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Bile acid-induced necrosis in primary human hepatocytes and in patients with obstructive cholestasis

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ABSTRACT

Accumulation of bile acids is a major mediator of cholestatic liver injury. Recent studies indicate bile acid composition between humans and rodents is dramatically different, as humans have a higher percent of glycine conjugated bile acids and increased chenodeoxycholate content, which increases the hydrophobicity index of bile acids. This increase may lead to direct toxicity that kills hepatocytes, and promotes inflammation. To address this issue, this study assessed how pathophysiological concentrations of bile acids measured in cholestatic patients affected primary human hepatocytes. Individual bile acid levels were determined in serum and bile by UPLC/QTOFMS in patients with extrahepatic cholestasis with, or without, concurrent increases in serum transaminases. Bile acid levels increased in serum of patients with liver injury, while biliary levels decreased, implicating infarction of the biliary tracts. To assess bile acid-induced toxicity in man, primary human hepatocytes were treated with relevant concentrations, derived from patient data, of the model bile acid glycochenodeoxycholic acid (GCDC). Treatment with GCDC resulted in necrosis with no increase in apoptotic parameters. This was recapitulated by treatment with biliary bile acid concentrations, but not serum concentrations. Marked elevations in serum full-length cytokeratin-18, high mobility group box 1 protein (HMGB1), and acetylated HMGB1 confirmed inflammatory necrosis in injured patients; only modest elevations in caspase-cleaved cytokeratin-18 were observed. These data suggest human hepatocytes are more resistant to human-relevant bile acids than rodent hepatocytes, and die through necrosis when exposed to bile acids. These mechanisms of cholestasis in humans are fundamentally different to mechanisms observed in rodent models.

Introduction

Bile acids (BAs) are the primary constituent of bile and are known to be cytotoxic to hepatocytes (Malhi et al., 2010). The predominant hypothesis for the development of cholestatic liver injury is that BAs accumulate in hepatocytes, and subsequently in serum, during cholestasis, which exposes hepatocytes to potentially cytotoxic levels of BAs (Spivey et al., 1993; Jaeschke et al., 2002; Guicciardi et al., 2013). This paradigm is supported by well-characterized mechanisms of apoptosis in rat hepatocytes (Malhi et al., 2010; Guicciardi et al., 2013) and human hepatoma lines that have been transfected with the sodium taurocholate cotransporting polypeptide (NTCP) to facilitate BA uptake (Faubion et al., 1999; Rust et al., 2009). A majority of these studies use glycochenodeoxycholic acid (GCDC) as a model hydrophobic bile acid to induce apoptosis, as it is the major BA present in serum of cholestatic patients (Spivey et al., 1993; Trottier et al., 2012). In contrast, studies in the bile duct ligation (BDL) model of cholestasis suggest BA concentrations in mice may not be directly toxic (Zhang et al., 2012). Instead, BAs act as pro-inflammatory signals, which trigger CXC chemokine formation in hepatocytes (Allen et al., 2011). These chemokines together with osteopontin derived from biliary epithelial cells recruit neutrophils to areas of biliary leakage in the liver during obstructive cholestasis (Allen et al., 2011; Yang et al., 2014b). In support of this hypothesis,

Abbreviations: LCA, lithocholic acid; UDCA, ursodeoxycholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; CA, cholic acid; TCA, taurocholic acid; GCDC, glycochenodeoxycholic acid; TCDC, taurochenodeoxycholic acid; GCA, glycocholic acid; GDCA, glycodeoxycholic acid; TDCA, taurodeoxycholic acid; NTCP, sodium taurocholate cotransporting polypeptide; ICAM-1, intercellular adhesion molecule; ERCP, endoscopic retrograde cholangiopancreatography; BDL, bile duct ligation; HMGB1, high mobility group box-1; UPLC/QTOF, ultra-performance liquid chromatography quadrupole time of flight mass spectrometry; CK18, cytokeratin-18

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mice deficient in either intercellular adhesion molecule-1 (ICAM-1) or CD18 are highly protected against both BDL-induced neutrophil recruitment and injury, implicating neutrophils as a major contributor to the pathogenesis (Gujral et al., 2003a, 2004a). Thus, BDL-induced liver injury in mice is caused by a neutrophilic inflammatory response (Woolbright and Jaeschke, 2012); BAs support this mechanism by generating chemotactic mediators in hepatocytes (Copple et al., 2010). However, the relevance of this injury mechanism, identified in the mouse BDL model, for the human pathophysiology of obstructive cholestasis remains unclear.

While there have been a substantial number of studies done in rodent models and transfected hepatoma lines, few studies have been performed to assess how BAs affect primary human hepatocytes. Early studies confirmed BA toxicity in human hepatocytes using GCDC, and protection by ursodeoxycholic acid (UDCA) (Galle et al., 1990). Of note, the concentrations required for even low levels of cell death were substantially higher than what is typically used in rat hepatocyte models (Galle et al., 1990). However, many of the mechanisms of BA toxicity established in rat hepatocytes have yet to be investigated in primary human hepatocytes. In particular, it remains unclear how human hepatocytes respond to pathophysiological concentrations of relevant BAs measured in human patients. Therefore, the objective of the current study was to determine BA composition and concentrations in serum and bile of patients with extrahepatic cholestasis and then expose primary human hepatocytes to these BAs. We hypothesized human hepatocytes would be more resistant to bile acid-induced apoptosis than rodent hepatocytes. Furthermore, our goal was to assess if pathophysiologically relevant concentrations of human BAs measured during obstructive cholestasis could cause direct cytotoxicity, or induce pro-inflammatory mediator formation in primary human hepatocytes.

Materials and methods

Criteria for cholestatic patients. Patients admitted to the University of Kansas Hospital were enrolled in an institutional review board (IRB) approved protocol. Inclusion criteria included subjects undergoing planned endoscopic retrograde cholangiopancreatography (ERCP) for medical diagnosis and potential treatment of cholestasis (Supplementary Table 1). Uninjured patients were defined as patients with ALT < 50 U/L and ALP < 110 U/L. Injured patients were defined as patients with ALT ≥ 50 U/L and ALP ≥ 110 U/L and defined cholestasis as evidenced by ERCP. Blood and bile samples were collected during the ERCP procedure. Bile dilution was held to an absolute minimum in this study. Injection of contrast dyes and other agents occurred after the initial suction of bile for collection. We estimate overall dilution would be no greater than 10% in any single sample. This study adhered to the Helsinki Declaration and all studies were done under informed consent.

Isolation and culture of human hepatocytes. Primary human hepatocytes were freshly isolated from liver resections by the Biospecimen Core in the Department of Pharmacology, Toxicology and Therapeutics at the University of Kansas Medical Center. All human tissues were obtained with informed consent from each patient, according to ethical and institutional guidelines. The study was approved by the Institutional Review Board at the University of Kansas Medical Center. Cells were isolated using a multi-step collagenase procedure as described in detail (Xie et al., 2014). Media consisted of Williams' Medium E (Life Technologies, Grand Island, NY) supplemented with L-glutamine (2 mM) (Life Technologies), HEPES (10 mM), insulin (10^{-7} M), dexamethasone (10^{-7} M), penicillin (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL). Media was not supplemented with fetal bovine serum as initial experiments did not show an effect on cell death if serum was present or not (data not shown). After an initial 3 h attachment period, cultures were washed with phosphate-buffered saline (PBS) and then culture medium, vehicle or media containing the indicated concentration of the bile acid or inhibitor was added along with the appropriate vehicle.

Isolation and culture of rat hepatocytes. Sprague-Dawley rats (200–230 g body weight) were acquired from Jackson Laboratories (Bar Harbor, ME). A three-step collagenase perfusion method was used to isolate hepatocytes. After the induction of anesthesia, the peritoneal cavity was opened, and a 20G catheter was inserted into the inferior vena cava. The liver was perfused in situ via the inferior vena cava after cutting the portal vein for 10 min with calcium and magnesium free HBSS containing 0.1 mM EGTA followed by a washout step using calcium and magnesium free HBSS without EGTA. The final perfusion step consisted of Eagle's Minimum Essential Medium containing 25 mM HEPES buffer and 0.025 mg/mL of Liberase TM (Roche, Basel, Switzerland) and continued until the liver showed signs of digestion. The remaining portion was cut into smaller pieces with scissors to release remaining cells. The cell suspension was sequentially filtered through nylon gauze and collected in 50 mL conical tubes. The cells were centrifuged for 5 min at 50 ×*g* and 4 °C and then resuspended in fresh cold Dulbecco's Minimum Essential Medium with 25 mM HEPES. This was repeated 3 times in order to isolate the hepatocyte fraction. Hepatocyte viability was assessed using a hemocytometer and the trypan blue exclusion assay. After an initial 3-h attachment period, cultures were washed with PBS and then culture medium (controls) or media containing the indicated concentrations of bile acids were added. Inhibition studies using the pancaspase inhibitor z-VAD-fmk (10 μM) (Enzo Life Sciences, Ann Arbor, MI) were carried out by pretreating for one hour with the indicated concentration of inhibitor and then adding the indicated treatment.

Murine studies. C57BL/6J mice (20–25 g bodyweight) 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 Animal Use Committees of the University of Kansas Medical Center. Bile duct ligation (BDL) was performed as described in detail (Woolbright et al., 2013). In addition, some mice were also treated with galactosamine and endotoxin for 6 h as described previously (Jaeschke et al., 1998).

Bile acid measurements. Bile acid measurements were performed as previously described in detail (Woolbright et al., 2014a). In brief, bile samples were first diluted 1:50 in water, whereas serum samples were used as is. The samples were prepared by mixing 20 μL of serum or bile with 80 μL of methanol and the resulting mixtures were centrifuged at 14,000 ×*g* for 10 min to remove protein. The supernatants were injected to UPLC-QTOFMS for analysis. Chromatographic separation of bile acids was achieved using a 100 mm × 2.1 mm (Acquity 1.7 μm) UPLC BEH C-18 column (Waters, Milford, MA). TOFMS was calibrated with sodium formate and monitored by the intermittent injection of lock mass leucine encephalin in real time. TOFMS was operated in a negative mode with electrospray ionization. The concentration of bile acids was calculated based on corresponding standard curves of six different concentrations ranging from 100 ng/mL to 25 μg/mL.

Western blotting. Liver sections were homogenized in a CHAPS based buffer, and then centrifuged at 14,000 rpm to remove cellular debris. The BCA assay (Pierce Scientific, Rockford, IL) was used to quantify protein levels. Equal quantities of protein were loaded into an Invitrogen Mini Blot electrophoresis system and transferred onto PVDF paper. The blot was probed using a caspase-3 antibody (Cell Signaling, Danvers, MA) and then visualized using a goat anti-rabbit HRP conjugated antibody (Santa Cruz Biotechnology Santa Cruz, CA).