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Lithocholic acid feeding results in direct hepato-toxicity independent of neutrophil function in mice

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HIGHLIGHTS

- Lithocholic acid (LCA) feeding results in significant hepatotoxicity and neutrophil recruitment.
- Ablation of neutrophil function and activity does not protect against LCA induced injury.
- Tauro-LCA and tauro-chenodeoxycholic acid accumulate to toxic levels after LCA feeding.
- Direct bile acid toxicity can occur when toxic bile acid species are administered.
- Species differences in bile acid composition may determine mechanistic differences in injury.

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ABSTRACT

Lithocholic acid (LCA) supplementation in the diet results in intrahepatic cholestasis and bile infarcts. Previously we showed that an innate immune response is critical for cholestatic liver injury in the bile duct ligated mice. Thus, the purpose of this study was to investigate the role of neutrophils in the mechanism of liver injury caused by feeding mice a diet containing LCA. C57BL/6 mice were given control or 1% LCA containing diet for 24–96 h and then examined for parameters of hepatotoxicity. Plasma ALT levels were significantly increased by 48 h after LCA feeding, which correlated with both neutrophil recruitment to the liver and upregulation of numerous pro-inflammatory genes. The injury was confirmed by histology. Deficiency in intercellular adhesion molecule-1 (ICAM-1) expression or inhibition of neutrophil function failed to protect against the injury. Bile acid levels were quantified in plasma and bile of LCA-fed mice after 48 and 96 h. Only the observed biliary levels of taurochenodeoxycholic acid and potentially tauro-LCA caused direct cytotoxicity in mouse hepatocytes. These data support the conclusion that neutrophil recruitment occurs after the onset of bile acid-induced necrosis in LCA-fed animals, and is not a primary mechanism of cell death when cholestasis occurs through accumulation of hydrophobic bile acids.

Abbreviations: BA, bile acid; CA, cholic acid; GCDCA, glycochenodeoxycholic acid; LCA, lithocholic acid; αMCA, α-muricholic acid; βMCAβ, -muricholic acid; DCA, deoxycholic acid; TCDCA, taurochenodeoxycholic acid; GCA, glycocholic acid; TCA, taurocholic acid; CDCA, chenodeoxycholic acid; TLCA, taurolithocholic acid; FXR, farnesoid X receptor; Egr-1, early growth response factor 1; ICAM-1, intercellular adhesion molecule-1; iNOS, inducible nitric oxide synthase; mKC, mouse keratinocyte chemoattractant; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-10, interleukin-10; TNF-α, tumor necrosis factor α; Cox-2, cyclooxygenase-2; VCAM-1, vascular adhesion molecule-1; HO-1, heme oxygenase-1; hsp86, heat shock protein-86; Gpx-1, glutathione peroxidase-1; MT-1, metallothionein-1; MIP-2, macrophage inflammatory protein 2; TSP-1, thrombospondin 1; TNFR1, tumor necrosis factor alpha receptor 1.

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1. Introduction

Cholestasis occurs as a result of a number of different pathologies including obstruction of the common bile duct by gall stones, biliary atresia, compression of the common bile duct from tumor growths such as cholangiocarcinoma, or during intrahepatic cholestasis of pregnancy (Zollner and Trauner, 2008; Jüngst and Lammert, 2013). During cholestasis, bile acids accumulate in hepatocytes, which have the potential to cause cytotoxicity (Guicciardi and Gores, 2002; Perez and Briz, 2009). Studies with rat hepatocytes have shown that high concentrations of toxic bile acids such as glycochenodeoxycholic acid (GCDCA) or taurolithocholic acid (TLCA) result in hepatocellular apoptosis mediated by mitochondrial and lysosomal dysfunction (Spivey et al., 1993; Botla et al., 1995; Graf et al., 2002). However, more recent data suggest these bile acids might not reach concentrations during obstructive cholestasis that result in hepatocellular toxicity *in vivo* (Trottier et al., 2011, 2012; Zhang et al., 2012), leading to alternate hypotheses for mechanisms of injury (Woolbright and Jaeschke, 2012).

Liver injury induced by obstructive cholestasis (bile duct ligation, BDL) in rodents is characterized by areas of focal necrosis (bile infarcts) and extensive neutrophil accumulation (Kountouras et al., 1984; Saito and Maher, 2000; Gujral et al., 2003). Animals with impaired neutrophil function developed significantly less liver injury after BDL suggesting neutrophils caused the majority of the cell damage (Gujral et al., 2003, 2004b). Prerequisite for neutrophil-induced liver injury is the activation and recruitment of neutrophils into sinusoids and a chemotactic signal for extravasation into the parenchyma (Jaeschke and Smith, 1997; Jaeschke and Hasegawa, 2006). Recent studies demonstrated that cleaved osteopontin in bile is a critical chemotactic factor for the early neutrophil-induced injury mechanisms after BDL (Yang et al., 2014). The increased biliary pressure during obstructive cholestasis results in rupture of cholangioles causing the leakage of bile back into the parenchyma, which is responsible for the focal nature of the liver damage (Fickert et al., 2002). In addition, the exposure of hepatocytes to high levels of the typical bile acids present in mice, i.e. taurocholic acid (TCA), β-muricholic acid (βMCA) and TβMCA, does not cause directly cell death (Allen et al., 2011; Zhang et al., 2012). However, these bile acids trigger intercellular adhesion molecule-1 (ICAM-1) gene expression in hepatocytes and CXC chemokine formation that provides an additional chemotactic gradient for neutrophil recruitment (Allen et al., 2011). Thus, the acute liver injury during obstructive cholestasis in mice is a neutrophilic inflammatory injury triggered by the biliary leakage of osteopontin and the generation of CXC chemokines by hepatocytes exposed to bile acids. Given the strong evidence for a neutrophil-mediated liver injury after BDL (Gujral et al., 2003, 2004b; Kim et al., 2006; O'Brien et al., 2013; Licata et al., 2013), it remains unclear if bile acids directly cause cell death under pathophysiologically relevant conditions *in vivo*.

Recently, lithocholic acid (LCA) feeding was used as a model of liver injury (Fickert et al., 2006). LCA is a hydrophobic bile acid generated by bacterial reduction of chenodeoxycholic acid (CDCA) in the gut (Hofmann, 2004). The accumulation of high biliary concentrations of LCA and its metabolites after feeding this bile acid results in the precipitation of LCA in cholangioles and the clogging of the biliary system resembling the obstructive cholestasis caused by BDL (Fickert et al., 2006). In addition to the focal areas of necrosis, an extensive accumulation of neutrophils was also observed (Fickert et al., 2006). This raises the question whether in this model, similar to BDL, a neutrophilic inflammatory response is also the main mechanism of liver injury or if the increased levels of the hydrophobic bile acid LCA can trigger direct cell death in hepatocytes. This issue may be clinically important as hydrophobic bile acids such as LCA and CDCA and their conjugates are more prevalent in humans (Trottier et al., 2011) than in mice (Zhang et al., 2012). Moreover, there are concerns that LCA may derive from UDCA (Sinakos et al., 2010; Hofmann, 2004), in particular when used at high doses in cholestatic conditions such as primary sclerosing cholangitis where it has been associated with adverse clinical outcomes (Lindor et al., 2009; Sinakos et al., 2010). Notably, LCA may also result in sclerosing cholangitis in some mouse strains (Fickert et al., 2006). Thus, the objective of this investigation was to evaluate the pathophysiological role of neutrophils in the LCA feeding model and, after analysis of LCA levels in bile and serum, assess the relevance of these concentrations for cytotoxicity in cultured mouse hepatocytes.

2. Materials and methods

2.1. Animals and experimental protocol.

C57Bl/6J, gp91*phox*−/− (NOX-2)-deficient, and ICAM-1-deficient mice on C57Bl/6 background were purchased from Jackson Laboratories (Bar Harbor, ME). All animals received humane care according to the criteria outlined in *Guide for the Care and Use of Laboratory Animals*. All experimental protocols were approved by the Institutional Animal Care and Usage Committees of the University of Kansas Medical Center. Mice were fed either 1% LCA mixed into control diet (Purina, St. Louis) or normal control diet and allowed food and water *ad libitum* for 0–96 h. There was no statistical difference in total intake of food for animals on each diet across experiments. Mice treated with the NADPH oxidase (NOX 2) inhibitor diphenylene iodonium chloride (DPI) (Sigma, St. Louis, MO) were given subcutaneously 10 mg DPI/kg daily (in 0.2 ml of 5% glucose). Some mice were treated with 700 mg/kg galactosamine and 100 μg/kg Salmonella abortus equi endotoxin (Sigma) for 6 h as positive control for apoptotic cell death (Jaeschke et al., 1998). Animals were sacrificed by cervical dislocation after isoflurane anesthesia. Blood and liver samples were collected at this time. Plasma was used for determination of alanine transaminase (ALT) activities and bile acid concentrations. Pieces of the liver were snap-frozen in liquid nitrogen for mRNA and protein analyses or fixed in phosphate-buffered formalin for immunohistochemistry and histology. ALT activities were determined in plasma by using the Pointe Scientific Serum ALT kit (Canton, MI) according to the manufacturer's instructions. Plasma alkaline phosphatase (ALP) activities were determined with an ALP kit from Thermo Scientific (Waltham, MA).

2.2. Immunohistochemistry

Formalin-fixed tissue samples were embedded in paraffin and 5 μm sections were cut. Sections were stained with hemotoxylin and eosin (H&E) and evaluated for necrosis by the pathologist (A. Farhood) or stained by the TUNEL assay according to manufacturer's protocol (Roche, Basel, Switzerland).

2.3. Western blotting

Frozen liver samples were homogenized and centrifuged at 14,000 × *g* for 10 min. Protein concentrations were normalized using the BCA assay, and then loaded into an Invitrogen Mini-Blot system for gel electrophoresis (Invitrogen, Carlsbad, CA). An anti-ICAM-1 antibody (Santa Cruz Biotechnology, Dallas, TX) was used at 1:1000 dilution and the proteins were visualized using a horseradish peroxidase conjugated secondary antibody.

2.4. Caspase activity

Quantification of hepatic caspase-3 activity was performed as previously described in detail (Jaeschke et al., 1998). Liver tissue was homogenized and protein concentration was normalized by BCA protein assay (Thermo Scientific, Waltham, MA). The homogenate was assayed with a fluorogenic substrate cleavable by caspase-3 (Enzo Life Sciences, Plymouth Meeting, PA) for change in fluorescence intensity over time using a Spectramax Gemini fluorescence plate reader (Molecular Devices, Sunnyvale, CA). The enzyme activity that was inhibitable by a pancaspase inhibitor was reported as caspase-3 activity.

2.5. Hepatic neutrophil sequestration

Neutrophil accumulation in the livers was assessed by staining tissue sections with an anti-neutrophil antibody (Ly-6b) (AbD Serotec, Raleigh, NC) followed by visualization with the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA). The numbers of neutrophils present in sinusoids and extravasated into parenchymal tissue were counted in either 10 or 20 high-power fields (HPF). The sum of the sinusoidal and extravasated neutrophils was expressed as total neutrophil count in liver.