Retrieval of the Rat Canalicular Conjugate Export Pump Mrp2 is Associated with a Rearrangement of Actin Filaments and Radixin in Bile Salt-induced Cholestasis

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Abstract

Bile salts may initiate or aggravate cholestasis in man. Infusion of Taurochenodeoxycholate (TCDCA) represents a model of bile salt-induced cholestasis in rat. The events leading to cholestasis are incompletely understood. The canalicular conjugate export pump Mrp2 is the major driving force for the bile salt-independent bile flow. Redistribution of Mrp2 has been suggested to cause reduction in bile flow in others models of acute cholestasis (i.e. endotoxin, phalloidin, GSH-depletion). We have studied the effects of TCD-CA on the distribution of Mrp2 and P-glycoproteins with respect to changes in the actin cytoskeleton and actin associated proteins radixin and ZO-1. Bile duct cannulated rats were infused with TCDCA (0.1 and 0.4 μmol/min/100g body weight) and bile flow was measured. After 30 min livers were removed and distribution of Mrp2, P-glycoproteins, actin, actin-associated radixin and ZO1 were studied by immunofluorescence analysis. TCDCA at subcholestatic amounts (0.1 μmol/min/100 g body weight) led to distortion and dilation of the canaliculi which was apparent in actin, ZO-1, and Mrp2 fluorescence. Administration of higher amounts of TCDCA (0.4 μmol/min/100g body weight) led to a reduction of bile flow to 31 % of control bile flow. Radixin, which localized strictly to the plasmamembrane in controls, was detected in intracellular structures partially colocalizing with actin aggregates especially at the sinusoidal membranes as visualized by double-immunofluorescence staining. Mrp2 appeared in pericanalicular membrane structures in cholestatic animals whereas P-glycoproteins remained unchanged under these conditions.
Conclusions: Bile salt-induced cholestasis is associated with changes of the actin cytoskeleton and actin binding protein radixin and a retrieval of the canalicular export pump Mrp2.

Abbreviations: Bsep, rat bile salt export pump (Abcb11); CA, cholic acid; DPPIV, dipeptidyl-peptidase IV; Mrp2, multidrug resistance associated protein 2 (Abcc2); TCDCA, Taurochenodeoxycholate

INTRODUCTION

Substantial retention of bile salts can occur in cholestatic liver disease which by themselves may initiate or aggravate cholestasis. In chronic cholestasis, bile salts may lead to a decrease in bile salt secretory rate by down-regulation of membrane transport proteins in a model of bile salt feeding in rat [1]. Moreover, bile salts may aggravate and maintain cholestasis by inducing apoptosis and necrosis in hepatocytes [2, 3]. However, these mechanisms can not readily explain the very early reduction in bile flow observed in animal models of acute bile salt-induced cholestasis in which cholestasis occurs within minutes after intravenous administration of cholestatic doses of bile salts [4, 5].

The multidrug resistance protein 2 (Mrp2, Abcc2) has been identified as the hepatocyte canalicular conjugate export pump mediating adenosine triphosphate (ATP)-dependent transport of a wide range of amphiphilic anionic conjugates into bile [6]. Mrp2-dependent secretion of solutes largely contributes to the bile salt-independent fraction of bile flow accounting for about half of the bile flow in rat [7]. Redistribution of Mrp2 has been suggested to cause reduction in bile flow in several models of acute cholestasis (i.e phalloidin, endotoxin, GSH-depletion, estradiol-17beta-D-glucuronide) [8-11]. Retrieval of Mrp2 may be accompanied with morphological changes at the canalicular membrane including rearrangement of microfilaments [9] and the protein radixin [12], which has been shown to crosslink actin filaments and integral membrane proteins including Mrp2 [13].

Therefore, the aim of present study was to determine the dose-dependent effect of Taurochenodeoxycholate on bile flow, hepatocyte canalicular morphology, actin and actin-binding protein radixin and the subcellular localization of Mrp2. Our results show that acute bile salt-induced cholestasis may be in part attributed to alteration of the canalicular architecture involving disintegration and dissociation of microfilaments and radixin leading to a retrieval of Mrp2 from the canalicular membrane into the cytosol.

METHODS

Animals
Experiments were performed with male Wistar rats (Charles River Wiga, Sulzfeld, Germany) weighing 200 to 250 g. All experiments were carried out under general anesthesia. Animals were kept according to the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23, revised 1985).

Bile collection

Animals were anesthetized with Rompun (Bayer, Germany; 15 mg/kg) and Ketanest (Parke-Davis, Morris Plains, NJ; 100 mg/kg), and the common bile duct was cannulated with a polypropylene tube (outer diameter 0.6 mm). Bile was collected in 10-min intervals into preweighed tubes and bile flow was calculated by weight of the samples. After reaching constant bile flow (at 20 min), rats were infused either with saline solution (group A, controls) or with TCDCA in 0.9% NaCl at a rate of 0.1 (group B) or 0.4 (group C) μmol/min/100g body weight via the portal vein. At 30 min, when bile flow was reduced in group C animals, organs were removed and processed as described below. Bile flow values are expressed as mean ± SD of experiments from four animals per group.

Antibodies

The rabbit polyclonal antibody EAG15 was raised against a 12-amino acid peptide from the carboxyl-terminus of Mrp2 [14]. The mouse monoclonal antibody C219 was purchased from Centocor (Malvern, PA) and reacts with rat P-glycoproteins including Mdr1a, Mdr1b, Mdr2, and Bsep. The rabbit polyclonal antibody TK89 raised against the C-terminal halves of radixin was kindly provided by Dr S. Tsukita, Kyoto University, Kyoto, Japan. The monoclonal antibodies Be 9.2, directed against rat canalicular ecto-ATPase [15], and De 13.4 [16] directed against rat dipeptidyl-peptidase IV (DPPIV), were gifts from Dr. Werner Reutter, Berlin. Rat monoclonal antibody against ZO-1 was purchased from Chemicon International Inc. (Temecula, Ca). TRITC-conjugated phalloidin was purchased from Sigma (St. Louis, MO). Cy2-conjugated and Cy3-conjugated secondary antibodies were purchased from Dianova (Hamburg, Germany). Nuclei were stained with Hoechst 33258 (Hoechst, Frankfurt, Germany).

Immunofluorescence microscopy

For immunofluorescence microscopy, livers were removed and immediately deep-frozen in isopentane precooled in liquid nitrogen and stored at -80°C. Tissue sections (2-4 μm) were prepared with a cryotome (FrigoCut 2800E; Leica, Nussloch, Germany), air dried for 2 hours, and fixed for 10 min with cold acetone (-20 °C). For actin staining, sections were fixed at room temperature in 3% paraformaldehyde containing 100 mmol/L 1,4piperazinebis (ethane sulfonic acid), 5 mmol/L ethylene glycol-bis (b-aminoethyl ether)-N,N,N8,N8-tetraacetic acid, and 2 mmol/L MgCl$_2$, at pH 6.8. For double-label immunofluorescence microscopy, the primary antibodies were applied simultaneously for 60 min. Sections were washed 3-times for 10 min with PBS and incubated with the combined secondary antibodies for 30 min. After washing 3-times with PBS, sections were rinsed with distilled water, air dried and mounted with Moviol (Hoechst, Frankfurt, Germany). Micrographs were taken with a Zeiss Axiophot (Carl Zeiss, Jena, Germany) on Kodak Elite II 400 ASA films (Kodak, Rochester, NY) exposed twice using the appropriate filter combinations for excitation of the secondary antibodies. Antibodies were diluted in PBS containing 5% fetal calf serum.

RESULTS

Effect of TCDCA infusion on bile flow

To study the effects of TCDCA-infusion on bile flow, rats were infused either with saline solution (group A, controls) or with TCDCA in 0.9% NaCl at a rate of 0.1 (group B) or 0.4 (group C) μmol/min/100g body weight via the portal vein. TCDCA at subcholestatic amounts (0.1 μmol/min/100 g body weight) did not lead to significant changes in bile flow over a period of 30 minutes (Fig. 1, groups A and B). Administration of higher amounts of TCDCA (0.4 μmol/min/100g body weight) led to a reduction of bile flow to 31 % of control bile flow which was significant at 20 and 30 minutes (Fig. 1, group C; P < .05).

Effect of TCDCA Infusion on canalicular morphology, actin, and ZO-1

The effects of TCDCA infusion on bile canalicular morphology, Mrp2 distribution, actin cytoskeleton and microfilament-associated radixin and ZO-1 was studied in groups of rats infused with 0.1 and 0.4 μmol/min/100 g body weight TCDCA (groups B and C). Livers were removed after 30 minutes of bile salt infusion (or saline infusion) and organs were processed as described under materials and methods. In control rats, Mrp2 fluorescence was largely restricted to the bile canalicular membrane. Actin staining is pronounced in the vicinity of canaliculi (pericanalicular staining) and to lesser extend at the basolateral membrane whereas ZO-1 staining is solely associated
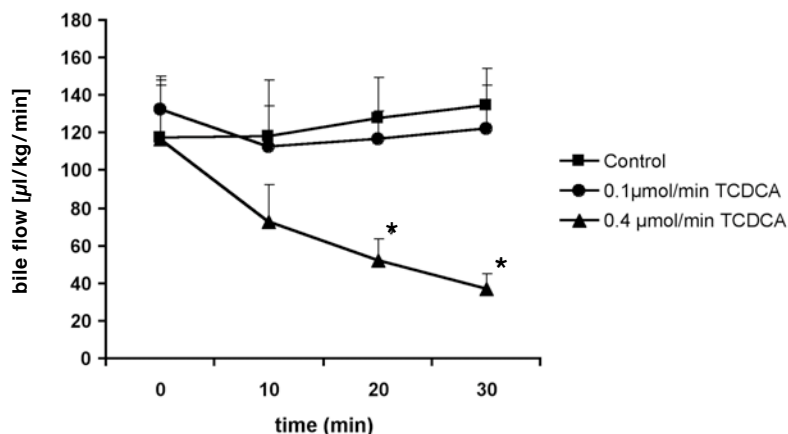

Fig. 1. Sequential changes in the bile flow in three groups of rats. Group A: ■, control rats infused with saline solution; Group B: ●, TUDCA, 0.1 μmol/min/100g body weight; Group C: ▲, TUDCA, 0.4 μmol/min/100g body weight; *significantly different from corresponding values in group A (P < .05)
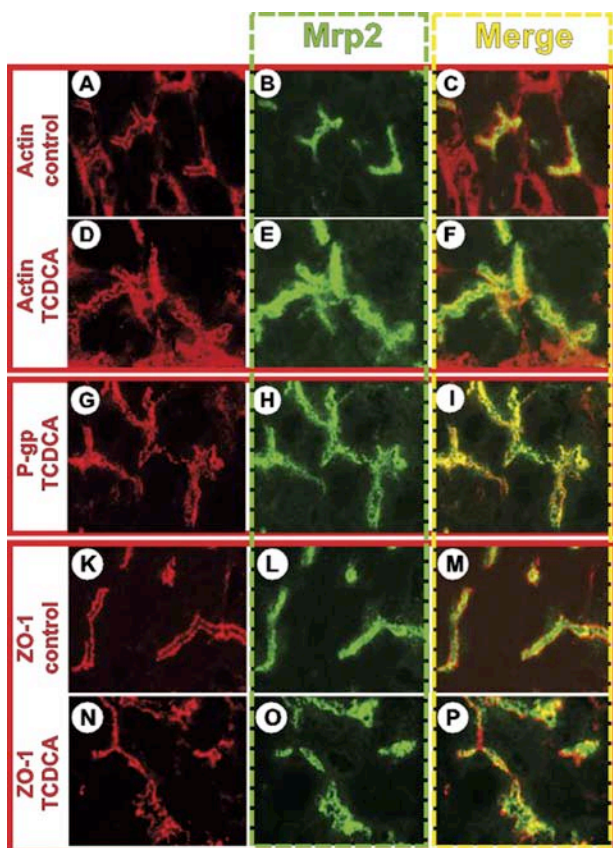
Fig. 2. Changes of the canalicular morphology after TCDCA treatment. Cryosections of livers from control (A-C, K-M) and TCDCA-treated rats (D-I, N-P) were double stained for Mrp2 (B, E, H, L, O) with either actin (A, D), P-glycoproteins (G) or ZO-1 (K, N). TCDCA treatment leads to deformation and widening of canaliculi (E-I, N-P). Deformation affects the actin cytoskeleton (D, F) and tight junction morphology (N, P). Although changes of canalicular morphology after low dose TCDCA-treatment (0.1 μmol/min/100g body weight) can be readily observed, bile flow did not significantly differ in both groups.
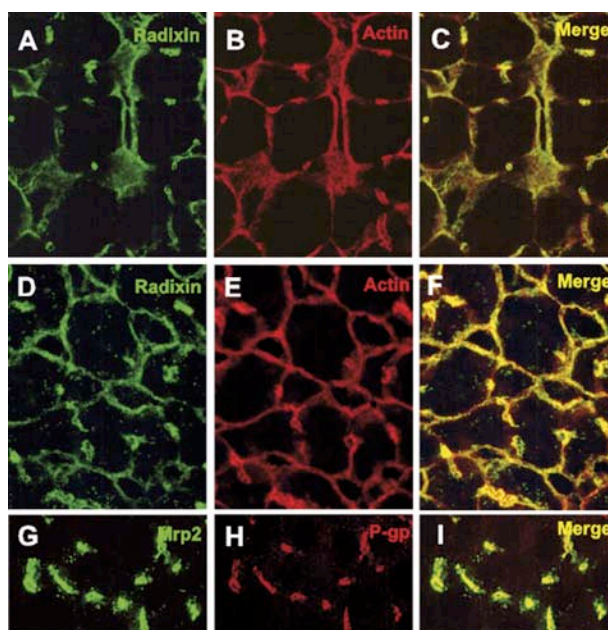

Fig. 3. A-F: Immunofluorescence staining of actin (red) and actin-associated radixin (green) in control (A-C) and TCD-CA-treated (D-F) rat livers. In control livers, actin (A) and radixin (B) colocalize at the plasma membrane of hepatocytes as revealed by the yellow color on merged image (C). In TCDCA-treated rat livers, radixin containing structures are scattered throughout the hepatocytes (D, E) indicating a disturbance of the actin-radixin-membrane interaction upon TCDCA treatment. G-I: Immunofluorescence staining of Mrp2 (G; green) and P-glycoproteins (H, red) in TCDCA-treated rat livers. Mrp2 fluorescence can be visualized in vesicular-like structures surrounding the canaliculus (G, I). These vesicular-like structures were negative for the P-glycoprotein staining as revealed by their green color in the merge image (I). In control livers, Mrp2 and P-glycoproteins are both strictly localized to the apical domains of hepatocytes (not shown).
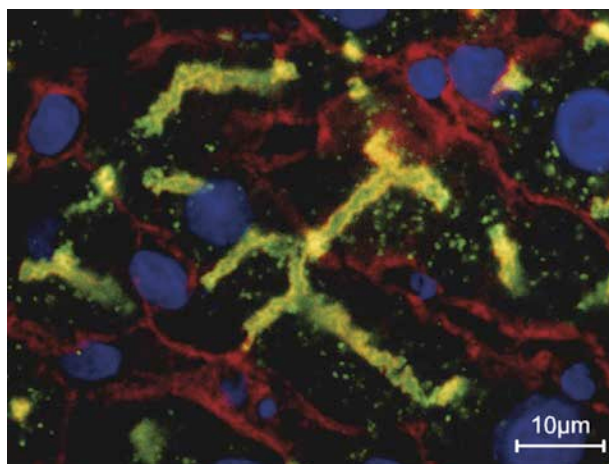
with tight junctions (pericanalicular). At subcholestatic amounts, TCDCA led to a widening and distortion of the bile canaliculi as revealed by staining for actin (Fig 2 A, F) and ZO-1 (Fig. 2 N, P) as compared to control animals (Fig 2 A, K). Occasionally canalicular outpouchings of the membrane were observed. The width of canaliculi increased from approximately 2 to 4 μm as estimated by fluorescence appearance. Morphological changes were also apparent on Mrp2 staining and P-glycoprotein (P-gp-) staining as revealed in Panels G and H of Figure 2, respectively. However, localization of export pumps Mrp2 and P-gps was largely confined to the canalicular membrane on double labeled slides as exemplified on merged images of Mrp2 with either actin, P-gp or ZO-1 (Fig. 2 F, I, P). Similarly, ecto-AT-Pase and DPPIV were confined to the canalicular domain under these conditions (not shown).

Redistribution of radixin and Mrp2 in TCDCA-induced cholestasis

Administration of higher amounts of TCDCA (0.4 μmol/min/100g body weight) led to a reduction of bile flow to 31 % of control bile flow (Fig.1). In these

Fig. 4. TCDCA-induced retrieval of Mrp2. In controls, Mrp2 fluorescence was largely restricted to the canalicular domain of rat hepatocytes (see Figure 2 A, C). In cholestatic rats, Mrp2 fluorescence (green) was located in intracellular vesicles in addition to the canalicular domain. Vesicles were predominantly located in the pericanalicular area. Note the distortion of canaliculi revealed by the actin staining (red / yellow) in the triple fluorescence. Nuclei are stained in blue.

animals (group C), radixin, which localized strictly to the plasmamembrane in controls (Fig. 3 A, C) was detected in intracellular structures partially colocalizing with actin aggregates especially at the sinusoidal membranes as visualized by double-immunofluorescence staining (Fig. 3 D, F). In controls, radixin fluorescence staining was smooth with regular appearance whereas in cholestatic animals the fluorescence staining was irregular with a fuzzy punctuate pattern. In cholestatic rats, considerable Mrp2-fluorescence staining was observed in intracellular vesicular membrane structures (Fig. 4). In contrast to Mrp2, P-glycoprotein fluorescence (i.e. Bsep and Mdr-Pgp), ecto-ATPase and DPPIV remained largely unchanged under these conditions (Fig.3 G-I, ecto-ATPase and DPPIV not shown), indicating that retrieval of bile canalicular export pumps is specifically observed for Mrp2 in association with a redistribution of radixin.

DISCUSSION

Cholestasis, defined as impairment of bile formation, is accompanied by retention of bile salts, which by themselves can cause hepatic injury and cholestasis. The cholestatic potential of bile salts depends to some extent upon the chemical nature of the bile salts and may also be dose dependent. Dehydrocholate and taurocholate are both efficient choleretic agents [17], whereas the monohydroxy bile salt, taurolithocholate, produces a dose-dependent inhibition of bile flow [18, 19] which may be prevented by infusion of micelle forming primary bile salts [20-22]. In addition, a dose-related cholestatic response to the dihydroxy bile salt, chenodeoxycholate, has been demonstrated in the isolated perfused rat liver as well as in in vivo experiments [23, 24]. In the present study we have chosen taurochenodeoxycholate (TCDCA) to induce cholestasis as TCDCA represents one of the principal bile salts accumulating in cholestasis.

Infusion of TCDCA at a rate of 0.1 μmol/min/100 g body weight did not lead to significant changes in bile flow over a period of 30 minutes (Fig. 1, group B). However, under these conditions, TCDCA infusions lead to a widening and characteristic distortion of bile canaliculi associated with outpouchings of the canalicular membrane. Morphological changes were regularly observed at the level of membranes (Mrp2, P-gps) tight junctions (ZO-1), and cytoskeletal proteins (actin). These morphological changes resemble those observed by scanning electron microscopy after taurolithocholate infusion [25]. In their careful study, Layden et al have noted that distortion of bile canaliculi was uniquely observed after treatment of rats with bile salts and that these changes were absent in ethinyl estradiol-treated rats and bile duct-ligated rats, respectively, indicating that the observed morphological changes may represent a pathognomonic feature induced by bile salts related to their direct toxic effect on cell membranes [25]. In the present study we have demonstrated, that theses distinctive abnormalities may already occur after administration of subcholestatic amounts of bile salts and must therefore not necessarily affect bile formation and function of bile canalicular export pumps Mrp2 and Bsep.

TCDCA infusion at a rate of 0.4 μmol/min/100 g body weight was sufficient to significantly reduce bile flow in rats to 31% of control animals (Fig. 1; group C). In cholestatic animals, immunofluorescence staining of actin-associated radixin was disrupted and irregular and radixin was partially located inside the hepatocytes (Fig. 3 D, F) This is in contrast to the normal regular pericanalicular and perisinusoidal staining of radixin in control rats (Fig. 3 A, C). Moreover, in cholestatic animals, Mrp2 was retrieved from the canalicular membrane into intracellular membrane compartments. Redistribution of membrane transport proteins (i.e. endocytic retrieval) was uniquely observed for the conjugate export pump Mrp2 whereas P-glycoprotein fluorescence was not altered under these conditions (Fig. 3). Moreover, fluorescence staining of ecto-ATPase and DPPIV, two canalicular ecto-enzymes with a single transmembrane domain was not affected under these conditions (not shown) indicating that dissociation of radixin from actin and canalicular membrane solely affects Mrp2 with respect to redistribution (i.e. endocytic retrieval).

Redistribution of Mrp2 and/or Bsep from the canalicular membrane into the cytosol (i.e. endocytic retrieval) may be considered a decisive step leading to decrease in bile flow under conditions of acute cholestasis. The ATP-dependent export pumps Mrp2 and Bsep represent the major driving forces of bile flow [6, 26]. Retrieval of these export pumps will inevitably result in diminution of bile flow due to reduced transport capacity at the bile canalicular membrane. Retrieval of the conjugate export pump Mrp2 in association with a redistribution of radixin has been described in other forms of cholestasis in rats and humans, respectively [12, 27, 28]. Furthermore, loss of radixin in radixin knockout mice resulted in a loss of Mrp2 from the canalicular membrane [13]. These results gave rise to the hypothesis that radixin, a cross linker between actin and membrane proteins, is necessary for the proper location (and function) of Mrp2 in the bile canalicular membrane. In the present study we provide evidence that retrieval of Mrp2 in association with redistribution of radixin contributes to bile salt-induced cholestasis. Since accumulation of bile salts represents a major consequence of cholestasis per se the described mechanism is likely to be involved in initiating or aggravating various cholestatic liver diseases.

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