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ORIGINAL ARTICLE

TUDCA prevents cholestasis and canalicular damage induced by ischemia-reperfusion injury in the rat, modulating PKCα–ezrin pathway

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Summary

Cholestasis, induced by liver ischemia-reperfusion injury (IRI), is characterized by dilatation of bile canaliculi and loss of microvilli. Tauroursodeoxycholic acid (TUDCA) is an anti-cholestatic agent, modulating protein kinase C (PKC) α pathway. PKC reduces ischemic damage in several organs, its isoform α modulates ezrin, a key protein in the maintenance of cell lamellipoidal extensions. We evaluated the effects of TUDCA on cholestasis, canalicular changes and PKCα–ezrin expression in a rat model of liver IRI. Livers flushed and stored with Belzer solution or Belzer + 10 mM TUDCA (4 °C for 6 h) were reperfused (37 °C with O_2) with Krebs–Ringer bicarbonate + 2.5 μmol/min of Taurocholate or TUDCA. Bile was harvested for bile flow assessment. Liver tissue was employed for Electron Microscopy (EM) and for PKCα and ezrin immunoblot and immunofluorescence. The same experiments were conducted with the PKCα inhibitor Go-6976. TUDCA-treated livers showed increased bile flow (0.25 ± 0.17 vs. 0.042 ± 0.02 μl/min/g liver, $P < 0.05$) and better preservation of microvilli and bile canalicular area at EM. These effects were associated with increased PKCα and ezrin expression ($P = 0.03$ and $P = 0.04$ vs. control respectively), as also confirmed by immunofluorescence data. PKCα inhibition abolished these TUDCA effects. TUDCA administration during IRI reduces cholestasis and canalicular damage in the liver modulating PKCα–ezrin pathway.

Introduction

Biochemical markers of cholestasis are usually elevated early after orthotopic liver transplantation (OLT) in humans [1]. Concurrently, bile lipid secretion and in particular bile acid output is greatly depressed [2–4]. Experimental data in rodents suggest that cold and warm ischemia may play a major role in the impairment of transport systems involved in the maintenance of normal bile flow [5–7]. While conventional hematoxilin–eosin staining invariably demonstrates cholestasis in human liver after ischemia-reperfusion injury (IRI), the ultrastructural electron microscopy study reveals peculiar anatomical changes of bile canaliculi [8]. These are mainly represented by increase of canalicular space with relevant loss of microvilli. The proposed pathway of these alterations involves radical oxygen species production, resulting in oxidative changes of cytoskeleton causing the disruption of actin microfilaments. In a previous study, our group had demonstrated that use of tauroursodeoxycholic acid (TUDCA) during graft cold storage and reperfusion was able to greatly reduce ultrastructural bile canalicular

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damage after human OLT [9]. TUDCA is an hydrophilic bile acid with cytoprotective and membrane-stabilizing properties [10,11]. In addition, it has been demonstrated in experimental models to improve IRI damage in both hepatocytes and bile duct cells [12,13]. Our previous pilot study did not elucidate the mechanisms of TUDCA-induced preservation of bile canaliculi after IRI. While a possible beneficial modulation of immunologic factors seemed unrealistic [14], the protection from more hydrophobic bile acids was also unlikely, given the profound bile acids pool depletion occurring after OLT [2–4]. Thus the speculative explanations were that TUDCA protection in liver IRI was derived from a 'genuine membrane protective effect', as previously demonstrated by other authors in isolated hepatocytes [15]. In the current study, we reassessed this issue in order to investigate the possible mechanisms involved in TUDCA prevention of functional and morphological abnormalities associated with IRI-induced cholestasis. Our results suggest that activation of protein kinase C (PKC) α by TUDCA may be an important factor in this setting. This pathway, in fact, was in our experiments implicated both in the maintenance of choleresis as in the reduction of canalicular changes. These effects were associated with the preservation of ezrin protein expression and tissue distribution.

Materials and methods

Animal model

Surgical procedure and cold ischemia

All procedures were in compliance with the standard indications for animal care and treatment of our institution deriving from the NIH 'Guide for the Care and Use of Laboratory Animals'. Male Wistar rats (≅200 g) were obtained from Charles River (Milan, Italy). Animals were allowed free access to rodent food and to a 12-h day–night rhythm before experiments. Normal control rats were sacrificed shortly after anesthesia (sodium pentobarbital 50 mg/kg body weight, intraperitonally), the others underwent the following protocol of IRI and treatment. The abdomen was incised, the common bile duct cannulated (polyethylene PE-10 tubing), and the pancreatoduodenal collateral branch of portal vein ligated. After systemic heparinization, the portal vein and the inferior vena cava were cannulated with an 18-gauge and a 16-gauge Teflon intravenous catheter, respectively. Livers were flushed and stored at +4 °C with 50 cc of Belzer solution (control), or Belzer + TUDCA 10 mM (treated). Livers were then stored for 6 h at +4 °C in Belzer solution (cold ischemia time). This time span, used in previous animal researches [16,17], corresponded to mean cold ischemia time of grafts in our earlier TUDCA human study [9].

Warm reperfusion

Livers were re-perfused for 1 h with Krebs–Ringer bicarbonate (KRB) solution (NaCl 118 mM, KCl 4.8 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.9 mM, D-glucose 5.5 mM) at 37 °C, pH 7.4, gassed with O_2–CO_2 (95–5%) at 3.5 ml/min/g liver. Taurocholic acid (in control rats) or TUDCA (in treated) were added in the perfusion media, to reproduce a normal plasmatic bile acid concentration of 20 μM/l [18]. In the experiments with inhibited PKCα, the treated as well as the control livers received both cold and warm solutions added with Go-6976 (0.2 μM) [19]. Grafts were transferred in a temperature-controlled chamber. Bile flow was assessed gravimetrically. Aliquots of effluent were harvested every 5 min to assess lactate dehydrogenase (LDH) release. At the end of the experiments, tissue specimens were harvested for western blot analysis, electron microscopy and immunofluorescence.

Biochemical analysis

Total bile acid concentration and LDH were determined spectrophotometrically using a Beckman DU-640 apparatus (Fullerton, CA, USA) employing commercially available kits (Bio-Sud Spa, Riardo, Italy) and following the instructions of the vendor.

Transmission Electron Microscopy

Liver specimens for Electron Microscopy were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 h at 4 °C. After washing three times with the same buffer, samples were postfixed with 1% OsO_4 solution, dehydrated in graded ethanol solutions, and embedded in Spurr resin. Ultra thin sections were cut with a Reichert Ultracut Microtome and stained with uranyl acetate and lead citrate. Tissue specimens from three different experiments were observed with a Philips CM 12 electron microscope (Philips, Eindhoven, the Netherlands). Pictures were taken at 30 000× magnification. For morphometric study, 10 bile canaliculi for each experiment were randomly examined by an expert pathologist (GP) and the number of bile microvilli/μm^2 of canalicular area calculated [8].

Immunofluorescence

Frozen sections (5 μm) from three separate experiments were fixed in absolute methanol (30 min at −20°C), and washed three times in phosphate-buffered saline (PBS) (pH 7,4). Sections were then blocked with 10% serum, 0.5% bovine serum albumin and 0.5% of Triton X-100 in PBS for 30 min. Slides were washed again with PBS three times for 5 min and incubated overnight with primary antibody.

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After washing with PBS, slides were incubated with the secondary antibody (Alexa Fluor conjugated) for 45 min in the dark. Sections were washed again with PBS, a coverslip was mounted, and sections were examined with a BRC 600 laser scanning confocal microscope at 560 nm emission.

Immunoblot analysis

Western blot analysis for PKCα and ezrin was performed as previously described [20]. In brief, whole liver tissue specimens were homogenized in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate, 2 mM ethylediaminetetracetic acid, 10 mM NaF, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). PKCα translocation to membrane was obtained determining its content both in a triton soluble (membrane) and a triton insoluble (cytoskeletal) fraction as previously described [20,21]. Briefly homogenates were dissolved in Triton extraction buffer (0.1% Triton X-100, 5 mM Tris, 2 mM ethylene glycol tetra-acetic acid, 300 mM sucrose, 200 μM PMSF, 10 μM aprotinin, 10 μM leupeptin; pH 7.4). After centrifugation (10 min at 15 000 *g*), the supernatant corresponded to Triton-soluble fraction. Proteins (50 μg) were then resolved on a sodium dodecyl sulfate 7.5% polyacrylamide gel electrophoresis and blotted on a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with a 5% solution of nonfat dry milk in TBST (50 mM Tris, 150 mM NaCl with 0.05% Tween 20). After overnight incubation (+4 °C) with PKCα or ezrin primary antibody in TBST, membranes were washed five times in TBST and incubated for 1 h (room temperature) with secondary antibody. Excess antibody was removed washing five times with TBST and proteins were visualized using chemiluminescence (ECL Plus Kit; Amersham Life Science). The intensity of bands by scanning video densitometry employing a ChemiImager 4000 low light imaging system, (Alpha Innotech Corp, San Leandro, CA, USA).

Reagents

All reagents were from Sigma-Aldrich (Milan, Italy) unless otherwise stated. Purified mouse anti PKCα antibody and secondary HRP-labeled antibody were from Becton-Dickinson (Milan, Italy). Anti-ezrin antibody was from Zymed (Milan, Italy). Go-6976 was purchased from Calbiochem (WWR International, Milan, Italy).

Statistical analysis

Data were analyzed using the NCSS 2000 software package (Kaysville, UT, USA). Differences between groups were evaluated with the Student's *t*-test for unpaired data. A $P < 0.05$ was considered statistically significant.

Results

LDH release during reperfusion

Sinusoidal efflux of LDH (mU/min × g liver) during warm reperfusion was studied in five min aliquots as a biochemical marker of cytolysis. Maximum values were observed as soon as reperfusions were begun (0.88 ± 0.22 in controls, vs. 1.25 ± 0.5 for TUDCA). After 5 min LDH release decreased in both groups, being 0.31 ± 0.15 in control and 0.28 ± 0.17 in treated livers. Enzyme efflux presented only small fluctuations thereafter, demonstrating the viability of the livers during the rest of the experiment. There were no differences in the LDH release profile between control and TUDCA-treated livers.

Bile flow and BA secretion: effect of IRI, TUDCA and PKCα inhibition

Choleresis was evaluated through the estimation of bile flow and total bile acid secretion during one h warm reperfusion (Fig. 1). TUDCA greatly reduced the impairment in bile flow caused by IRI as indicated by the comparison with control ($P = 0.03$). Also BA secretion was improved by TUDCA administration; however the difference with controls did not reach statistical significance. When the PKCα inhibitor Go-6976 was administered, the effect on bile flow of TUDCA was abolished (Fig. 1), whereas the bile flow and BA secretion did not change in control (respectively 0.04 ± 0.02 and 0.21 ± 0.1; data not shown in figure).

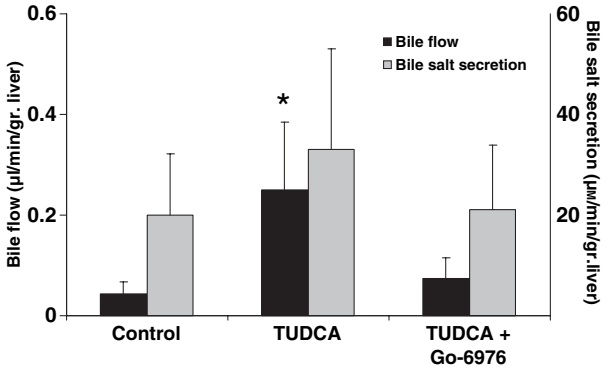

Figure 1 Bile flow and bile salt secretion after IRI in control livers and liver perfused with TUDCA or TUDCA + Go-6976. TUDCA infusion determines a statistically significant increase in bile flow in comparison with controls (*$P < 0.03$). A similar effect was obtained for bile salt secretion but statistical significance is not achieved. Addition of Go-6976 to TUDCA reverted these effects.

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PKCα protein expression and activation

Protein kinase C α was assessed by both immunoblot analysis (Fig. 2) and immunofluorescence (Fig. 3). In order to evaluate whether the changes of PKCα expression were associated with its increased activation (migration to membrane), protein analysis was conducted on triton-soluble (membrane) and insoluble (cytosol) fractions (Fig. 2). Fractions analysis demonstrated that TUDCA treatment enhanced PKCα expression in cytosol and its translocation to membrane as activated membrane-associated form. At immunofluorescence (Fig. 3) TUDCA livers showed enhanced staining of PKCα, suggesting a prevalent localization at the membrane level (Fig. 3, panel c). As expected, this TUDCA-induced effect was abolished by Go-6976 (Figs 2 and 3, panel d), whereas the inhibitor did not change PKCα expression in controls (western blot, data not shown).

Electron Microscopy

The ultra-structural observation of bile canaliculi evidenced several differences between groups. Whereas the normal rats had bile canaliculi with a well-defined area

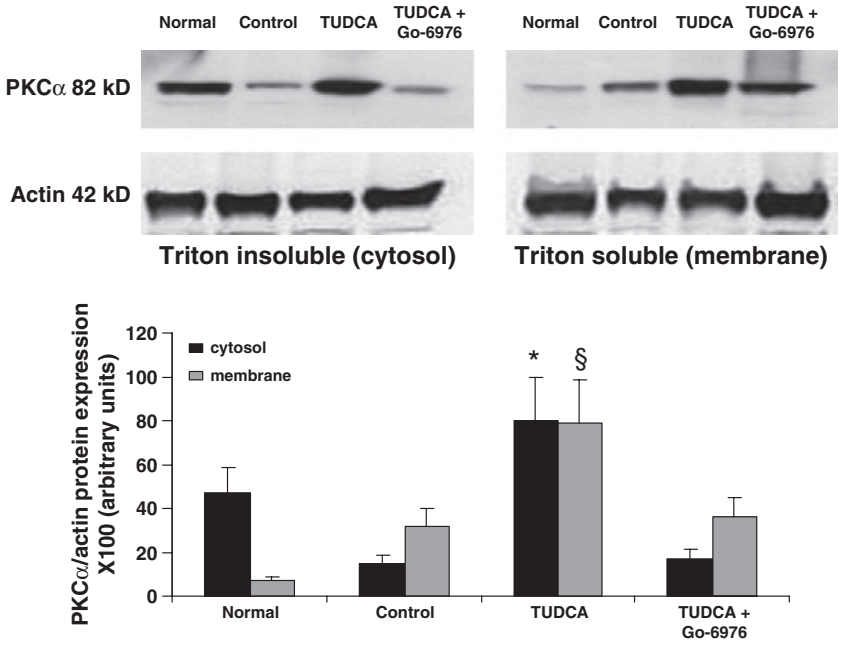

Figure 2 PKCα protein expression in cytosol and membrane of normal liver and after IRI in control and liver perfused with TUDCA or TUDCA + Go-6976. TUDCA treatment increased PKCα expression in both cytosol and membrane fraction. The enhanced distribution in membrane fraction denotes protein activation (mild gray bar). Results are mean values ± SD of three experiments. $^{*}P < 0.05$ versus control and TUDCA + Go-6976. $^{\S}P < 0.05$ versus all other groups.

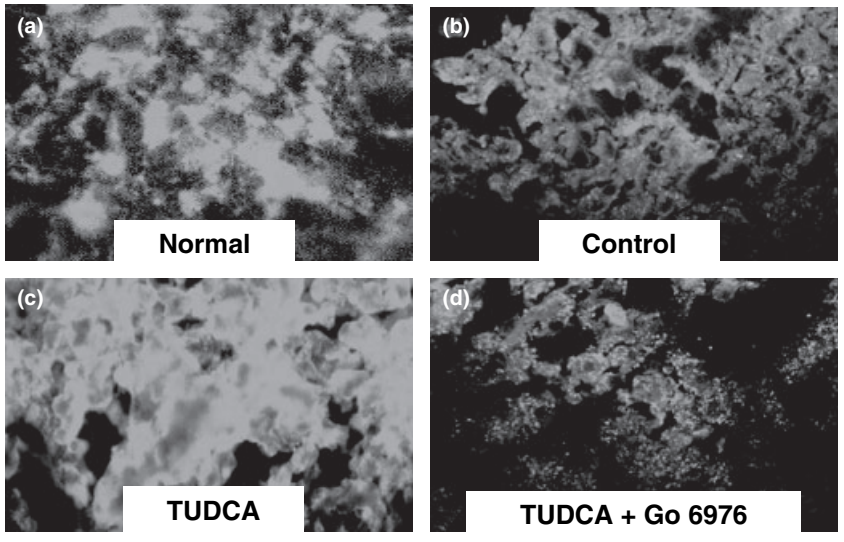

Figure 3 Immunofluorescence staining for PKCα in normal liver (panel a), and after IRI in control livers (panel b) and liver perfused with TUDCA (panel c) or TUDCA + Go-6976 (panel d). An enhanced expression and membrane distribution of PKCα, is suggested in TUDCA-treated livers in comparison with other groups. Original magnification 400×.

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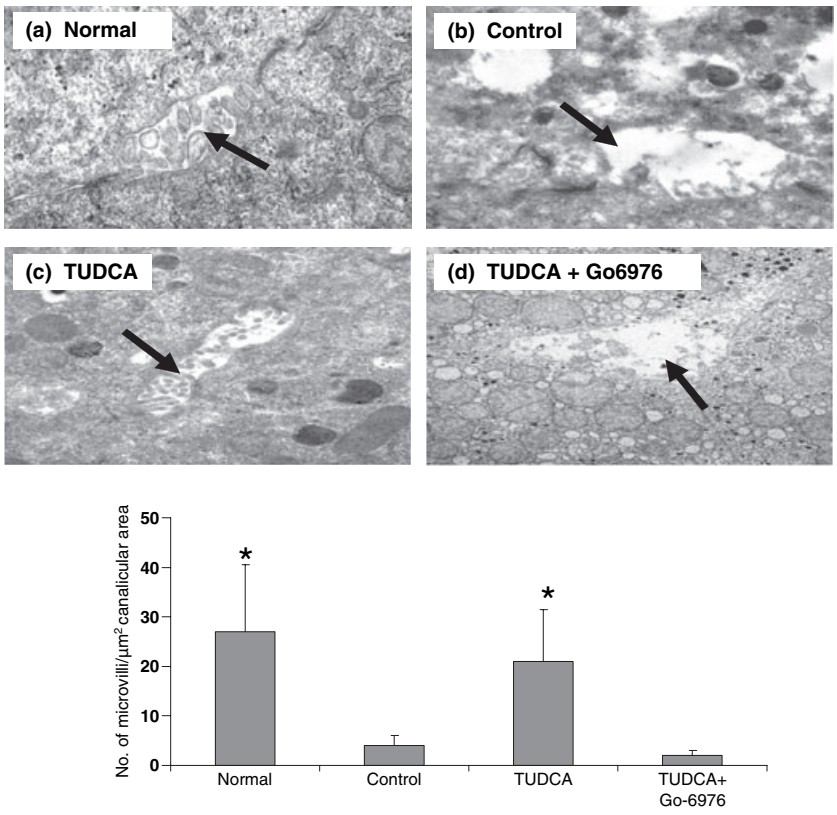

Figure 4 Transmission Electron Microscopy study of bile canaliculi (black arrows) in normal liver and after IRI in control livers (panel b) and liver perfused with TUDCA (panel c) or TUDCA+Go-6976 (panel d). TUDCA preserved canalicular profile and microvillar structures similar to those of normal liver. This effect was abolished by the co-infusion of the PKCα inhibitor Go-6976 (panel d). Original magnification 30 000×. The graph shows in bars the calculated number of bile microvilli/μm^2 of canalicular area for each group (10 bile canaliculi in three different experiments). In TUDCA-treated livers the amount of microvillar structures was well preserved and similar to normal. *$P < 0.05$ in comparison with control and TUDCA + Go-6976.

and large presence of microvilli (Fig. 4, panel a), control liver presented major alterations of canalicular profile, where normal microvillar structures were rarely observed (Fig. 4, panel b). Similar alterations were observed when Go-6976 was administered to controls (data not shown). TUDCA treatment (Fig. 4, panel c) was able to largely prevent the alterations induced by IRI and a microvillar distribution similar to normal liver was observed. TUDCA administration was however ineffective when Go-6976 was simultaneously infused (Fig. 4, panel d). The graph in Fig. 4 shows the calculated number of bile microvilli/μm^2 of canalicular area in each group. In TUDCA-treated livers an amount of microvillar structures similar to normal liver was maintained ($P < 0.05$ in comparison with control and TUDCA + Go-6976).

Ezrin protein expression

Figure 5 shows the immunoblot results of ezrin protein expression obtained in the different treatment groups. Whereas the control rats exhibited an ezrin expression less than a half of normal liver, TUDCA treatment greatly increased the rate of expression of this protein. The co-administration of the inhibitor Go-6976, simulta-

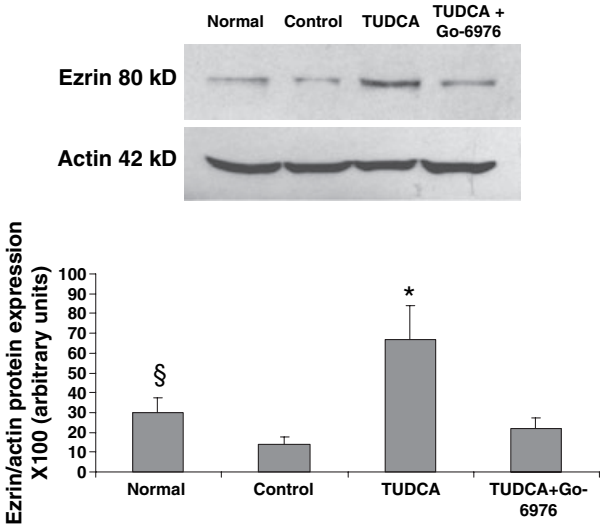

Figure 5 Ezrin protein expression in normal liver and after IRI in control and liver perfused with TUDCA or TUDCA + Go-6976. TUDCA (black bar) induced a significant increase of ezrin expression in comparison with other groups. Results are mean values ± SD of three experiments. *Difference statistically significant ($P < 0.05$) in comparison with normal, control and TUDCA + Go-6976. §$P < 0.05$ versus control.

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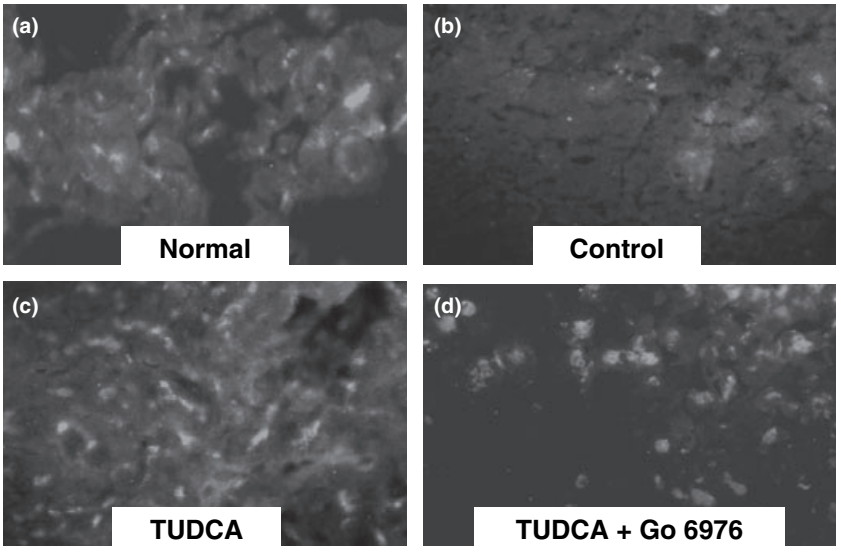

Figure 6 Immunofluorescence staining for ezrin in normal liver (panel a), and after IRI in control livers (panel b) and liver perfused with TUDCA (panel c) or TUDCA + Go-6976 (panel d). TUDCA determined an ezrin expression in whole liver similar to normal ones, thus preventing the altered distribution induced by IRI (see control, panel b). Go-6976 (panel d) reverted this effect. Original magnification 400×.

neously with TUDCA, reverted this effect, even though the inhibitor did not change ezrin expression in controls (data not shown). Immunofluorescence data showed a clear reduction of ezrin-staining in control rats (Fig. 6, panel b), whereas it was similar to normal livers in TUDCA group (Fig. 6, panel c). Consistently with immunoblot results, addition of Go-6976 counteracted the TUDCA effect on ezrin distribution (Fig. 6, panel d), confirming the key role of a functional PKCα in this process.

Discussion

Ursodeoxycholic acid (UDCA) and its taurine conjugate TUDCA are known to exert beneficial effect in human cholestatic liver diseases such as primary sclerosing cholangitis and primary biliary cirrhosis [22–24]. In the latter, UDCA treatment is able to reduce the risk of death and of end-stage disease requiring liver transplantation [25]. The molecular mechanisms involved in the therapeutic effect of these drugs are not completely elucidated yet. However, experimental evidences suggest that the anti-cholestatic properties of UDCA and TUDCA are related to the reduction of bile acid toxicity on liver epithelial cells and stimulation of hepatobiliary secretion through a PKCα dependent pathway [26]. PKC is a multigene family contributing to different cell functions. Multiple isoforms of PKC are expressed in mammalian cells in the so called 'immature form'. The activation (phosphorylation) requires translocation to selected sites of the cell, where these kinases exert their specific action [27]. The PKC pathway plays an essential role in counteracting ischemic damage in several tissues. For instance, a beneficial effect has been claimed for PKC activation on myocardial stunning during ischemic preconditioning (a brief period of warm ischemia prior to a longer one) [28]. Moreover, intestinal ischemic preconditioning completely reverts jejunal mucosal injury through PKC system activation [29]. In the liver, as also in other organs, protective strategies against ischemic damage are ineffective after inhibition of PKC pathway [30]. Several important properties of epithelial liver cells are dependent on the PKC isoform α, such as regulation of growth, as demonstrated in cholangiocarcinoma [31] and hepatocarcinoma cell lines [32], and modulation of bile secretion [33]. In addition, PKCα co-localizes and interacts with ezrin [34–36], a key protein in the organization of canalicular membrane and in the maintenance of apical microvilli in various tissues [37–39], including murine liver cells [40,41]. The major role of ezrin in villus morphogenesis has been clearly demonstrated in the intestine. Ezrin-deficient (ez-/-) mice do not survive after birth and exhibit major alteration in the intestinal mucosa, showing unorganized multilobar structures instead of multiple individual villar units [42]. This protein seems to be particularly vulnerable to ischemic or oxidative stress, and its degradation leads to modification of cell shape and loss of normal organization of apical membrane domain [40,43]. With this background, we undertake this study to evaluate whether TUDCA administration may modulate IRI-induced cholestasis in rat liver, and the possible mechanisms involved. As previously observed by other authors in a similar murine model [44], we confirmed the choleretic effect of TUDCA administration and the lack of a specific effect on cytolytic damage, as suggested by the observation of similar LDH-release between control and TUDCA. We extended this finding showing that this process most likely requires an increased expression and membrane translocation of PKCα. In fact, when the

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inhibitor Go 6976 was employed, PKCα activation was blocked together with bile flow. Even if a TUDCA/PKCα strict interaction and the resulting choleresis have already been described in another model of cholestasis [33], the efficiency of this system following IRI, has not been tested before. In addition, in our experimental setting, TUDCA not only normalizes but even up-regulates PKCα (Figs 2 and 3). With regard to TUDCA prevention of IRI-induced hepatic morphological alterations, an early report employing routine light microscopy failed to find any improvement, thus questioning a possible beneficial effect of this bile acid in the OLT setting [44]. However, in a more recent ultra-structural study [9], our group showed that use of TUDCA during cold storage of human liver, was able to significantly decrease bile canaliculi alterations including the loss of microvilli. In the present study, we readdressed this issue to investigate the possible mechanisms of these effects, bearing in mind that ezrin: (i) is a key protein in the maintenance of cellular microvillar structures; (ii) it is preferentially degraded in cells undergoing oxidative stress; and (iii) it has been demonstrated to be a downstream effector of PKCα. In keeping with these considerations, in our experiments TUDCA-preservation of canalicular environment after IRI was associated to the maintenance of adequate ezrin protein expression and tissue distribution (Figs 5 and 6). This paralleled the activation of PKCα. In this perspective, while some authors claim that the stimulation of PKCα–ezrin pathway may be involved in promoting cell migratory phenotypes as precursors of cancer metastases [34–36], our study suggests that up-regulation of this circuit may be beneficial in preserving cellular functions in particular conditions of damage. In regard to TUDCA–PKCα interaction, this was demonstrated to enhance insertion of canalicular transporter proteins, such as Bsep and Mrp2, into the apical membrane [33,45]. A possible contribution of this mechanism in the reduced injury and increased bile flow observed in our experiments. In addition we understand that our data on rodent may not be indisputably extrapolated to human, as species-derived differences may exist. However morphological alterations of canaliculi with reduction or impairment of microvilli are frequent ultrastructural findings in human cholestasis [46,47]. This supports the concept of a key role of these structures in normal bile formation [48,49], as their qualitative or quantitative impairment is associated with altered apical protein and transporter distribution, with major changes in secretory process [33,50]. In this perspective, the possible involvement of PKCα–ezrin pathway on functional and morphological features in different cholestatic conditions may be hypothesized. This issue however, requires further investigations.

Authorship

LB: study design, writer, animal procedure. GT: study design. MAR: electron microscopy. CL: animal procedure, experiments. GP: electron microscopy. AV: electron microscopy. CA: immunostaining. CT: animal procedure, experiments. MC: animal procedure, experiments. LT: experiments, revision of manuscript. FD: animal procedure, experiments. MA: study design.

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