Cathepsin B inactivation attenuates hepatocyte apoptosis and liver damage in steatotic livers after cold ischemia-warm reperfusion injury

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Baskin-Bey, E. S., A. Canbay, S. F. Bronk, N. Werneburg, M. E. Guicciardi, S. L. Nyberg, and G. J. Gores. Cathepsin B inactivation attenuates hepatocyte apoptosis and liver damage in steatotic livers after cold ischemia-warm reperfusion injury. Am J Physiol Gastrointest Liver Physiol 288: G396–G402, 2005. First published October 7, 2004; doi:10.1152/ajpgi.00316.2004.—Hepatic steatosis predisposes the liver to cold ischemia-warm reperfusion (CI/WR) injury by unclear mechanisms. Because hepatic steatosis has recently been associated with a lysosomal pathway of apoptosis, our aim was to determine whether this cell-death pathway contributes to CI/WR injury of steatotic livers. Wild-type and cathepsin B knockout (Ctsb−/−) mice were fed the methionine/choline-deficient (MCD) diet for 2 wk to induce hepatic steatosis. Mouse livers were stored in the University of Wisconsin solution for 24 h at 4°C and reperfused for 1 h at 37°C in vitro. Immunofluorescence analysis of the lysosomal enzymes cathepsin B and D showed a punctated intracellular pattern consistent with lysosomal localization in wild-type mice fed a standard diet after CI/WR injury. In contrast, cathepsin B and D fluorescence became diffuse in livers from wild-type mice fed MCD diet after CI/WR, indicating that lysosomal permeabilization had occurred. Hepatocyte apoptosis was rare in both normal and steatotic livers in the absence of CI/WR injury but increased in wild-type mice fed an MCD diet and subjected to CI/WR injury. In contrast, hepatocyte apoptosis and liver damage were reduced in Ctsb−/− and cathepsin B inhibitor-treated mice fed the MCD diet following CI/WR injury. In conclusion, these findings support a prominent role for the lysosomal pathway of apoptosis in steatotic livers following CI/WR injury.

THE DEMAND FOR LIVER TRANSPLANTATION continues to be in excess of available deceased donor livers. This discrepancy has stimulated research to improve the function of marginal donor livers at risk of initial poor function (IPF) or primary nonfunction (PNF) (3). A prominent risk factor for the development of IPF or PNF is moderate macrovesicular steatosis (2). Moderate macrovesicular steatosis is associated with nonalcoholic fatty liver disease (NAFLD). NAFLD is extremely common in Western societies. In fact, it is estimated that 9–26% of potential donors have NAFLD (22). Because donor livers with NAFLD (>30% macrovesicular steatosis) are not used by many transplant centers, the presence of NAFLD limits the donor pool. Although, moderate macrovesicular steatosis results in enhanced cold ischemia and warm reperfusion (CI/WR) injury (10), the cellular mechanisms underlying CI/WR injury of steatotic livers remain unclear. Insight into the mechanisms mediating CI/WR injury of steatotic livers could help develop rational therapies to attenuate this injury, thereby potentially permitting use of these organs for liver transplantation.

Recently, data from our laboratory have shown that hepatic steatosis is associated with lysosomal permeabilization (9). This lysosomal disruption has been implicated in cellular injury including apoptosis (14) and is mediated by lysosomal proteases released into the cytosol. These lysosomal proteases are a part of a family of proteases referred to as cathepsins. Although, there are eleven known cathepsins in mammalian lysosomes, cathepsin B and D are the most prominent and stable at physiological pH (14). Cathepsins released from lysosomes into the cytosol trigger the mitochondrial pathway of apoptosis and thus are injurious to the liver (15). Despite this information, the role of the lysosomal pathway in hepatic steatosis-associated CI/WR injury is unexplained.

The overall objective of the current study was to examine the role of lysosomal permeabilization in CI/WR injury of steatotic livers. To address this objective, two fundamental questions were formulated. In steatotic livers after CI/WR injury: 1) is lysosomal integrity reduced? And if so, are lysosomal cathepsins B and D released into the cytosol? 2) Does liver cell apoptosis occur? And if so, is it cathepsin B dependent? The results indicate that lysosomal integrity is reduced in steatotic livers after CI/WR injury, allowing for release of cathepsin B and D into the cytosol, which potentiate hepatocyte apoptosis and liver injury. Genetic and pharmacological inactivation of cathepsin B attenuates injury-associated hepatocyte apoptosis. An understanding of these mechanisms may lead to therapeutic strategies to enhance the use of donor livers with NAFLD for transplantation, such as employment of cathepsin B inhibitors.

MATERIALS AND METHODS

Animal models. The care and use of the animals for these studies were reviewed and approved by the Institutional Animal Care and Use Committee. C57/BL wild-type (Jackson laboratories, Bar Harbor, ME) and cathepsin B knockout mice (20–25 g body wt) were fed a methionine/choline deficient diet (MCD; Harland Tech Lab, Madison, WI). This diet rapidly induces steatosis and steatohepatitis in rodents (11). Identical groups of animals received standard rodent chow (n = 7 per group).

Oil red O staining and fat quantification. Cryostat sections of liver were cut (20 μm), air dried, then stained with oil red O as per standard techniques (16). Slides were then viewed under microscopy (Axioplan 2, Carl Zeiss, Oberkochen, Germany). Digital pictures were captured through a video archival system using a digital TV camera system (Axiocam high-resolution color, Carl Zeiss). With an automated software analysis program (KS400), the percent fat (red color in area/field area × 100) of digital photomicrographs were quantified.

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CI/WR of the liver. Wild-type and cathepsin B−/− (Ctsb−/−) mice were anesthetized with pentobarbital sodium (50 mg/kg ip injection). After a midline abdominal incision, the portal vein (PV) was cannulated and 30 ml of University of Wisconsin solution (UW) at 4°C were infused following incision of the inferior vena cava. To permit efficient perfusion of the liver, the liver was removed in block with the PV cannula in place and subsequently stored in a protective plastic apparatus suspended in 40 ml of UW at 4°C for 24 h. The livers were reperfused in a recirculating, isolated, perfusing liver apparatus en- 
apparatus suspended in 40 ml of UW at 4°C for 24 h. The livers were reperfused in a recirculating, isolated, perfusing liver apparatus enclosed in a humidified, temperature-controlled cabinet, as previously described (24). The livers were perfused through the PV catheter with oxygen-saturated Krebs-Ringer-HEPES (KRH) solution at 37°C for 1 h. The portal venous flow was maintained at ~30 ml/min to provide optimal oxygenation in the absence of red blood cells (12).

In vivo loading of lysosomes with fluorescein dextran and assessment of lysosomal integrity. Mice were injected (20 mg/100 g ip) with fluorescein dextran (40,000 MW; Sigma-Aldrich, St. Louis, MO). Twenty-four hours later, the animals were anesthetized, livers were procured, and they were either homogenized directly or stored in UW for 24 h at 4°C and reperfused at 37°C for 1 h as described above. The livers were subsequently homogenized as described previously (1). Briefly, the livers were placed in a Teflon glass Potter-Evihem homogenizer (Curtin Matheson Scientific, Houston, TX), and homogenate buffer (in mM: 70 sucrose, 220 mannitol, 1 EGTA, and 10 HEPES, pH 7.4 at 4°C) was added to make a 10% homogenate solution. The livers were gently homogenized at a speed of 800 rpm using six complete up and down strokes with a wall-mounted, speed-controlled motor and Teflon pestle (Glas-Col, Terre Haute, IN). The homogenate was placed in 50-m1 conical plastic tubes and centrifuged at 600 g for 15 min at 4°C. The supernatant was collected and centrifuged in 2-m1 conical plastic tubes at 13,000 g for 15 min. The pellet was resuspended with KRH solution. Protein quantification was performed using the Bradford assay (Sigma-Aldrich) (25). Fluorescence was measured in the supernatant and the resuspended pellet using a fluorescent spectrophotometer (Sequoia-Turner, model 450, Mountain View, CA) using excitation and emission wavelengths of 490 and 515 nm, respectively. We measured lysosomal integrity by calculating the ratio of the fluorescence of the pellet compared with the supernatant. Because lysosomes are found in the pellet, a high ratio suggests intact lysosomes, and conversely, a low ratio is consistent with disrupted lysosomes.

Histology and electron microscopy. For histological review of hematoxylin and eosin (H&E) liver sections, the liver was diced into 5 × 5-mm sections, fixed in 4% paraformaldehyde for 48 h, and then embedded in paraffin (Curtin Matheson Scientific, Houston, TX). Tissue sections (4 μm) were prepared with a microtome (Reichert Scientific Instruments, Buffalo, NY) and placed on glass slides. H&E staining was performed according to standard techniques. For transmission electron microscopic (TEM) review, the livers were fixed in 1% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2, at 4°C for 15 min. The livers were then rinsed for 30 min in three changes of 0.1 M phosphate buffer, followed by 1 h in post-fix, in phosphate-buffered 1% OsO4. After being rinsed three times with distilled water for 30 min, the livers were stained with 2% uranyl acetate for 30 min at 60°C. Next, the livers were rinsed again in distilled water, dehydrated in progressive concentrations of ethanol followed by 100% propylene oxide, and embedded in Spurr’s resin. Sections (90 nm) were cut on an LKB Ultratome (Mager Scientific, Dexter, MI), placed on 200-nm mesh copper grids, and stained with lead citrate. Electron photomicrographs were taken (JEOL, model 12000, Peabody, MA) at 60 kV.

Immunofluorescence. Paraffin-embedded liver tissue was cut, deparaffinized, and hydrated as previously described by us in detail (5). The primary antibody used was rabbit polyclonal antibody (Neo-Markers, Fremont, CA) for cathepsins B and D, which are predicated by the manufacturer for staining formalin-fixed, paraffin-embedded tissues. The secondary antibody used was FITC anti-rabbit IgG (NeoMarkers, Fremont, CA).

Terminal transferase-mediated dUTP nick end-labeling assay and immunofluorescence detection of activated caspases 3/7. Terminal transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed using a commercially available kit, following the manufacturer’s instructions (In Situ Cell Death Detection Kit; Roche Diagnostics, Indianapolis, IN). Hepatocyte apoptosis in liver sections was quantitated by counting the number of TUNEL-positive cells in 30 random microscopic high-power fields (×100). Immunofluorescence analysis for activated caspase 3/7 was performed using a rabbit anti-caspase polyclonal antibody as previously described in detail (24). Briefly, liver tissue was paraaffin embedded and cut, deparaffinized, and hydrated. The samples were incubated with primary antibody at a dilution of 1:50 for 2 h. Specimens were incubated with the secondary antibody (FITC goat anti-rabbit IgG; Molecular Probes, Eugene, OR) at a dilution of 1:100 for 1 h. The samples were mounted and viewed by a fluorescent microscope using excitation and emission wavelengths of 490 and 515 nm, respectively.

Pharmacological cathepsin B inhibition. R-3032 obtained from Celera Genomics (South San Francisco, CA) is a reversible cathepsin B inhibitor. The dose was based on preliminary data demonstrating that R-3032 extracellular concentrations of 10 μM were required to maximally inhibit TNF-α-induced apoptosis in isolated cultured murine hepatocytes (data not shown). The equilibrium constant for inhibition (K1) for cathepsin B is 0.02 μM, whereas the K1 for cathepsin K is 89 μM, cathepsin L is 12 μM, and cathepsin S is 3 μM. The K1 for cathepsin B is at least 2 logs lower than it is for these other lysosomal cathepsin proteases, and the drug does not inhibit caspases. Thus the drug is a selective cathepsin B inhibitor. In selected experiments, mice were treated with R-3032 (4.9 mg/kg in 30:70 polyethylene glycol 400/H2O at pH 7.5) intraperitoneally 2 h before liver procurement as previously described (4). R-3032 (10 μM) was also placed in UW and reperfusion media.

Statistics. All data represent at least four independent experiments and are expressed as the means ± SD unless otherwise indicated. Differences between groups were compared using ANOVA for repeated measures and a post hoc Bonferroni test to correct for multiple comparisons.

RESULTS

Are lysosomes more fragile in steatotic livers and after CI/WR injury? To assure similar development of steatosis in wild-type and Ctsb−/− mice on the MCD diet, the percentage of total fat was quantified in liver specimens. Both wild-type and Ctsb−/− animals developed >40% fat after 2 wk on the MCD diet. With the use of oil red O stain and computer image analysis, the percentage of total fat was quantified. Wild-type and Ctsb−/− liver specimens had similar fat content (WT 44 ± 9% and Ctsb−/− 48 ± 3%; data not shown). Thus the MCD diet induces similar hepatic steatosis in both groups of mice.

Lysosomal integrity was decreased in steatotic livers and after CI/WR injury in both chow- and MCD diet-fed wild-type mice (Fig. 1). In chow-fed wild-type mice without CI/WR injury, lysosomes were intact with a lysosomal pellet-to-supernatant ratio of 14:1, whereas in chow-fed wild-type animals that underwent CI/WR injury, the ratio dropped considerably to 0.6:1 (P < 0.001). In contrast, in wild-type mice fed the MCD diet alone, there was evidence for decreased lysosomal integrity (ratio 5:1) that was further enhanced in livers subjected to CI/WR injury (ratio 0.5:1; P < 0.01). These data suggest that the hepatic lysosomes have reduced integrity in steatotic livers and are more susceptible to CI/WR injury. This assay does not distinguish between disruption of lysosomes in Ctsb mice.
situ or during the mechanical homogenization process, and therefore, the results required verification in intact tissue.

Does lysosomal breakdown occur in situ in steatotic livers after CI/WR injury? To ascertain whether lysosomal breakdown occurred in situ, the cellular compartmentalization of cathepsin B and D in liver sections was examined by immunofluorescence (Fig. 2). Under basal conditions, the cellular distribution of cathepsin B and D fluorescence was punctate, consistent with lysosomal localization. Cathepsin B and D remained punctate in animals fed the MCD diet but manifested a more diffuse cellular pattern following CI/WR injury, consistent with the redistribution of the proteins from the lysosomes to the cytosol; this did not occur in animals fed a chow diet. Thus in situ release of cathepsin B and D from lysosomes into the cytosol only occurs in steatotic livers after CI/WR injury. However, these data do not determine whether this process contributes to liver damage or whether it is an epiphenomenon.

Fig. 1. Lysosomal integrity is reduced in steatotic livers and after cold ischemia-warm reperfusion (CI/WR) injury. Wild-type mice were injected intraperitoneally with fluorescein-tagged dextran and studied 24 h later. Lysosomal integrity was assessed by fractionating the liver into an organelle-containing pellet and cytosolic supernatant fractions and by calculating the ratio of the fluorescence of the pellet compared with the supernatant. Lysosomes are in the pellet, and a high ratio suggests intact lysosomes, and conversely, a low ratio is consistent with disrupted lysosomes. In chow-fed, wild-type animals, lysosomes were intact with a lysosomal pellet to supernatant ratio of 14:1. In contrast, in chow-fed animals that underwent CI/WR injury, the ratio dropped to 0.6:1. In mice fed the methionine/choline-deficient (MCD) diet without CI/WR injury, decreased lysosomal integrity (ratio 5:1) was evident. Lysosomal integrity was further enhanced after the MCD mice were subjected to CI/WR injury (ratio 0.5:1). **P < 0.01; *P < 0.001.

Fig. 2. In situ release of cathepsin B and D occurs in steatotic livers after CI/WR injury. Paraffin-embedded liver sections were analyzed by immunofluorescence for cathepsins B and D and viewed by confocal microscopy. Photomicrographs of liver sections from animals under basal conditions (A–D) and after CI/WR injury (E–H) are shown. A–B and E–F: liver sections from animals fed a chow diet; C–D and G–H: liver sections from animals fed the MCD diet. In animals fed a chow diet under basal conditions, the hepatocellular distribution of cathepsin B and D was punctate consistent with lysosomal localization. Cathepsin B and D remained punctate in livers from animals fed the MCD diet but manifested a diffuse cellular pattern of fluorescence following CI/WR injury, consistent with the redistribution of cathepsins from the lysosomes to the cytosol; this did not occur in animals fed a chow diet.

Does liver cell apoptosis occur in steatotic livers after CI/WR injury by a cathepsin B-dependent mechanism? To determine whether lysosomal disruption contributes to liver damage, liver cell apoptosis was quantified. Initially apoptosis was assessed in liver tissue specimens using the TUNEL assay. Minimal TUNEL-positive cells were observed in liver specimens obtained from chow fed, wild-type, and $Ctsb^{-/-}$ mice after CI/WR injury [5.3 ± 1.3 and 1.6 ± 1.1 TUNEL-positive cells high power field (HPF); Fig. 3] compared with wild-type and $Ctsb^{-/-}$ mice without CI/WR injury (2.1 ± 1.8 and 1.1 ± 1.4 TUNEL-positive cells/HPF; data not shown). However, the number of TUNEL-positive cells doubled in wild-type animals fed the MCD diet alone (26.1 ± 2.4) compared with $Ctsb^{-/-}$ animals (12.1 ± 2.9; P < 0.001; data not shown). Wild-type mice fed the MCD diet after CI/WR injury showed increased TUNEL-positive cells (59.2 ± 17.7) in liver specimens (Fig. 3). The number of TUNEL-labeled cells was markedly reduced in liver specimens obtained from $Ctsb^{-/-}$ animals (9.5 ± 4.3; P < 0.001) fed the MCD diet after CI/WR compared with liver...
specimens from wild-type animals under the same conditions. Although TUNEL-positive cells usually reflect apoptosis, the assay can be relatively nonspecific. Therefore, we confirmed the occurrence of apoptosis by performing immunofluorescence for activated caspase 3/7 in the liver tissue specimens (Fig. 4). Wild-type and Ctsb−/− animals fed a chow diet following CI/WR injury had a slightly higher number of caspase 3/7-positive cells in liver specimens compared with those obtained from animals fed a chow diet alone (4.2 ± 1.5 and 1.4 ± 1.5; P = 0.4) Wild-type animals fed MCD diet alone had an increased number of caspase 3/7-positive cells (10.9 ± 1.4) compared with liver specimens from Ctsb−/− animals (3.3 ± 0.5; P < 0.001). Caspase 3/7-positive cells were notably reduced in liver tissue specimens from Ctsb−/− animals fed a MCD diet following CI/WR injury (2.8 ± 2.6) compared with liver specimens from wild-type animals (26.7 ± 4.7; P < 0.001). These data are remarkable for demonstrating that liver cell apoptosis is potentiated in steatotic livers following CI/WR injury and that genetic inactivation of cathepsin B reduces this apoptosis.

We used TEM analysis to 1) confirm that the TUNEL-positive liver cells were apoptotic by morphological analysis, and 2) identify the liver cell type undergoing apoptosis. TEM identified apoptotic hepatocytes in steatotic livers after CI/WR injury (Fig. 5). The classic morphological features of apoptosis, including nuclear chromatin margination, plasma membrane blebbing, and cytoplasmic condensation, were readily identified. Apoptosis was identified in hepatocytes of steatotic livers of wild-type animals after CI/WR injury and was decreased in liver specimens from Ctsb−/− animals. Thus, after CI/WR injury of steatotic livers, the initial injury is apoptosis of hepatocytes, which is reduced by genetic inactivation of cathepsin B.

Because developmental and compensatory processes may occur in genetically manipulated mice, we also determined whether a pharmacological cathepsin B inhibitor is salutary during CI/WR injury of steatotic livers. The ability of the cathepsin B inhibitor (R-3032) to reduce hepatocellular apoptosis and liver damage when administered to the animal added to the UW and the reperfusion media was examined. TUNEL-
positive cells in liver specimens were reduced in MCD-fed animals subjected to CI/WR injury after R-3032 treatment (44.6 ± 9.8 TUNEL-positive cells/HPF) compared with animals without treatment (59.2 ± 17.7 TUNEL-positive cells/HPF; P = 0.02; data not shown). R-3032 also reduced caspase 3/7-positive cells in liver specimens from MCD-fed animals by 38% (26.7 ± 4.7 positive cells per HPF after CI per WR with no inhibitor compared with 16.5 ± 5.1 positive cells per HPF after CI per WR injury with R-3032; data not shown). TEM analysis revealed a decrease in hepatocyte apoptosis from liver specimens of R-3032-treated animals (Fig. 5). These data demonstrate that R-3032 is effective in reducing liver cell apoptosis and that apoptosis in steatotic livers after CI/WR injury is cathepsin B dependent.

Is liver damage in steatotic livers following CI/WR attenuated by cathepsin B inactivation? Liver injury was assessed by histopathological examination of H&E-stained liver sections (Fig. 6). Typical areas of ischemic damage, both hepatocyte apoptosis and necrosis, were observed in zone 3 (pericentral region) of the hepatic acinus in liver specimens from wild-type animals fed the MCD diet following CI/WR injury. These findings were minimal in liver specimens from Ctsb−/− animals and were decreased in liver sections from R-3032-treated wild-type animals. Consistent with the occurrence of apoptosis and necrosis, reperfusate alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) values were also increased in wild-type livers after CI/WR injury. These indices of liver damage were reduced in Ctsb−/− livers (P < 0.01; ALT: 775 ± 250 vs. 72 ± 30 U/l; P < 0.01; LDH: 1,348 ± 505 vs. 168 ± 65 U/l; P < 0.05). Thus inhibition of hepatocyte damage by the lysosomal pathway was associated with a reduction in liver injury.

**DISCUSSION**

The principal findings of this study relate to the mechanisms of CI/WR injury in steatotic livers. These results demonstrate that in an MCD model of hepatic steatosis subjected to in vitro CI/WR injury 1) lysosomes are fragile, 2) lysosomes undergo permeabilization releasing cathepsin B and D into the cytosol, 3) hepatocyte apoptosis is increased and, in part, cathepsin B dependent, and 4) hepatic damage is attenuated by pharmacological or genetic inactivation of cathepsin B. These data suggest inhibition of the lysosomal pathway of cell injury is salutary in CI/WR injury of steatotic livers.

We used the MCD animal model of steatosis for this study. Mice fed the MCD diet develop NAFLD with inflammation as opposed to single steatosis, which occurs in genetic models of hepatic steatosis, such as ob/ob mice (20). The presence of inflammation mimics the human NAFLD syndrome, in which inflammation frequently accompanies steatosis. Thus the MCD diet may be a better model than the genetic models. Nonetheless, mice fed the MCD diet are similarly susceptible to CI/WR injury as are the ob/ob mice (6). The injury in both models...
observed after CI/WR injury is hepatocellular as opposed to the endothelial cell injury observed in nonsteatotic livers (7, 24, 26). Regardless of the model, hepatic steatosis predisposes the liver to hepatocellular CI/WR injury, and in this context, the MCD model is a rational model to explore the mechanism responsible for this injury.

We used an in vitro perfused rodent liver apparatus to simulate WR after cold storage. This model approximates the first stage of CI/WR injury, namely injury produced by restoration of flow and oxygen. This experimental approach has been widely used to examine the cellular and tissue mechanism of CI/WR injury (12, 24). The model does have several limitations. The later stages of CI/WR injury are not simulated. The role of platelets, neutrophils, and other inflammatory cells are not examined in this model, which are involved in the later stages of CI/WR injury. Nonetheless, this model does permit analysis and dissection of the early stages of CI/WR injury that often trigger the later stages of injury.

There are various possible mechanisms that may contribute to the lysosomal permeabilization observed in steatotic livers after CI/WR injury. Recent data from our laboratory have shown that hepatic steatosis is associated with lysosomal permeabilization. Both in vivo and in vitro, the development of lipid vacuoles with elevated concentrations of cellular free fatty acids results in lysosomal permeabilization with release of lysosomal proteases into the cytosol (9). Perhaps, intracellular concentrations of free fatty acids increase in CI/WR injury. The free fatty acids may directly or indirectly mediate lysosomal disruption. In vivo models suggest free fatty acids may activate Bax, a proapoptotic member of the Bcl-2 family, which, in turn, mediates lysosomal destabilization (9, 14). Alternatively, enhanced formation of reactive oxygen radicals accompanying CI/WR injury may also cause lysosomal breakdown (17, 18, 29). Further elucidation of the mechanisms}

causing lysosomal permeabilization may help in developing rational therapies to attenuate CI/WR injury in steatotic livers.

After lysosomal permeabilization, several proteases are released into the cytosol, some of which have been implicated in the apoptotic process. Cathepsin B, D, and L are the most abundant lysosomal cathepsins (14). Cathepsin B is the most stable cysteine protease at physiological pH and is essential in bile acid-induced hepatocyte apoptosis (4) and TNF-α-induced hepatocyte apoptosis (15). Cathepsin D is the second most stable cathepsin at physiological pH and has been implicated in apoptosis induced by TNF-α (8) and oxidant stress (19). Cathepsin L, the least stable at physiological pH, is an important regulator of ultraviolet light-induced apoptosis (28). Little more is known about other cathepsins and their role in apoptosis. Because cathepsin B is the most stable and abundant cysteine protease, it would have the greatest destructive potential in cellular compartments outside the lysosome. Indeed, in this study, we did observe a decrease in hepatocyte apoptosis with genetic and pharmacological inactivation of cathepsin B. Other cathepsins, such as cathepsin D and L, may also contribute to cell injury. However, cathepsin B also appears to contribute to lysosomal permeabilization (14) and, therefore, may be the best pharmacological target to prevent lysosomal-mediated liver injury.

In the current study, hepatocyte apoptosis was predominantly observed in steatotic livers after CI/WR injury. Although, Selzner and Clavien (27) have had similar observations, their data suggested that hepatocyte necrosis was the predominant mode of cell injury following CI/WR in steatotic livers. However, as emphasized by Lemasters (21), apoptosis and necrosis are not distinct forms of cell death, but they likely represent a continuum of cell injury. Apoptosis represents the genetic program triggered by injury, and necrosis represents a more severe manifestation of this process, occurring when
ATP drops to levels insufficient to support apoptosis. Within this framework, we may simply have observed a milder form of CI/WR injury than that imposed by Clavien and co-workers. Because mitochondrial dysfunction is generic to both apoptosis and necrosis and lysosomal dysfunction is upstream of mitochondrial membrane permeabilization (13), inhibition of the mitochondrial pathway should attenuate both forms of cell injury. Consistent with this context, cathepsin B inactivation not only reduced hepatocyte apoptosis but also zone 3 ischemic liver damage. Thus the lysosomal pathway appears to be an integral mechanism mediating CI/WR injury in the steatotic liver.

In summary, liver donors with NAFLD (>30% macrovesicular steatosis) are not used by many transplant centers, thereby decreasing the donor pool. Hepatic steatosis associated with NAFLD results in enhanced CI/WR injury and graft failure. Lysosomal fragility with resultant release of cathepsin B and D into cytosol induces hepatocyte apoptosis following CI/WR injury of steatotic livers. Genetic and pharmacological attenuation of cathepsin B may potentially be a therapeutic strategy to increase the use of donor livers with NAFLD. In addition, the use of cathepsin B inhibitors prevents hepatocyte apoptosis following CI/WR injury.

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