Differential Roles of Unsaturated and Saturated Fatty Acids on Autophagy and Apoptosis in Hepatocytes

Shuang Mei, Hong-Min Ni, Sharon Manley, Abigail Bockus, Karen M. Kassel, James P. Luyendyk, Bryan L. Copple, and Wen-Xing Ding

Department of Pharmacology, Toxicology and Therapeutics, the University of Kansas Medical Center, Kansas City, Kansas

Received May 21, 2011; accepted August 18, 2011

ABSTRACT

Fatty acid-induced lipotoxicity plays a critical role in the pathogenesis of nonalcoholic liver disease. Saturated fatty acids and unsaturated fatty acids have differential effects on cell death and steatosis, but the mechanisms responsible for these differences are not known. Using cultured HepG2 cells and primary mouse hepatocytes, we found that unsaturated and saturated fatty acids differentially regulate autophagy and apoptosis. The unsaturated fatty acid, oleic acid, promoted the formation of triglyceride-enriched lipid droplets and induced autophagy but had a minimal effect on apoptosis. In contrast, the saturated fatty acid, palmitic acid, was poorly converted into triglyceride-enriched lipid droplets, suppressed autophagy, and significantly induced apoptosis. Subsequent studies revealed that palmitic acid-induced apoptosis suppressed autophagy by inducing caspase-dependent Beclin 1 cleavage, indicating cross-talk between apoptosis and autophagy. Moreover, our data suggest that the formation of triglyceride-enriched lipid droplets and induction of autophagy are protective mechanisms against fatty acid-induced lipotoxicity. In line with our in vitro findings, we found that high-fat diet-induced hepatic steatosis was associated with autophagy in the mouse liver. Potential modulation of autophagy may be a novel approach that has therapeutic benefits for obesity-induced steatosis and liver injury.

Introduction

Lipotoxicity refers to cellular toxicity in the presence of excessive free fatty acids (Malhi and Gores, 2008). Fatty acid-induced lipotoxicity in hepatocytes plays an essential role in the pathogenesis of nonalcoholic fatty liver disease (Malhi and Gores, 2008; Neuschwander-Tetri, 2010). Fatty acids are chemically classified as saturated and unsaturated (monounsaturated and polyunsaturated), and their structure affects their biological functions. Palmitic acid (PA), a saturated fatty acid, and oleic acid (OA), a monounsaturated fatty acid, are two of the most abundant fatty acids present in the diet and in serum (Baylin et al., 2002). Saturated and unsaturated fatty acids differentially regulate apoptosis in various experimental systems in which saturated fatty acids are the more toxic lipid species (Listenberger et al., 2003; Ricchi et al., 2009). Although the mechanisms underlying the cytotoxicity of fatty acids are largely unknown, it has been suggested that the conversion of fatty acids to triglyceride (TG) may reduce the cytotoxicity. For example, PA is poorly converted to TG and more toxic, whereas OA is readily converted to TG and less toxic (Listenberger et al., 2003). Another possibility is that different fatty acids may differ in their potential to activate endogenous cellular protective pathways. However, this has not been explored in detail.

Macroautophagy (referred to as autophagy hereafter) is a major intracellular degradation system. Autophagy is usually activated in response to the deprivation of nutrients or growth factors (Kuma et al., 2004). Autophagy also plays a role in the pathogenesis of a number of human diseases, including obesity and steatosis (Singh et al., 2009a; Zhang et

ABBREVIATIONS: PA, palmitic acid; OA, oleic acid; TG, triglyceride; LC3, light chain 3; PE, phosphatidylethanolamine; CQ, chloroquine; BAF, bafilomycin A1; Beclin 1, Bcl-2-interacting protein 1; PI3K, phosphatidylinositol 3-kinase; p70S6K, 70-kDa ribosomal protein S6 kinase-1; 4EBP1, translational initiation factor 4E binding protein-1; BSA, bovine serum albumin; 3-MA, 3-methyladenine; NAC, N-acetylcysteine; TNF-α, tumor necrosis factor-α; Z-VAD-fmk, N-benzoxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone; TMRM, tetramethylrhodamine methyl ester; PBS, phosphate-buffered saline; GFP, green fluorescent protein; ANOVA, analysis of variance; EM, electron microscopy; AV, autophagosomes; LD, lipid droplets; ROS, reactive oxygen species; mTOR, mammalian target of rapamycin; ActD, actinemia D.
Materials and Methods

Reagents. Antibodies used in this study were cleared caspase-3, phosphorylated 70-kDa ribosomal protein S6 kinase-1 (p70S6K) (T389), total p70S6K, phosphorylated translational initiation factor 4E binding protein-1 (4EBP1) (85D), total-4EBP1, and perilipin from Cell Signaling Technology (Danvers, MA), β-actin from Sigma-Aldrich (St. Louis, MO), and horseradish peroxidase-labeled secondary antibodies from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). The rabbit polyclonal anti-LC3B antibody was made using a peptide representing the NH2-terminal 14 amino acids of human LC3B and an additional cysteine (PSEKTFQRTFEQC) as described previously (Ding et al., 2009). The following chemicals were all from Sigma-Aldrich: delipidated bovine serum albumin (BSA), OA (unsaturated fatty acid), PA (saturated fatty acid), 3-methyladenine (3-MA), CQ, rapamycin, N-acetylcysteine (NAC), tumor necrosis factor-α (TNF-α), and Z-VAD-fmk. The fluorescence probes tetramethylrhodamine methyl ester (TMRM), Bodipy 493/503, propidium iodide, and Hoechst 33342 were all from Invitrogen (Carlsbad, CA).

Cell Culture and Treatment. HepG2 cells, a human hepatoma cell line that was obtained from American Type Cell Culture (Manassas, VA), were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and other standard supplements. All cultures were maintained in a 37°C incubator with 5% CO2. All cell culture materials were obtained from Invitrogen. OA-BSA and PA-BSA conjugates were prepared as described previously (Choi et al., 2009). In brief, a 20 mM solution of OA or PA in 0.01 N NaOH was incubated at 70°C for 30 min, and fatty acid soaps were then complexed with 5% BSA in PBS at a 7:1 molar ratio of fatty acid to BSA. The OA-BSA or PA-BSA conjugate was administered to the cultured cells. BSA was used as a vehicle control.

Mice and Experimental Diets. Male C57BL/6j mice (The Jackson Laboratory, Bar Harbor, ME) were fed either a control diet (Harlan Teklad 8604; Dyets Inc., Bethlehem, PA) or a Western diet (diet 100244; Dyets Inc.), which provides approximately 40% of calories from milk fat, for 3 months. All studies were approved by the University of Kansas Medical Center Animal Care and Use Committee and comply with National Institutes of Health guidelines. Mice were fasted overnight before sample collection. Total liver lysates were prepared using radioimmunoprecipitation assay buffer (1% NP40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl [lauryl] sulfate) with fresh protease inhibitors. Preparation of liver sections for histology and hematoxylin and eosin staining were performed as described previously (Luyendyk et al., 2010).

GFP-LC3 Adenovirus Infection. Adenovirus expressing GFP-LC3B (human; Ad-GFP-LC3) was used as described previously (Ding et al., 2009). HepG2 cells (105/well in a 12-well plate with microscopic cover glasses; Thermo Fisher Scientific, Waltham, MA) were infected with adenoviral GFP-LC3 (100 viral particles/cell) in Dulbecco’s modified Eagle’s medium overnight followed by treatment with BSA or BSA-conjugated fatty acids.

Fluorescence Microscopy. For fluorescence microscopy, cells were cultured in 12-well plates with microscopic cover glasses. After designated treatments, cells were fixed with 4% paraformaldehyde in PBS. All the cellular images were obtained using an inverted Nikon Eclipse 200 fluorescence microscope. For quantification of autophagic cells, GFP-LC3 punctated dots were determined from triplicates by counting a total of more than 60 cells. For the intracellular lipid droplets, cells were stained with Bodipys 493/503 (0.1 μM) for 15 min at room temperature before the analysis. Apoptotic cell death was determined by nuclear staining with Hoechst 33342 (5 μg/ml) for fragmented and condensed nuclei. For mitochondria membrane potential, live cells were stained with TMRM (50 nM) for 15 min followed by fluorescence microscopy.

Electron Microscopy. Liver tissue or HepG2 cells were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), followed by 1% OsO4. After dehydration, thin sections were cut and stained with uranyl acetate and lead citrate.
Oleic Acid Induces Autophagy in HepG2 Cells. Because it has been reported that the biological functions of saturated and unsaturated fatty acids are different (Lindenthal et al., 2003), we first determined the effect of oleic acid, an unsaturated fatty acid that is most abundant in the diet, serum, and liver tissue (Baylin et al., 2002; Xu et al., 2011), on autophagy in HepG2 cells (a human hepatoma cell line) expressing GFP-LC3 by infecting them with adenovirus GFP-LC3. GFP-LC3 behaves in a manner similar to that of endogenous LC3 and has been widely used to monitor autophagy (Hosokawa et al., 2006). GFP-LC3 is located in the cytosol and displays a diffuse pattern. Upon autophagy induction, GFP-LC3 translocates to the preautophagosome and autophagosome membranes, displaying a punctuated pattern after its conjugation with PE (Hosokawa et al., 2006; Suzuki and Ohsumi, 2010). The number of GFP-LC3 puncta can be quantified and represent the number of autophagosomes and autolysosomes. To assess whether OA, a monounsaturated fatty acid, would affect autophagy, we first examined the changes in the GFP-LC3 pattern in OA-treated HepG2 cells. HepG2 cells were infected with an adenovirus GFP-LC3 and treated with OA in the presence or absence of CQ, which inhibits lysosomal degradation by increasing lysosomal pH (Ni et al., 2011). Compared with the vehicle-treated cells, the number of GFP-LC3 puncta increased in OA-treated cells in a concentration-dependent manner and further increased in the presence of CQ (Fig. 1, A–C), suggesting that OA induces autophagy in HepG2 cells. In addition to the changes in GFP-LC3 puncta, we also determined the autophagic flux by examining the changes in endogenous LC3-II in the presence of lysosomal inhibitors. We found that the LC3-II levels were not affected by OA treatment. Treatment with either CQ or BAF (Fig. 1D), which inhibit lysosomal functions by either increasing lysosomal pH or suppressing vacuolar type H+ ATPase (Klionsky et al., 2008), increased levels of LC3-II. Although OA cotreatment tended to increase LC3-II levels in the presence of CQ, this difference did not achieve statistical significance. Taken together, these data indicate that OA induces autophagy in HepG2 cells.

Results

Oleic Acid Induces Autophagy and Apoptosis.

Although OA (18:1) and PA (16:0) represent extreme unsaturated and saturated fatty acids, there is a possibility that their differential effects on autophagy could also be due to the different carbon length in addition to their saturating status. We thus determined the effect of another unsaturated fatty acid, palmitoleate (16:1), which has the same carbon length as PA, on autophagy. We found that palmitoleate induces autophagy in HepG2 cells on the basis of the autophagic flux assay, either assessing for the GFP-LC3 puncta formation or the levels of LC3-II changes (Supplemental Fig. 2). These results suggest that the saturate status rather than the carbon length contribute to the differential effects of saturated and unsaturated fatty acids on autophagy.

Oleic Acid but Not Palmitic Acid Increases the Number of Autophagosomes and Neutral Lipid Storage.

To further confirm the differential roles of OA and PA on autophagy, we performed EM studies on OA- and PA-treated HepG2 cells. OA treatment significantly increased the number of double-membrane autophagosomes (AV), and most of them had enveloped cytosolic contents (Fig. 3A, e, arrows).
and lipid droplets (LD) (Fig. 3A, c and d, arrow). In addition to increasing the number of AV, OA treatment also increased the number of LD, which are featured as phospholipid monolayer membrane structures with electron lucent content (Fig. 3A, c, d, and f). In contrast, AV and LD are barely detectable in BSA-treated control (Fig. 3A, a) or PA-treated cells (Fig. 3, A, b, B, and C). Fluorescence microscopy studies using the fluorescent dye Bodipy493/503 for neutral lipids further confirmed that OA increased the number of LD significantly more than PA although PA also slightly increased LD numbers in HepG2 cells (Fig. 3, D and E). Similar results were found in primary mouse hepatocytes (Supplemental Fig. 3). These data indicate that OA but not PA increases the number of autophagosomes in HepG2 cells. Moreover, OA also increases more LD than PA in HepG2 cells and normal hepatocytes.

ROS but not mTOR Contributes to OA-Induced Autophagy in HepG2 Cells. Because increased oxidative stress is often observed in human and experimental models of steatohepatitis (Chalasani et al., 2004; Seki et al., 2005), we next determined the levels of ROS production in OA- or PA-treated cells in the presence or absence of the antioxidant NAC. NAC has been widely used as an antioxidant and has been shown to block autophagy in many experimental models (Scherz-Shouval et al., 2007; Ding et al., 2010b). We found that PA significantly increased the level of ROS production in HepG2 cells compared with the control cells. OA also increased the level of ROS production but not to a degree of statistical significance. However, NAC significantly decreased the levels of ROS in both PA- and OA-treated cells (Supplemental Fig. 4). We then determined the effects of NAC on OA-induced GFP-LC3 puncta. We found that OA-induced GFP-LC3 puncta formation was significantly suppressed by NAC (Fig. 4, A and B). Because Beclin 1/class-III PI3 kinase complex is important in regulating autophagosome formation (He and Levine, 2010), we next determined the effects of 3-MA, a class-III PI3 kinase inhibitor, on OA-induced GFP-LC3 puncta formation. We found that OA-induced GFP-LC3 puncta were also suppressed by 3-MA (Fig. 4, A and C). Taken together, these data suggest that OA-induced autophagy requires increased oxidative stress and the Beclin 1-Vps34 PI3 kinase complex.

We next determined whether mTOR, one of the key molecular signaling pathways regulating autophagy, would also be involved in fatty acid-induced autophagy. We found that neither PA nor OA treatment suppressed mTOR activity as determined by the level of phosphorylated 4EBP1 and p70S6K at different time points (Fig. 4D) and various concentrations (Fig. 4E). It seemed that OA treatment even increased the phosphorylated level of p70S6K after 12 and 24 h of treatment although it slightly decreased the phosphorylated 4EBP1 levels (Fig. 4D). The reasons for the different changes for p70S6K and 4EBP1 are not clear although they both are mTOR downstream substrates. In contrast, a high dose of PA (500 μM) reduced both total and phosphorylated 4EBP1, which is probably mediated by its cytotoxicity (see below). Taken together, these data suggest that OA-
induced autophagy is mediated by ROS but not by mTOR suppression in HepG2 cells.

**PA but Not OA Induces Apoptosis in HepG2 Cells.**
Because we found that OA and PA differentially regulated autophagy, we next determined whether OA and PA would also differentially regulate apoptosis in HepG2 cells. We found that PA significantly increased the number of apoptotic cells in a time- and dose-dependent manner, whereas cells treated with OA were barely affected (Fig. 5, A and B). The apoptotic nuclear changes in PA-treated cells were evident by the typical fragmented and condensed nuclear morphology (Fig. 5A, arrows), similar to apoptosis induced by TNF-α/H9251 plus actinomycin D (ActD), a widely used model to trigger death receptor activation-induced apoptosis (Ding and Yin, 2004; Ding et al., 2004). Meanwhile, PA but not OA treatment also increased the number of cells with depolarized mitochondria (Fig. 5, D and E), suggesting that PA-induced apoptosis is mediated by the mitochondrial apoptotic pathway.

**Caspase-Mediated Beclin 1 Cleavage Is Associated with the Suppression of Autophagy in PA-Treated Cells.** Increasing evidence suggests that apoptosis may suppress autophagy by caspase-mediated cleavage of essential autophagy proteins (Luo and Rubinsztein, 2010; Li et al., 2011a). Because we observed that OA and PA differentially regulate autophagy and apoptosis, we next determined whether PA-induced caspase activation would cleave Beclin 1, an essential autophagy protein serving as a key component in the Beclin 1-Vps34 PI3 kinase complex and in turn suppress autophagy in PA-treated cells. Indeed, we found that PA but not OA induced Beclin 1 cleavage in a time-dependent manner (Fig. 6A). We consistently observed a 50-kDa cleaved Beclin 1 in PA or TNF-α/H9251/ActD-treated cells as reported previously (Li et al., 2011a). Occasionally, an additional cleaved Beclin 1 band at approximately 45 kDa was also detected (Fig. 6A). However, this cleaved band could be less stable because it was not always detectable in our experiments. We further found that PA as well as TNF-α/H9251-induced Beclin 1 cleavage and apoptosis were inhibited by a general caspase inhibitor, Z-VAD-fmk, further supporting the notion that PA-induced Beclin 1 cleavage and apoptosis are caspase-dependent (Fig. 6, D and E). Furthermore, in the presence of Z-VAD-fmk, the PA-induced number of GFP-LC3 puncta was significantly increased (Supplemental Fig. 5). These findings suggest that the cleavage of Beclin 1 by PA-induced caspase activation may suppress autophagy in PA-treated HepG2 cells as we observed in Fig. 2.

**Autophagy Attenuates Fatty Acid-Induced Apoptosis and Accumulation of Lipids.** We next determined whether autophagy would play a protective role against fatty acid-induced apoptosis. We found that suppression of autophagy by using two pharmacological autophagy inhibitors, 3-MA, which suppresses the upstream PI3K (Beclin 1-Vps34 complex), and CQ, which inhibits the downstream lysosomal function, by increasing lysosomal pH, significantly increased OA-induced apoptosis (Fig. 7A). Interestingly, we further found that OA attenuated PA-induced apoptosis.
This finding can be partially explained by OA-induced autophagy in HepG2 cells. Autophagy has been shown to remove excessive lipid droplets in hepatocytes and in alcohol-treated mouse liver (lipophagy) (Singh et al., 2009a; Ding et al., 2010a). Therefore, we next determined whether autophagy would also influence OA- and PA-induced accumulation of lipids in HepG2 cells. Perilipin is a lipid droplet-associated protein that is localized at the surface of the lipid droplet and whose protein levels may correlate with the number of lipid droplets (Ducharme and Bickel, 2008). We next determined the protein level of perilipin after OA and PA treatment in HepG2 cells. We found that both OA and PA increased the protein levels of perilipin in a dose-dependent manner (Fig. 7B). These results are generally in agreement with our earlier findings in Fig. 5 and previous reports (Listenberger et al., 2003; Ricchi et al., 2009). Of interest, we found that suppression of autophagy by CQ further increased both OA- and PA-induced expression of perilipin, supporting the current notion that autophagy may help to remove lipid droplets (Fig. 7C). In line with the findings for the perilipin changes, OA also significantly increased the total cellular TG level compared with that of control cells or PA-treated cells. Suppression of autophagy by CQ significantly increased TG levels in OA-treated cells (Fig. 3).

(Supplemental Fig. 6). Fig. 3. OA but not PA increases the number of autophagosomes and lipid droplets in HepG2 cells. A, HepG2 cells were treated with BSA vehicle control (a), PA (500 μM, b), or OA (500 μM, c–f) for 6 h, and the cells were further processed for EM. Arrows denote autophagosomes. N, nuclei; LD, lipid droplet; m, mitochondria. The number of autophagosomes (B) and lipid droplets (C) per cell section was determined (mean ± S.D.) from more than 30 different cells. #, p < 0.01 (one-way ANOVA with the Scheffé post hoc test). D, HepG2 cells were treated with BSA vehicle control (a), OA (500 μM, b), or PA (500 μM, c) for 6 h and fixed with 4% paraformaldehyde. The cells were further stained with Bodipy 493/503 (0.1 μM) for lipid droplets and Hoechst 33342 (0.5 μg/ml) for the nuclei followed by fluorescence microscopy. E, the number of lipid droplets per cell was quantified, and data are presented as the mean ± S.E. from at least three independent experiments. #, p < 0.01 (one-way ANOVA with the Scheffé post hoc test).
Furthermore, suppression of autophagy by 3-MA also increased OA-induced accumulation of LD (Supplemental Fig. 7A). In contrast, induction of autophagy by rapamycin tended to reduce TG levels in OA-treated cells (Supplemental Fig. 7B). Of interest, CQ alone also increased TG levels in HepG2 cells, suggesting that even inhibition of basal level
autophagy could also increase the level of TG (Fig. 7D). The level of TG was higher in the PA and CQ treatment groups than that with PA treatment alone but was almost identical to that with CQ treatment alone. The lack of an additional increase of TG by CQ in CQ- and PA-treated cells is probably due to the already low autophagy activity induced by PA treatment in these cells (Fig. 7D). Taken together, these findings suggest that autophagy can attenuate fatty acid-induced apoptosis and the accumulation of lipids.

**Induction of Steatosis and Autophagy in High-Fat Diet Mouse Liver.** To determine the effects of a high-fat diet on steatosis and autophagy in the mouse liver, C57BL/6 mice were fed a high-fat diet or a control diet for 12 weeks. Macro- and microvesicular steatosis was evident in livers of mice fed with the high-fat diet (Fig. 8A). EM studies further confirmed that both the number and size of the hepatic lipid droplets were markedly increased in livers of mice fed a high-fat diet (Fig. 8B, b). Moreover, we also often found double-membrane autophagosomes that enwrapped damaged mitochondria in livers from mice fed a high-fat diet (Fig. 8B, c–e). Results from Western blot analysis indicate that there was an increased level of LC3-II in livers from mice fed a high-fat diet (Fig. 8, C and D), suggesting a possible increased number of autophagosomes or autolysosomes in high-fat diet-fed mouse livers. Taken together, these data suggest that a high-fat diet increases steatosis and may induce autophagy in mouse liver.

**Discussion**

Hepatic lipotoxicity is closely associated with the progression of fatty liver disease. However, the mechanisms by which excess fatty acids induce hepatotoxicity are not completely understood. Furthermore, the mechanisms by which hepatocytes tolerate lipotoxicity remain largely unknown. In the present study, we found that saturated and unsaturated fatty acids differentially regulate autophagy and apoptosis in HepG2 cells. Monounsaturated OA was readily converted to
TG-enriched lipid droplets, induced autophagy, and was resistant to apoptosis in HepG2 cells. In contrast, saturated PA was only slightly converted to TG-enriched lipid droplets resulting in the induction of apoptosis without the activation of autophagy. We demonstrated that there is cross-talk between fatty acid-induced apoptosis and autophagy, in which saturated PA-induced apoptosis suppresses autophagy by caspase-mediated cleavage of Beclin 1. Conversely, autophagy also attenuated fatty acid-induced apoptosis and accumulation of lipids. We further found that a high-fat diet induced marked steatosis and autophagy in the mouse liver. Overall, the results reveal a novel mechanism underlying the differential role of saturated versus unsaturated fatty acids in hepatotoxicity and could suggest new therapeutic approaches for treating fatty liver diseases by modulating autophagy.

Induction of apoptosis by excessive free fatty acids is a key pathological feature of nonalcoholic fatty liver disease and correlates with progressive inflammation and fibrosis. The accumulation of TG-enriched lipid droplets was once thought to be the underlying cause of liver injury and insulin resistance in tissues, but it has recently been suggested that the accumulation of lipid droplets is a parallel phenomenon and accumulation of lipids. We further found that a high-fat diet induced marked steatosis and autophagy in the mouse liver. Overall, the results reveal a novel mechanism underlying the differential role of saturated versus unsaturated fatty acids in hepatotoxicity and could suggest new therapeutic approaches for treating fatty liver diseases by modulating autophagy.

Fig. 6. PA but not OA induces caspase-mediated Beclin 1 cleavage. A, HepG2 cells were treated with OA or PA (500 μM) for 6, 12, or 24 h. Total cell lysates were subjected to immunoblot analysis using an anti-Beclin 1 antibody. B, HepG2 cells were treated with vehicle control (5% BSA), OA (500 μM), PA (500 μM), or TNF-α (10 ng/ml) plus ActD (0.2 μg/ml) for 24 h. Total cell lysates were subjected to immunoblot analysis for Beclin 1 and (E) apoptotic cell death was analyzed by nuclear staining with Hoechst 33342 (mean ± S.E., n = 3). #, p < 0.01 (one-way ANOVA with the Scheffé post hoc test). D, HepG2 cells were treated with vehicle control (5% BSA), PA (500 μM), PA (500 μM) plus Z-VAD-fmk (50 μM), TNF-α (10 ng/ml) plus ActD (0.2 μg/ml), or TNF-α (10 ng/ml) plus ActD (0.2 μg/ml) with Z-VAD-fmk (50 μM) for 24 h. Total cell lysates were subjected to immunoblot analysis for Beclin 1 and (E) apoptotic cell death was analyzed by nuclear staining with Hoechst 33342 (mean ± S.E., n = 3). #, p < 0.01 (one-way ANOVA with the Scheffé post hoc test).
versed alcohol-induced liver injury in a rat model (Nanji et al., 1995). This paradox could be due to cytochrome P450 2E1 activity being suppressed by saturated but not by unsaturated fatty acids and CYP2E1 being the key enzyme that promotes alcohol-induced liver injury (Nanji et al., 1995).

Why would saturated and unsaturated fatty acids have different effects in autophagy induction? In mammalian cells, the mTOR pathway is the most studied pathway regulating autophagy. Many diverse signals such as growth factors and amino acids activate mTOR to suppress autophagy. In contrast, rapamycin suppresses mTOR and induces autophagy in various cell lines. Inhibition of mTOR leads to reduced phosphorylation of two of its downstream effectors, p70S6K and 4EBP1. How the suppression of mTOR leads to autophagy induction is not completely known, but data suggest that suppression of mTOR is probably coupled to the activation of the ULK1-ULK2 (mammalian orthologs of yeast Atg1) complex, which may recruit other autophagy proteins to the isolation membrane, the origin of the autophagosome membrane (Mizushima, 2010). In this study, we found that OA has little effect on mTOR suppression, suggesting that OA-induced autophagy could be mTOR-independent. Several stimuli have been shown to induce autophagy independent of mTOR such as lithium, carbamazepine, and valproic acid, all of which reduce intracellular inositol levels (Ravikumar et al., 2010). Although the effect of OA on intracellular inositol levels is not known, we found that OA-induced autophagy requires ROS formation and the classic PI3 kinase complex because an antioxidant (NAC) and a PI3 kinase inhibitor (3-MA) suppress OA-induced autophagy. Unlike OA, PA increased apoptosis and caspase-3 activation in HepG2 cells, whereas there is no or even decreased autophagy induction. Cleavage of Beclin 1 during apoptosis has been shown to block Beclin 1-dependent autophagy (Luo and Rubinsztein, 2010). Indeed we found that PA induced caspase-mediated cleavage of Beclin 1. Thus, our results support the emerging notion that there is cross-talk between apoptosis and autophagy, in which autophagy and apoptosis counteract each other. Therefore, the different capacity for apoptosis induction by PA and OA may determine their different effects on autophagy induction.

In addition to protecting against cell death, autophagy has recently been shown to regulate lipid homeostasis by removing excess lipid droplets (Singh et al., 2009a). Liver-specific
knockout of Atg7, an essential autophagy gene regulating conjugation of LC3 with PE, leads to steatosis in the mouse liver (Singh et al., 2009a). Our previous studies also demonstrated that activation of autophagy reduced alcohol-induced steatosis in an acute mouse model (Ding et al., 2010a, 2011). In the present study, we found that autophagy also regulates fatty acid-induced lipid accumulation. Suppression of autophagy by CQ increases the accumulation of lipids in hepatocytes, whereas induction of autophagy by rapamycin tends to decrease TG contents in OA-treated hepatocytes. These findings suggest that modulation of autophagy may provide a novel therapeutic approach for not only alcoholic liver disease but also general obesity-induced steatosis.

In conclusion, we found that unsaturated and saturated fatty acids differentially regulate apoptosis and autophagy in hepatocytes. Unsaturated OA promotes the formation of TG-enriched lipid droplets, induces autophagy, and has little effect on lipoapoptosis. Saturated PA is poorly converted into TG-enriched lipid droplets, induces lipoapoptosis, and decreases autophagy. Induction of autophagy protects against fatty acid-induced lipotoxicity. Our data also support the emerging concept that autophagy and apoptosis are two antagonistic events that tend to inhibit each other. The modulation of autophagy represents a novel approach that may have therapeutic benefits for obesity-induced steatosis and liver injury.

Acknowledgments

We thank Barbara Fegley (University of Kansas Medical Center Electron Microscopy Research Laboratory) for excellent assistance with the EM studies. We thank Drs. Hao Zhu and Ming Xu for technical support for the triglyceride measurement.

Authorship Contributions

Participated in research design: Ding and Luyendyk.
Conducted experiments: Mei, Ni, Manley, Bockus, Kassel, Copple, and Ding.
Contributed new reagents or analytic tools: Luyendyk.
References
Supplemental Materials and Methods

**Primary hepatocytes culture.** As described previously (Ding et al., 2004), murine hepatocytes were isolated by a retrograde, nonrecirculating perfusion of livers with 0.05% Collagenase Type IV (Sigma). Cells were cultured in William’s medium E with 10% fetal bovine serum but no other supplements for 2 hrs for attachment. Cells were then cultured in the same medium without serum overnight before treatment. All cells were maintained in a 37°C incubator with 5% CO₂.

**Treatment with unsaturated fatty acid: palmitoleate (PO,16:1).** HepG2 cells culture and palmitoleate (PO)/BSA conjugate were prepared as described in the Materials and Methods (main text). Briefly, a 20 mM solution of PO in 0.01 N NaOH was incubated at 70 ºC for 30 min, and fatty acid soaps were then complexed with 5% BSA in PBS at a 7:1 molar ratio of fatty acid to BSA. The PO/BSA conjugate was administered to the cultured cells. BSA was used as a vehicle control.

**Measurement of ROS production.** Intracellular ROS was measured with the fluoroprobe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as described previously (Ding et al., 2004). Briefly, HepG2 cells were treated with OA (500 µm) or PA (500 µm) in the presence or absence of NAC (10 mM) for 6 hrs. The cells were further
incubated with 2.5 μM DCFH-DA for 30 min at 37 °C. After DCFH incubation, ROS analysis was carried out with ROS assay kits (Green Fluorescence) (Cell BIOLABS, INC.) in a 96-well plate as modified from the manufacturer’s instruction to quantitatively measure cellular ROS level using an Infinite M200 plate reader (Tecan, Durham, NC).

Supplementary Figure Legends

**Figure S1. Differential effects of OA and PA on autophagy induction in primary cultured mouse hepatocyte cells.** Primary mouse hepatocyte cells were first infected with Ad-GFP-LC3 overnight and then treated with vehicle control (5% BSA), OA (500 μM) or PA (500 μM) in the absence or presence of CQ (20 μM) for 6 hrs followed by fluorescence microscopy. Representative GFP-LC3 images were shown in (A). The numbers of GFP-LC3 dots per cell (mean ± SE, n=3) were determined from 3 independent experiments and more than 20 cells were counted in each experiment (B-C). (D) Total cell lysates were subjected to immunoblot analysis with anti-LC3 and anti-β-Actin antibodies. Densitometry analysis for the expression level of LC3-II was performed using Image J software which was further normalized with its loading control (β-Actin).

**Figure S2. PO induces autophagy in HepG2 cells.** HepG2 cells were first infected with Ad-GFP-LC3 (100 viral particles per cell) overnight and then treated with vehicle control (5% BSA), PO (500 μM), PO plus CQ (20 μM) or CQ (20 μM) alone for 6 hrs followed by fluorescence microscopy. Representative GFP-LC3 images were shown
in (A). The number of GFP-LC3 dots per cell was determined (B). Data are presented as mean ± SE from three independent experiments by counting more than 20 cells in each individual experiment. *: p<0.05; #: p<0.01 (one way ANOVA with Scheffe's post-hoc test). (C) HepG2 cells were treated with vehicle control (5% BSA), PO (500 µM), PO plus CQ (20 µM), CQ (20 µM) alone for 6 hrs. Total cell lysates were subjected to immunoblot analysis with anti-LC3 and anti-β-Actin antibodies. Densitometry analysis for the expression level of LC3-II was performed using Image J software which was further normalized with its loading control (β-Actin). Digital data are presented as the ratio of the vehicle control (mean ± SE) from at least three independent experiments.

**Figure S3. Fatty acid-treatment increases lipid droplet in primary mouse hepatocytes.** (A) Primary mouse hepatocytes were treated with BSA vehicle control OA (500 µM), PA (500 µM) and PO (500 µM) for 6 hrs and fixed with 4% paraformaldehyde. The cells were further stained with Bodipy 493/503 (0.1 µM) for lipid droplets and Hoechst 33342 (0.5 µg/mL) for the nuclei followed by fluorescence microscopy. (B) The number of lipid droplets per cell was quantified and data are presented as mean ± SE from at least three independent experiments. #: p<0.01 (one way ANOVA with Scheffe's post-hoc test).

**Figure S4. Effects of NAC on fatty-acid-induced ROS production in HepG2 cells.** HepG2 cells were treated with vehicle control (5% BSA), OA (500 µM), PA (500 µM) in the presence or absence of NAC (5 mM) for 6 hrs. The cells were further incubated with 2.5 µM DCFH-DA for 30 min at 37 °C. After DCFH incubation, ROS
analysis was carried out with the ROS assay kits (Green Fluorescence) (Cell BIOLABS, INC.) in a 96-well plate as modified from the manufacturer’s instruction.

**Figure S5. Effects of ZVAD on PA-induced GFP-LC3 puncta formation in HepG2 cells.** (A) HepG2 cells were first infected with Ad-GFP-LC3 (100 viral particles per cell) overnight and then treated with vehicle control (5% BSA), PA (500 μM), PA plus ZVAD (50 μM) or ZVAD (50 μM) alone for 6 hrs followed by fluorescence microscopy. (B) The number of GFP-LC3 dots per cell (mean ± SE) was quantified from three independent experiments and more than 20 cells were counted in each individual experiment. *: p<0.05; #: p<0.01 (one way ANOVA with Scheffe's post-hoc test).

**Figure S6. OA protects against PA-induced apoptosis.** HepG2 cells were treated with OA (500 μM), PA (500 μM) or OA (500 μM) plus PA (500 μM) for 24 hrs. Apoptotic cell death was analyzed by nuclear staining with Hoechst 33342 (mean ± SE, n=3).

**Figure S7. Effects of autophagy modulation on fatty acid-induced lipid accumulation.** (A) HepG2 cells were treated with vehicle control (5% BSA), OA (500 μM), OA plus 3MA (10 mM) or 3MA (10 mM) for 6 hrs. Lipid droplets were analyzed by staining with Bodipy 493/503(0.1 μM) and the number of lipid droplets per cell was quantified (mean ± SE) from three independent experiments. (B) HepG2 cells were treated with vehicle control (5% BSA), OA (500 μM) in the presence or absence of rapamycin (Rap, 10 μM) for 6 hrs. The cellular TG contents were quantified (mean ± SE) from three independent experiments.
Supplemental Figure 1

(D) OA (500 µM) + CQ (20 µM)

<table>
<thead>
<tr>
<th></th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CQ</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

LC3-I
LC3-II

1 0 1.9 0.8 5.1 2.8 4.2 2.2 LC3-II/β-Actin

<table>
<thead>
<tr>
<th></th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CQ</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

PA (500 µM) + CQ (20 µM)

<table>
<thead>
<tr>
<th></th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CQ</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

LC3-I
LC3-II

1 0 0.8 0.1 3.2 0.2 4.0 1.1 LC3-II/β-Actin

β-Actin
Supplemental Figure 2

A

Control PO PO+CQ CQ

Supplemental Figure 2 B

GFP-LC3 Puncta Per Cell

Control PO PO+CQ CQ

Supplemental Figure 2 C

PO (500 µM) CQ (20 µM)

PO (500 µM)

CQ (20 µM)

LC3-I

LC3-II

β-Actin

β-Actin
Supplemental Figure 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DCF Fluorescence (Fold of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
</tr>
<tr>
<td>OA</td>
<td>1.2</td>
</tr>
<tr>
<td>OA+NAC</td>
<td>1.0</td>
</tr>
<tr>
<td>PA</td>
<td>1.6</td>
</tr>
<tr>
<td>PA+NAC</td>
<td>1.4</td>
</tr>
<tr>
<td>NAC</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Significance:
- # indicates a significant difference compared to Control.
**Supplemental Figure 5**

**A**

- Control
- PA
- PA+ZVAD
- ZVAD

**B**

- GFP-LC3 Puncta Per Cell

- Control
- PA
- PA+ZVAD
- ZVAD

Histogram showing the number of GFP-LC3 puncta per cell for each condition.
Supplemental Figure 6

The graph shows the percentage of apoptotic cells across different conditions: Control, OA, PA, and OA+PA. The x-axis represents the different treatments, and the y-axis shows the percentage of apoptotic cells. The PA condition has the highest percentage of apoptotic cells, significantly higher than the Control and OA conditions, indicated by the symbol #.
Supplemental Figure 7

A

**No of LDs Per Cell**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>OA</th>
<th>OA+3MA</th>
<th>3MA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bar</strong></td>
<td><img src="image1" alt="Graph" /></td>
<td><img src="image2" alt="Graph" /></td>
<td><img src="image3" alt="Graph" /></td>
<td><img src="image4" alt="Graph" /></td>
</tr>
</tbody>
</table>

B

**TG (µg/mg Protein)**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>OA</th>
<th>OA+Rap</th>
<th>Rap</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bar</strong></td>
<td><img src="image5" alt="Graph" /></td>
<td><img src="image6" alt="Graph" /></td>
<td><img src="image7" alt="Graph" /></td>
<td><img src="image8" alt="Graph" /></td>
</tr>
</tbody>
</table>