Bax inhibition protects against free fatty acid-induced lysosomal permeabilization

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smal permeabilization is a key feature of hepatocyte lipotoxicity, yet the mechanisms mediating this critical cellular event are unclear. This study examined the mechanisms involved in free fatty acid (FFA)-induced lysosomal permeabilization and the role of Bax, a Bcl-2 family member, in this event. Exposure of liver cells to palmitate induced Bax activation and translocation to lysosomes. Studies to suppress Bax activation either by pharmacological approaches or small interfering-RNA-mediated inhibition of Bax expression showed that lysosomal permeabilization is Bax dependent. In addition, palmitate treatment resulted in a significant decrease in Bcl-XL, a Bax antagonist. Moreover, forced Bcl-XL expression blocked lysosomal permeabilization. Lysosomal permeabilization by FFA was ceramide and caspase independent. Finally, paradigms that inhibit lysosomal permeabilization also reduced apoptosis. In conclusion, these data strongly support a regulatory role for Bax in FFA-mediated lysosomal permeabilization and subsequent cell death.

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OBESITY AND TYPE 2 DIABETES have reached epidemic proportions in most of the Western world (14). Both conditions are strongly associated with nonalcoholic fatty liver disease (NAFLD), an increasingly recognized form of chronic liver disease in both adults and children that can progress to end-stage liver disease (2, 5, 9, 37). The clinicopathological picture resembles that of alcohol-induced liver injury but occurs in persons who consume little or no alcohol (4). Nonalcoholic steatohepatitis (NASH) represents a stage within the spectrum of NAFLD and is characterized by the accumulation of fat in the liver (steato-
sis) along with inflammation and different degrees of scarring or fibrosis (16, 23). NASH is a potentially serious condition, as the mechanisms of lysosomal permeabilization in lipotoxicity are of significant biomedical importance, given the role of this process in liver injury in NAFLD.

The overall objective of this study was to identify the mechanisms by which FFA causes lysosomal permeabilization and, in particular, to define the role of Bcl-2 family members in this process. To address this objective, we formulated the following questions: 1) Does FFA induce Bax activation upstream of lysosomes? 2) Is lysosomal permeabilization dependent on Bax translocation? 3) Do anti-apoptotic Bcl-2 members prevent FFA-induced lysosomal permeabilization? 4) Do caspase activation and/or de novo ceramide synthesis contribute to FFA-induced lysosomal permeabilization? For these studies, we employed an in vitro cell model of hepatocyte steatosis. Our results implicate Bax in FFA-induced lysosomal permeabilization.

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destabilization and ctsb redistribution to the cytosol. These observations have direct implications for attenuation of liver injury and disease progression in NAFLD and provide further insight into FFA cytotoxic signaling in liver cells.

MATERIALS AND METHODS

Cell lines and culture. Mouse hepatocytes were isolated from wild-type C57BL/6 male mice (Jackson Laboratories, Bar Harbor, ME), purified by Percoll gradient centrifugation, cultured as described by us in detail (13), and used 4 h after isolation. A human, well-differentiated hepatoblastoma cell line, HepG2, and a rat hepatoma cell line, McNtcp.24, were cultured as previously described (11, 36). Previous studies from our laboratory showed that these cells are sensitive to FFA-induced lysosomal permeabilization in a dose- and time-dependent manner with kinetics comparable to those observed in primary mouse hepatocytes (13). Cultured cells and primary mouse hepatocytes were incubated with 0.05–0.5 mM saturated FFA (palmitate; Sigma, St. Louis, MO), which covers the physiological to pathological range (31, 39), in media containing 1% BSA. We have previously demonstrated that incubation of liver cells with FFA results in significant steatosis as measured by Nile red staining (13). In selective experiments, cells were incubated with the fatty acid mixture in the presence or absence of 50–200 μM of the Bax inhibitor (CN 196810; EMD Biosciences, La Jolla, CA), a cell-permeable pentapeptide based on the Ku70-Bax inhibiting domain that interacts with Bax and prevents its conformational change (28, 38), the pan-caspase inhibitor zVAD-fmk (10–20 μM; Enzyme Systems Products, Livermore, CA), or the ceramide synthase inhibitor fumonisin B1 (50 μM; Sigma) for up to 24 h. The ctsb-green fluorescent protein (ctsbgFP) expression vector was transfected into HepG2 cells using Lipofectamine (13). Confocal microscopy was performed with an inverted Zeiss laser-scanning confocal microscope (Zeiss LSM 510; Carl Zeiss, Thornwood, NJ). Cells were counted in 30 random microscopic fields (∼400) as previously described by us in detail (10, 11).

Small interfering-RNA and transfection. We obtained the human Bax mRNA sequence from the National Center for Biotechnology Information Entrez nucleotide database. The sequence was designed and synthesized using software (available online from http://www.ambion.com) and a Silencer siRNA construction kit from Ambion (Austin, TX) according to the manufacturer’s instructions. The sequence of the double-stranded RNA used was AACCATCATGGGCTGGACATT. The target sequence was searched with National Information Entrez nucleotide database. The sequence was designed by us in detail (10, 11).

Immunoblot analysis. Whole-cell lysates were prepared by sonication in a digestion buffer containing 1% Nonidet P-40, 50 mM HEPES (pH 7.4), 150 mM NaCl, 0.5 mM EDTA, 25 mM β-glycerophosphate, 1 mM Na3VO4, and 1 mM sodium fluoride. After centrifugation, supernatants were mixed with sample buffer containing 10% SDS, 20% glycerol, 50 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol, and 0.1% bromophenol blue, and heated for 5 min at 95°C. Proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with appropriate primary antibodies. The membrane was stained by 10.220.33.2 on November 7, 2017 http://ajpgi.physiology.org/ Downloaded from

LysoTracker Red loading and immunofluorescence. Cells were loaded with LysoTracker Red (DND-99; Molecular Probes, Eugene, OR), a fluorescent dye that loads predominantly into lysosomes, by incubating the cells in probe-containing medium at a final concentration of 50 nM for 30 min at 37°C. Alternatively, cells were fixed with freshly made 4% paraformaldehyde in PBS for 15 min at 4°C. Immunofluorescence detection of the active “apoptotic” conformation of Bax was performed by using a monoclonal antibody (MAb 6A7; BD Biosciences) that selectively binds to an NH2-terminal epitope that is exposed only after Bax activation (18, 19). Cells were then imaged by using an inverted laser-scanning confocal microscope (model LSM S10; Carl Zeiss). The percentage of cells displaying positive staining was counted in 30 random fields as previously described by us in detail (10).

Generation of McNtcp.24 cells overexpressing Bcl-XL. Plasmid constructs for Bcl-XL expressing the hygromycin-resistance gene were kindly provided by Dr. Jennifer Pietenpol (Vanderbilt Cancer Center, Nashville, TN). McNtcp.24 cells, a rat hepatoma cell line, were stably transfected with Bcl-XL. DNA transfections were performed when cells were ∼40% confluent. This cell line was selected because of its high transfection efficiency (∼70% with GFP) (27). The cells were transfected by addition of 20 μg of the DNA plasmid to each 100-mm petri dish. Serum-free medium containing a 1:9 ratio of DNA-single cationic lipid (Perfect transfection kit; Invitrogen) was used, and cells were incubated in the mixture for 8 h. Next, transfected cells were cultured in serum-supplemented medium for 36 h. After recovery from transfection, cells were grown in selective medium containing 200 μg/ml of hygromycin B (Gibco, Gaithersburg, MD). The antibiotic was added every 3 days. A culture dish with nontransfected McNtcp.24 cells treated with hygromycin B was used as a control for hygromycin selection. After 3 wk of transfection, resistant clones were isolated using cloning cylinders (Belco Glass, Vineland, NJ) and transferred for expansion and analysis. Bcl-XL expression was assessed by immunoblot analysis. Stable transfectants were grown in medium containing 100 μg/ml of hygromycin B.

Apoptosis quantification. Apoptosis in cell culture was quantified by assessing the characteristic nuclear changes of apoptosis (i.e., chromatin condensation and nuclear fragmentation) using the nuclear binding dye 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes) and fluorescence microscopy (11).

Immunoblot analysis. Immunoblot analysis was performed using whole-cell lysates as previously described (11). Samples were resolved by 12% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with appropriate primary antibodies. The membrane was incubated with peroxidase-conjugated secondary antibodies (1:10,000 dilution; Biosource International, Camarillo, CA), and the bound antibody was visualized using a chemiluminescent substrate (ECL, Amersham, IL) and Kodak X-OMAT film (Eastman Kodak, Rochester, NY). Primary antibodies were rabbit polyclonal Bax antibody (1:500 dilution; BD PharMingen), mouse monoclonal Bax antibody (1:200 dilution MAb 6A7; BD Biosciences), mouse monoclonal Bcl-XL antibody (1:200 dilution; BD PharMingen), and goat anti-β-actin (1:2,000).

Data analysis. All data are expressed as the mean with SD in parentheses unless otherwise indicated. Differences between groups were compared by an ANOVA analysis followed by a post hoc Bonferroni test to correct for multiple comparisons. Differences were considered to be statistically significant at P < 0.05.

RESULTS

Does palmitate induce Bax activation upstream of lysosomes? We first determined whether the cytotoxic FFA palmitate induces Bax activation and, if so, whether this occurs independent of ctsb activation. Isolated hepatocytes from C57BL/6 male mice were incubated with 0.2 mM palmitate in media with serum albumin as a carrier in the presence or
absence of the ctsb-selective inhibitor E-64 for 3 h. At the end of incubation, cells were fixed and subjected to immunofluorescence staining for active Bax by using a conformation-specific anti-Bax monoclonal antibody (6A7) that selectively binds to an NH$_2$-terminal epitope which is exposed only after Bax activation (18). Active Bax was not identified in untreated cells but was readily detectable in cells after 3 h of treatment with palmitate (Fig. 1A). Similar results were obtained by incubating, in the same manner, HepG2 cells, a human, well-differentiated hepatoblastoma cell line, and McNtcp.24 cells, a rat hepatoma cell line (data not shown). Pretreatment with the ctsb inhibitor E-64 did not inhibit Bax conformational changes (Fig. 1A). These data suggest that palmitate induces Bax activation in liver cells independent of ctsb. Next, we assessed the cellular localization of active Bax following palmitate treatment. For this, HepG2 cells were incubated in the presence or absence of palmitate for up to 6 h and loaded with LysoTracker Red for another 30 min. Cells were then fixed and subjected to immunofluorescence detection of active Bax (Fig. 1B). Overlay images demonstrated that during palmitate treatment, active Bax fluorescence significantly overlapped with LysoTracker Red, suggesting colocalization with lysosomes (Fig. 1B). These results suggest that during FFA treatment, active Bax localizes at least in part to lysosomes.

Is lysosomal permeabilization dependent on Bax translocation? If translocation of Bax to lysosomes is sufficient and/or necessary to induce lysosomal permeabilization and release of ctsb into the cytosol, then inhibition of Bax activation should block FFA-induced lysosomal permeabilization. To test this hypothesis, we used two different approaches. In the first approach, siRNA was used to silence Bax expression in HepG2 cells. By this approach, we were able to reduce Bax expression to background levels, as assessed by immunoblot analysis (Fig. 2A). Untreated cells displayed punctuate fluorescence consis-

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**Fig. 1.** Bax, a Bcl-2 family member, is activated upstream of cathepsin B (Cat B) and localizes in lysosomes during palmitate treatment. Isolated hepatocytes from C57BL/6 male mice were incubated with 0.2 mM palmitate in media with serum albumin as a carrier in the presence or absence of the Cat B selective inhibitor E-64 for 3 h. Cells were then fixed and subjected to immunofluorescence detection of active Bax by using a monoclonal antibody (MAb 6A7; BD Biosciences) that selectively binds to an NH$_2$-terminal epitope that is exposed only after Bax activation. A: cells were then imaged by using an inverted laser-scanning confocal microscope. Palmitate treatment resulted in significant Bax activation regardless of the presence or absence of the Cat B selective inhibitor (inh). B: to assess the cellular localization of active Bax. HepG2 cells were loaded with LysoTracker Red (DND-99, Molecular Probes, Eugene, OR), a fluorescent dye that loads predominantly into lysosomes, for 30 min at 37°C. Cells were then fixed, subjected to immunofluorescence detection of active Bax, and imaged by confocal microscopy as described above. Following palmitate treatment, active Bax fluorescence overlaps in part with LysoTracker Red, suggesting colocalization. FFA, free fatty acid.
tent with localization of LysoTracker Red in lysosomes (Fig. 2B). In contrast, cells treated with palmitate manifested diffuse fluorescence consistent with lysosomal permeabilization and redistribution of the dye to the cytosol (Fig. 2B). Cells transfected with the Bax siRNA displayed predominantly punctuate fluorescence despite treatment with palmitate, indicating that this maneuver prevented lysosomal permeabilization (Fig. 2B). Quantification of the number of cells displaying a diffuse vs. punctuate pattern of fluorescence demonstrated a dramatic reduction in cells with diffuse fluorescence in the Bax siRNA-transfected cells compared with cells treated with palmitate alone (Fig. 2C). Consistent with their ability to prevent lysosomal permeabilization, palmitate-induced apoptosis was also markedly decreased by transfection with Bax siRNA compared with cells treated with FFA alone (Fig. 3). In our second approach, cells were transfected with a ctsb-GFP construct. Forty-eight hours after transfection, cells were pretreated with 200 μM of a selective Bax inhibitor for an additional 30 min, followed by incubation with or without 0.2 mM palmitate in media containing 1% BSA. Untreated cells transfected with ctsb-GFP displayed punctuate fluorescence consistent with lysosomal localization (Fig. 4A). In contrast, cells transfected with ctsb-GFP and treated with palmitate manifested diffuse fluorescence consistent with a redistribution of the protease from lysosomes to the cytosol (Fig. 4A). Moreover, cells preincubated with the Bax inhibitor followed by treatment with palmitate displayed predominantly a punctate fluorescent pattern (Fig. 4, A and B). The decrease in lysosomal permeabilization,

Fig. 2. Genetic inhibition of Bax prevents FFA-induced lysosomal permeabilization. Bax expression in HepG2 cells was silenced by small interfering-RNA (siRNA) as described in MATERIALS AND METHODS. After transfection of HepG2 cells with Bax siRNA, an immunoblot for Bax and β-actin was performed (A). Following 48 h of transfection with the Bax siRNA constructs, cells were loaded with LysoTracker Red (LTR) for 30 min at 37°C and incubated with 0.2 mM palmitate in media with BSA for 24 h. B: cells were then imaged by confocal microscopy. C: cells were scored as either having a predominantly punctuate or diffuse fluorescent pattern. Bax inhibition significantly reduced palmitate-initiated lysosomal permeabilization (*P < 0.01 vs. palmitate-treated cells).

Fig. 3. Bax inhibition reduces palmitate-induced apoptosis. Bax expression in HepG2 cells was silenced by siRNA as described in MATERIALS AND METHODS. After transfection of HepG2 cells with Bax siRNA, cells were incubated with 0.2 mM palmitate in media with BSA for 24 h. A: apoptosis was quantified by using the DNA-binding dye 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) and fluorescence microscopy. Bax inhibition significantly reduced palmitate-associated liver cell apoptosis (*P < 0.001 vs. palmitate-treated cells).
cathepsin B release, and apoptosis caused by either genetic or pharmacological inhibition of Bax strongly suggest that FFA-induced lysosomal permeabilization is Bax dependent.

Do anti-apoptotic Bcl-2 members prevent FFA-induced lysosomal permeabilization? Although hepatocytes do not express Bcl-2, they richly express Bcl-XL, an anti-apoptotic member of the Bcl-2 family which antagonizes Bax activity. Next, we assessed the effect of palmitate on Bcl-XL protein expression to find that palmitate induced a significant decrease in the amount of Bcl-XL protein after 6 h of incubation. In contrast, there was no change in expression of Bax (Fig. 5). Thus palmitate exposure results in specific loss of Bcl-XL. To ascertain whether a loss of cellular Bcl-XL is necessary for FFA-induced lysosomal permeabilization and apoptosis, we established a stable McNtcp rat hepatoma cell line overexpressing Bcl-XL. As assessed by immunoblot analysis, this stably transfected cell line demonstrated a significant increase in Bcl-XL protein content compared with untransfected cells (Fig. 6A). Bcl-XL-overexpressing cells were markedly resistant to palmitate-induced lysosomal permeabilization (Fig. 6, B and C). FFA-induced apoptosis was also significantly decreased in Bcl-XL-overexpressing cells (Fig. 7). Indeed, palmitate-induced apoptosis was 42% (SD 2) in wild-type cells but was only 7% (SD 3) in Bcl-XL-transfected cells (P < 0.01). These data suggest that the loss of cellular Bcl-XL observed during palmitate treatment is a requisite for its lysosomal permeabilization and subsequent cell death.

Does caspase activation and/or de novo ceramide synthesis contribute to FFA-induced lysosomal permeabilization? Caspase activation and de novo ceramide synthesis from FFA surplus have been implicated in lipotoxicity in nonhepatic cell lines (31, 32). Also, both caspase activation and ceramide and its metabolite sphingosine have been shown to be involved in an intracellular signaling cascade resulting in lysosomal permeabilization (17, 35, 36). To address whether FFA-induced lysosomal permeabilization involves caspase activation and/or ceramide, we incubated FFA in the absence or presence of a pan-caspase inhibitor z-VAD-fmk (20 μM) or 50 μM of a ceramide synthase inhibitor (fumonisin B1). Cells were then loaded with LysoTracker Red and imaged by fluorescence microscopy. Both inhibition of caspase activation and ceramide synthesis failed to protect against FFA-induced lysosomal permeabilization with these cells displaying a pattern of diffuse LysoTracker Red fluorescence similar to those treated with FFA alone (Fig. 8). These results suggest FFA-induced
lysosomal permeabilization is independent of caspase activation and de novo ceramide biosynthesis.

**DISCUSSION**

The principal findings of this study relate to the mechanisms involved in FFA cytotoxic signaling through lysosomes. The results demonstrate that during exposure of a human or rat hepatoma cell line to the long-chain saturated fatty acid palmitate, 1) Bax is activated upstream of lysosomes; 2) lysosomal permeabilization and redistribution of ctsb into the cytosol is blocked by both genetic and pharmacological inhibition of Bax; 3) inhibition of lysosomal permeabilization by these maneuvers also is associated with a reduction in cellular apoptosis; 4) FFA treatment of liver cells is associated with decreased Bcl-XL protein expression; and 5) overexpression of Bcl-XL prevents FFA-induced lysosomal permeabilization. These results strongly support a regulatory role for Bcl-2 proteins in the FFA-induced lysosomal permeabilization process.

Net accumulation of lipids in the liver (steatosis) is a prerequisite for the development of NAFLD (8). Although the mechanisms responsible for disease progression from simple steatosis to steatohepatitis to cirrhosis are poorly understood, FFAs may play a role in the pathogenesis and progression of NAFLD (7). Not only are circulating FFAs increased in patients with NAFLD, but their levels also correlate with disease severity (26). FFAs are potentially cytotoxic, and in vitro studies have implicated increased cellular FFA levels as a trigger of apoptotic cell death (29, 34).
have demonstrated that incubation of liver cells with FFA results in lysosomal membrane permeabilization and release of ctsb, a lysosomal protease and a specific mediator of apoptosis, into the cytosol (13). Furthermore, we were able to show that the lysosomal permeabilization process is preceded by cytosol-to-lysosome translocation of Bax, a proapoptotic member of the Bcl-2 family that induces channel formation in membranes (13). Our current data extend these observations by defining the mechanisms of lysosomal permeabilization induced by palmitate and in particular the role of Bax and other Bcl-2 members in this modification of organelle function. Consistent with prior observations that during FFA treatment of liver cells, Bax translocates to lysosomes preceding lysosomal permeabilization, this study shows palmitate induces Bax conformational activation, which occurs regardless of the presence or absence of a ctsb-selective inhibitor, suggesting Bax activation is ctsb independent. More importantly, Bax silencing by siRNA, or its pharmacological inhibition, significantly prevented FFA-induced lysosomal permeabilization, placing Bax upstream of ctsb activation and proving Bax is essential for the lysosomal permeabilization process. The precise mechanisms by which palmitate induces Bax activation will require further investigation but is not specific to palmitate, because other long-chain FFA (e.g., stearate) have a similar effect, and blocking palmitoylation of proteins by means of using a palmitoylation inhibitor (4-bromopalmitate) does not prevent it (data not shown).

Bcl-2 and its close homolog Bcl-X_L are anti-apoptotic members of the Bcl-2 family that are known to antagonize Bax channel formation activity (6). To address the possible role of these anti-apoptotic members of the Bcl-2 family in FFA-induced lysosomal permeabilization, we first measured Bcl-X_L protein expression. Palmitate treatment significantly decreased the amount of the anti-apoptotic protein Bcl-X_L. Conversely, we demonstrated that enforced expression of Bcl-X_L prevented palmitate-induced lysosomal permeabilization. Taken together, these data strongly suggest a regulatory role for Bax-Bcl-X_L in FFA-induced lysosomal permeabilization.

Accumulating evidence suggests a central role of lysosomes in apoptotic cell death (15). Lysosomal permeabilization appears to be an early event that precedes other features characteristic of the cell death process (15). Our previous work suggests that lysosomal permeabilization followed by redistribution of ctsb to the cytosol may play an important role in lipid-induced hepatotoxicity and disease progression in NAFLD (13). Consistent with our prior observation, we have shown in the present study that maneuvers preventing lysosomal permeabilization also markedly reduced apoptotic cell death. The mechanisms by which lysosomal permeabilization and ctsb activation may result in hepatocyte apoptosis during FFA signaling will require further study.

In summary, the current studies further elucidate the subcellular mechanisms responsible for activation of the lysosomal pathway of apoptosis during FFA signaling. The results support a model in which FFA induces lysosomal breakdown via an alteration in the ratio of proapoptotic and anti-apoptotic Bcl-2 family members. Targeting Bax or its natural inhibitor Bcl-X_L would appear to be a viable strategy to prevent FFA-induced lysosomal permeabilization and hepatocyte lipotoxicity.

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