Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells

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We (41) have recently demonstrated that hepatic steatosis characterized by increased saturated fatty acids leads to increased caspase-3 activity, ER stress, and liver injury in rats. Thus the presence of increased saturated fatty acid delivery to or accumulation in the liver may constitute an intrinsic second hit in the steatotic liver. Previous studies (7, 13, 22, 24, 32, 36) in Chinese hamster ovary (CHO) cells, cardiac myocytes, pancreatic β-cells, breast cancer cell lines, and hematopoietic precursor cell lines have demonstrated that toxicity from accumulation of long-chain fatty acids is specific to or made more severe by saturated fatty acids. The nature of the lipid species and/or signaling molecule(s) that provokes saturated fatty acid-induced apoptosis appears to differ across cell types but includes ceramide, reactive oxygen species, and nitric oxide (22, 36).

In the present study, we examined the direct effects of saturated and unsaturated fatty acids on liver cell homeostasis and apoptosis. In addition, we examined the role of ceramide in saturated fatty acid-induced ER stress and apoptosis. The data demonstrate that the saturated fatty acids steerate and palmitate potently induced ER stress and apoptosis via mechanisms that appear to be independent of ceramide accumulation.

METHODS

Cell culture. H4IE liver cells (American Type Culture Collection, Manassas, VA), a rat liver hepatoma cell line, were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin sulfate. Experiments were performed at 80–100% cell confluence.

Experimental agents. Fatty acids (Sigma Chemical, St. Louis, MO) were complexed to bovine serum albumin at a 2:1 molar ratio (22). Two agents were used to induce ER stress: thapsigargin (450 nM), a tumor-promoting sesquiterpene lactone that induces ER stress via inhibition of the ER-associated calcium ATPase (14, 39), and tumicamycin (6 μg/ml), which inhibits protein glycosylation (11). Fumonisin B1 (50 μM; Sigma) was used to inhibit ceramide synthetase (36). Disipramine (50 μM; Sigma) was used to inhibit acidic sphingomyelinase (18).

RNA isolation and analysis. Total RNA was extracted with TRIzol reagent, using the manufacturer’s protocol (Invitrogen, Carlsbad, CA). For analysis of XBP1 splicing, a two-step protocol was used for reverse transcription PCR using Superscript II reverse transcriptase and Taq polymerase (42). For real-time PCR, reverse transcription was performed using 0.5 μg of DNase-treated RNA, Superscript II

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Results were similar regardless of the control gene cyclophilin (control genes), according to the procedures of Muller et al. (28). PCR efficiency was between 90 and 105% for all primer and probe sets and linear over five orders of magnitude. The specificity of products generated for the target gene relative to the β2-microglobulin and cyclophilin (control genes), according to the procedures of Muller et al. (28). Results were similar regardless of the control gene used; therefore, data in the results section are reported using β2-microglobulin.

Immunoblot analysis. Cells were washed with PBS and harvested using a lysis buffer containing 20 mM HEPES, pH 7.4, 1% Triton X-100, 10% glycerol, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 mM β-mercaptoethanol, 3 mM benzamidine, 10 μM leupeptin, 5 μM pepstatin, and 10 μg/ml aprotinin. Equivalent amounts of protein (50–100 μg) were subjected to SDS-PAGE and transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Piscataway, NJ), and the membranes were incubated with antibodies against IRE1α (Santa Cruz Biotechnology, Santa Cruz, CA), total and phosphorylated eukaryotic initiation factor-2α (eIF2α; Cell Signaling, Waverly, MA), CCAAT/enhancer-binding protein homologous protein (CHOP; Santa Cruz Biotechnology, Santa Cruz, CA), glucose-regulated protein 78 (GRP78; Stressgen, Victoria, BC, Canada), and albumin (Sigma) concentrations were determined by a modification of the diacylglycerol kinase assay using [γ-32P]ATP and quantification of the radioactive spot on the cleavage of 7-amino-4-trifluoromethylcoumarin (BioVision, Mountain View, CA). Cells were washed with PBS and harvested using a lysis buffer containing 20 mM HEPES, pH 7.4, 1% Triton X-100, 10% glycerol, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 mM β-mercaptoethanol, 3 mM benzamidine, 10 μM leupeptin, 5 μM pepstatin, and 10 μg/ml aprotinin. Equivalent amounts of protein (50–100 μg) were subjected to SDS-PAGE and transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Piscataway, NJ), and the membranes were incubated with antibodies against IRE1α (Santa Cruz Biotechnology, Santa Cruz, CA), total and phosphorylated eukaryotic initiation factor-2α (eIF2α; Cell Signaling, Waverly, MA), CCAAT/enhancer-binding protein homologous protein (CHOP; Santa Cruz Biotechnology, Santa Cruz, CA), glucose-regulated protein 78 (GRP78; Stressgen, Victoria, BC, Canada), and growth arrest and DNA damage-inducible protein 34 (GADD34; Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were detected using horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence reagent (Pierce, Rockford, IL). Density was quantified using a UVMP bioimaging system (UVP, Upland, CA).

Determination of caspase activity, apoptosis, and cell viability. Activity of the caspase-3 class of cysteine proteases was determined with the colorimetric caspase-3 activation assay, which uses a caspase-specific peptide that is conjugated to the color reporter molecule p-nitroanaline (R&D Systems, Minneapolis, MN). Caspase-9 activity was determined using a fluorometric procedure that was based on the cleavage of 7-amino-4-trifluoromethylcoumarin (BioVision, Mountain View, CA). Caspase activities were normalized to cell lysate protein concentration. DNA laddering was evaluated using a modification of the protocols of Bilalik et al. (22) and Logenberger et al. (22). In some experiments, apoptosis was determined using the Cell Death Detection ELISA kit (Roche Diagnostics, Penzberg, Germany). The assay is based on the quantitative sandwich enzyme immunoassay principle, using mouse monoclonal antibodies directed against DNA and histones. This allows specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Trypan blue exclusion was used to examine cell viability. Following the addition of a 0.4% (wt/vol) trypan blue solution, cells were counted using a hemocytometer with light microscopy. Cells staining with trypan blue dye were counted as nonviable and were reported as a percent of the total cells visualized.

Metabolite analysis. Glucose (Sigma), free fatty acid (Wako NEFA C test kit), and albumin (Sigma) concentrations were determined by standard techniques. The pH of the medium was not significantly affected by any of the experimental conditions. Ceramide concentration was determined by a modification of the diacylglycerol kinase assay using [γ-32P]ATP and quantification of the radioactive spot corresponding to ceramide 1-phosphate (36).

Data analysis and statistics. Statistical comparisons were calculated using analysis of variance and post hoc comparisons among means using the Scheffé’s or Tukey’s test. To avoid the assumption of a normal distribution gene expression, data were also analyzed using nonparametric analysis of variance, the Kruskal-Wallis test. Results from this analysis provided results similar to those obtained with ANOVA. Statistical significance was set at P < 0.05. All data are reported as means ± SD.

**RESULTS**

Saturated fatty acids induce ER stress and apoptosis in liver cells. Disruption of ER homeostasis, collectively termed ER stress, activates the unfolded protein response (UPR), a signaling pathway that links the ER lumen with the cytoplasm and nucleus (15). The UPR is initiated by three ER transmembrane proteins, IRE1, RNA-dependent protein kinase-like ER eIF-2α kinase (PERK), and activating transcription factor 6 (ATF6) (33). Activation of IRE1 promotes the splicing of X-box-binding protein-1 (XBP1) mRNA and subsequent transcription of molecular chaperones (e.g., GRP78) and genes involved in ER-associated degradation [e.g., ER mannosidase (DEME)] (35). PERK activation leads to phosphorylation of the α-subunit of the translation initiation factor eIF2 and subsequent attenuation of translation initiation, as well as increased ex-
Fig. 2. Caspase-3 activity (A), DNA laddering (B), and incorporation of trypan blue in H4IIE cells (C) following 6 and 16 h of LG or in the presence of Th (450 nM), O500, P500, L500, or S500. Data in graphs are means ± SDEV for n = 6–8 for each time point. In B, a representative gel is shown for 6- and 16-h incubations from a total n = 6–8. In C, data are expressed as % cells in which trypan blue was incorporated relative to the total cells counted. *Significantly different from LG and O500; †significantly different from LG and L500.

Fig. 3. Caspase-3 and caspase-9 activity (A) and XBP1u and XBP1s and DNA laddering (B) in H4IIE cells following 16 h of LG or in the presence of O500 or P500. FMK (Z-Val-Ala-Asp-fluoromethylketone) is a general caspase inhibitor. Data in graphs are presented as means ± SDEV for n = 4. Gels presented in B are representative of a total n = 4. *Significantly different from LG and O500.
pression and selective translation of activating transcription factor 4 (ATF4) (14, 35). Increased expression of GADD34, a member of the growth arrest and DNA damage family of proteins, is involved in dephosphorylation of eIF2α and, therefore, reversal of translational attenuation (33). Failure of the UPR to reestablish ER homeostasis can lead to apoptosis (15, 33).

Incubation with thapsigargin (positive control), palmitate (500 μM), or stearate (500 μM) resulted in phosphorylation of IRE1α (Fig. 1A), splicing of XBP1 (Fig. 1B), and phosphorylation of eIF2α (Fig. 1C; for clarity of presentation data for stearate and linoleate are not shown). Thapsigargin, palmitate, or stearate also significantly increased the expression of ATF4, CHOP, GRP78, and GADD34 mRNA (see Supplemental Table S2). Oleate or linoleate had no effect on these markers of ER stress (Fig. 1 and Supplemental Table S2).

Thapsigargin, palmitate, or stearate increased caspase-3 activity after 6 and 16 h (Fig. 2A), induced DNA laddering after 16 h (Fig. 2B), and reduced cell viability after 16 h (Fig. 2C). Oleate or linoleate did not increase caspase-3 activity, induce DNA laddering, or reduce cell viability (Fig. 2, A–C). Total fatty acid disappearance (nmol·mg protein⁻¹·h⁻¹) from the medium was not significantly different when liver cells were exposed to oleate (3.2 ± 0.6), palmitate (2.7 ± 0.5), linoleate (3.1 ± 0.5), or stearate (2.6 ± 0.4).

**Saturated fatty acid-induced apoptosis is caspase dependent.** The presence of the general caspase inhibitor Z-Val-Ala-Asp- fluoromethylketone (26) prevented thapsigargin- and palmitate-induced caspase-3 and -9 activation (Fig. 3A) and DNA laddering (Fig. 3B) but did not have a significant effect on XBP1 splicing (Fig. 3B) or other markers of the ER stress response pathway, such as phosphorylation of IRE1α and eIF2α and increased expression of ATF4, CHOP, GRP78, and GADD34 mRNA (data not shown).

**Chemical- vs. palmitate-induced ER stress target distinct genes.** Gene profiling was used to compare thapsigargin- and tunicamycin-induced ER stress, chemical agents that induce ER stress via distinct mechanisms (see METHODS), with that of palmitate-induced ER stress. Of the 10 genes examined, four responded similarly among all three treatments (Table 1). In contrast, palmitate had no effect on six genes that were increased by both thapsigargin and tunicamycin (Table 1).

**Saturated fatty acid-induced ER stress and apoptosis occurs independently of ceramide.** Palmitate increased ceramide concentrations in H4IIE cells (Fig. 4A), and coinubation of palmitate with fumonisin B1 [an inhibitor of ceramide synthetase (22, 36)] prevented palmitate-induced ceramide accumulation (Fig. 4A). In contrast, coinubation of palmitate with disipramine (an inhibitor of acidic sphingomyelinase) did not prevent palmitate-induced accumulation of ceramide (Fig. 4A). The presence of fumonisin B1 or disipramine had no effect on palmitate-induced CHOP gene expression (Fig. 4B) or other markers of ER stress (data not shown). In addition, the presence of fumonisin B1 (Fig. 4C) or disipramine (data not shown) had no effect on caspase-3 activity or DNA laddering.

**Unsaturated fatty acids protect against saturated fatty acid-induced ER stress and apoptosis.** Oleic acid rescued palmitate-induced apoptosis in CHO cells (21). Therefore, we next examined whether oleic acid or linoleic acid rescued palmitate-induced ER stress and apoptosis in liver cells. Titration of oleic acid or linoleic acid reduced palmitate-induced upregulation of CHOP, GRP78, and GADD34 mRNA (Fig. 5A), and DNA laddering (Fig. 5B).

**DISCUSSION**

Fatty liver occurs frequently in obesity and type 2 diabetes and is the first stage of NAFLD, a chronic disease syndrome that can lead to NASH and end-stage liver disease (1, 3, 43). However, the factors that lead to progression from steatosis to steatohepatitis are poorly understood. We have recently demonstrated that hepatic steatosis characterized by increased saturated fatty acids leads to increased caspase-3 activity, ER stress, and liver injury in rats (41). In the present study, we examined the direct effects of saturated and unsaturated fatty acids on liver cell ER homeostasis and apoptosis. The data demonstrate that the saturated fatty acids stearate and palmitate disrupt ER homeostasis and induce apoptosis via mechanisms that appear to be independent of ceramide accumulation. Because titration of unsaturated fatty acids reduces or prevents saturated fatty acid-induced ER stress and apoptosis, it appears that the composition of fatty acids delivered to and accumulated within the liver is an important determinant of liver cell ER homeostasis and susceptibility to injury.

Accumulation of unfolded proteins in the lumen of the ER activates a highly regulated, adaptive program termed the unfolded protein response (UPR) (33, 35). Activation of the UPR upregulates protein-folding capacity and degradation pathways in the ER and inhibits protein synthesis (33, 35). It has been proposed that chronic ER stress may contribute to the attrition of β-cell function and to impaired regulation of glucose homeostasis in diabetes (12, 29, 30, 34). A recent study (31) has identified ER stress as a molecular link between obesity, the deterioration of insulin action in liver and adipose tissue, and, potentially, the development of type 2 diabetes. However, the cellular signals linking obesity to ER stress have not been identified. Data from the present study demonstrate

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**Table 1. Comparison of gene responses to thapsigargin, tunicamycin, and palmitate in H4IIE liver cells**

<table>
<thead>
<tr>
<th></th>
<th>Thapsigargin</th>
<th>Tunicamycin</th>
<th>Palmitate</th>
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<tbody>
<tr>
<td>CHOP</td>
<td>14.7 ± 2.0*</td>
<td>25.5 ± 3.3*</td>
<td>9.8 ± 1.4*</td>
</tr>
<tr>
<td>GRP78</td>
<td>8.2 ± 1.3*</td>
<td>9.0 ± 1.2*</td>
<td>2.5 ± 0.4*</td>
</tr>
<tr>
<td>ATF4</td>
<td>3.3 ± 0.5*</td>
<td>3.6 ± 0.6</td>
<td>2.6 ± 0.3*</td>
</tr>
<tr>
<td>GADD34</td>
<td>16.7 ± 2.2*</td>
<td>13.3 ± 2.9</td>
<td>10.9 ± 1.8</td>
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Values are means ± SD for 3–5 independent experiments, each of which was performed in duplicate. Values are represented as the change from control cells, as described in METHODS. Incubations were for 16 h using 450 nM thapsigargin, 6 μg/mL tunicamycin, or 500 μM palmitate. CHOP, CCAAT/enhancer-binding protein homologous protein; GRP78, glucose-regulated protein 78; ATF4, activating transcription factor 4; GADD34, growth arrest and DNA damage-inducible gene 34; IRE1, inositol-requiring endoplasmic reticulum-to-nucleus signaling protein-1; XBP1, X-box-binding protein-1; EDEM, DNA damage-inducible gene 34; IRE1, activating transcription factor 4; GADD34, growth arrest and DNA damage-inducible gene 34; IRE1, inositol-requiring endoplasmic reticulum-to-nucleus signaling protein-1; XBP1, X-box-binding protein-1; EDEM, endoplasmic reticulum mannosidase; GRP75, glucose-regulated protein-75. *Significantly different from control cells (P < 0.05); †significantly different from thapsigargin and tunicamycin.

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Fig. 4. Ceramide concentration (A), CCAAT/enhancer-binding protein homologous protein (CHOP) mRNA (B), and caspase-3 activity and DNA laddering (C) in H4IIE cells following 16 h of LG or in the presence of Th (450 nM), O500, or P500. Data in graphs are presented as means ± SD for n = 4. In B, LG in the absence of fumonisin B1 (FB1) is set to 1. Gel in C is representative of a total n = 4. *Significantly different from LG and O500.

Fig. 5. CHOP, glucose-regulated protein-78 (GRP78), and growth arrest and DNA damage-inducible protein-34 (GADD34) mRNA (A) and DNA laddering in H4IIE cells (B) following 16 h of LG or in the presence of palmitate alone or in combination with oleate or linoleate. In A, data are expressed as the change from LG, which was set to 1. Data in graphs are presented as means ± SD for n = 4. In B, the gel presented is representative of a total n = 4. *Significantly different from LG.
that palmitate and stearate induce ER stress and activate multiple components of the UPR in liver cells.

The UPR is activated when the influx of nascent, unfolded polypeptides exceeds the folding capacity of the ER (35). Thus long-chain saturated fatty acids likely induce the UPR via accumulation of unfolded proteins. In the liver cell, apoptosis and microsomal triglyceride transfer protein may be one specialized client protein-chaperone pair that may be affected by fatty acid composition (35). The induction of ER stress by palmitate and stearate may result from changes to the size and/or composition of the ER membrane. Changes in the lipid composition of the ER membrane can reduce calcium stores and impair protein glycosylation and folding (10, 37, 38). In INS-1 cells, palmitate was converted in the ER to tripalmitin, which resulted in severe disruption of the cellular architecture (27). Future studies will examine the specific mechanisms used by saturated fatty acids to induce the UPR in liver cells.

Previous studies have demonstrated that toxicity from accumulation of long-chain fatty acids is specific to or made more severe by saturated fatty acids (7, 13, 22, 24, 32, 36). The present study demonstrates that long-chain saturated fatty acids induce apoptosis and reduce cell viability in liver cells. When considered with recent data demonstrating increased caspase-3 activity, ER stress, and liver injury in rats with hepatic steatosis characterized by increased saturated fatty acids (41), these data suggest that the composition of fatty acids delivered to or stored within the liver is an important determinant of ER homeostasis and liver cell integrity.

De novo ceramide synthesis has been directly linked to fatty acid-induced apoptosis in β-cells (36). Because palmitate and stearate are precursors of de novo ceramide synthesis, we examined the role of ceramide in saturated fatty acid-induced ER stress and apoptosis in liver cells. Although palmitate increased de novo ceramide synthesis (on the basis of the observation that fumonisin B1, but not disipramine, prevented ceramide accumulation), this was not required for saturated fatty acid-induced ER stress or apoptosis. Notably, palmitate-induced apoptosis also occurred via a ceramide-independent pathway in CHO cells (22).

Cellular models that investigate the effects of individual fatty acid species are far removed from in vivo conditions, where a mixture of fatty acids is always present. Consistent with previous studies (21, 23), oleate or linoleate reduced or prevented palmitate-induced ER stress and apoptosis in liver cells. Thus fatty acid-mediated induction of ER stress and cytotoxicity in cellular models must be studied using both individual and combined fatty acid species (4, 27). The presence of increased saturated fatty acids in serum cholesterol esters has been observed in individuals with type 2 diabetes (40). The sources of fatty acids stored in liver and secreted via lipoproteins in patients with NAFLD has recently been estimated (8). Of the triglyceride accounted for in the liver, ~60% arose from nonesterified fatty acids, 26% from de novo lipogenesis, and 15% from the diet. In addition, de novo lipogenesis was increased in the fasting state in these patients. In future studies, it will be important to examine the relationship between circulating and intrahepatic fatty acid composition and liver damage in patients with NAFLD.

Notably, palmitate did not induce a number of genes that were significantly upregulated by both thapsigargin, which induces ER stress via inhibition of the ER-associated calcium ATPase, and tunicamycin, which induces ER stress via inhibition of protein glycosylation. The lack of induction of these genes may contribute to palmitate-induced apoptosis. It has been suggested that increased XBP1 transcription, mediated by ATF6 cleavage, is required for sustained activation of the UPR and management of ER stress (20, 35). The lack of induction of two genes that encode chaperone proteins, calreticulin and GRP78, as well as EDEM mRNA, which encodes a protein critical to ER-associated degradation, also suggests that the adaptive response to palmitate-induced ER stress may be impaired. Alternatively, palmitate may regulate only selective components of the UPR or not induce a sufficient enough stress to require activation of the entire UPR. Future studies will determine whether palmitate-induced ER stress is causally linked to apoptosis and whether saturated fatty acids interfere with ATF6 cleavage or binding to the XBP1 promoter and/or ER-associated degradation.

In total, our data support the notion that the composition of fatty acids presented to or stored within the liver are an important determinant of ER homeostasis and cellular integrity. In this cellular model, saturated fatty acid-induced ER stress and apoptosis appear to occur independently of ceramide accumulation. Other potential intracellular signals linking saturated fatty acids to ER stress and apoptosis include reactive oxygen species and physicochemical properties of cellular saturated fatty acids.

GRANTS
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REFERENCES


