Different fatty acids inhibit apoB100 secretion by different pathways: unique roles for ER stress, ceramide, and autophagy

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Abstract Although short-term incubation of hepatocytes with oleic acid (OA) stimulates secretion of apolipoprotein B100 (apoB100), exposure to higher doses of OA for longer periods inhibits secretion in association with induction of endoplasmic reticulum (ER) stress. Palmitic acid (PA) induces ER stress, but its effects on apoB100 secretion are unclear. Docosahexaenoic acid (DHA) inhibits apoB100 secretion, but its effects on ER stress have not been studied. We compared the effects of each of these fatty acids on ER stress and apoB100 secretion in McArdle RH7777 (McA) cells: OA and PA induced ER stress and inhibited apoB100 secretion at higher doses; PA was more potent because it also increased the synthesis of ceramide. DHA did not induce ER stress but was the most potent inhibitor of apoB100 secretion, acting via stimulation of autophagy. These unique effects of each fatty acid were confirmed when they were infused into C57BL6J mice. Our results suggest that when both increased hepatic secretion of VLDL apoB100 and hepatic steatosis coexist, reducing hepatic steatosis but at the expense of increased VLDL secretion. In contrast, increasing autophagy might reduce VLDL secretion without causing steatosis.—Caviglia, J. M., C. Gayet, T. Ota, A. Hernandez-Ono, D. M. Conlon, H. Jiang, E. A. Fisher, and H. N. Ginsberg. Different fatty acids inhibit apoB100 secretion by different pathways: unique roles for ER stress, ceramide, and autophagy. J. Lipid Res. 2011. 52: 1636–1651.

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The secretion of apolipoprotein B100 (apoB100) by hepatocytes is regulated mainly by the fatty acids and triacylglycerols (1–3). Oleic acid (OA) can increase the secretion of apoB100 both in hepatocyte cell lines and in mice, mainly by decreasing the intracellular degradation of apoB100 (4, 5). However, at high physiologic doses for longer periods of exposure, OA induces endoplasmic reticulum (ER) stress, which in turn inhibits the secretion of apoB100 (6). OA-induced ER stress decreases apoB100 secretion both by reducing its translation and by increasing its degradation by proteasomal and nonproteasomal pathways (6). Recent work suggests that one of the nonproteasomal pathways for apoB100 degradation may be via autophagy (7, 8).

The secretion of apoB100 is especially sensitive to OA-induced ER stress, as OA reduces the apoB100 secretion at lower concentrations than those necessary to affect other proteins secreted by hepatocytes, like apolipoprotein AI or albumin (6). This may be due to the complex structure of apoB100, a relatively large protein that requires complex posttranslational processing, including lipidation and glycosylation, for proper folding and secretion (5). This possibility is supported by the significantly lower sensitivity of apoB48 to ER stress-mediated degradation (6). Moreover, since OA ordinarily increases the secretion of apoB100, which increases the workload for the ER, this in turn can induce ER stress. Thus, it has been proposed that apoB100 itself can mediate the induction of ER stress by OA (3, 9).

Different fatty acids have different effects on ER stress. Palmitic acid (PA) is a more potent inducer of ER stress than OA: In cultured cells, short incubation with PA cause ER stress, whereas OA requires longer incubation to produce

Abbreviations: DHA docosahexaenoic acid; eIF2α, ER, endoplasmic reticulum; eukaryotic initiation factor 2a; GRP78, 78-kDa glucose-regulated protein; LC, light chain 3; McA, McA-RH7777; Myr, myriocin; OA, oleic acid; PA, palmitic acid; PBA, 4-phenylbutyric acid; PERK, ER membrane protein PKR-like ER kinase; SCD1, stearoyl-CoA desaturase 1; siRNA, small interfering RNA; TG, triglyceride.

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the same effect (6, 10). PA, but not OA, stimulates the synthesis of ceramide and increases reactive oxygen species (10, 11), either of which may induce ER stress (11–13). Polyunsaturated fatty acids induce lipid peroxidation and decrease apoB100 secretion, although it is not known whether ER stress is involved (7, 14). In this study, we compared the effects of the monounsaturated fatty acid OA, the saturated fatty acid PA, and the omega-3 polyunsaturated fatty acid docosahexaenoic acid (DHA), on ER stress and apoB100 secretion, and we studied the mechanisms involved.

EXPERIMENTAL PROCEDURES

Growth of cells

McArdle RH7777 (McA) cells were obtained from ATCC. McA cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) that contained penicillin (100 U/ml), streptomycin (100 µg/ml), 10% fetal bovine serum, and 10% horse serum (Invitrogen, Carlsbad, CA).

Cell studies

For steady-state studies, McA cells were incubated for 0-16 h in DMEM containing OA (0.7501, Sigma, St. Louis, MO), PA (P0500, Sigma), or DHA (U-84A, Nu-check) (0.2 to 1.2 mM) bound to 1.5% BSA; control incubations had only BSA. When apoB metabolism was being studied, cells were further incubated in methionine/cysteine-free DMEM for an additional 2 h, and then labeled with [35S]methionine for 2 h. The latter two incubations were also in the absence or presence of varying concentrations of OA, PA, or DHA. After labeling, media were transferred to tubes containing a mixture of protease inhibitors (30 µl/ml protease inhibitors, 1 mM benzamidine, 5 mM EDTA, 1% NP-40, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 10 nM okadaic acid, and 1 mM phenylmethylsulfonyl fluoride. Samples were processed for medium apoB by immunoprecipitation and SDS-PAGE as described below (4).

Lipid extraction, cell triglyceride, and protein mass determination

For lipid extraction, cells were washed with phosphate buffered saline (PBS) and then extracted twice with hexane/isopropanol (3/2, v/v) (2 ml/well) at room temperature for 30 min. The extraction solution was collected and dried under a stream of N<sub>2</sub> gas. Then triglyceride (TG) mass in cell extracts was determined using a TG determination kit (Trig/GB from Roche Diagnostics). The cellular proteins, which remained in the culture vessels, were solubilized with 0.1 N NaOH, and protein levels were determined with a BCA kit (Pierce, Rockford, IL).

Immunoprecipitation

Immunoprecipitation of apoB, apoA-I, or albumin in medium was carried out according to the method of Dixon et al. (4). The antibodies used were goat anti-human apoB, goat anti-human apoA-I (Calbiochem, San Diego, CA) and anti-human albumin (Sigma). Protein G agarose (Invitrogen) was added to the reaction solution to precipitate apoB, 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 by SDS-PAGE. The gel was treated with autolfluor (National Diagnostics, Atlanta, GA) and, after drying, exposed to X-ray film (Kodak X-Omat AR) at −80°C and quantified as incorporation of [35S] into apoB48 and apoB100 by autoradiography. The areas of the gels corresponding to apoB for each sample were isolated and the radioactivity incorporated was quantified by liquid scintillation counting.

Immunoblots

After in vitro treatment of McA with OA, PA, or DHA, cells were washed twice with PBS, lyed using either SDS buffer [2% SDS, 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue] or 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 protease inhibitors] and then boiled for 5 min. At the end of the in vivo experiments, mice were euthanized, and liver samples were collected and snap frozen. Protein extracts were prepared with a lysis buffer containing 25 mM Tris-HCl (pH 7.4), 2 mM Na3VO4, 10 mM NaF, 10 mM Na4P2O7, 1 mM EGTA, 1 mM EDTA, 1% NP-40, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 10 nM okadaic acid, and 1 mM phenylmethylsulfonyl fluoride. Equal amounts of protein extracts were separated on an 8% SDS-PAGE gel and electrotransferred to 0.45 µm nitrocellulose membrane using a Bio-Rad Laboratories mini-transfer tank. Membranes were incubated with primary antibodies overnight, and the protein bands were detected with HRP-conjugated secondary antibodies and SuperSignal West Pico enhanced chemiluminescent solution (Pierce). Membranes were stripped with Restore Western blot stripping buffer (Pierce) for 15 min at room temperature and reprobed with antibodies to β-actin or GAPDH to control for differences in loading. The primary antibodies used were anti-GRP78, anti-phospho-eIF2α (Stressgen, Victoria, British Columbia, Canada), anti-eIF2α, anti-phospho-ERK (Cell Signaling, Beverly, MA), anti-GAPDH, and monoclonal anti-β-actin (Sigma). All immunoblots were quantified by densitometry.

Phenyl butyric acid treatment of McA cells

Phenyl butyric acid (PBA) is known to protect against OA-induced ER stress (6, 15). McA cells were preincubated with serum-free DMEM containing 1 mM PBA for 6 h and then incubated with OA, PA, or DHA (0, 0.4, 0.8, and 1.2 mM) for 6 or 16 h. The cells were incubated with methionine/cysteine-free DMEM and labeled with [35S]methionine at the end of the 6 h treatment, and apoB secretion was measured as described above. In parallel experiments using the same conditions, ER stress level was measured. After 6 h incubation, proteins were extracted for immunoblots as described above.

Myriocin treatment of McA cells

Myriocin (Sigma) is known as a specific inhibitor of ceramide biosynthesis (16). McA cells were incubated with OA or PA in the presence or absence of myriocin (50 µM) for 16 h. The cells were then incubated in methionine/cysteine-free DMEM and labeled with [35S]methionine for 2 h. In these two latter steps, the cells were also incubated in the presence or absence of myriocin. Immunoprecipitation of apoB, apoA-I, or albumin in medium or cellular lysate was carried out. In parallel experiments using the same conditions, cells were lysed and analyzed by immunoblotting for GRP78 and phosphorylation of eIF2α. Lipids were extracted from parallel sets of cells, and ceramide levels in McA cells were determined using the diacylglycerol kinase method (17) or by liquid chromatography-mass spectrometry (18).

Transfection of small interfering RNAs

Stearoyl-CoA desaturase (SCD1) converts PA to palmitoleic acid, reducing utilization of PA for ceramide synthesis (19).
Two pairs of small interfering RNAs (siRNA) specific to SCD1 (target sequence: CAGCACCTTCTTGAGATACACTCTG) and a nonspecific control were synthesized by Invitrogen, annealed, and transfected into McA cells using Lipofectamine 2000 (Invitrogen). At 48 h after transfection, McA cells were incubated with OA or PA for 16 h. Immunoprecipitation of apoB in medium or cellular lysate was carried out as described above. In parallel experiments using the same conditions, cells were lysed and analyzed by immunoblotting for GRP78 and phosphorylation of eIF2α, and cellular ceramide levels were determined as described above.

Measurement of autophagy
McA cells were incubated with OA, PA, DHA, or tunicamycin as described previously. The cells were treated with or without the lysosomal inhibitors leupeptin (100 μM) and ammonium chloride (20 mM) (20, 21), which were added 8 h before the cells were harvested. Autophagy was assessed by analyzing the expression of microtubule-associated protein light chain 3 (LC3). The expression of LC3-I and LC3-II was detected by immunoblotting using an anti-LC3B antibody (Cell Signaling, Danvers, MA) and quantified by densitometry.

Quantitative real-time PCR
Total RNA samples were used for cDNA synthesis with oligo d(T) primers using a commercial kit (Invitrogen). The resulting cDNA samples were then quantified for each test gene using target gene-specific primers. Quantitative real-time PCR was done using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to the protocols provided by the manufacturer. Detection of specific products was performed in triplicate using the Mx4000 Multiplex Quantitative PCR system (Stratagene, La Jolla, CA). Using the standard curve method, the relative quantitation of specific PCR products for each primer set was generated. For normalization, GAPDH or β-actin was amplified from each sample. The primers used in real-time PCR were as described previously (6).

Analysis of XBP1 processing
XBP1 cDNA encompassing the region of restriction site was amplified by PCR using previously described primers (22). The PCR product was incubated with the PstI restriction enzyme for 5 h at 37°C, followed by separation of the restriction digests on a 2% agarose gel with ethidium bromide. The gels were photographed under UV transillumination and quantified by densitometry. The amount of 601-bp material (indicative of XBP1 activation and, thus, of ER stress) was expressed as a percentage of the total amount of amplified material, considering the sum of the two bands in each lane as 100%.

Animals
The animals studied were male C57BL/6J mice, ages 12-14 weeks, purchased from the Jackson Laboratory. All mice were maintained on a 12 h light/12 h dark cycle (light cycle was 7 AM to 7 PM) and on a regular rodent chow diet (5053 PicoLab Rodent Diet 20, Purina Mills). Animal studies were approved by the Institutional Animal Care and Use Committee of Columbia University College of Physicians and Surgeons.

Infusion study. A silicone catheter was inserted in the jugular vein with surgical procedures as described previously (6, 23). The mice were then randomly assigned to receive intravenous infusion of 6 mM OA (O7501, Sigma), 6 mM PA (P5585, Sigma), or 6 mM DHA (D2534, Sigma) bound to fatty acid-free albumin. On the morning of the experiment, food was removed, and the silicone tubing was flushed with saline and connected to a KDS 220 infusion pump (KD Scientific). The infusion line was suspended from the top of the cage and allowed the mice complete freedom of movement. Infusion was carried out at a rate of 2.5 µl/min for 6 or 9 h. In some studies, mice were euthanized at this time and their livers obtained for other measurements. In parallel studies, Triton WR1339 and 35S-methionine were administered at this time and blood samples were obtained from the retro-orbital plexus over the next 2 h for determination of apoB secretion (see below). Fatty acids were not infused after administration of Triton WR1339.

Fatty acid solutions. Albumin-bound OA was prepared as previously described (4). The albumin-bound DHA solution was prepared the same way. For PA, a modified approach was needed because of the lower solubility of this fatty acid. Briefly, 1 g of PA powder was dissolved in 2.5 ml of ethanol, and the mixture was sonicated and warmed at 51°C to allow for complete solubilization. A filtered saline-BSA solution was added, and the mixture was stirred overnight at 51°C. The pH was then adjusted to 7.8, and the solution was aliquoted and stored at –20°C. The ratio of OA, PA, or DHA to BSA was 5:1.

Determination of in vivo apoB secretion rates. After each fatty acid infusion, in vivo secretion rates of apoB were determined as described previously (23). Mice were injected intravenously with 500 mg/kg Triton WR1339 (25301-02-4, Sigma) in 0.9% NaCl. Lipoprotein clearance from plasma is completely inhibited in mice under these conditions (24), and the accumulation of VLDL lipids and apolipoproteins in plasma after injection of Triton can be used to estimate their rates of secretion into the plasma compartment. ApoB secretion rates were measured by injecting each mouse with 200 μG of [35S]methionine (1,175 Ci/mmol; PerkinElmer Life Sciences) together with Triton. Blood samples were taken at 60 and 120 min after injection, and the accumulation of [35S]methionine-labeled apoB was used to determine the rates of VLDL apoB secretion. Plasma samples were subjected to 4% SDS-PAGE, and the gel was dried and exposed to X-ray films to visualize labeled apoB. The apoB100 and apoB48 bands on the film were scanned for densitometry measurements. Total protein synthesis was measured by trichloroacetic acid (TCA) precipitable radioactivity in plasma, and the plasma volume loaded for each sample was adjusted accordingly.

Treatments with PBA and myriocin
PBA at a dose of 1 g/kg body weight/day was administered orally by gavage, twice a day, for eight days prior to the infusion of PA, as described previously (6); control mice received water. Myriocin (300 μg/kg body weight/day) was administered intraperitoneally for four days prior to the PA infusion, while controls received the corresponding vehicle DMSO.

Statistics
All data are presented as means and standard deviations. Differences in the mean values between two groups were assessed by two-tailed Student’s t-test. Differences in mean values among more than two groups were determined by ANOVA with Tukey's post hoc test to look for differences between specific groups. P < 0.05 was considered to be statistically significant.

RESULTS

Incubation of McA cells with OA, PA, or DHA results in dose-dependent increases in cell TG
To compare the effects of different fatty acids on apoB100 secretion and examine the mechanisms for those
effects, we incubated McA cells for 16 h with OA, PA, or DHA at concentrations ranging from 0.2 to 1.2 mM. Incubation with OA, PA, or DHA resulted in an increase in cell TG from control levels of 42.4 ± 8.4 µg/mg cell protein in the absence of any fatty acids to levels of 259.3 ± 22.2 µg/mg cell protein in the presence of 1.2 mM OA, 166.1 ± 20.9 µg/mg cell protein in the presence of 1.2 mM PA, and 253.4 ± 52.7 µg/mg cell protein in the presence of 1.2 mM DHA (Fig. 1). We estimated toxicity by reductions in TCA precipitable radioactivity and trypan blue exclusion in several experiments at concentrations of 1.2 mM PA and DHA; accordingly, we only show data up to concentrations of 0.8 mM for those two fatty acids in this and all succeeding experiments. Incubation with PA led to less accumulation of cell TG compared with OA or DHA ($P < 0.05$, PA versus OA or DHA) at 0.4 and 0.8 mM.

**OA and PA, but not DHA, induce ER stress in McA cells**

We demonstrated previously that hepatic ER stress was induced by loading McA cells with OA at high physiologic concentrations for more than 6 h (6). To determine whether other fatty acids could induce hepatic ER stress, we incubated McA cells with OA (0.2-1.2 mM), PA (0.2-0.8 mM), or DHA (0.2-0.8 mM) for 16 h and evaluated ER stress by examining GRP78 protein expression and phosphorylation of eIF2α (Fig. 2). Incubation of McA cells with OA for 16 h caused increasing GRP78 protein expression (Fig. 2A, left) and phosphorylation of eIF2α (Fig. 2A, right) compared with incubation in the absence of fatty acids ($P < 0.05$ at 0.4, 0.8, 1.2 mM). Incubation of McA cells with PA (0.2-0.8 mM) caused greater ER stress than OA at 0.4 and 0.8 mM for GRP78, and at all doses for eIF2α ($P < 0.05$ versus OA). Of note, DHA (0.2-0.8 mM) did not stimulate ER stress at any dose, as judged by the levels of GRP78 and phosphorylated eIF2α. Incubation of McA cells with 0.4 mM OA for 3-16 h resulted in increases in markers of ER stress only at the 16 h point (Fig. 2B).

**PA, but not OA, increases ER stress and inhibits apoB100 secretion via a ceramide-mediated pathway**

PA is a well-characterized precursor of ceramide, which in turn has been implicated as an inducer of both ER stress and apoptosis (13). We first confirmed that PA, unlike OA, caused an increase in the level of ceramide in McA cells. Incubation of McA cells for 16 h with 0.4 or 0.8 mM PA significantly increased level of ceramide compared with either cells not receiving any fatty acids or cells receiving the same concentrations of PA (Fig. 4A, left). Incubation of McA cells with DHA for 16 h did not affect ceramide concentrations (data not shown). When myriocin, an inhibitor of serine palmitoyltransferase (16), which...
Suppression of SCD1 results in higher levels of ceramide, increased ER stress, and greater inhibition of apoB100 secretion after incubation of McA with PA

PA is a precursor of ceramide, and it is also a substrate for TG synthesis, particularly after conversion to palmitoleic acid by SCD1 (25). To examine further the role of ceramide in PA-associated inhibition of apoB100 secretion, we reduced SCD1 mRNA by approximately 90% by transfecting McA cells with siRNA to SCD1 (Fig. 5A) to shunt PA away from TG synthesis and toward ceramide generation. Indeed, cells transfected with SCD1 siRNA generated higher levels of ceramide after 16 h incubation with PA compared with cells transfected with scrambled siRNA; the effect of SCD1 siRNA treatment was reversed by incubation of the cells with myriocin catalyzes a key step in the conversion of PA to ceramide, was added to the incubation media, the PA-induced increase in ceramide was mostly abrogated (Fig. 4A, right). Effects of 16 h incubation with 0.4 mM PA on ER stress, as assessed by measurements of cellular GRP78 and phosphorylation of eIF2α, was concomitantly reduced by addition of myriocin to the incubation media, whereas myriocin treatment had no effect on the pattern of either OA- or DHA-mediated inhibition of apoB100 secretion at a concentration of 0.8 mM was significantly, although not completely, reversed. Myriocin treatment had no effect on the pattern of either OA- (Fig. 4C) or DHA-mediated inhibition of apoB100 secretion (data not shown).
Fatty acids inhibit apoB secretion by different pathways

PBA has modest effects on PA-induced inhibition of apoB100 secretion

We have previously shown that pretreatment of McA cells with PBA, which may act as a chemical chaperone (15), could significantly reduce OA-induced ER stress (6). We studied, therefore, the effect of PBA on PA-induced ER stress. PBA modestly inhibited ER stress, as demonstrated by decreased levels of phospho-PERK (Fig. 6A), which was associated with a partial reversal of the inhibition of apoB100 secretion at high concentrations of PA (Fig. 6B). Importantly, the ability of PBA to partially reverse the effects of PA on apoB100 secretion was not associated with a concomitant reduction in ceramide concentrations (Fig. 6C).
on markers of hepatic ER stress with the effects of 6 h infusion of OA. PA infusion increased phosphorylation of eIF2α/H8251 more than 2-fold compared with OA (226 ± 46 versus 100 ± 28%,
\( P < 0.0005 \) versus OA). GRP78 protein expression also increased more with PA (136 ± 18 versus 100 ± 31%,
\( P < 0.05 \) versus OA) (Fig. 7A). This greater stimulation of ER stress was associated with suppression of apoB100 secretion by the liver compared with OA: After 6 h of PA infusion, secretion of newly synthesized apoB100 after injection of Triton WR1339 was decreased compared with OA (84 ± 8% versus 100 ± 17% and 81 ± 21% versus 100 ± 11%,
\( P < 0.05 \) versus OA at 60 and 120 min, respectively) (Fig. 7B). A nonsignificant trend toward a similar decrease in the appearance of newly synthesized apoB48 was also observed. Surprisingly, in contrast to our observation

Additionally, combined treatment with PBA and myriocin also reversed the decrease in apoB100 secretion induced by PA, but this effect was not greater than the effect of either PBA or myriocin alone (data not shown).

**Intravenous infusion of PA increases hepatic ER stress markers more than OA and inhibits secretion of apoB in wild-type mice**

We previously reported that intravenous infusion of 6 mM OA in C57BL/6J mice increased hepatic markers of ER stress to a greater extent after 9 h than after 6 h and that the greater stimulation of ER stress at 9 h was associated with a loss of the OA-induced increases in apoB secretion observed a 6 h (6). In the present study, we compared the effects of an intravenous infusion of 6 mM PA for 6 h on markers of hepatic ER stress with the effects of 6 h infusion of OA. PA infusion increased phosphorylation of eIF2α more than 24-fold compared with OA (226 ± 46 versus 100 ± 28%,
\( P < 0.0005 \) versus OA). GRP78 protein expression also increased more with PA (136 ± 18 versus 100 ± 31%,
\( P < 0.05 \) versus OA) (Fig. 7A). This greater stimulation of ER stress was associated with suppression of apoB100 secretion by the liver compared with OA: After 6 h of PA infusion, secretion of newly synthesized apoB100 after injection of Triton WR1339 was decreased compared with OA (84 ± 8% versus 100 ± 17% and 81 ± 21% versus 100 ± 11%,
\( P < 0.05 \) versus OA at 60 and 120 min, respectively) (Fig. 7B). A nonsignificant trend toward a similar decrease in the appearance of newly synthesized apoB48 was also observed. Surprisingly, in contrast to our observation

![Graph A](image1.png)

**Graph A**

![Graph B](image2.png)

**Graph B**

![Graph C](image3.png)

**Graph C**
Fig. 5. Enhanced PA-induced ceramide accumulation, ER stress, and PA-mediated inhibition of apoB100 by knockdown of SCD1. After efficient knockdown of SCD1 mRNA by siRNA (A), McA cells were incubated with no FA, OA (0.4 or 0.8 mM), or PA (0.4 or 0.8 mM) in the presence or absence of myriocin (Myr, 50 μM) for 16 h. Then ceramide content was measured by DAG kinase assay followed by thin layer chromatography and autoradiography, and GRP78 and phosphorylation of eIF2α were evaluated by Western blot. The cells were incubated for 2 h in methionine/cysteine-free DMEM, and then labeled with [35S]methionine for 2 h. (B) Ceramide levels in McA cells were further increased with PA (0.8 mM) by knockdown of SCD1 (P < 0.05 SCD1 versus control siRNA). Inhibition of ceramide synthesis with myriocin reduced PA-induced ceramide
that pretreatment of mice with PBA for seven days reversed the loss of OA-induced increases in apoB after 9 h (6), pretreatment of mice with PBA prior to infusion for 6 h with PA did not reduce the levels of ER stress markers or increase apoB secretion (data not shown).

Reducing ceramide in liver increases apoB secretion in mice receiving an infusion of PA

As shown in McA cells treated with myriocin (Fig. 4), preventing the PA-induced increase in ceramide significantly reversed the PA-associated increase in ER stress and inhibition of apoB100 secretion. To investigate whether ceramide has a similar role in vivo, we treated mice with myriocin for four days and then performed PA infusion for 9 h, a protocol designed to exaggerate the inhibition of apoB secretion by PA. As expected, myriocin decreased liver ceramide by 58% (129 ± 14 versus 54 ± 10%, P < 0.05 versus controls) (Fig. 8A). In the livers of the mice treated with myriocin, GRP78 protein expression and XBP1 splicing (another marker of ER stress) decreased compared with controls (76 ± 11 versus 100 ± 15%, P < 0.05 and 15 ± 2 versus 19 ± 2%, P < 0.05 myriocin-treated mice versus controls for GRP78 and XBP1 splicing, respectively) (Fig. 8B, C). In mice treated with myriocin, the secretion of apoB100 was 31% more than secretion in controls (131 ± 10 versus 100 ± 21%, P < 0.05 versus controls at 60 min) The secretion of apoB48 showed a nonsignificant trend toward a similar increase (Fig. 8D). At 120 min, the secretion of apoB100 maintained a nonsignificant trend toward higher rates, but there was no effect of myriocin on apoB48 secretion.

Intravenous infusion of DHA does not alter hepatic ER stress markers more than OA, but it inhibits secretion of apoB in wild-type mice

Infusion of 6 mM DHA for 6 h had the same effect on hepatic ER stress markers as 6 mM OA (Fig. 9A). However, consistent with our previous data (14), a 6 h infusion of DHA inhibited both apoB100 and apoB48 secretion (apoB100 for DHA versus OA: 85 ± 9% versus 100 ± 10% and 76 ± 15% versus 100 ± 14%, P < 0.05 at 60 and 120 min, respectively; apoB48 for DHA versus OA: 76 ± 7% versus 100 ± 8% and 82 ± 17% versus 100 ± 11%, P < 0.05 at 60 and 120 min, respectively) (Fig. 9B).

DHA, but not OA or PA, increases autophagic flux in McA cells

We previously demonstrated that DHA inhibited apoB100 secretion at least in part via induction of autophagy (7). To determine whether autophagy plays a role in the inhibition of apoB100 secretion by either OA or PA, we incubated McA cells with OA, PA, or DHA and measured the induction of autophagy using the standard assay of LC3-I conversion to LC3-II in the absence or presence of lysosomal inhibitors (20, 26). Incubation of cells for 16 h with 5 μg/ml tunicamycin, which was used as a control, increased the LC3-II/LC3-I ratio both in the presence and absence of lysosomal inhibitors (Fig. 10). DHA increased this ratio after only 4 h of incubation at 0.6 mM, confirming our previous results (7). The results with tunicamycin and DHA indicated increased flux of substrates through the autophagic pathway (26). Incubation of McA cells with 0.8 mM PA for 6 h increased the LC3-II/LC3-I ratio in the absence, but not in the presence, of lysosomal inhibitors, a finding that indicated decreased autophagic flux (20, 26). Finally, OA in concentrations as high as 0.8 mM did not induce autophagy in incubation as long as 16 h.

DISCUSSION

Hepatic steatosis is an increasing problem around the world because of its association with insulin resistance and obesity. Although increases in hepatic TG content result mainly from increased delivery of plasma fatty acids as substrate for TG synthesis (27), there are at least two other mechanisms that contribute significantly to the development of hepatic steatosis, and both are the result of fatty acid induction of ER stress. First, ER stress has been shown to stimulate de novo fatty acid synthesis in the liver, adding to the burden of excess fatty acid delivery to the liver (28–30). Second, ER stress reduces the secretion of apoB100 lipoproteins, interfering with the ability of hepatocytes to export the excess TG that has accumulated (6,9). TG itself does not appear to be the mediator of fatty acid-induced ER stress in the liver (31,32) and may instead constitute a neutral form of storage for fatty acids. Indeed, in the present studies, PA was not incorporated into TG as well as OA (Fig. 1), but it induced greater ER stress and more inhibition of apoB secretion (Figs. 2 and 3). This finding is consistent with prior work demonstrating reduced lipotoxicity associated with increased incorporation of saturated fatty acids into TG (31–33). At the opposite end of the spectrum, DHA was incorporated into TG as well as OA (Fig. 1), but it did not induce ER stress at all (Figs. 2 and 3). These findings suggest that either fatty acids or some of their metabolites, but not TG, are the inducers of ER stress. Of particular interest, therefore, is how fatty acids induce ER stress and whether all fatty acids affect apoB secretion similarly.

It is well established that delivery of moderate concentrations of fatty acids to hepatocytes actually stimulates the secretion of apoB100 both in cell culture (4,34) and animal models (23). However, we recently demonstrated that high physiologic concentrations of the monounsaturated fatty acid OA, particularly when provided over longer...
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Although both PA and DHA were more potent inhibitors of apoB100 secretion than OA, PA had a dual effect similar to OA, increasing apoB100 secretion at relatively low concentrations and decreasing apoB100 secretion at higher concentrations, whereas DHA inhibited apoB100 secretion without having any stimulatory effect (Fig. 3). PA, like OA, induced ER stress, but much of the inhibition of apoB100 secretion by PA was associated with an increase in the synthesis of ceramide; OA induced ER stress and inhibited apoB secretion without altering levels of ceramide (Fig. 4). A striking finding was that DHA, the most potent inhibitor of apoB100 secretion, did not cause ER stress (Fig. 4); as we reported previously, DHA inhibited secretion of apoB100 by stimulating autophagy (7).

Though we and others have demonstrated OA-induced ER stress (6, 36), this has not been a universal observation (37, 38). Furthermore, the mechanism for OA-induced

Fig. 6. Effect of PBA on ER stress and apoB100 secretion in McA cells treated with PA. McA cells were preincubated with or without 1 mM PBA for 6 h and then incubated with no FA or PA (0.2 mM to 0.8 mM) for additional 6 h in the presence or absence of PBA. (A) Phospho- PERK was analyzed as marker of ER stress. (B) The secretion of apoB100 was measured as described in Fig. 3. (C) Ceramide content was measured as described in Fig. 4. Plotted data correspond to means ± SD; n = 4 per group, from two independent experiments. Statistical analysis was performed using two-way ANOVA. *P < 0.05 compared with the incubation in the absence of PA, †P < 0.05 compared with the incubation in the absence of PA but with PBA, ‡P < 0.05 compared with incubation with the same concentration of PA but without PBA.

periods, induced ER stress and decreased the secretion of apoB100 (6). In the present studies, we compared the effects of OA to those of PA, a saturated fatty acid, and DHA, a polyunsaturated fatty acid, regarding their ability to induce ER stress and inhibit apoB secretion. The relevance of these studies derives from the fact that PA is both the major component of the plasma fatty acid pool and the product of de novo lipogenesis, whereas DHA has been shown to inhibit apoB100 secretion in both mice (14), and humans (35). In addition, there are very limited data on the effects of PA, which is known to induce ER stress, on apoB secretion (see below). Furthermore, although it is clear that DHA inhibits apoB secretion in association with increased autophagy (7), its effect on ER stress had not been studied.

In the present investigation, we found that, although all three fatty acids inhibit apoB100 secretion both in vitro and in vivo, there were significant quantitative and qualitative differences between the effects of either PA or DHA and those of OA. Thus, although both PA and DHA were more potent inhibitors of apoB100 secretion than OA, PA had a dual effect similar to OA, increasing apoB100 secretion at relatively low concentrations and decreasing apoB100 secretion at higher concentrations, whereas DHA inhibited apoB100 secretion without having any stimulatory effect (Fig. 3). PA, like OA, induced ER stress, but much of the inhibition of apoB100 secretion by PA was associated with an increase in the synthesis of ceramide; OA induced ER stress and inhibited apoB secretion without altering levels of ceramide (Fig. 4). A striking finding was that DHA, the most potent inhibitor of apoB100 secretion, did not cause ER stress (Fig. 4); as we reported previously, DHA inhibited secretion of apoB100 by stimulating autophagy (7).

Though we and others have demonstrated OA-induced ER stress (6, 36), this has not been a universal observation (37, 38). Furthermore, the mechanism for OA-induced
ER stress is unclear. Su et al. proposed that, in hepatocytes, OA induces ER stress by increasing the translocation of newly synthesized apoB into the secretory pathway and, thus, the amount of apoB processed by the ER (9). On the basis of our prior studies, we can rule out increases in lipid peroxides and reactive oxygen species as the mechanism for OA-induced ER stress (14). Additionally, in the present studies, incubation with OA did not generate lipid peroxides, as measured by malondialdehyde (data not shown). We can also eliminate, based on the present investigations, OA-mediated increases in ceramide as the mechanism. Thus, although the identification of the explicit mechanisms underlying OA-mediated ER stress will require further experimentation, the present studies have eliminated several suspected pathways. We have also significantly extended our prior work, where we observed cotranslational degradation of apoB100 (6), by demonstrating that autophagy, which can participate in the degradation of apoB100 (7, 8, 39), did not play a role in OA-stimulated degradation of apoB100. Further investigations of the links between OA-induced ER stress and degradation of apoB are still needed.

In contrast to the paucity of mechanistic data for OA’s effects, the ability of saturated fatty acids to cause ER stress
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Inhibitor of the de novo synthesis of ceramide, decreased the concentration of ceramide and reduced ER stress (Fig. 4). Furthermore, siRNA-mediated suppression of SCD1, by reducing the conversion of PA to palmitoleic acid, increased ceramide and ER stress (Fig. 5). The importance of desaturation of palmitate is supported by the demonstration that genetically induced increases or decreases in SCD1 activity were associated with reduced or increased ER stress, respectively, in a pancreatic cell line (45).

Dashti et al. studied the effects of PA on apoB secretion. DASHI et al. studied the effects of low concentrations of PA in HepG2 cells by pretreating the cells with 0.1 mM PA for five days and then collecting media after 16 h of additional treatment with 0.2 mM PA (46, 47). They found no stimulation of apoB secretion by PA, but it is difficult to compare their results with those in our studies. Importantly, there have been no prior studies of the simultaneous effects of PA on both ER stress and the secretion of apoB100 from hepatocytes. In the present studies, we show that PA is a more potent inhibitor of the de novo synthesis of ceramide, decreased the concentration of ceramide and reduced ER stress (Fig. 4). Furthermore, siRNA-mediated suppression of SCD1, by reducing the conversion of PA to palmitoleic acid, increased ceramide and ER stress (Fig. 5). The importance of desaturation of palmitate is supported by the demonstration that genetically induced increases or decreases in SCD1 activity were associated with reduced or increased ER stress, respectively, in a pancreatic β-cell line (45).

As noted above, there are very limited data on the effects of PA on apoB secretion. Dashti et al. studied the effects of low concentrations of PA in HepG2 cells by pretreating the cells with 0.1 mM PA for five days and then collecting media after 16 h of additional treatment with 0.2 mM PA (46, 47). They found no stimulation of apoB secretion by PA, but it is difficult to compare their results with those in our studies. Importantly, there have been no prior studies of the simultaneous effects of PA on both ER stress and the secretion of apoB100 from hepatocytes. In the present studies, we show that PA is a more potent inhibitor of the de novo synthesis of ceramide, decreased the concentration of ceramide and reduced ER stress (Fig. 4). Furthermore, siRNA-mediated suppression of SCD1, by reducing the conversion of PA to palmitoleic acid, increased ceramide and ER stress (Fig. 5). The importance of desaturation of palmitate is supported by the demonstration that genetically induced increases or decreases in SCD1 activity were associated with reduced or increased ER stress, respectively, in a pancreatic β-cell line (45).
increase in the ratio of LC3-II to LC3-I, an indicator of autophagic activity, in PA-treated McA cells, the ratio was not elevated in cells treated with lysosomal inhibitors (Fig. 10). These results suggested that the increase in the ratio of LC3-II to LC3-I seen in the absence of lysosomal inhibitors was due to defective rather than increased autophagy; i.e., the production of LC3-II was normal, but flux of LC3-II to the lysosome, where it is degraded, was reduced (26). It must be noted, however, that when the levels of LC3-II with and without lysosomal inhibitors are not congruent, the results are difficult to interpret (20, 26). We cannot rule out, therefore, that palmitate inhibition of apoB100 secretion was mediated, in part, by inducing autophagy (48, 49). However, we have never seen intracellular aggregates of nascent apoB in cells incubated with OA (7) or with PA (Kummrow and Fisher, unpublished observations).

The complex nature of the effects of fatty acids on apoB secretion is illustrated best by our data with DHA. We showed previously that DHA, via increased lipid peroxidation and reactive oxygen species, causes aggregation of inhibitor of apoB100 secretion that OA and that this greater potency derives from PA-mediated increases in ER stress that are both ceramide dependent and independent. Thus, both myriocin treatment (Fig. 4) and PBA treatment (Fig. 6) partially reversed PA-mediated ER stress and the inhibition of apoB100 secretion; the interaction between PBA and myriocin appears to be complex, as the effects of each were not additive when they were used in combination. In any case, ceramide seemed more closely related to inhibition of apoB100 secretion: When we treated mice with myriocin before infusing PA, there were reductions of liver ceramide, lower levels of ER stress markers, and reversal of the inhibition of apoB100 secretion in vivo (Fig. 8), whereas pretreatment with PBA did not alter the effects of PA infusion. Together, these results are in accord with prior work demonstrating a role for ceramide in the induction of ER stress by PA (33), and they indicate that this mechanism is involved in the inhibition of the secretion of apoB100 by PA.

Because of our prior work with DHA (7), we examined the effects of PA on autophagy. Although we observed an increase in the ratio of LC3-II to LC3-I, an indicator of autophagic activity, in PA-treated McA cells, the ratio was not elevated in cells treated with lysosomal inhibitors (Fig. 10). These results suggested that the increase in the ratio of LC3-II to LC3-I seen in the absence of lysosomal inhibitors was due to defective rather than increased autophagy; i.e., the production of LC3-II was normal, but flux of LC3-II to the lysosome, where it is degraded, was reduced (26). It must be noted, however, that when the levels of LC3-II with and without lysosomal inhibitors are not congruent, the results are difficult to interpret (20, 26). We cannot rule out, therefore, that palmitate inhibition of apoB100 secretion was mediated, in part, by inducing autophagy (48, 49). However, we have never seen intracellular aggregates of nascent apoB in cells incubated with OA (7) or with PA (Kummrow and Fisher, unpublished observations).

The complex nature of the effects of fatty acids on apoB secretion is illustrated best by our data with DHA. We showed previously that DHA, via increased lipid peroxidation and reactive oxygen species, causes aggregation of
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ability to increase the synthesis of ceramide. In contrast, DHA, which was the most potent of the three fatty acids in terms of inhibiting apoB secretion, did not induce ER stress but, rather, targeted apoB100 for autophagic degradation. In states of insulin resistance, in which plasma fatty acids are usually elevated (53, 54), the liver is exposed to a mixture of different fatty acids. Therefore, several mechanisms may contribute to the alterations in lipoprotein secretion and the hepatosteatosis characteristics of these diseases. PA is the major plasma fatty acid that is delivered from adipose tissue and also the major product of hepatic lipogenesis; it is also the most potent of the three fatty acids we studied in terms of induction of ER stress. Our results provide new insights into potential approaches to the prevention or treatment of hepatosteatosis. For example, approaches that reduce ER stress without affecting the availability of OA or PA may improve hepatic steatosis by increasing VLDL TG secretion, albeit at the expense of increased plasma levels of TG and apoB100. On the other hand, approaches that increase the delivery of DHA to the liver may be effective at reducing both hepatic TG content and the secretion of apoB by stimulating autophagy. This possibility is supported both by recent evidence that autophagy may protect against hepatic steatosis (21, 52) and the well-established utility of the omega-3 fatty acids as a TG-lowering therapeutic agent (35, 55).

Fig. 10. Increased markers of autophagy after incubation of McA cells with PA and DHA. McA cells were incubated with OA (0.4 and 0.8 mM) for 16 h, with PA (0.4 and 0.8 mM) for 8 h, with DHA (0.4 mM) for 4 h, or with tunicamycin (5 μg/ml) for 6 or 16 h. The cells were treated without (A) or with (B) lysosomal inhibitors (leupeptin and ammonium chloride). LC3-I and LC3-II were detected by immunoblotting, quantified by densitometry, and the ratios LC3-I/LC3-II were calculated (C). Statistical analysis was conducted using ANOVA with Tukey’s posthoc test (data for each condition at each time point are from duplicates).
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