Biliary Bicarbonate Secretion Constitutes a Protective Mechanism against Bile Acid-Induced Injury in Man

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Abstract

Background: Cholangiocytes expose a striking resistance against bile acids: while other cell types, such as hepatocytes, are susceptible to bile acid-induced toxicity and apoptosis already at micromolar concentrations, cholangiocytes are continuously exposed to millimolar concentrations as present in bile. We present a hypothesis suggesting that biliary secretion of HCO_3^- in man serves to protect cholangiocytes against bile acid-induced damage by fostering the deprotonation of apolar bile acids to more polar bile salts. Here, we tested if bile acid-induced toxicity is pH-dependent and if anion exchanger 2 (AE2) protects against bile acid-induced damage. **Methods:** A human cholangiocyte cell line was exposed to chenodeoxycholate (CDC), or its glycine conjugate, from 0.5 mM to 2.0 mM at pH 7.4, 7.1, 6.7 or 6.4, or after knockdown of AE2. Cell viability and apoptosis were determined by WST and caspase-3/-7 assays, respectively. **Results:** Glycochenodeoxycholate (GCDC) uptake in cholangiocytes is pH-dependent. Furthermore, CDC and GCDC (pK$_a$ 4–5) induce cholangiocyte toxicity in a pH-dependent manner: 0.5 mM CDC and 1 mM GCDC at pH 7.4 had no effect on cell viability, but at pH 6.4 decreased viability by >80% and increased caspase activity almost 10- and 30-fold, respectively. Acidification alone had no effect. AE2 knockdown led to 3- and 2-fold enhanced apoptosis induced by 0.75 mM CDC or 2 mM GCDC at pH 7.4. **Discussion:** These data support our hypothesis of a biliary HCO_3^- umbrella serving to protect human cholangiocytes against bile acid-induced injury. AE2 is a key contributor to this protective mechanism. The development and progression of cholangiopathies, such as primary biliary cirrhosis, may be a consequence of genetic and acquired functional defects of genes involved in maintaining the biliary HCO_3^- umbrella.

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Introduction

Human biliary HCO_3^- secretion accounts for 25–40% of total bile flow and by far exceeds that of rodents [1]. The specific function of this pronounced HCO_3^- secretion in humans is not understood. However, impaired function of anion exchanger 2 (AE2) and impaired HCO_3^- secretion have been recognized as potential pathogenetic factors in primary biliary cirrhosis [2–7]. We have recently specu-

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lated that the pronounced biliary HCO_3^- secretion in humans constitutes a protective mechanism of cholangiocytes against the toxicity of bile salts [8]. Hydrophobic bile salts induce cytotoxicity in many cell types, including hepatocytes, already at low micromolar concentrations [9–12]. In striking contrast, human biliary epithelial cells are exposed to high millimolar concentrations of hydrophobic bile salts under physiologic conditions [13] without any signs of cytotoxicity.

A major route of cytotoxicity induced by bile salts is apoptosis, which is mediated via intracellular signaling pathways [9–12]. Penetrability of bile salts is determined by their polarity and protonation [14]. Glycine conjugates account for the majority of bile salts in human bile, have a pK_a of approximately 4 [15] and at physiologic pH are protonated, apolar and thus cell permeable at considerable, micromolar amounts. Even small changes in local biliary pH close to the apical membrane of cholangiocytes have a dramatic effect on the sensitivity of cholangiocytes towards glycine-conjugated bile salt toxicity. In rodents, which have a more hydrophilic, less toxic bile salt pool with mainly taurine conjugates [13] (pK_a of approx. 1–2) [15], changes in biliary pH would have a minor, negligible effect on bile salt protonation and toxicity. pH dependency of cell penetration and toxicity by bile salts has been established in gastric and esophageal mucosa cells [16–18]. It was the aim of the present study to test the concept of pH dependency of bile salt-induced toxicity in human cholangiocytes and to explore the role of AE2 expression in the protection against hydrophobic bile salt-induced cholangiotoxicity.

Methods

Two cholangiocarcinoma cell lines and an immortalized human cholangiocyte cell line were stimulated with the hydrophobic bile salt chenodeoxycholate (CDC) or its glycine and taurine conjugates at different pH from 7.4 to 6.4. Bile salt uptake was determined by intracellular accumulation of ^{14}C-glycochenodeoxycholate (GCDC), and cholangiotoxicity was determined by metabolic activity (WST-1) and apoptosis assays (caspase-3/-7 activity). Cholangiocellular bile salt uptake and bile salt-induced apoptosis were also tested after knockdown of AE2 in immortalized human cholangiocytes by transduction with specific shRNA.

Results

Cholangiocellular Bile Salt Uptake Is pH-Dependent
As calculated from the Henderson-Hasselbalch equation, protonation of chenodeoxycholate (CDC, pK_a ap-

prox. 4) and its glycine conjugate GCDC (pK_a approx. 4.2) are highly sensitive to changes of pH around the physiologic range, whereas TCDC (pK_a approx. 2) stays virtually unaffected. Accordingly, intracellular accumulation of ^{14}C-GCDC in immortalized human cholangiocytes increased exponentially when pH was decreased from 7.4 to 6.4 during bile salt incubation.

Bile Salt-Induced Cholangiocellular Toxicity Is pH-Dependent
In both cholangiocarcinoma cell lines and immortalized human cholangiocytes, CDC- and GCDC-induced cholangiocyte toxicity, but not TCDC-induced, were pH-dependent and increased gradually when pH was lowered from 7.4 to 7.1, 6.7 or 6.4. In immortalized cholangiocytes, no bile salt-induced toxicity was observed even at low millimolar concentrations of CDC (0.5 mM), GCDC (1.5 mM) or TCDC (2.0 mM) at pH 7.4. At a lower pH of 6.4, however, 0.5 mM CDC and 1 mM GCDC led to a decrease in cell viability by >80% and increased caspase-3/-7 activity almost 10- and 30-fold, respectively. Acidification alone had no effect on cell viability or apoptosis. TCDC-induced cell damage did not increase within the pH-range studied, reflecting its relative inertness in protonation in this range.

Knockdown of AE2 Renders Cholangiocytes More Sensitive to Bile Salt-Induced Toxicity
AE2 knockdown in immortalized cholangiocytes led to a 3- and 2-fold increase of apoptosis induced by 0.75 mM CDC or 2 mM GCDC at pH 7.4. When increasing pH to 8.0 during bile salt exposure, AE2 knockdown cells were rescued from bile salt toxicity. Again, TCDC-induced toxicity was not affected by AE2 knockdown.

Conclusion and Outlook

Our in vitro data indicate that penetration of bile salts into cholangiocytes is pH-dependent, as predicted from their pK_a values. Accordingly, we demonstrated that bile salt-induced toxicity and apoptosis in cholangiocytes is determined by extracellular pH and that alkalization of bile close to the apical membrane of cholangiocytes via HCO_3^- secretion might be a key protective mechanism against bile salt toxicity. By shRNA-mediated knockdown, AE2 was confirmed as a candidate gene for formation of the postulated 'biliary HCO_3^- umbrella' [8].

Our findings may delineate why HCO_3^- secretion is highly pronounced in man compared to rodents, as pro-

Fig. 1. The biliary HCO_3^- umbrella. Several signaling pathways regulate the formation of the postulated biliary HCO_3^- umbrella [8]. Dysfunction of any of the delineated elements [20] might contribute to fibrosing/sclerosing cholangitis. Established and experimental therapeutic agents such as UDCA or norUDCA stabilize the biliary HCO_3^- umbrella. ACh = Acetylcholine; ATP = adenosine trisphosphate; CA = carboanhydrase; CaCl = Ca^{++}-dependent chloride channel; cAMP = cyclic adenosine monophosphate; CDCA = chenodeoxycholic acid; CFTR = cystic fibrosis transmembrane conductance regulator; ER = endoplasmic reticulum; P2Y = purinergic P2Y receptors; SR = secretin receptor; TGR5 = G protein-coupled bile acid receptor.
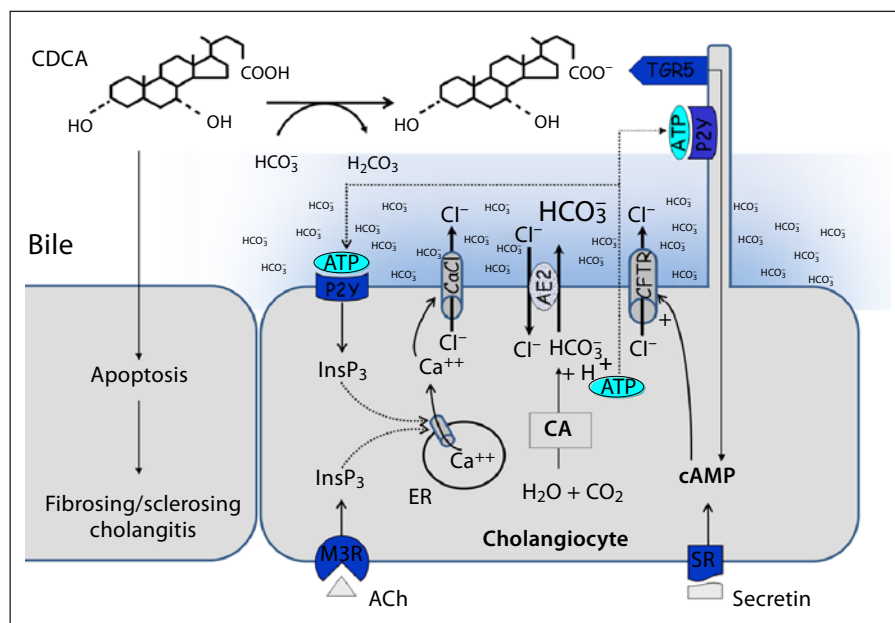
tonation of the mainly glycine-conjugated bile salt pool in man, but not of the less toxic mainly taurine-conjugated rodent bile salt pool, is sensitive to changes in biliary pH within the physiologic range [8].

Our hypothesis of a human biliary 'HCO_3^- umbrella', supported by the in vitro data provided here, provides a unifying link between the molecular pathogenesis and progressive course of different cholangiopathies [8]. Genetic variations of the candidate HCO_3^--secreting enzyme AE2 have been associated with improved prognosis of primary biliary cirrhosis [2], and mutations of the bile salt sensor TGR5, which mediates biliary HCO_3^- secretion, have been associated with PSC [19]. Altered maintenance of a transmembrane Cl^- gradient, driving HCO_3^- secretion, or altered direct flux of HCO_3^- via CFTR might contribute to liver disease associated with cystic fibrosis. Finally, disrupted acetylcholine signaling after liver transplantation, physiologically stimulating HCO_3^- secretion, may contribute to the formation of nonanastomotic biliary strictures following liver transplantation (fig. 1). Further experimental work is underway to (dis-)prove these speculations.

Disclosure Statement

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