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Basal efflux of bile acids contributes to drug-induced bile acid-dependent hepatocyte toxicity in rat sandwich-cultured hepatocytes

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ABSTRACT

The bile salt export pump (BSEP or Bsep) functions as an apical transporter to eliminate bile acids (BAs) from hepatocytes into the bile. BSEP or Bsep inhibitors engender BA retention, suggested as an underlying mechanism of cholestatic drug-induced liver injury. We previously reported a method to evaluate BSEP-mediated BA-dependent hepatocyte toxicity by using sandwich-cultured hepatocytes (SCHs). However, basal efflux transporters, including multidrug resistance-associated proteins (MRP or Mrp) 3 and 4, also participate in BA efflux. This study examined the contribution of basal efflux transporters to BA-dependent hepatocyte toxicity in rat SCHs. The apical efflux of [^{3}H]taurocholic acid (TC) was potently inhibited by 10 μM cyclosporine A (CsA), with later inhibition of basal [^{3}H]TC efflux, while MK571 simultaneously inhibited both apical and basal [^{3}H]TC efflux. CsA-induced BA-dependent hepatocyte toxicity was 30% at most at 10 μM CsA and ~60% at 50 μM, while MK571 exacerbated hepatocyte toxicity at concentrations of ≥50 μM. Quinidine inhibited only basal [^{3}H]TC efflux and showed BA-dependent hepatocyte toxicity in rat SCHs. Hence, inhibition of basal efflux transporters as well as Bsep may precipitate BA-dependent hepatocyte toxicity in rat SCHs.

1. Introduction

Drug-induced liver injury (DILI) is a potentially serious adverse event leading to the dropout of candidate compounds from drug development and the withdrawal of pharmaceuticals from clinical use (Kaplowitz, 2001; Schuster et al., 2005). DILI can severely damage the liver, resulting in liver transplantation in worst case scenarios. Hence, it is essential to promptly identify, remove, and/or assign alerts for possible risk compounds of DILI at all stages of the drug development process. Recently, the accumulation of bile acids (BAs) within hepatocytes was suggested as an underlying mechanism of cholestatic DILI (Byrne et al., 2002; Fattinger et al., 2001; Kostrubsky et al., 2006; Stieger et al., 2000). The bile salt export pump (human BSEP or rat Bsep), localized on the apical side of the hepatocyte plasma membrane, plays a major role in the excretion of BAs from the liver into the bile (Meier and Stieger, 2002). Therefore, control of BSEP function is an important factor for the regulation/dysregulation of hepatic BA content.

Several genetic mutations of BSEP are associated with progressive familial intrahepatic cholestasis type 2 (PFIC2) and cause severe intracellular accumulation of BAs within the liver (Strautnieks et al., 1998). Thus, BSEP dysfunction is quite likely to be related to liver injury. In fact, many researchers have reported that most drugs causing cholestatic DILI also potently inhibit BSEP (Dawson et al., 2012; Morgan et al., 2010; Pedersen et al., 2013; Warner et al., 2012). Accordingly, several methods have been developed for the determination of BSEP inhibition, including the widely used membrane vesicle assay. Nonetheless, ample experimental evidence indicates that the membrane vesicle assay might misestimate the clinical risk of cholestatic DILI, because this cell-free

Abbreviations: BA, bile acid; BEI, biliary excretion index; BSEP/Bsep, bile salt export pump; CsA, cyclosporine A; DILI, drug-induced liver injury; DMSO, dimethyl sulfoxide; $E_2$17βG, estradiol-17β-D-glucuronide; FBS, fetal bovine serum; GFP, green fluorescent protein; HBSS, Hank's balanced salt solution; HEK, human embryonic kidney; IC_{50}, half-maximal inhibitory concentration; LDH, lactate dehydrogenase; MRP/Mrp, multidrug resistance-associated protein; NTCP/Ntcp, Na^+-taurocholate co-transporting polypeptide; OATP/Oatp, organic anion-transporting polypeptide; PFIC2, progressive familial intrahepatic cholestasis type 2; qPCR, quantitative polymerase chain reaction; SCH, sandwich-cultured hepatocyte; SD, standard deviation; TC, taurocholic acid; WME, Williams' Medium E.

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system lacks certain molecular players related to BA disposition (e.g., metabolic enzymes and uptake transporters) (Dawson et al., 2012). To overcome this shortcoming, an alternative protocol using sandwich-cultured hepatocytes (SCHs) was established (Swift et al., 2010).

We recently utilized rat SCHs for constructing an *in vitro* BA-dependent hepatocyte toxicity assay system to mimic cholestatic DILI (Ogimura et al., 2011). We determined that recognized potent BSEP or Bsep inhibitors induced BA-dependent hepatocyte toxicity in SCHs, and successfully observed the influence of cytochrome P450-mediated drug metabolism on this toxicity. However, selected drugs (i.e., imipramine and quinidine), which were not previously regarded as BSEP inhibitors, also showed BA-dependent hepatocyte toxicity (Ogimura et al., 2011). These findings imply that BSEP-independent mechanisms might also underlie BA-dependent hepatotoxicity, at least to some extent.

Multidrug resistance-associated proteins 3 and 4 (human MRP3 or rat Mrp3 and human MRP4 or rat Mrp4) are additional BA efflux transporters. Unlike BSEP or Bsep, these transporters are localized on the basal side of the hepatocyte membrane (Akita et al., 2002; Jemnitz et al., 2010; Rius et al., 2003). Liver injury caused by bile duct ligation in mice was reportedly attenuated by the induction of Mrp3 (Teng and Piquette-Miller, 2007), and worsened by the genetic depletion of Mrp4 (Mennone et al., 2006). Therefore, Mrp3 and 4 seemingly protect hepatocytes from cellular accumulation of toxic BAs when Bsep function is abolished or compromised.

Evidence from clinical cases stresses the importance of MRP3 and 4. For example, protein expression levels of MRP3 were strongly increased in intensive care unit cholestasis patients, together with decreased protein expression levels of BSEP (Vanwijngaerden et al., 2011). On the other hand, MRP4 protein levels were induced in PFIC2 patients (Keitel et al., 2005) and primary biliary cirrhosis patients (Zollner et al., 2007). Hence, the importance of MRP3 and 4 as compensatory BA efflux transporters under cholestatic conditions is now increasingly recognized.

The current study focused on the involvement of basal BA efflux transporters, including Mrp3 and 4, on BA-dependent hepatocyte toxicity in rat SCHs. We hypothesized that Bsep-mediated BA-dependent hepatocyte toxicity might be aggravated in the face of basal efflux transporter blockade. Cyclosporine A (CsA) and MK571 were chosen as test compounds, because CsA and MK571 showed an inhibitory effect against both human BSEP and MRP3 or 4 in earlier work (Morgan et al., 2013). Moreover, we examined to demonstrate an example of BA-dependent hepatocyte toxicity caused by selective inhibitor of basal BA efflux transporters. For this study, quinidine was chosen as test compound, because quinidine did not inhibit human BSEP but inhibits human MRP4 (Morgan et al., 2013). Our results now indicate that basal efflux transporters in addition to Bsep may contribute to BA-dependent hepatocyte toxicity in rat SCHs.

2. Material and methods

2.1. Animals

Sprague Dawley rats (SLC Japan Inc., Tokyo, Japan), 7–8-weeks-old, were used throughout the study. The animals were treated humanely in accordance with the guidelines issued by the National Institutes of Health (Bethesda, MD, USA). In addition, all procedures were approved by the Animal Care Committee of Chiba University (Chiba, Japan).

2.2. Materials and cells

BAs and test compounds were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Sigma–Aldrich (St. Louis, MO, USA), or Calbiochem (Darmstadt, Germany). Williams' Medium E (WME), antibiotic–antimycotic solution, and GlutaMAX™ were purchased from Invitrogen (Carlsbad, CA, USA). Insulin was purchased from Sigma–Aldrich. Matrigel and ITS premix culture supplement were purchased from BD Biosciences (San Jose, CA, USA). Collagenase and dexamethasone were purchased from Wako Pure Chemical Industries, Ltd. Bsep-expressing Sf9 membrane vesicles were purchased from Genomembrane (Kanagawa, Japan). [^{3}H]TC (5 Ci/mmol) was purchased from Perkin–Elmer (Waltham, MA, USA). [^{3}H]estradiol-17 β-D-glucuronide ($E_2$17βG) (50 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). Sf9 cells were maintained as a suspension culture at 27 °C in serum-free EX-CELL 420 medium (JRH Biosciences, Inc., Lenexa, KS, USA). Human embryonic kidney (HEK) 293 and HEK293A cells were cultured at 37 °C with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL). All other chemicals and solvents were of analytical grade, unless otherwise noted.

2.3. Preparation of membrane vesicles expressing Mrp3 or 4

Preparation of membrane vesicles from Sf9 cells infected with recombinant Mrp3 baculovirus (Akita et al., 2002) was conducted as reported previously (Ninomiya et al., 2005). Rat Mrp4 adenovirus expression vector was constructed as follows. Rat Mrp4 cDNA was amplified from the excised rat liver by using the forward primer 5′-aaaaagcaggctCCCGGG**ACC**ATGCTGCCGGTGCACACC-3′, which included the Kozak sequence (**ACC**) and the SmaI restriction site (CCCGGG), and the reverse primer 5′-agaaagctgggtGTCGACTC ACAATGCTGTTTCAAATATCG-3′, which included the SalI site (GTCGAC). The amplified fragment was inserted into the pDONR™ 221 vector (Invitrogen), and its sequence was confirmed. The vector was inserted into the pAd/CMV/V5-DEST™ vector (Invitrogen) to finally produce the adenovirus expression vector. The final expression vector was digested with PacI, transfected into 5.0×10^5 HEK293A cells by using DNA-Lipofectamine™ 2000 (Invitrogen), and amplified until the appropriate titer was reached.

Next, HEK293 cells were infected with recombinant Mrp4 adenoviral stock at a multiplicity of infection of 2 and incubated for 48 h. Preparation of membrane vesicles from the infected HEK293 cells was carried out by using the same procedures as those previously described for Sf9 membrane vesicles (Ninomiya et al., 2005). Membrane vesicles were also prepared from green fluorescent protein (GFP)-expressing Sf9 insect cells and lacZ-expressing HEK293 cells and employed as negative controls for ATP-dependent transport.

2.4. Membrane vesicle transport assay

The membrane vesicle transport study was performed by using the rapid filtration technique, as described previously (Ninomiya et al., 2005). Membrane vesicles were prepared from Sf9 insect cells (10 μg) or HEK293 cells (10 μg) and incubated for 5 min at 37 °C with transport buffer (10 mM Tris–HCl, 4 mM ATP or AMP, 250 mM sucrose, and 10 mM $MgCl_2$) containing test compounds. The test compounds (CsA, MK571, and quinidine) were dissolved in dimethyl sulfoxide (DMSO, final concentration = 1%) prior to use. [^{3}H]TC (1 μM for Bsep) and [^{3}H]$E_2$17βG (1 μM for Mrp3 and 4) were used as the substrates. The transport reaction was terminated by the addition of 1 mL of ice-cold buffer (10 mM Tris–HCl, 250 mM sucrose, and 100 mM NaCl). The terminated reaction mixture was passed through a 0.45-μm membrane filter (Advantec Mfs, Inc., Dublin, CA, USA) and then washed twice with 5 mL of stop buffer. Radioactivity of all samples was quantified using a LSC-6100 liquid scintillation counter (Hitachi Aloka Medical,