; Fig. 1) can be largely quantitatively accounted for by a corresponding reduction in apoB-100 degradation.

DISCUSSION

The impact of dietary fatty acids on plasma levels of lipids and lipoproteins has been investigated in a long line of clinical investigations that extend back to at least the 1950s. Many of the classic early studies were carried out by Ahrens, Connor, Hegsted, Keys and their colleagues (28). By performing extensive clinical studies, Hegsted (29) and Keys (30) were able to develop equations from regression analysis to estimate the quantitative contributions to plasma cholesterol levels of dietary factors, such as fatty acid type, individual fatty acid species, and cholesterol. In general, the major findings from those analyses have been amply confirmed since the original publications, and briefly summarized, they include the following: *1*) monounsaturated fatty acids had relatively neutral effects; *2*) the strongest dietary influence on plasma cholesterol was the percentage of calories as SFA; of the SFA species, MA and PA had the greatest effect on elevating plasma cholesterol levels, and stearic acid the least (which has been attributed to the conversion of stearic to OA by stearoyl CoA desaturase); and, *3*) PUFA had a lowering effect on plasma cholesterol levels.

As the influences of dietary fatty acids on plasma lipid and lipoprotein levels became established, efforts were made to identify the biological bases for these effects. One proposed mechanism has been the regulation of LDL clearance, with SFA and PUFA tending to down and upregulate LDLR activity, respectively (31). We hypothesized that another mechanism may be effects on apoB-100-lipoprotein production by the liver, and tested this at the cellular level. Although the extrapolation of results obtained with cell culture models, whether from primary hepatocytes or transformed cell lines, to the in vivo situation must be done cautiously and with recognition of the fact that the type of cell and the precise incubation conditions are critical variables, it was quite encouraging that the basic pattern of the effects of SFA (including stearic), PUFA, and monounsaturated fatty acids on apoB-100-lipoprotein levels was conserved between the present cellular and the past clinical and animal dietary studies.

We choose the McA cell line for these studies, rather than a model of human hepatic lipoprotein metabolism, the hepatocarcinoma cell line HepG2. Indeed, HepG2 cells have been frequently employed in studies of the effect of fatty acids on apoB-lipoprotein secretion (32), but they have a number of features distinct from what has been observed in perfused rat livers or in rodent primary hepatocytes (33). In particular, they are highly dependent on a relatively high concentration of exogenous fatty acids, such as OA, to maintain an adequate supply of "lipid ligands" for lipoprotein assembly (33, 34). Even with robust stimulation of TG synthesis, there is still deficient delivery of nascent TG to the "secretion-coupled" pool of microsomal TG that provides the substrate for the initial (and, perhaps, the later) lipidation of apoB-100 (34), resulting in a low level of secretion of VLDL particles.

In contrast, in McA cells the addition of OA to the culture medium does not increase apoB secretion (17, 23, 35) but does increase the lipid loading of apoB-lipoproteins so that majority of the secreted apoB-100 is in the VLDL fraction. In these respects, McA cells more closely resemble primary human hepatocytes (36). The physiological relevance of McA cells is further supported in the present studies by their mimicking, as noted above, the effects of dietary fatty acids seen in clinical and animal studies (28).

While McA cells are capable of secreting lipoproteins in all density classes, the density of the apoB-100-containing lipoproteins changed dramatically depending on the fatty acid used for incubation. Incubation with OA resulted in the secretion of primarily VLDL-apoB-100 lipoproteins, while incubation with MA caused the secretion of much denser apoB-100-containing lipoproteins. It is notable that the formation and secretion of a dense apoB-containing lipoprotein has also been reported in rat liver after perfusion of PA (37), again reflecting the relevance on the findings in vitro to the mammalian liver.

Our results could not be explained by differences in TG synthesis or in a preferential utilization of TG pools (i.e., pre-formed or nascent). Nor is it likely that extracellular hydrolysis of lipoprotein-associated TG (reported in studies of HepG2 cells) (38) was the basis for the density differences, given that *1*) the effect of MA was specific, *2*) a more dense particle with MA treatment was predicted from the independent measurements of the secretion of labeled apoB-100 and TG, and *3*) the amount of hepatic lipase secreted by McA cells is low (<2 ng/mg cell protein/4 h) (39).

A more plausible explanation is that the recruitment of TG into larger lipoproteins is decreased in MA-treated cells. This is not likely to be the same phenomenon reported by Ginsberg et al. in HepG2 cells (34). In those studies, low rates of apoB-100 and TG secretion were associated with reduced delivery of nascent TG to the "secretion-coupled pool". Presumably, then, a significant fraction of apoB-100 did not successfully complete the first step of lipidation and was targeted to the ubiquitin proteasome pathway for degradation (5).

The results of the present studies, however, suggest that in the presence of MA, apoB-100 successfully completes the first step of lipidation because: *1*) the first step is known to result in the formation of an HDL-density apoB-lipoprotein, consistent with the peak density of ~1.12g/ml of the apoB-100-lipoproteins in the conditioned media of the MA-treated cells (Fig. 4); and *2*) interference with the first step leads to *increased* apoB-100 degradation (5), not *decreased* (Fig. 6). It is more likely, then, that the recruitment of TG to complete the second step of lipidation is impaired in the MA-treated cells. Because the regulation of the second lipidation step is not yet defined in detail, it is premature to speculate on what aspect of this process is regulated by fatty acid type. Nonetheless, based on our present results and our published studies on n-3 fatty acid-induced apoB degradation (23), it is clear that VLDL assembly can be regulated by fatty acid type and that the underlying mechanisms are different than those that affect the intracellular degradation of apoB.

We have previously shown in rat primary hepatocytes and McA cells (17, 22, 23) that incubation with the n-3 fatty acids EPA or DHA increased intracellular degradation of apoB-100. In the present study, we have shown that incubation with MA decreases intracellular degradation of labeled apoB-100. By comparing the results of the steady-state and pulse-chase experiments, the MA-associated increase in labeled apoB-100 accumulation (Fig. 1) could be largely accounted for by decreased degradation (Fig. 6). One possible explanation for this is suggested by Woolett et al. (13, 14), who have shown that feeding SFA to hamsters led to a down-regulation of the LDLR. This would be expected to result in an increase in the net secretion of apoB-containing lipoproteins (because of decreased re-uptake of newly secreted apoB-lipoproteins, as shown in vitro (40) and in vivo (41). Against this possibility is that blockade of the LDLR by heparin (42, 43) did not alter the relative differences in the conditioned media apoB-100 content after incubation with MA and OA (data not shown).

What is the nature of the degradative pathway regulated by fatty acid type? We and others have defined the role of the ubiquitin-proteasome pathway for apoB-100 when HepG2 cells are maintained in fatty acid-deficient medium, which results in a low level of TG synthesis (5). Unlike that situation, there is no limitation of TG synthesis in the present study (Fig. 2A). Not surprisingly, treatment of McA cells with a proteasome inhibitor did not ablate the difference in apoB-100 secretion between incubations with MA and OA (M. Pan, E. Fisher, unpublished observations). We have recently reviewed the data from a number of published studies on apoB degradation (5, 44). Taken together, the results suggest that in certain metabolic states (e.g., choline deficiency, acute administration of insulin, supplementation with n-3 fatty acids), there is a post-ER degradative process that targets the more buoyant, heavily lipidated, apoB lipoprotein particles. Thus, the increased density of a lipoprotein enriched in MA or PA may, allow that particle to escape such a pathway.

This "escape" may be related to a change in a global property of the lipoprotein or in specific conformational changes, such as in the domains of apoB-100 exposed on the surface. Conformational changes are likely based on

the studies in hepatic cells showing that apoB length normally dictates the buoyancy of the lipoprotein particle (45). This means that the incorporation of lipids enriched in MA somehow overrides this relationship because a peak density of 1.12 g/ml (Fig. 4) ordinarily would be expected for a particle containing an apoB species considerably shorter than apoB-100.

In summary, our results imply that type of fatty acid supplied to the liver profoundly influences the formation and net secretion of apoB-100-containing lipoproteins. OA and MA differentially affect the formation of larger lipoproteins in spite of the comparable stimulation of cellular TG synthesis. Our results also support the existence of a non-proteasomal intracellular degradation pathway with lower activity toward apoB-100 associated with the dense lipoproteins formed when cells are incubated with MA. Overall, the ability of certain SFA to increase apoB-100-lipoprotein production may represent a mechanism in addition to reduced LDLR-mediated clearance by which diets enriched in SFA raise plasma lipoprotein levels in animals and humans.

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