FREE FATTY ACIDS INDUCE JNK DEPENDENT HEPATOCYTE LIPOAPOPTOSIS

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Running title: Hepatocyte lipoapoptosis occurs via JNK-dependent Bax-activation

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Abbreviations: FFA, free fatty acid; JNK, C-jun N-terminal kinase; TNF-α, tumor necrosis factor-alpha; NAFLD, non alcoholic fatty liver disease; NASH, non alcoholic steatohepatitis.

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Elevated serum free fatty acids (FFA) and hepatocyte lipoapoptosis are features of non-alcoholic fatty liver disease (NAFLD). However, the mechanism by which FFA mediate lipoapoptosis is unclear. Because JNK activation is pivotal in both the metabolic syndrome accompanying NAFLD and cellular apoptosis, we examined the role of JNK activation in FFA-induced lipoapoptosis. Multiple hepatocyte cell lines and primary mouse hepatocytes were treated in culture with mono-unsaturated fatty acids and saturated fatty acids. Despite equal cellular steatosis, apoptosis and JNK activation were greater during exposure to saturated versus monounsaturated FFA. Inhibition of JNK, pharmacologically as well as genetically, reduced saturated FFA mediated hepatocyte lipoapoptosis. Cell death was caspase dependent, and associated with mitochondrial membrane depolarization and cytochrome c release indicating activation of the mitochondrial pathway of apoptosis. JNK-dependent lipoapoptosis was associated with Bax activation, a known mediator of mitochondrial dysfunction. As JNK can activate Bim, a BH3 domain only protein capable of binding to and activating, Bax, its role in lipoapoptosis was also examined. siRNA targeted knockdown of Bim attenuated both Bax activation and cell death. Collectively, the data indicate that saturated FFA induce JNK dependent hepatocyte lipoapoptosis by activating the proapoptotic Bcl-2 proteins Bim and Bax which trigger the mitochondrial apoptotic pathway.

Non Alcoholic Fatty Liver Disease (NAFLD) is associated with obesity and affects a third of the population of the United States. Because, a subset of patients with NAFLD progresses to non-alcoholic steatohepatitis (NASH) and end stage liver disease (1), NAFLD has emerged as a substantial public health concern. This syndrome is associated with hepatocyte steatosis and elevated serum free fatty acids (2). However, the cellular mechanisms linking elevated serum free fatty acids and hepatocyte steatosis to tissue injury remain obscure. Liver cell apoptosis is a prominent feature of NASH and correlates with disease severity (3). The toxicity of lipids, or lipotoxicity, and specifically lipid-induced apoptosis, or lipoapoptosis, is a potential mechanism relating apoptosis to NASH. In support of this concept, FFA have been reported to cause cellular steatosis, and enhance expression of the apoptosis effectors, tumor necrosis factor-α (TNF-α) and Fas (4), (2). However, direct evidence for hepatocyte lipoapoptosis by individual FFA’s is lacking, as is the potential mechanism by which they may engage the cellular apoptotic machinery.

C-jun N-terminal kinase (JNK) is activated by various stress signals, and interestingly, also in obesity (5,6). The JNK pathway contributes to stress induced apoptosis in several cell types, including pancreatic β-cells, neurons, and hepatocytes (7), (8), (9). Interactions between FFA and JNK signaling may, therefore, be a potential link between obesity and lipoapoptosis. JNK has three isoforms of which only two are expressed in the liver, JNK 1 and JNK 2(10). Sustained JNK 2 and JNK 3 activation are cytotoxic in neurons (11), whereas JNK 1 potentiates hepatocyte apoptosis by the toxic bile acid deoxycholate (9). Thus, JNK isoform cytotoxicity appears to be cell type and stimulus specific. JNK may induce cell injury through both transcriptional and non-transcriptional mechanisms. Growth factor withdrawal, UV light, and oxidative stress associated JNK-mediated apoptosis require activation of the transcription factor c-jun/AP-1 (12-15). In contrast, JNK may also induce apoptosis in a transcription independent process by activating pro-apoptotic members of the bcl-2 family, including Bim and Bax or inactivating Bcl-2 and Bcl-xL, anti-apoptotic members of this family (16,17) (18-20). Activation of the pro-apoptotic proteins or inhibition of their anti-apoptotic counterparts triggers mitochondrial dysfunction and cell death.

The dual objectives of this study were to determine if individual free fatty acids induce hepatocyte apoptosis and if the observed lipoapoptosis is JNK dependent. We induced steatosis in cultured hepatocytes and demonstrated apoptotic cell death with free fatty acid treatment. Sustained JNK activation was a prominent feature of this lipoapoptosis, and inhibition of JNK abrogated hepatocyte lipotoxicity.
**EXPERIMENTAL PROCEDURES**

**Cells.** Mouse hepatocytes were isolated from C57/Bl6 wild type, JNK 1 −/−, JNK 2 −/− (Jackson Laboratories, Bar Harbor, Maine), and cathepsin B −/− mice (21) by collagenase perfusion (22). After percoll purification hepatocytes were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing high glucose (25mM), 100,000 Units/L penicillin, 100 mg/L streptomycin and 10% fetal bovine serum. HepG2, a well-differentiated human hepatoblastoma cell line, frequently used for studies of lipid metabolism (23), Huh7, a human hepatoma cell line and MRH7777, a rat hepatocellular carcinoma cell line, were also cultured in DMEM containing penicillin/streptomycin and 10% fetal bovine serum.

**Fatty acid treatment:** Oleic acid (Cat # O 1008), palmitoleic acid (Cat # P 9417) palmitic acid (Cat # P 0500), and stearic acid (Cat # S 4751) were all obtained from Sigma pharmaceuticals (St. Louis, MO). Palmitic acid and stearic acid were dissolved in isopropanol at a concentration of 20 mM or 40 mM. Oleic acid and palmitoleic acid, commercially available in liquid form, were diluted in isopropanol to obtain 20 mM and 40 mM stock solutions. The concentration of the vehicle, isopropanol, was 1% in final incubations. DMEM containing 1% bovine serum albumin was used in all experiments. The concentration of fatty acids used ranged from 50-400 µM.

**Fatty acid treatment: Oleic acid (Cat # O 1008), palmitoleic acid (Cat # P 9417) palmitic acid (Cat # P 0500), and stearic acid (Cat # S 4751).**

**Fat quantitation by Nile Red.** Free fatty acid treated cells were fixed with 3.7% paraformaldehyde for 15 minutes at room temperature. Intracellular neutral lipid was stained with Nile Red (0.2 mg/ml) for 5 minutes at room temperature (24). Images were acquired by confocal microscopy with an inverted Zeiss laser scanning confocal microscope (Zeiss LSM 510, Carl Zeiss Inc., Thornwood, NJ). Cellular fluorescence was quantitated using Zeiss KS400 image analysis software (Carl Zeiss, Inc., Oberkochen, Germany). Nile red staining, reflecting lipid content, was expressed as an increase in total cellular fluorescence intensity per cell (pixels above threshold x fluorescence intensity).

**Quantitation of Mitochondrial Membrane Potential (Δψ).** Cells were cultured on 35-mm glass bottomed dishes (MatTek Corp., Ashland, MA). Δψ was measured using a fluorescence quenching assay based on the concept of resonance energy transfer (26). The normal negative mitochondrial membrane potential results in selective uptake of the cationic Mitofluor green, which covalently binds mitochondrial proteins. On loss of mitochondrial membrane potential, Mitofluor green is still retained within mitochondria, TMRM, also a fluorescent cation, loads into mitochondria based on Δψ but rapidly diffuses out of mitochondria upon mitochondrial depolarization. Mitofluor green fluorescence emission at fluorescein wavelengths is effectively quenched by TMRM via resonance energy transfer in fully polarized mitochondria. However, as Δψ is lost, TMRM diffuses out of mitochondria resulting in enhanced Mitofluor green fluorescence. Cells were treated with free fatty acid for 24 hours prior to loading with 200 nmol/l Mitofluor green for 20 minutes at 37°C in media. Then the cells were loaded with 1 µmol/l TMRM for 20 minutes at 37°C. After initial fluorescence measurement (Y), the nonfluorescent uncoupler 1799 (50 nmol/L) was added. This uncoupler results in mitochondrial
depolarization and loss of TMRM allowing determination of maximal Mitofluor green fluorescence (X), from depolarized mitochondria. Change in fluorescence (X-Y) was calculated for control (Xc-Yc) and treated (Xt-Yt) cells, and expressed as a percent change of control (Xt-Yt / Xc-Yc *100). By this assay percent decrease in fluorescence intensity correlates with loss of Δψ. Fluorescence was recorded using an inverted fluorescent microscope, and images collated using a cooled, charge-coupled device camera (Photometerics, Tucson, AZ) and digitized by Metafluor software (Universal Imaging, Westchester, Penn)

**Immunocytochemistry.** Cells on coverslips were fixed with 4% paraformaldehyde in PBS. Permeabilization was performed with 0.0125% CHAPS in PBS, 0.05% Saponin in PBS, and 0.5% Triton-X-100 in PBS for Bax, cytochrome c and NF-κB immunostaining respectively. Primary antibodies were rabbit anti-NF-κB RelA/p65 (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-universal Bax (1:50-1:100 dilution, Exalpha Biologicals, Watertown, MA), mouse anti-cytochrome c (1:200 dilution, Pharmingen, San Diego, CA). Secondary antibodies were TMRM conjugated anti-mouse (4µg/ml), FITC conjugated anti-mouse (4µg/ml) antibodies (Molecular Probes, Eugene, OR) and Cy3 conjugated antirabbit antibody (1:1000, Jackson Immunological Research Labs, Inc.) Prolong antifade (Molecular probes) was used as mounting medium. Images were acquired by Confocal Microscopy with an inverted Zeiss laser Scanning Confocal Microscope (Zeiss LSM 510, Carl Zeiss Inc., Thornwood, NJ).

**Cell Fractions.** Whole cell, cytosolic and mitochondrial fractions were prepared from HepG2 cells approximately 70% confluent prior to treatment with free fatty acids. To prepare whole cell lysates, cells were placed on ice, the medium aspirated and the cells were rinsed once with ice-cold phosphate buffered saline (PBS). The cells were then scraped in 1 ml of lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 6 mM deoxycholic acid, 1% NP 40, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 1 mM NaF, and protease inhibitor mix (Complete Protease Inhibitor Cocktail; Roche Diagnostics, Mannheim, Germany). microcentrifuge tubes and incubated for 30 minutes at 4°C. Whole cell lysates were centrifuged at 13,000×g for 15 mins at 4°C to remove insoluble material. For subcellular fractions, cultured cells were collected by scraping in homogenization buffer (70 mM Sucrose, 220 mM Mannitol, 3 mM EDTA, 5 mM MOPS, pH 7.4), transferred to an ice-cold dounce homogenizer. After 100 up and down passes in the homogenizer (on ice), the crude homogenate was centrifuged at 600 x g for 10 minutes at 4°C to remove debris. The post-nuclear supernatant was centrifuged at 7000 x g for 10 minutes at 4°C to pellet mitochondria from cytosol. The supernatant from this step is the cytosolic fraction and the pellet is the mitochondrial fraction. The mitochondrial pellet was rinsed in wash buffer (5 mM MOPS, 100 mM KCl, pH 7.4), collected by centrifugation and resuspended in lysis buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1mM PMSF, 1% Triton-X, 1% NP-40 and a complete protease inhibitor tablet (Roche). After incubating for 30 minutes at 4°C with gentle shaking, the mitochondrial lysate was centrifuged at 13,000 x g for 10 minutes at 4°C to remove insoluble protein. The protein content of samples was estimated by the Bradford assay (Sigma, Saint Louis, MO). The mitochondrial, cytosolic and whole cell lysates were subject to immunoblot analysis as described below.

**Immunoblot Analysis.** Protein was electrophoretically resolved by SDS-PAGE and immobilized on PVDF membrane. 5% non-fat dairy milk in Tris-buffered saline (20 mM Tris, 150 mM NaCl, pH 7.4) with 0.1% Tween-20 was used to block non-specific binding sites. Primary antibodies were: JNK, phospho-JNK, c-jun, phospho c-jun, p38 MAPK, ERK (1:1000, Cell Signaling Technology, Beverly, MA); phospho p38 (1:2000), phospho ERK (1:2500, Promega Corporation, Madison, WI); Bid (1:1000, R & D Systems, Minneapolis, MN), cytochrome c (1:500, Pharmingen, San Diego, CA); Bak, Bak (1:500), cytochrome c oxidase II (1:200, Santa Cruz Biotechnology, Santa Cruz, CA); Bim (1:1000, Chemicon, Temecula, CA); Bim (1:1000, BD Pharmingen, San Jose, CA). Peroxidase conjugated secondary antibodies (1:3000, Biosource International, Camarillo, CA) were used to detect antigen-antibody complexes. Immune complexes were visualized using a
chemiluminescent substrate (ECL, Amersham, IL), and Kodak XOMAT film (Eastman Kodak, Rochester, NY). Immunoreactive areas were quantitated by densitometry, using an imaging densitometer (Model GS-700, Bio-Rad, Hercules, CA) and the Molecular Analyst software program (Bio-Rad) to calculate the ratio of Bim-EL to γ-tubulin.

Caspase-3/7 Activity. Cells were plated in 96 well plates (Corning Inc., Corning, NY). Caspase activity assay was performed using commercially available Apo-ONE homogeneous Caspase-3/7 assay (Promega Corporation, Madison, WI) according to the manufacturers’ instructions. Briefly, this assay involves cleavage of a profluorescent caspase-3/7 consensus substrate, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-aspartic acid amide) conjugated to rhodamine 110 (Z-DEVD-R110) on its C-terminal side. Proteolytic cleavage liberates rhodamine 110 unquenching its fluorescence. Fluorescence was measured using excitation and emission wavelengths of 498 and 521 nm, respectively.

JNK and Phospho-JNK immunoreactivity by ELISA. JNK and phospho-JNK immunoreactivity was measured using a commercially available Fast Activated Cell-based ELISA (FACE, Active Motif, Carlsbad, CA). Briefly cells were plated in 96 well plates. Cells were fixed with 4% formaldehyde followed by quenching of endogenous peroxidase with 1% H2O2 and 0.1% Sodium Azide in PBS containing 0.1% Triton X-100. Each well was incubated with antibody specific to total JNK or phospho-JNK. HRP-conjugated secondary antibody was subsequently added and developed with the commercial reagent. Absorption was measured at 450 nm using a spectrophotometer. Cell number was quantitated by crystal violet staining. JNK and phospho-JNK immunoreactivity were normalized to cell number.

Bim gene silencing by siRNA. RNA interference was used to silence Bim gene expression in Huh7 and HepG2 cells. A 21-nucleotide double stranded siRNA, 5'-AAT TAC CAA GCA GCC GAA GAC-3', targeting human Bim was designed using proprietary software at www.ambion.com and synthesized using Silencer siRNA construction kit (Ambion Inc., Austin, TX). Transient gene silencing was attained by transfection of siRNA into cells using siPORT lipid transfection reagent according to the manufacturers instructions. Scrambled siRNA was used as a control. Gene silencing was verified by detecting protein with immunoblot analysis after transient transfection of Huh7 cells with siRNA. Briefly, cells grown in 6-well dishes were transiently transfected with 35 nM siRNA using 4 µl/ml siPORT Lipid (Ambion Inc.) in a total transfection volume of 0.5 ml of OptiMEM (Invitrogen Corp.). After incubation at 37°C/5% CO2 for 4 h, 1 ml of normal growth medium was added. Transfected cells were then analyzed for apoptosis, Bax activation by immunofluorescence or by immunoblot as described elsewhere in methods.

Real-time polymerase chain reaction: Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen). Maloney leukemia virus reverse transcriptase (Invitrogen) and random primers (Invitrogen) were used to reverse transcribe RNA into cDNA. The cDNA template was quantified using real-time PCR (LightCycler, Roche Molecular Biochemicals, Mannheim, Germany) using SYBR green (Molecular Probes) as fluorophore. The PCR primers for Bim were forward 5’-AGATCCCCGCTTTTCATCTT-3’ and reverse 5’-AGGACTTGGGTTTGTGGTG-3’; the primers for human cytochrome P450 (CYP) 2E1 were forward 5’-CTACAAGGGCGGTAAGGAAG-3’ and reverse 5’-GGGAGTGCTGAGTAGGTGGA-3’; the primers for murine CYP2E1 were forward 5’-TTCATCAACCTCGTCCCTTC-3’ and reverse 5’-AGGCCCTTCTCAAACACACAC-3’. Commercially available 18S rRNA primers (Ambion, Austin, TX) were used as internal control. The expected PCR product was electrophoresed in 1% agarose gel and eluted into Tris.HCl using a DNA elution kit (Gel extraction kit, Qiagen, Valencia, CA). The concentration of DNA in the extracted PCR product was measured (copies/µl) spectrophotometrically at 260nm. A standard curve was generated using the extracted PCR product. The inverse linear relationship between copy and cycle numbers was then determined. Each resulting standard curve was then used to calculate the copy number per microliter in each experimental sample. The target mRNA level was expressed relative to the 18S rRNA level for each sample. The PCR conditions and primers were optimized to produce a single
PCR product of the correct base pair size.

Reagents. Tetramethyl rhodamine methyl ester (TMRM), Mitofluor and Mitotraker green were from Molecular Probes (Eugene, OR). Nile Red, 4',6-diamidino-2-phenylindole (DAPI), Percoll, stearic acid, palmitic acid, oleic acid, and palmitoleic acid were from Sigma Pharmaceuticals (St. Louis, MO). Z-VAD-fmk was from Enzyme Systems (Livermore, CA). Mitogen activated protein kinase inhibitors, U0126, PD98059, SP600125 and JNK peptide inhibitor were from Calbiochem (La Jolla, CA). Caspase inhibitor, IDN-6556, was from IDUN Pharmaceuticals, Inc. (San Diego, CA).

Statistical Analysis. All data are expressed as the mean ± SEM unless otherwise indicated. Differences between groups were compared using students t-test.

RESULTS

FFA treatment induces cellular steatosis: We first confirmed our cell-culture model of cellular steatosis by incubating HepG2 cells with various FFA in medium containing 1% bovine serum albumin. Specifically, HepG2 cells were incubated with oleic acid (C18:1), palmitoleic acid (C16:1), both monounsaturated fatty acids, and stearic acid (C18:0), palmitic acid (C16:0), both saturated fatty acids, for 18 hours. Intracellular lipid vacuoles visible under phase contrast microscopy were confirmed by Nile Red staining (Fig. 1A). To confirm equal steatotic effects of saturated and unsaturated fatty acids, cellular steatosis, expressed as a percent of total cellular area, was quantified (Fig. 1B). Percent steatosis was 9.5±2.6% in oleic acid treated cells, 14.7±1.9% in palmitic acid, 10.1±0.5% in palmitoleic acid, 12.4±1.8% in stearic acid and 3.7±0.4% in untreated controls. Thus, all the individual FFA produced similar cellular steatosis.

Biochemical confirmation of apoptosis was obtained by demonstrating DNA cleavage and caspase activation, both biochemical hallmarks of apoptosis (28). In HepG2 cells, FACS analysis demonstrated 15.6±1.1% death in control cells; this was increased to 19.8±1.3% (p=0.04) with oleic acid treatment and 58±2.7% (p<0.001) with palmitic acid treatment for 24 hours. Fatty acid treatment also led to robust caspase 3/7 activation (Fig. 2E). Consistent with the morphologic assessment of apoptosis, caspase 3/7 activity was greater in palmitic acid (7.1 fold) and stearic acid (6.5 fold) treated HepG2 cells as compared with oleic acid treated cells (2.6 fold). The pancaspase inhibitor zVAD-fmk, attenuated cell death by all FFA examined, in a concentration dependent manner (Fig. 2F). The structurally dissimilar pancaspase inhibitor, IDN 6556, also reduced cell death by 50% in HepG2 cells incubated with palmitic acid or stearic acid (p<0.01, data not shown). Taken together, these data demonstrate that FFA induce caspase-dependent apoptosis.

FFA mediated apoptosis occurs via the mitochondrial pathway of apoptosis. Mitochondrial dysfunction with loss of the mitochondrial membrane potential (∆ψ) and release of cytochrome c into the cytosol are prominent features of the mitochondrial pathway of apoptosis. Indeed, ∆ψ was 26±14% of the initial value following treatment with palmitic acid and 25±11% of the initial value following treatment with stearic acid for 18 hours. Upon treatment with fatty acids, cytochrome c was also released from mitochondria into the cytosol, as demonstrated by a change from punctate to diffuse...
cytoplasmic staining in 19% of oleic acid treated HepG2 cells, 98% of palmitic acid treated cells and 96% of stearic acid treated cells (Fig. 3A and B). Release of cytochrome-c into the cytosol by palmitic acid and stearic acid was confirmed by immunoblot analysis of cytosolic fractions (Fig. 3C). These data suggest that saturated FFA mediate apoptosis by engaging the mitochondrial pathway of apoptosis.

Mitochondrial dysfunction in hepatocytes may be initiated via either death receptor (extrinsic) or intracellular stress (intrinsic) pathways. Death receptor mediated mitochondrial dysfunction occurs by caspase 8 cleavage of the BH3-only Bcl-2 family protein, Bid, to generate an active smaller C-terminal fragment referred to as tBid (29). However, FFA treatment did not result in Bid cleavage, whereas Bid cleavage to tBid was readily identified in TRAIL plus actinomycin D treated HepG2 cells (Fig. 4A, lane 10). Selective pharmacologic inhibition of caspase 8 with IETD-fmk, 10 and 30 µM, also did not diminish palmitic acid or stearic acid toxicity (data not shown). Lysosomal permeabilization has also been reported to trigger mitochondrial dysfunction in hepatocytes. Cathepsin B, a lysosomal cysteine protease, is a key mediator of this pathway (21). However, oleic acid, palmitic acid, and stearic acid treatment induced 31.9±2.3%, 67±9.4%, and 60.3±12.1% apoptosis in cathepsin B-/- hepatocytes (Fig. 4B), respectively, which was similar to that observed in wild type hepatocytes [28.6±2.9%, 64.9±6.2%, and 54.1±2.6% apoptosis with oleic acid, palmitic acid, and stearic acid treatment (p=NS)]. Thus, free fatty acid induced apoptosis is not associated with Bid cleavage nor dependent on cathepsin B. These data suggest FFA engagement of the mitochondrial pathway of apoptosis excludes death receptor and lysosomal initiated events.

JNK levels remained unchanged during FFA treatment (Fig. 5A). Stearic acid treatment led to a significant increase in phospho-JNK levels and activity by both immunoblot analysis and ELISA in HepG2 cells. A robust increase in phospho-JNK was observed, by immunoblot analysis, as early as 3 hours following treatment with stearic acid (Fig. 5B). This increase in JNK activity was sustained and persisted at 24 hours. Quantitatively, as assessed by an ELISA assay, phospho-JNK was increased 2.4, 2.5 and 6.3 fold (compared with untreated cells), at 3, 6, and 24 hours, respectively, following treatment with stearic acid (Fig. 5C). Oleic acid treatment, on the other hand, led to only modest 1.6 and 1.8 fold increase in phospho-JNK at 3, 6 and 24 hours, respectively. In primary mouse hepatocytes phospho-JNK was increased 2.3, 2.4 and 2.7 fold (compared with untreated cells), at 3, 6, and 24 hours, respectively, following treatment with stearic acid (Fig. 5C). Oleic acid treatment, on the other hand, led only to a modest increase in phospho-JNK activity of 1.7, 1.7 and 1.6 fold at 3, 6 and 24 hours, respectively. P44/42 MAP kinase, another member of the MAP kinase family was phosphorylated following stearic acid treatment (Fig. 5B). In contrast, p38 MAPK was not phosphorylated following exposure to stearic acid. Thus, both JNK and p44/42 MAPK were strongly activated by pro-apoptotic free fatty acids.

To define whether JNK or p44/42 MAPK contributes to lipoapoptosis in our model, inhibitor studies were performed. A cell permeable, small peptide JNK inhibitor (JNKI) reduced oleic acid toxicity by 56% (p<0.05), palmitic acid toxicity by 70% (p<0.001), and stearic acid toxicity by 45% (p<0.05) (Fig. 6A). To further confirm this observation, SP600125, a structurally dissimilar JNK inhibitor was also employed (30). This compound also reduced oleic acid toxicity by 45% (p<0.005) and palmitic acid and stearic acid toxicity by 70% (p<0.05) (Fig. 6A). In order to determine the role of the two JNK isoforms expressed by hepatocytes in our model of lipoapoptosis, hepatocytes were isolated from mice deficient in JNK 2 (JNK2-/-) and JNK 1 (JNK1-/-). Stearic acid and oleic acid induced cell death was reduced in JNK2 -/- mice (p<0.05) (Fig. 6B). In contrast, hepatocytes from JNK 1-/- mice were not resistant to oleic acid or stearic acid cytotoxicity (Fig. 6C). Finally, the role of p44/42 MAPK...
MAPK in this apoptotic pathway was assessed using the inhibitors, PD 98059 and U0126. At concentrations which block p44/42 MAPK activity, neither inhibitor reduced palmitic acid nor stearic acid mediated lipoapoptosis (data not shown). Collectively, these observations suggest that FFA-mediated lipoapoptosis is, in part, JNK dependent, and at least in murine hepatocytes, the JNK2 isoform mediates this cytotoxicity.

JNK engages the mitochondrial pathway of apoptosis by activating Bax. Bax, a pro-apoptotic BH3 family protein, can be activated by JNK (31). Upon activation, Bax undergoes conformational change exposing an amino-terminal epitope, which is specifically recognized by the 6A7 monoclonal antibody (32). Indeed, FFA treatment led to Bax activation (Fig. 7A) as demonstrated by immunofluorescence microscopy for active Bax. Pre-treatment of HepG2 cells with JNK peptide inhibitor and SP 600125 prevented stearic acid and palmitic acid induced Bax activation (Fig 7B). Bax activation led to mitochondrial permeabilization and release of cytochrome c. Indeed, cytochrome c release was decreased 61% by SP600125 and 73% by the JNK peptide inhibitor (Fig. 7C) in stearic acid treated cells, confirming inhibition of the mitochondrial pathway of apoptosis. These data place Bax activation and mitochondrial permeabilization downstream of JNK activation in stearic acid induced lipoapoptosis.

Multidomain proteins of the Bcl-2 family, such as Bax, are known to be activated by BH-3 only Bcl-2 family proteins. Of the 8 known BH-3 only proteins only Bim and Bid bind and activate Bax (33). As Bid activation was not observed (Fig. 4A), we focused on Bim in FFA-mediated lipoapoptosis. We first examined Bim expression in free fatty acid treated HepG2 cells. Of three known isoforms, Bim-EL, Bim-L and Bim-S, only the first two were detected consistently in HepG2 cells. Consistent with its toxicity profile, stearic acid led to an early and sustained increase in cellular Bim levels (Fig. 8A). Quantitative densitometry showed that stearic acid treatment led to a 2±0.3-fold increase in cellular Bim-EL levels at 6 hours, whereas oleic acid treatment to a 1.2±0.2-fold increase (Fig. 8B). The increase in Bim protein was also associated with an increase in Bim mRNA. Indeed, as assessed by real-time PCR, stearic acid treatment led to a 2-fold increase in Bim mRNA expression in HepG2 cells and 3-fold increase in Huh7 cells; in contrast an increase was not observed in oleic acid treated cells (data not shown). The observed increase in Bim is not a result of cell death as it occurred within 6 hours of treatment, well before detectable apoptosis. Thus, stearic acid-induced JNK-dependent lipoapoptosis is associated with Bim induction. Whether Bim induction is an epiphenomenon of stearic acid treatment or plays a role in stearic acid induced lipoapoptosis, was further dissected by siRNA mediated Bim gene silencing in Huh7 and HepG2 cells. A reduction in Bim expression was cytoprotective; indeed, stearic acid-induced lipoapoptosis was reduced by 55% (Fig. 8C). Though oleic acid toxicity was minimal per se, it was slightly reduced by decreasing Bim expression using siRNA (Fig. 8C). Furthermore, Bax activation, the effector of mitochondrial permeabilization in this model, was Bim dependent (Fig 8D). In cells with targeted Bim knockdown, stearic acid induced Bax activation was reduced by 83% (p<0.001, Fig 8E). Collectively, these data suggest that JNK mediates lipoapoptosis via Bim-dependent Bax activation.

DISCUSSION

The principal findings of this study relate to the mechanism of FFA-induced hepatocyte lipoapoptosis. The data demonstrate that: i) saturated FFA are more cytotoxic than monounsaturated FFA; ii) FFA cytotoxicity occurs via caspase-dependent apoptosis; and iii) JNK triggers the mitochondrial pathway of apoptosis by Bim-dependent Bax activation. These data provide mechanistic insight into the cellular mechanism of hepatocyte lipoapoptosis, a model relevant to human NASH.

In the current study, FFA directly induced apoptosis in hepatocytes. Saturated FFA were substantially more toxic than monounsaturated FFA despite causing a similar magnitude of cellular steatosis. Our data are consistent with observations in disparate cell types, such as pancreatic β cells, myocardium, skeletal muscle, neurons and endothelial cells (34,35), where saturated FFA, palmitic acid and stearic acid, also exhibit greater cytotoxicity than monounsaturated FFA, oleic acid and palmitoleic acid. Our observations are in contrast to alcohol-mediated
hepatotoxicity where feeding animals unsaturated FFA potentiates hepatotoxicity (36). The mechanisms of alcohol-induced hepatotoxicity may well be quite different from those of NAFLD despite the fact that both are characterized by steatosis. Our data, indicating that specific FFA have distinct inherent toxic potential, suggest that characterizing the FFA profile in serum and/or liver tissue may predict which individuals with NAFLD will develop more severe liver disease.

Formation of reactive intermediates (ROS), alternative lipid metabolic pathways, such as ceramide synthesis, modulation of death receptor expression and direct activation of cellular pro-apoptotic machinery are putative mechanisms by which lipoapoptosis is thought to occur (2-4,37,38). However, none of these observations and concepts sufficiently explain how FFA engage the core cell death machinery. The current data address this question. The results demonstrated that FFA-mediated lipoapoptosis occurs by caspase-dependent mechanism by activating the mitochondrial pathway of apoptosis. Indeed, caspase 3/7 activity was readily identified and FFA lipapoptosis was attenuated by caspase inhibition. The caspase activation coincided with mitochondrial dysfunction and was not blocked by inhibiting the death receptor pathway with a selective caspase 8 inhibitor nor by disrupting the lysosomal pathway by genetic deletion of cathepsin B. Collectively, these data indicate that cytotoxic FFA directly engage the mitochondrial cell death pathway.

FFA are known to affect several cellular signaling pathways, including the MAPK cascades (39,40). In the hepatocyte cell lines studies, stearic acid activated both p44/42 and JNK MAPK. Consistent with the pro-apoptotic properties of JNK and the pro-survival effects of p44/42 (15), only inhibition of JNK abrogated FFA-induced lipoapoptosis. These data link the known contribution of JNK to obesity associated insulin resistance with the lipoapoptosis observed in this syndrome. In this regard JNK activation by FFA may not only contribute to impaired insulin signaling but also contribute to cellular injury. Therefore, JNK would appear to play a pivotal role in obesity related liver injury. Cellular responses to JNK activation are also governed by isoform specific actions. In neurons, JNK3 mediates apoptosis (41) and cell differentiation, depending on context (42). Similarly, in non-neuronal cells, JNK 1 and 2 have pro-apoptotic functions or pro-survival functions in a cell, stimulus and context dependent manner (5,9,43,44). In experimental dietary obesity, JNK1 promotes weight gain and insulin resistance (5) and also promotes bile acid-mediated hepatocyte apoptosis (9). Our data suggest a predominant role for the JNK2 isoform in FFA-mediated lipoapoptosis, as JNK2-/- hepatocytes, but not JNK1-/- hepatocytes were resistant to cell death. Thus, even in hepatocytes, cytotoxicity by JNK isoforms is stimulus-specific.

Mitochondrial release of cytochrome-c is regulated by the pro-apoptotic multidomain members of the Bcl-2 family, Bax and Bak. Bax is necessary for mitochondrially mediated apoptosis depending on cellular context (45,46). For example, in hepatocytes Bax mediates ethanol- and bile salt-induced mitochondrial permeabilization and apoptosis (47,48). Consistent with the crucial role of Bax in hepatocyte apoptosis, stearic acid treatment led to Bax activation and subsequent cytochrome c release. Furthermore, pharmacologic inhibition of JNK prevented both Bax activation and downstream events leading to amelioration of stearic acid induced lipoapoptosis. BH-3 domain proteins are the sensors of cellular stress and are essential for engaging the mitochondrial pathway of apoptosis. To date, only Bim and Bid have been shown to directly activate Bax (33). As Bid was not activated in our model, we focused on Bim. FFA-induced JNK activation was linked mechanistically to the mitochondrial pathway of apoptosis by Bim induction. Indeed, we observed an early and sustained increase in cellular Bim protein levels following treatment of HepG2 cells with stearic acid and amelioration of apoptosis in Bim silenced cells. These observations are consistent with data from other models where JNK-dependent Bim induction as well as phosphorylation with downstream activation of Bax mediated apoptosis has been reported (8,19,20,49). Thus, the current data support a model where JNK induces hepatocyte lipoapoptosis via a, Bim-mediated, Bax-dependent mitochondrial pathway of cell death.

Hepatocyte lipoapoptosis is a significant public health issue given the current obesity epidemic and the relationship between apoptosis
and liver disease in this syndrome (3). Our cellular model is relevant to this syndrome as toxic FFA are thought to contribute to hepatocyte lipoapoptosis in man. Our data indicate that FFA cause sustained JNK activation leading to engagement of the core mitochondrial pro-apoptotic machinery with Bim-mediated Bax activation. These observations identify JNK as a potential therapeutic target to ameliorate FFA cytotoxicity, especially the employment of JNK inhibitors (50).
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FIGURE LEGENDS

**Figure 1:** Characterization of FFA induced intracellular steatosis. Nile red staining was performed on HepG2 cells treated with 200 µM each, saturated fatty acids, palmitic acid and stearic acid, and monounsaturated fatty acids, oleic acid and palmitoleic acid for 18 hours. (A) Representative fluorescent photo-micrographs (X 10) are shown. (B) Cellular steatosis was quantified in 4 random low power fields for each condition with automated software. The percent of fat (fat area /field area x 100) of digital photomicrographs was calculated. Each individual fatty acid induced cellular steatosis and this was statistically significant.

**Figure 2:** FFA treated cells undergo apoptosis. HepG2, primary mouse hepatocytes (PMH), and MRH 7777 cells were each treated with 200 µM FFA for 24 hours (except where indicated otherwise). Oleic acid (●), palmitoleic acid (□) palmitic acid (△), and stearic acid (○) were used to treat cells. Except Fig. D all data are from HepG2 cells. DAPI stained apoptotic nuclei were quantitated using fluorescence microscopy. Four hundred random cells were counted for each condition. Each experiment was done in triplicate. Data are mean ± SEM. (A) FFA toxicity in HepG2 cells is time-dependent. (B) FFA toxicity in HepG2 cells is concentration-dependent. (C) Saturated fatty acids, palmitic acid and stearic acid, are more apoptotic than monounsaturated fatty acids, oleic acid and palmitoleic acid in HepG2 cells. (D) Saturated fatty acids, palmitic acid and stearic acid, are more apoptotic than monounsaturated fatty acid oleic acid in PMH and MRH7777 cells. (E) FFA treated HepG2 cells demonstrate biochemical activation of caspase 3/7. Greater caspase activation is observed with saturated fatty acids. (F) Pan-caspase inhibitor, zVAD-fmk, effectively reduces FFA toxicity in a concentration-dependent fashion.
**Figure 3:** FFA treatment leads to mitochondrial dysfunction and release of cytochrome c. HepG2 cells were treated with oleic acid, palmitic acid and stearic acid, 200 µM each, for 18 hours. (A) Immunofluorescence for cytochrome c was performed (original magnification, X 100). Control cells demonstrate distinct punctate staining consistent with mitochondrial compartmentation of cytochrome c. FFA treatment leads to release of cytochrome c, demonstrated by diffuse cytoplasmic staining. (B) Cytochrome c release was quantitated by counting the number of diffuse cells in each condition. One hundred random cells were counted. (C) Cytosolic fractions prepared from FFA treated cells was analyzed by SDS-PAGE. Oleic acid (OA), palmitic acid (PA) and stearic acid (SA) treatment led to release of mitochondrial cytochrome c into the cytosol. Absence of mitochondrial contamination of cytosol was demonstrated by probing for cytochrome c oxidase II (Cyto. Oxidase), which was present in mitochondrial fractions (not shown) and absent in cytosolic fraction. Actin served as a control for protein loading.

**Figure 4:** FFA apoptosis does not occur via death receptor pathway or lysosomal pathway of apoptosis. (A) Western blot analysis was performed for Bid cleavage using whole cell lysates from HepG2 cells treated with 200 µM oleic acid, palmitic acid and stearic acid at time points shown in the figure. TRAIL and actinomycin D (ActD) were used to verify that this pathway is intact in HepG2 cells. Fatty acid treatment did not cleave Bid into the active c-terminal peptide tBid., whereas, predictably, TRAIL + ActD were effective in activating this pathway. (B) Lysosomal dependence was assessed using mice deficient in cathepsin B. Cellular apoptosis was assessed by DAPI staining. 400 total cells were counted for each condition. Data are the mean from 3 experiments, each done in triplicate. Percent cell death is shown on the Y axis (error bars are SEM). Fatty acid treatment (on X axis), 200 µM each, was for 24 hours. There is no reduction in free fatty acid mediated toxicity in hepatocytes from Cathepsin B knock out mice.

**Figure 5:** Oleic acid and stearic acid treatment lead to JNK activation. HepG2 cells and primary mouse hepatocytes were treated with 200 µM oleic acid and stearic acid for the indicated times. Whole cell lysates were analyzed for protein expression using antibodies specific to total and active (phospho) members of the MAP Kinase family members. γ-tubulin was used as a control for protein loading for Figures A and B. (A) Primary mouse hepatocytes were treated with stearic acid show a robust activation of JNK. An increase in phospho-JNK levels was not observed by immunoblot analysis in oleic acid treated cells (though by quantitative ELISA, there was a slight [< 2fold] increase). (B) Stearic acid treatment led to marked JNK activation in HepG2 cells and oleic acid-induced JNK activation was not as marked. There was no associated c-jun and p38 MAP Kinase activation. P44/42 was activated, but to a lesser extent. (C) Primary mouse hepatocytes and HepG2 cells were treated with 200 µM oleic acid and stearic acid for the indicated times. ELISA for total and phospho-JNK was performed using the FACE JNK ELISA Kit. Fold change in phospho-JNK activity relative to total-JNK activity is shown. In both cell types, stearic acid lead to an early and sustained increase in phospho-JNK activity.

**Figure 6:** JNK inhibition or absence confers resistance to FFA mediated apoptosis. HepG2 cells were treated with FFA in the presence of pharmacologic JNK inhibitors. Hepatocytes from JNK2--mice, and JNK1-- mice were treated with FFA. Following 24 hours of treatment, cell death was assessed morphologically. DAPI stained apoptotic nuclei were quantitated using fluorescence microscopy. Four hundred random cells were counted for each condition. Each experiment was done in triplicate. Data are mean ± SEM. (A) Pharmacologic inhibition of JNK activity in HepG2 cells by SP600125 and a small peptide inhibitor of JNK (JNKI) abrogated FFA toxicity. (B) Stearic acid cytotoxicity was significantly reduced in JNK2--hepatocytes. (C) The absence of JNK1-- did not prevent FFA toxicity in primary mouse hepatocytes.
**Figure 7:** JNK leads to lipoapoptosis via direct activation of Bax. (A) HepG2 cells were examined by immunofluorescence microscopy for activation of Bax and following treatment with 200 µM oleic acid, palmitic acid and stearic acid. Staurosporine treated cells were used as positive control. The top panel shows representative photo-micrographs of negative and positive controls. The bottom panel shows that FFA treatment led to bax activation, this effect was greater with palmitic acid and stearic acid treatment than with oleic acid treatment. (B) HepG2 cells were examined by immunofluorescence microscopy for activation of Bax following treatment with 200 µM stearic acid and palmitic acid in the presence of SP600125 and JNK peptide inhibitor. The top panel confirms that oleic acid did not lead to significant Bax activation. The middle panel shows that stearic acid activated Bax in a JNK-dependent manner. The bottom panel shows that palmitic acid activated Bax in a JNK-dependent manner. (C) HepG2 cells were examined by immunofluorescence microscopy for cytochrome c release following treatment with 200 µM stearic acid in the presence of SP600125 and JNK peptide inhibitor (JNK PI). Cytochrome c release was quantitated by counting the number of diffuse cells in each condition. One hundred random cells were counted. JNK inhibition reduced stearic acid-induced cytochrome c release.

**Figure 8:** Stearic acid toxicity is Bim dependent. (A) HepG2 cells were treated with 200 µM oleic acid and stearic acid for the indicated time. Whole cell lysates were analyzed for Bim protein expression. γ-tubulin was used as a control for protein loading. Stearic acid treatment led to an early and sustained increase in Bim levels. (B) Quantitative densitometry was performed on 4 independent Bim immunoblots from HepG2 cells treated with oleic acid (200 µM, 6 hours) and stearic acid (200 µM, 6 hours). Bim-EL expression was normalized to γ-tubulin expression. Data are expressed as fold change compared to controls. A significant increase in Bim-EL was observed in stearic acid treated cells. (C) Bim protein expression was silenced in Huh7 cells using siRNA. Western blot for Bim-EL expression is shown in the inset with γ-tubulin as control for protein loading. Stearic acid (SA) induced lipoapoptosis was reduced > 50% by Bim gene silencing in Huh 7 cells. Oleic acid (OA) toxicity was minimal and slightly reduced in siBim treated cells. (D) Activated Bax was detected by immunofluorescence microscopy in HepG2 (shown here) and Huh7 cells (not shown). Following targeted knockdown of Bim expression, stearic acid induced Bax activation was abrogated in both cell lines. (E) Stearic acid induced Bax activation in Bim silenced cells was quantitated by manual counting of four or more random confocal photomicrographs. Bax activation in stearic acid treated cells was Bim dependent.
Figure 1

A

Control  Oleic acid  Palmitic acid

Palmitoleic acid  Stearic acid

B

Intracellular steatosis (% area)

Control  Oleic acid  Palmitic acid  Palmitoleic acid  Stearic acid

*p<0.01
Figure 2

A. Apoptosis (%) over time with different fatty acids: Stearic acid, Palmitic acid, Palmitoleic acid, and Oleic acid.

B. Apoptosis (%) as a function of fatty acid concentration: Stearic acid, Palmitic acid, Palmitoleic acid, and Oleic acid.

C. Apoptosis (%) for different fatty acids: Control, Oleic acid, Palmitic acid, Palmitoleic acid, and Stearic acid.

D. Apoptosis (%) for Primary Mouse Hepatocytes and MRH 7777 cells: Oleic acid, Palmitic acid, Palmitoleic acid, and Stearic acid.

E. Caspase 3/7 activity (Fold increase) for different fatty acids: Control, Oleic acid, Palmitic acid, and Stearic acid.

F. Apoptosis (%) as a function of ZVAD-fmk concentration: Stearic acid, Palmitic acid, Palmitoleic acid, and Oleic acid.
Figure 3

A

Control

Oleic acid

Palmitic acid

Stearic acid

B

Diffuse cells (%)

0 20 40 60 80 100

Control Oleic acid Palmitic acid Stearic acid

*p<0.01

C

Cytochrome c

Cyto. Oxidase

Actin

Ctrl OA PA SA
Figure 5

A

Primary mouse hepatocytes

| Time (hr) | Control | Oleic acid | Stearic acid |
|-----------|---------|------------|--------------|
| 0         |         | 3          | 6            |
| 3         |         |            |              |
| 6         |         |            |              |

Phospho JNK

JNK

γ-tubulin

B

HepG2 cells

| Total Protein | Phospho Protein |
|---------------|-----------------|
| Time (hr)     | Control | OA | SA | Control | OA | SA |
| 0             |         | 3  | 6  |         | 3  | 6  |
| 3             |         |    |    |         | 3  |    |
| 6             |         |    |    |         | 6  |    |

JNK

p38 MAPK

ERK

γ-tubulin

C

Primary mouse hepatocytes

Control 3 hrs 6 hrs 24 hrs

Phospho-JNK (fold increase)

HepG2

Control 3 hrs 6 hrs 24 hrs

Phospho-JNK (fold increase)
Figure 6

A

Hep G2

- JNK peptide Inhibitor
- SP600125

* p<0.05

Control, Oleic acid, Palmitic acid, Stearic acid

B

Mouse Hepatocytes

- Wild Type
- JNK2-/-

* p<0.05

Control, Oleic acid, Palmitic acid, Stearic acid

C

Mouse Hepatocytes

- Wild Type
- JNK1-/-

Control, Oleic acid, Palmitic acid, Stearic acid
Figure 7

C

![Bar chart showing the percentage of diffuse cells.]

- Control
- Stearic acid
- SP600125
- JNK PI + stearic acid
- JNK PI + stearic acid
Figure 8

A

| Time (hrs) | Control | Oleic acid | Stearic acid |
|-----------|---------|------------|--------------|
| 0         | 2       | 4          | 6            | 24           |
| Bim-EL    |         |            |              |
| Bim-L     |         |            |              |
| γ-tubulin |         |            |              |

B

Relative Bim expression (fold increase over control)

Control | Oleic acid | Stearic acid

*p<0.05

C

Apoptosis (%)

Control | SA | OA | Control | SA | OA | Control | SA | OA | Scrambled siRNA

No siRNA | Bim siRNA | Scrambled siRNA

* p<0.001

* p<0.001
