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 Kassell, James P Luyendyk, Bryan L Copple, and Wen-Xing Ding*

Department of Pharmacology, Toxicology and Therapeutics, The University of Kansas Medical Center, Kansas City, KS 66160;
Running title: Fatty acids modulate autophagy and apoptosis

*Correspondence to: Wen-Xing Ding, Ph.D.; Department of Pharmacology, Toxicology and Therapeutics; The University of Kansas Medical Center MS 1018; 3901 Rainbow Blvd.; Kansas City, Kansas 66160; Phone: 913-588-9813, Fax: 913-588-7501; Email: wxding@kumc.edu

Abstract word #: 191; Introduction word #: 788; Discussion word #: 1269

Recommend section: Cellular and Molecular

Keywords: Autophagy; apoptosis; fatty acids; GFP-LC3; steatosis

List of Non-Standard Abbreviations:

- Beclin-1: Bcl-2-interacting protein 1;
- BSA: Bovine serum albumin;
- CQ: Chloroquine;
- DMSO: Dimethylsulfoxide;
- 4-EBP1: Translational initiation factor 4E binding protein-1;
- EM: Electron microscopy;
- FFA: Free fatty acid;
- LD: Lipid droplets;
- 3-MA: 3-methyladenine;
- mTOR: Mammalian target of rapamycin;
- NAC: N-acetylcysteine;
- NAFLD: Nonalcoholic fatty liver disease;
- OA: Oleic acid;
- PA: Palmitic acid;
- PE: Phosphatidylethanolamine;
- p70S6K: 70-kDa ribosomal protein S6 kinase-1;
- ROS: Reactive oxygen species;
- TG: Triglyceride;
Abstract

Fatty acid-induced lipotoxicity plays a critical role in the pathogenesis of non-alcoholic 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 crosstalk 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 (NAFLD) (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 al., 2009). To date, more than 30 Atg (autophagy) genes have been defined which participate in autophagy or autophagy-related processes (Klionsky et al., 2003; Mizushima, 2010). Mammalian microtubule-associated protein 1 light chain 3 (LC3), which is a homologue of yeast Atg8, is widely used as a marker to monitor the autophagy process. After its synthesis, LC3 is rapidly cleaved by Atg4 (an autophagy protein which has protein protease activity) and the cleaved form remains in the cytosol (called LC3-I) (Li et al., 2011b). Upon autophagy induction, LC3 is conjugated with phosphatidylethanolamine (PE) which is mediated by Atg7 (an E1-like protein) and the Atg12-Atg5-Atg16 complex (a complex which has E3-like activity). The conjugated form (called LC3-II) targets the autophagosomal membrane. LC3-II has been shown to have a membrane tethering function and may play a role in the elongation and closure of the autophagosome membrane. The changes of LC3-II in the presence and absence of lysosomal inhibitors such as chloroquine (CQ) or bafilomycin A1 (BAF) have been widely used as an autophagic flux assay (Rubinsztein et al., 2009; Mizushima et al., 2010; Ni et al., 2011).

Regulation of autophagosome formation is rather complicated in that it also involves numerous intracellular mediators such as the Beclin 1/Vps34 PI3 kinase complex (He and Levine, 2010). Notably, Bcl2/xL suppresses autophagy by directly interacting with Beclin 1, whereas other BH3-domain only Bcl-2 family proteins such as Bad promotes autophagy by disrupting the Bcl2/xL interaction and releasing Beclin 1 (He and Levine, 2010). Interestingly, recent data suggest that there is...
crosstalk between apoptosis and autophagy (Fimia and Piacentini, 2010). During apoptosis, Beclin 1 is cleaved by activated caspases resulting in the inhibition of autophagy (Djavaheri-Mergny et al., 2010; Luo and Rubinsztein, 2010).

In addition to acting as a cell survival mechanism against cell death, emerging evidence suggests that autophagy also regulates lipid homeostasis. Studies from the autophagy gene (Atg7) adipose tissue-specific knockout mice reveal that autophagy may regulate adipose mass and differentiation in mice (Singh et al., 2009b; Zhang et al., 2009). These knockout mice are generally lean with decreased white adipose mass, an increased number of mitochondria and enhanced insulin sensitivity (Singh et al., 2009b; Zhang et al., 2009). In contrast, liver-specific Atg7 knockout mice have an increased number of lipid droplets in hepatocytes (Singh et al., 2009a). Further experimental evidence suggests that autophagy may help to remove the excess lipid droplets in hepatocytes, a process termed as lipophagy (Singh et al., 2009a). We also recently reported that induction of autophagy by rapamycin significantly attenuates alcohol-induced steatosis in mice (Ding et al., 2010a). While it seems that autophagy may attenuate steatosis, it remains largely unknown how fatty acids modulate autophagy. It is also not known whether saturated or unsaturated fatty acids play different roles in autophagy. While some recent studies report that fatty acids may induce autophagy in INS-1 β-cells, no autophagic flux assays have been conducted in these studies and thus it is unclear whether there would be an increase of autophagic activity in these scenarios (Choi et al., 2009; Komiya et al., 2010).

In the present study, we determined the effects of saturated and unsaturated fatty
acids on autophagy and apoptosis in hepatocytes.

Methods

Reagents. Antibodies used in this study were cleaved caspase-3, phosphorylated-p70S6K (T389), total p70S6K, phosphorylated translational initiation factor 4E binding protein-1 (4EBP-1(S65)), total-4EBP-1 and perilipin (Cell Signaling), β-actin (Sigma-Aldrich), and horseradish peroxidase–labeled secondary antibodies (Jackson ImmunoResearch Lab). 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 (PSEKTFKQRRTFEQC) as described previously (Ding et al., 2009). The following chemicals: delipidated bovine serum albumin (BSA), oleic acid (OA, unsaturated fatty acid), palmitic acid (PA, saturated fatty acid), 3-methyladenine (3-MA), chloroquine (CQ), rapamycin, N-acetylcysteine (NAC), tumor necrosis factor-α (TNF-α), and ZVAD-fmk were all from Sigma-Aldrich. The fluorescence probes tetramethylrhodamine methyl ester (TMRM), Bodipy 493/503, propidium iodide (PI), and Hoechst 33342 were all from Invitrogen.

Cell culture and treatment. HepG2 cells, a human hepatoma cell line that was obtained from American Type of Cell Culture, were cultured in Dulbecco’s modified Eagle medium (DMEM) 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). Briefly, 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 conjugates were administered to the cultured cells. BSA was used as a vehicle control.

**Mice and experimental diets.** Male C57BL/6J mice (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 prior to sample collection. Total liver lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl (lauryl) sulfate) with fresh protease inhibitors. Preparation of liver sections for histology and hematoxylin and eosin (HE) 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 previously described (Ding et al., 2009). HepG2 cells (1×10^5/well in a 12-well plate with microscopic cover glasses, Thermo Fisher Scientific) were infected with adenoviral GFP-LC3 (100 viral particle per cell) in DMEM overnight followed by treatment with BSA or BSA conjugated fatty acids.

**Fluorescence Microscopy.** For fluorescence microscopy, cells were cultured in 12-well plates with microscope cover glasses. After designated treatments, cells were fixed with 4% paraformaldehyde (PFA) 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 Bodipy 493/503 (0.1 μmol/L) for 15 minutes 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 mol/L phosphate buffer (pH 7.4), followed by 1% OsO4. After dehydration, thin sections were cut and stained with uranyl acetate and lead citrate. Digital images were obtained using a JEM 1016CX electron microscope. Random images were chosen and the number of typical autophagosome and autolysosomes from each cell section were counted from more than 30 cells.

**Immunoblot Analysis.** Cells were washed in PBS and cell pellets were lysed in RIPA buffer. Thirty micrograms of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated with the indicated primary and secondary antibodies and developed with SuperSignal West Pico chemiluminescent substrate (Pierce). Densitometry analysis was performed using Image J software (National Institutes of Health). The relative levels of LC3-II in each group were normalized to their loading control.
Caspase-3 activity assay. This was determined as we described previously (Ding et al., 2004). Briefly, Caspase-3 activities were measured using 30 µg of proteins and 20 µM of fluorescent substrates (Ac-DEVD-AFC, Biomol). The fluorescence signals were detected by a fluorometer (Tecan GENios) at excitation and emission wavelengths of 400 nm and 510 nm, respectively.

Triglyceride (TG) Analysis. The level of TG was determined as described previously (Wobser et al., 2009). After treatment, cells were briefly washed with PBS and scraped off with a cell scraper. After centrifugation, the cell pellets were re-suspended in 200 µl ice-cold lysis buffer (18 mM Tris-HCl, 300 mM Mannitol, 50 mM EGTA, pH 7.6, with protease inhibitors) followed by sonication with a micro-tip. Ten microliters of cell extracts were taken out for the protein assay. The rest of the cell extracts were further mixed with 3 mL chloroform-methanol mix (2:1) and incubated for 1 hr at room temperature with occasional shaking to extract the lipid. Afterwards, 0.4 mL KCl (0.15 M) was added and mixed by vortex. These mixtures were centrifuged for 5 min at 3,000 g, the lower lipid phase was collected and dried by a vaccum at room temperature. The lipids were further dissolved in 20 µL Isopropanol with 10% Triton X100. Lipid analysis was carried out following the manufacturer’s instruction with a colorimetric assay kit (Sigma). Protein amount was determined with a protein assay kit (Thermo Scientific).

Statistical Analysis. Results are given as mean ± standard error of the mean. One-way anova analysis of variance and Scheffe’s post-hoc test was used for multiple comparisons and Student’s t test was used for comparison of two matched groups.
Results

Oleic acid induces autophagy in HepG2 cells

Because it has been reported that the biological functions of saturated and unsaturated fatty acids are different (Listenberger et al., 2003), we first determined the effect of oleic acid, an unsaturated fatty acid which 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 similar manner to 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 pre-autophagosome and autophagosome membranes, displaying a punctated pattern following its conjugation with PE (Suzuki and Ohsumi; Hosokawa et al., 2006). 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 of 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 chloroquine (CQ), which inhibits lysosomal degradation by increasing lysosomal pH (Ni et al., 2011). Compared to 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 (Figure 1 A-C), suggesting that OA induces autophagy in HepG2 cells. In addition to the changes of GFP-LC3 puncta, we also determined the autophagic flux by examining the changes of 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 (Figure 1 D), which inhibit lysosomal functions by either increasing lysosomal pH or suppressing vacuolar type H+-ATPase (V-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. All together, these data indicate that OA induces autophagy in HepG2 cells.

Palmitic acid does not activate autophagy in HepG2 cells

We next determined the effects of palmitic acid (PA), a saturated fatty acid which is the most abundant in the diet, serum and liver tissue (Baylin et al., 2002; Xu et al., 2011), on autophagy induction in HepG2 cells. In contrast to OA, we found that PA did not increase autophagy in HepG2 cells as examined by a series of autophagic flux assays including GFP-LC3 puncta (Fig 2 A-C) and the changes of endogenous LC3-II in the presence or absence of CQ or BAF (Figure 2 D). PA treatment alone slightly decreased the number of GFP-LC3 puncta at various concentrations although this did not achieve a statistic difference. However, the number of GFP-LC3 puncta after PA treatment in the presence of CQ was significantly lower than that of CQ.
treatment alone (Fig 2 A-C). Similarly, PA alone did not alter the level of endogenous LC3-II. Moreover, PA cotreatment did not significantly impact the levels of LC3-II in cells cotreated with CQ or BAF (Figure 2 D). Similar results were found in OA or PA treated primary cultured mouse hepatocytes (Supplemental Figure 1). These results suggest that unlike OA, PA does not activate autophagy in either HepG2 hepatoma cells or normal hepatocytes.

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 (PO,16:1) which has the same carbon length as PA, on autophagy. We found that PO induces autophagy in HepG2 cells based on the autophagic flux assay either assessing for the GFP-LC3 puncta formation or the levels of LC3-II changes (Supplemental Figure 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 (Figure 3A, panel e, arrows) and lipid droplets (LD) (Figure 3A, panels 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 (Figure 3A, panels c, d & f). In contrast, AV and LD are barely detectable in BSA treated control (Figure 3A, panel a) or PA-treated cells (Figure 3A, panel b; Figure 3B & 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 (Figure 3 D & E). Similar results were found in primary mouse hepatocytes (Supplemental Figure 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 N-acetylcysteine (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 to the control cells.
OA also increased the level of ROS production but did not to a degree of statistical significance. However, NAC significantly decreased the levels of ROS in both PA and OA-treated cells (Supplemental Figure 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 (Figure 4A & 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-methyladenine (3MA), 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 3MA (Figure 4A & 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 translational initiation factor 4E binding protein-1 (4EBP1) and 70-kDa ribosomal protein S6 kinase-1 (p70S6) kinase at different time points (Figure 4D) and various concentrations (Figure 4E). It seemed that OA treatment even increased the phosphorylated level of p70S6 kinase after 12 and 24 hours treatment although slightly decreased the phosphorylated 4EBP1 levels (Figure 4D). The reasons for the different changes for p70S6 kinase and 4EBP1 are not clear although they both are mTOR downstream substrates. In contrast, a high dose of PA (500 µM) reduced both the total and phosphorylated 4EBP1 which is
likely 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**

Since 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 (Figure 5A & B). The apoptotic nuclear changes in PA-treated cells were evident by the typical fragmented and condensed nuclear morphology (Figure 5A arrows), similar to apoptosis induced by tumor necrosis factor-α (TNF-α) plus actinomycin D (ActD), a widely used model to trigger death-receptor activation-induced apoptosis (Ding et al., 2004; Ding and Yin, 2004). Meanwhile, PA but not OA treatment also increased the number of cells with depolarized mitochondria (Figure 5 D& 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
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 (Figure 6A). We consistently observed a 50 kDa cleaved Beclin 1 in PA or TNF-α/ActD-treated cells as previously reported (Li et al., 2011a). Occasionally an additional cleaved Beclin 1 band around 45 kDa was also detected (Figure 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/ActD but not OA also induced caspase-3 cleavage (Figure 6B) and increased caspase-3 activity (Figure 6C). More importantly, PA and TNF-α/ActD-induced Beclin 1 cleavage and apoptosis were inhibited by a general caspase inhibitor, ZVAD-fmk, further supporting the notion that PA-induced Beclin 1 cleavage and apoptosis are caspase-dependent (Figure 6D & E). Furthermore, in the presence of ZVAD-fmk, the PA-induced number of GFP-LC3 puncta was significantly increased (Supplemental Figure 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 Figure 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, 3MA 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 (Figure 7A). Interestingly, we further found that OA attenuated PA-induced apoptosis (Supplemental Figure 6). This 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 (a term called 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 following 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 (Figure 7B). These results are generally in agreement with our earlier findings in Figure 5 and previous reports (Listenberger et al., 2003; Ricchi et al., 2009). Interestingly, 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 (Figure 7C). In line with the findings for the perilipin changes, OA also significantly increased total cellular triglyceride (TG) level compared to that of control cells or PA-treated cells. Suppression of autophagy by
CQ significantly increased TG levels in OA-treated cells (Figure 7D). Furthermore, suppression of autophagy by 3MA also increased OA-induced accumulation of LD (Supplemental Figure 7A). In contrast, induction of autophagy by rapamycin tended to reduce TG levels in OA-treated cells (Supplemental Figure 7B). Interestingly, CQ alone also increased TG levels in HepG2 cells, suggesting that even inhibition of basal level autophagy could also increase the level of TG (Figure 7D). The level of TG was higher in the PA and CQ treatment group than that of PA alone but was almost identical to the CQ treatment alone. The lack of additional increase of TG by CQ in CQ and PA treated cells is likely due to the already low autophagy activity induced by PA treatment in these cells (Figure 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 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 micro-vesicular steatosis was evident in livers of mice fed with the high fat diet (Figure 8A). EM studies further confirmed that both the number and size of the hepatic lipid droplets were markedly increased in livers of mice fed high fat diet (Figure 8B, panel b). Moreover, we also often found double membrane autophagosomes that enwrapped damaged mitochondria in livers from mice fed high fat diet (Figure 8B, panels c-e). Results from western blot analysis indicate that
there was an increased level of LC3-II in livers from mice fed high fat diet (Figure 8C & D), suggesting a possible increased number of autophagosomes or autolysosomes in high fat diet fed mouse livers. Taken together, these data suggest that 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 crosstalk 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 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 histological feature of NAFLD 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 may even play a protective role against the lipotoxicity from free fatty acids and other fatty acid-derived mediators (Malhi and Gores, 2008; Garbarino and Sturley, 2009; Neuschwander-Tetri, 2010). In CHO cells, OA is readily converted into TG and stored in lipid droplets resulting in less apoptosis, whereas PA is poorly incorporated into triglyceride and increases apoptosis (Listenberger et al., 2003). In the present study, we also found that the number of lipid droplets and the levels of TG are significantly higher in OA-treated HepG2 cells than in PA-treated cells. As a result, OA fails to induce apoptosis and even protects against PA-induced apoptosis in HepG2 cells (Figure 5C and Supplemental Figure 6). It has been reported that overexpression of stearoyl-CoA desaturase 1 (SCD1) that increases the level of unsaturated fatty acids and TG formation decreases PA-induced lipotoxicity (Listenberger et al., 2003). Conversely, impairing the formation of TG by knockout of acyl CoA:diacylglycerol transferase 1 (DGAT1) increases the lipotoxicity of OA (Listenberger et al., 2003). Collectively, our results together with other groups’ findings suggest that the formation of TG may not be the cause of fatty acid-induced lipotoxicity.

It is interesting that monounsaturated fatty acid OA but not the saturated fatty acid PA induces autophagy in HepG2 cells. This could be one additional important
mechanistic basis for why OA is less toxic than PA in HepG2 cells. Autophagy is recognized as a critical cell survival mechanism induced by nutrient or growth factor deprivation, hypoxia, ROS, DNA damage, protein aggregates, damaged organelles, or intracellular pathogens (Kroemer et al., 2010; Ravikumar et al., 2010). Although the mechanisms by which autophagy protects against cell death are not fully understood, it is generally thought that it involves multiple mechanisms including bulk protein degradation, recycling of misfolded and aggregate-prone proteins, relieving endoplasmic reticulum stress, and removing depolarized or permeabilized mitochondria (Ding et al., 2007a; Ding et al., 2007b; Kim and Lemasters, 2010; Kroemer et al., 2010). Fatty acids have been shown to induce apoptosis through activation of the pro-apoptotic protein Bax and subsequent mitochondrial damage (Malhi et al., 2006). In the present study, we found that saturated fatty acids increased the number of cells with depolarized mitochondria (Figure 5D & E). Moreover, suppression of autophagy enhanced fatty acid-induced apoptosis. It is possible that the impaired autophagy in PA-treated cells may exacerbate mitochondrial damage and further increase apoptosis. Although our present study and other previous reports found that saturate fatty acids (such as PA) are more toxic than unsaturated fatty acids (such as OA), it has also been reported that a diet enriched in saturated but not unsaturated fatty acids reversed alcohol-induced liver injury in a rat model (Nanji et al., 1995). This paradox could be due to the cytochrome P450 2E1 (CYP2E1) activity being suppressed by saturated but not by unsaturated fatty acids, and CYP2E1 is key enzyme which 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 the 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 the intracellular inositol levels is not known, we found that OA-induced autophagy requires ROS formation and the classical PI3 kinase complex because an antioxidant (NAC) and a PI3 kinase inhibitor (3MA) 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 crosstalk between apoptosis and autophagy, in which autophagy and apoptosis counteract each other. Therefore, the different capacity on 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; Ding et al., 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 acid 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.

Acknowledgements

We thank Ms Barbara Fegley (KUMC Electron Microscopy Research Laboratory) for her excellent assistance with the EM studies. We thank Drs. Hao Zhu and Ming Xu for their 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.

Performed data analysis: Mei, Ni, and Ding.

Wrote or contributed to the writing of the manuscript: Mei, Kassel, Luyendyk, and Ding.
References:


Footnotes:

This study was supported in part by the National Institute of Health (NIH) funds R21 AA017421 & P20 RR021940, and P20 RR016475 from the IDeA Networks of Biomedical Research Excellence (INBRE) program of the National Center for Research Resources (W.X.D). J.P.L was supported by the NIH funds R01 ES017537, P20 RR021940 and American Heart Association Scientist Development Grant (0835121G). No additional external funding received for this study.
Figure legends:

Figure 1. OA 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), OA (500 μM), OA plus CQ (20 μM) or CQ (20 μM) alone, or with various concentrations of OA (0, 125, 250 and 500 μM) 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-C). 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). (D) HepG2 cells were treated by vehicle control (5% BSA), OA (500 μM), OA plus CQ (20 μM), CQ (20 μM) alone, OA plus Baf (50 nM) or Baf (50 nM) 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 2. PA fails to induce autophagy in HepG2 cells. HepG2 cells were first infected with Ad-GFP-LC3 overnight and then treated by vehicle control (5% BSA), PA (500 μM), PA plus CQ (20 μM), CQ alone, or wither various concentrations of PA (0, 125, 250 and 500 μM) 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-C). 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). (D)
HepG2 cells were treated by vehicle control (5% BSA), PA (500 µM), PA plus CQ (20
µM), CQ (20 µM) alone, PA plus Baf (50 nM) or Baf (50 nM) 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 3. OA but not PA increases the number of autophagosomes and lipid
droplet in HepG2 cells. (A) HepG2 cells were treated with BSA vehicle control
(panel a), PA (500 µM, panel b) or OA (500 µM, panel c-f) for 6 hrs and the cells
were further processed for EM. Arrows denote autophagosomes. N, nuclei; LD, lipid
droplet; and M, mitochondria. The number of autophagosomes (B) and lipid droplets
(C) per cell section was determined (mean ± SD) from more than 30 different cells. #: p<0.01 (one way ANOVA with Scheffe's post-hoc test). (D) HepG2 cells were treated
with BSA vehicle control (panel a), OA (500 µM, panel b) or PA (500 µM, panel c) 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. (E) 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 4. NAC and 3MA suppress OA-induced autophagy 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), OA (500 μM), PA (500 μM), OA plus NAC (5 mM), PA plus NAC (5 mM), or NAC (5 mM), OA plus 3MA (10 mM), PA plus 3MA (10 mM) or 3MA (10 mM) alone for 6 hrs followed by fluorescence microscopy. (B-C) 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). (D) HepG2 were treated by OA or PA (500 μM) for 6, 12 and 24 hrs, and the expression levels of p-4EBP1/Total-4EBP1 and p-p70S6k/Total-p70S6k were determined by immunoblot analysis from at least three independent experiments. (E) HepG2 cells were treated with vehicle control (5% BSA), various concentrations (125, 250 and 500 μM) of OA and PA for 6 hrs. The expression levels of p-4EBP1/Total-4EBP1 and p-p70S6k/Total-p70S6k expression were determined by immunoblot analysis from at least three independent experiments. Densitometry analysis for the expression levels of p-4EBP1 and p-p70S6K was performed using Image J software which was further normalized with its loading control (β-Actin).

**Figure 5. Differential effects of OA and PA on apoptosis in HepG2 cells. (A)**

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 hrs. Apoptotic nuclei were analyzed by nuclear staining with Hoechst 33342 (1 μg/mL) for fragmented or
condensed nuclei (arrows). (B) HepG2 cells were treated with OA or PA (500 µM) for 6, 12, 24 hrs or various concentrations (125, 250 and 500 µM) of OA or PA (C), and the number of apoptotic nuclei was quantified (mean ± SE, n=3). #: p<0.01 (one way ANOVA with Scheffe’s post-hoc test). (D) 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 6 hrs. The cells were loaded with TMRM (50 nM) followed by fluorescence microscopy (arrow heads: cells with partially lost mitochondrial membrane potential; arrows: cells with completely lost mitochondrial membrane potential). (E) The number of cells with the loss (both partial and complete) of TMRM staining was quantified (mean ± SEM) from at least three independent experiments. #: p<0.01 (one way ANOVA with Scheffe’s post-hoc test).

Figure 6. PA but not OA induces caspase-mediated Beclin 1 cleavage. (A) HepG2 cells were treated by OA or PA (500 µM) for 6, 12, 24 hrs. 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 hrs. Total cell lysates were subjected to immunoblot analysis for Beclin 1 and caspase 3. (C) Total cell lysates (30 µg) were used for caspase-3 activity analysis (mean ± SE, n=3). *: p<0.05; #: p<0.01 (one way ANOVA with Scheffe’s post-hoc test). (D) HepG2 cells were treated with vehicle control (5% BSA), PA (500 µM), PA (500 µM) plus ZVAD-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 ZVAD-fmk (50 µM) for 24 hrs. 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 ± SE, n=3). #: p<0.01 (one way ANOVA with Scheffe's post-hoc test).

**Figure 7. Suppression of autophagy enhances fatty acid-induced cell death and lipid accumulation.** (A) HepG2 cells were treated with vehicle control (5% BSA), OA (500 μM), OA plus CQ (20 μM), CQ alone, OA plus 3MA (10 mM), or 3MA alone for 24 hrs, and apoptotic cell death was analyzed by nuclear staining with Hoechst 33342 (mean ± SE, n=3). *: p<0.05; #: p<0.01 (one way ANOVA with Scheffe's post-hoc test). (B) HepG2 cells were treated with vehicle control (5% BSA) or various concentrations (125, 250, 500) of OA or PA for 6 hrs or (C) treated with OA (500 μM) or PA (500 μM) in the presence or absence of CQ (20 μM) for 6 hrs. Total cell lysates were subjected to immunoblot analysis for perilipin-a. Densitometry analysis for the expression level of perilipin was performed using Image J software which was further normalized with its loading control (β-Actin). (D) HepG2 cells were treated as in (C) and cellular TG levels (mean ±SE, n=3) were quantified as described in the *Materials and Methods*, #: p<0.01 (one way ANOVA with Scheffe's post-hoc test).

**Figure 8. High fat diet induces steatosis and autophagy in mouse liver.** Male C57BL/6J mice were fed either a control diet or a western diet for 3 months. All the mice were starved for 16 hrs before they were sacrificed. (A) Representative photomicrograph of H&E-stained liver section from a mouse fed control diet (panel a) and from a mouse fed the high fat diet (panel b). Panel c was enlarged photomicrograph from panel b showing typical macro-vesicular hepatic steatosis (arrows). (B) Liver samples
were processed for EM. Control diet (panel a); high fat diet (panels b & c); panels d & e were enlarged photomicrographs from the boxed areas in panel c. Arrows: double membrane autophagosomes; N: nuclei, LD: lipid droplets; M: mitochondria. (C) Total liver lysates were subjected to western blot analysis using an anti-LC3 antibody. The same membrane was blotted for GAPDH as the loading control. (D) Densitometry analysis for the expression level of LC3-II was performed using Image J software which was further normalized with its loading control (GAPDH). Data are presented as the fold of the control diet mouse livers (mean ± SE, n=6). #: p<0.01, Student t test.
Figure 1

A) Images showing different conditions:
- Control
- OA
- OA+CQ
- CQ

B) Graph showing GFP-LC3 puncta per cell as a function of OA concentration (μM):
- OA concentration: 0, 125, 250, 500

C) Graph showing GFP-LC3 puncta per cell:
- Conditions: Control, OA, OA+CQ, CQ

Legend:
- #: Significant difference
- *: Not significant
Figure 1

D

<table>
<thead>
<tr>
<th></th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>1 ± 0</td>
<td>1.14 ± 0.04</td>
<td>2.24 ± 0.12</td>
<td>1.9 ± 0.27</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>1 ± 0</td>
<td>1.14 ± 0.04</td>
<td>2.82 ± 0.5</td>
<td>2.3 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

OA (500 μM) CQ (20 μM)

OA (500 μM) BAF (50 nM)
Figure 2

D

<table>
<thead>
<tr>
<th>Condition</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>PL (500 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CQ (20 μM)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>LC3-I</td>
</tr>
<tr>
<td>LC3-II</td>
<td>1±0</td>
<td>0.97±0.07</td>
<td>1.9±0.38</td>
<td>1.9±0.27</td>
<td>LC3-II/β-Actin</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>PL (500 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAF (50 nM)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>LC3-I</td>
</tr>
<tr>
<td>LC3-II</td>
<td>1±0</td>
<td>0.97±0.07</td>
<td>1.83±0.37</td>
<td>2.3±0.31</td>
<td>LC3-II/β-Actin</td>
</tr>
</tbody>
</table>

β-Actin
Figure 3

A

B

No of AV Per Cell Section

Control  OA  PA

#  #

C

No of LD Per Cell Section

Control  OA  PA

#  #
Figure 5

A

Control  OA  PA  TNFα/ActD

B

C

Apoptotic Nuclei (%) vs Time (hrs)

Apoptotic Nuclei (%) vs Concentration

control  OA  PA
Figure 7

[Graph showing TG (μg/mg Protein) levels with data points for different treatments.]

Legend:
- OA: -
- PA: -
- CQ: -

Conditions:
- +: Treatment
- -: Control

Significance:
- #: Statistical significance
Figure 8

C

<table>
<thead>
<tr>
<th></th>
<th>Control Diet</th>
<th></th>
<th>High Fat Diet</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse number</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>LC3I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC3II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>High Fat Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC3-II/GAPDH</td>
<td>1</td>
<td>3.5</td>
</tr>
</tbody>
</table>

#
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*

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

A

Control  OA  OA+CQ

CQ  PA  PA+CQ

B

GFP-LC3 Puncta Per Cell

Control  OA  OA+CQ  CQ

C

GFP-LC3 Puncta Per Cell

Control  PA  PA+CQ  CQ

# indicates significant difference between control and OA, OA+CQ or CQ.
Supplemental Figure 2

A

Control | PO | PO+CQ | CQ
---|---|---|---

Control | PO | PO+CQ | CQ

GFP-
LC3 Puncta Per Cell

B

C

PO (500 µM)
CQ (20 µM)

# LC3-II/β-Actin
## LC3-I

β-Actin
Supplemental Figure 4

DCF Fluorescence (Fold of Control)

Control      OA    OA+NAC   PA    PA+NAC   NAC

# # #
Supplemental Figure 5

A

Control    PA    PA+ZVAD    ZVAD

B

GFP-LC3 Puncta Per Cell

Control    PA    PA+ZVAD    ZVAD

Control          PA          PA+ZVAD        ZVAD
Supplemental Figure 6

The figure shows a bar chart indicating the percentage of apoptotic cells for different treatments: Control, OA, PA, and OA+PA. The y-axis represents the percentage of apoptotic cells, ranging from 0 to 80. The x-axis lists the treatments. The PA treatment has the highest percentage of apoptotic cells, significantly higher than the other treatments, as indicated by the hash marks (#) above the bars.