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Tauroursodeoxycholic acid reduces bile acid-induced apoptosis by modulation of AP-1

Thomas Pusl [a], Timo Vennegeerts [a], Ralf Wimmer [a], Gerald U. Denk [a], Ulrich Beuers [b], Christian Rust [a,*]

[a] Department of Medicine II - Grosshadern, University of Munich, Marchioninistrasse 15, 81377 Munich, Germany
[b] Department of Gastroenterology and Hepatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

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Abstract

Ursodeoxycholic acid (UDCA) is used in the therapy of cholestatic liver diseases. Apoptosis induced by toxic bile acids plays an important role in the pathogenesis of liver injury during cholestasis and appears to be mediated by the human transcription factor AP-1. We aimed to study if TUDCA can decrease taurolitholic acid (TLCA)-induced apoptosis by modulating AP-1. TLCA (20 μM) upregulated AP-1 proteins cFos (26-fold) and JunB (11-fold) as determined by quantitative real-time PCR in HepG2-Ntcp hepatoma cells. AP-1 transcriptional activity increased by 300% after exposure to TLCA. cFos and JunB expression as well as AP-1 transcriptional activity were unaffected by TUDCA (75 μM). However, TUDCA significantly decreased TLCA-induced upregulation of cFos and JunB. Furthermore, TUDCA inhibited TLCA-induced AP-1 transcriptional activity and reduced TLCA-induced apoptosis. These data suggest that reversal of bile acid-induced AP-1 activation may be relevant for the antiapoptotic effect of TUDCA in liver cells.

Keywords: AP-1; Apoptosis; cFos; Cholestasis; JunB; Liver; Signal transduction; Taurolithocholic acid; Tauroursodeoxycholic acid

Cholestatic liver diseases are characterized by elevated hepatocellular levels of hepatotoxic bile acids as a consequence of impaired hepatobiliary secretion [1]. Although mechanisms of liver damage in cholestasis are multifactorial, bile acid-induced apoptosis appears to play a major role in the pathogenesis of cholestatic diseases [2]. Indeed, hydrophobic bile acids have been shown to induce hepatocyte apoptosis *in vitro* and *in vivo* [3–6]. For instance, in primary rat hepatocytes the toxic bile acid taurolithocholic acid (TLCA) induced apoptosis in 40% of cells at a concentration of 100 μmol/L after 12 h of incubation [7].

AP-1 is a human transription factor involved in the regulation of cell proliferation, transformation and death and is a dimer consisting of proteins belonging to Jun-, Fos-, ATF- or MAF-families [8]. Interestingly, Jun-proteins like cJun, JunB and JunD are able to form homo- and heterodimers whereas Fos-proteins including cFos, FosB, Fra-1 and Fra-2 have only been found as heterodimers with Jun-proteins [8]. There is evidence, that AP-1 proteins, mostly from the Jun-family, control cell death by their ability to modulate the expression and function of cell cycle regulators [9]. We have recently shown that the toxic bile acid glycochenodeoxycholic acid (GCDCA) up-regulates AP-1 proteins cFos and JunB and increases transcriptional activity of AP-1 thereby contributing to the cytotoxicity of this bile acid [10].

The hydrophilic dihydroxy bile acid ursodeoxycholic acid (UDCA) is the only drug currently approved for the treatment of patients with primary biliary cirrhosis and is also used in patients with primary sclerosing cholangitis [11]. Several studies demonstrated that the taurine conjugate of UDCA (TUDCA) can reduce apoptosis *in vitro* by interrupting classic pathways of apoptosis [12,13]. However, the mechanisms by which UDCA reduces apoptosis

* Corresponding author. Fax: +49 89 7095 6000.
E-mail address: christian.rust@med.uni-muenchen.de (C. Rust).

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are not fully understood. Thus, the objective of the present study was to evaluate whether TUDCA can reduce TLCA-induced apoptosis via modulation of transcription of AP-1 proteins and activation of the AP-1 complex.

Materials and methods

Materials. Primary antibodies against phospho-JNK and phospho-p38 MAPK were purchased from Cell Signaling (Beverly, MA), antibodies against β-actin were from Sigma Chemical (St. Louis, MO). TUDCA, TLCA, TCA, methyl methane sulfonate (MMS) and all other reagents were obtained from Sigma Chemical.

Plasmids. The AP-1 regulated luciferase reporter gene *5xTRE jun2 pGl3* was kindly provided by Dr. Peter Hasselblatt (Research Institute of Molecular Pathology, Vienna, Austria). TK-*Renilla*-CMV plasmid was purchased from Promega (Madison, WI).

Cell culture. HepG2-Ntcp cells [14] were grown at 37 °C under 5% CO_2 in MEM (pH 7.4) containing 10% fetal bovine serum, 1% non-essential amino acids, 4 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mg/mL geneticin and 0.25 μg/mL amphotericin B.

Caspase assays. Caspase-3/7 activation was determined in subconfluent HepG2-Ntcp cells treated with TLCA, TUDCA or a combination of both at the indicated concentrations and time intervals. Activity assays were performed using a commercially available caspase-3/7 assay kit from Promega according to the manufacturer's instructions.

Quantitative RT-PCR. HepG2-Ntcp cells were stimulated with TLCA, TUDCA, TCA or a combination for 4 h. Total RNA was isolated using the commercially available Nucleo Spin RNA II kit (Machery-Nagel, Düren, Germany). 10 pg to 5 μg of total RNA were transcribed into cDNA by incubation with 200–500 ng oligo-d$(T)_{12\text{-}18}$ primer and 1 μl 10 mM dNTP-mix in a volume of 13 μl for 5 min at 65 °C. After cooling on ice, 4 μl of 5X First-Strand-buffer, 1 μl 0.1 M DTT, 1 μl RNase OUT and 1 μl Superscript III (Invitrogen, Karlsruhe, Germany) were added and incubated for 1 h at 50 °C. Degradation of enzyme and RNA was done by heating for 15 min at 70 °C and incubation with 1 μl RNase H (Invitrogen) for 20 min at 37 °C. For quantitative RT-PCR the PCR cycler Rotor Gene RG-3000 (Corbett Research, Australia) and the QuantiTect SYBR Green PCR-Kit (Qiagen, Hilden, Germany) were applied according to the manufacturer's protocols. Quantification was done by the relative quantification standard curve method using GAPDH as endogenous control. Primer sequences were as follows: JunB forward primer 5′-GCC ACC TCC CGT TTA CAC CA-3′, JunB reverse primer 5′-GGC ACG GTC TGC GGT TCC T-3′, cFos forward primer 5′-CAG TTA TCT CCA GAA GAA GAA GA-3′, cFos reverse primer 5′-CCG CTT GGA GTG TA CAG TCA-3′.

Luciferase reporter gene assay. HepG2-Ntcp cells were cotransfected with 33 ng of TK-Ren-CMV (as a control for transfection efficacy) and 166 ng of *5xTRE jun2 pGl3*. After 24 h, cells were stimulated with bile acids at the indicated concentrations. Both firefly and Renilla luciferase activities were quantified using a dual reporter gene assay from Promega according to the manufacturer's instructions and a TD 20/20-Luminometer (Software Turner Design Version 2.0.1, Turner Designs Inc., CA). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Immunoblot analysis. Subconfluent cells were treated with diluent, TLCA, TUDCA, TCA, methyl methane sulfonate (MMS) or a combination, washed with PBS, homogenized in ice-cold lysis buffer (20 mM Tris–HCl pH 8, 150 mM NaCl, 2 mM EDTA, 1% Triton, 100 μM vanadate, 10 mM NaF), incubated for 5 min on ice, sonicated and centrifuged for 5 min at 14,000*g* and 4 °C. The supernatant was resolved by 10% SDS–PAGE, transferred to Immobilon-P membranes (Millipore, Eschborn, Germany) and probed against phospho-JNK antibodies at a dilution of 1:1000 in 5% milk/TBS-T overnight. Peroxidase-conjugated second IgG antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) was incubated at a dilution of 1:10000. Membranes were stripped and reprobed with an antibody against β-Actin (1:3500) to ensure equal loading of protein.

Statistics. Results from at least 3 independent experiments were expressed as means ± SD. Differences between groups were compared using an analysis of variance for repeated measures (ANOVA).

Results

TUDCA reduces TLCA-induced apoptosis

TLCA (20 μmol/L) effectively induced apoptosis in the bile acid transporting human hepatoma cell line HepG2-Ntcp, as quantified by determination of caspase-3/7 activity (Fig. 1). TUDCA (75 μmol/L) alone did not cause hepatocyte apoptosis, but reduced TLCA-induced caspase-3/7 activity by 64% when both bile acids were added simultaneously (Fig. 1).

TUDCA reduces TLCA-induced AP-1 transcriptional activity

We recently demonstrated that the hydrophobic bile acid GCDCA activates the transcription factor AP-1 [10]. However, it remained unclear if other proapoptotic bile acids also can induce AP-1 trancriptional activity. Stimulation of HepG2-Ntcp cells with TLCA (20 μmol/L) for 1 h increased AP-1 transcriptional activity by 300 ± 90% as assessed by luciferase reporter gene assay ($p < 0.05$ vs. control) (Fig. 2). In contrast, neither TUDCA (75 μmol/L) nor taurocholic acid (TCA; 75 μmol/L), both hydrophilic bile acids, significantly altered AP-1 transcriptional activity. We next assessed whether TLCA-induced activation of AP-1 transcriptional activity can be modulated by TUDCA or TCA. Interestingly, only TUDCA (75 μmol/L) significantly reduced TLCA-induced transcriptional activity to levels observed with TUDCA alone, whereas TCA at equimolar concentrations had no effect (Fig. 2).

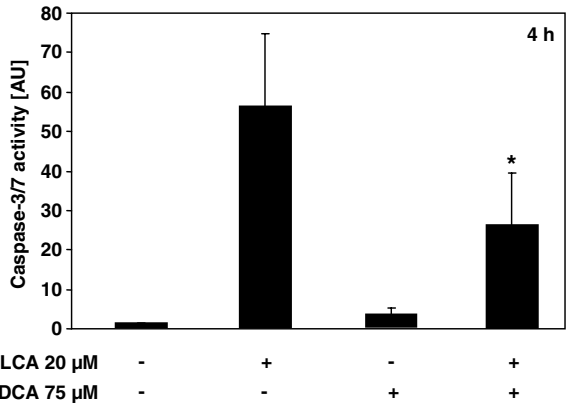

Fig. 1. TUDCA prevents TLCA-induced apoptosis. HepG2-Ntcp cells were treated with dilutent or TLCA (20 μmol/L) in the absence and presence of TUDCA (75 μmol/L) for 4 h. Apoptosis was quantified by measuring capase-3/7 activity. Results are expressed as means ± SD of three independent experiments (*$p < 0.05$ vs. TLCA).

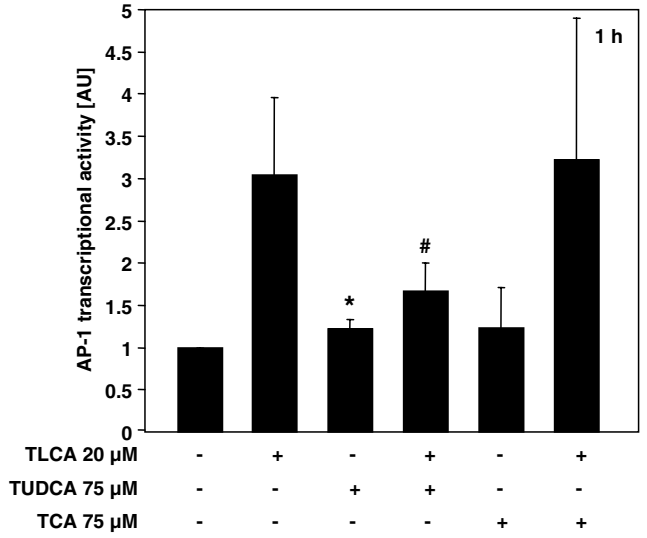

Fig. 2. TUDCA inhibits TLCA-induced AP-1 transcriptional activity. HepG2-Ntcp cells were cotransfected with TK-*Renilla*-luciferase-CMV and the AP-1 regulated luciferase reporter gene *5xTRE jun2 pGl3*. Cells were stimulated with diluent, TLCA (20 µmol/L), TUDCA (75 µmol/L), TCA (75 µmol/L) or a combination for 1 h. Cell lysates were prepared, and firefly and *Renilla* luciferase assays were performed. The ratio of firefly to Renilla luciferase was calculated to control for transfection efficiency and the resulting values were presented as arbitrary units (AU). Results are the mean ± S.D. of 6 independent experiments ($^{*}p < 0.05$ vs. TLCA).

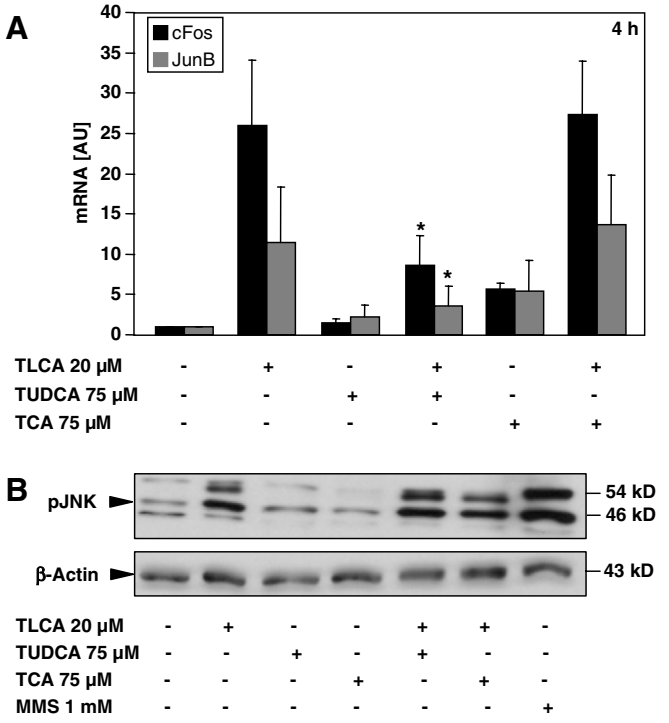

Fig. 3. TUDCA inhibits TLCA-induced transcription of AP-1 proteins, but does not prevent TLCA-induced JNK phosphorylation. (A) HepG2-Ntcp celss were treated with diluent or TLCA (20 µmol/L), TUDCA (75 µmol/L), TCA (75 µmol/L) or a combination for 4 h. Total RNA was isolated and transcribed into cDNA. Quantitative RT-PCR with specific primers for cFos and JunB was realized using GAPDH as an endogenous control and the standard curve method for calculation. Results are expressed as x-fold increase over control (arbitrary units, AU) and are the mean ± SD of 4 independent experiments ($^{*}p < 0.01$ vs. TLCA). (B) HepG2-Ntcp cells were stimulated with TLCA (20 µmol/L), TUDCA (75 µmol/L), TCA (75 µmol/L) or a combination for 4 h. Stimulation with MMS (1 mM) was used as positive control. Equivalent amounts of protein were immunoblotted with antibodies against phospho-JNK. Membranes were then stripped and reprobed with an anti-GAPDH antibody to ensure equal loading in an identical procedure. Representative blots from 3 independent experiments are shown.

TUDCA inhibits TLCA-induced transcription of AP-1 proteins, but does not prevent TLCA-induced JNK phosphorylation

AP-1 is a heterodimeric transcription factor mainly composed of proteins belonging to the Fos or Jun protein family. Transcription of cFos and JunB was analyzed by quantitative real-time PCR in HepG2-Ntcp cells. TLCA (20 µmol/L) induced a 26-fold increase of cFos mRNA and an 11-fold increase of JunB mRNA compared to unstimulated controls after 4 h ($p < 0.01$ vs. control, each) (Fig. 3A). In contrast, the hydrophilic bile acids TUDCA (75 µmol/L) and TCA (75 µmol/L) did not affect cFos or JunB mRNA levels (Fig. 3A). We next determined if TLCA-induced upregulation of AP-1 proteins can be modulated by hydrophilic bile acids. To address this question, HepG2-Ntcp cells were incubated with TLCA (20 µmol/L) and TUDCA (75 µmol/L) or TCA (75 µmol/L) for 4 h. TUDCA significantly reduced TLCA-induced upregulation of both, cFos and JunB, by 67% and 69%, respectively ($p < 0.05$ vs. control, each) (Fig. 3A). In contrast, TCA did not affect TLCA-induced up-regulation of cFos or JunB. Thus, inhibition of TLCA-induced upregulation of cFos and JunB appears to be specific for TUDCA.

Since activation of AP-1 may be influenced by MAP-kinases [15], phosphorylation of JNK was determined after TLCA stimulation. Indeed, TLCA (20 µmol/L) induced phosphorylation and activation of JNK as shown by immunoblotting (Fig. 3B). However, neither TUDCA nor TCA were able to reduce this TLCA-induced phosphorylation of JNK (Fig. 3B).

The AP-1 inhibitor curcumin reduces TLCA-induced apoptosis

A dose-dependent reduction in TLCA-induced apoptosis was observed when curcumin, a well-known inhibitor of AP-1 [16–18], was added. After 4 h, curcumin dose-dependently reduced TLCA-induced caspase-3/7 activity; 50 µmol/L resulted in a 90% reduction and 5 µmol/L in a 55% reduction in caspase-3/7 activity (Fig. 4).

Discussion

Hepatic retention of bile acids may lead to liver injury by hepatocyte apoptosis and eventually deterioration of cholestatic liver diseases. Better understanding of the mechanisms underlying bile acid-induced apoptosis and its prevention may provide new concepts for the medical treatment of cholestatic disorders. In the present study,

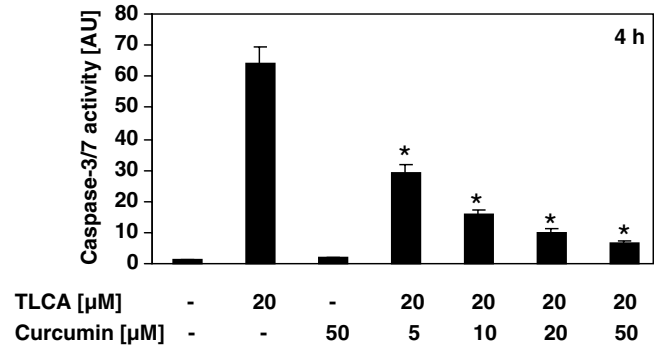

Fig. 4. Curcumin inhibits TLCA-induced apoptosis. HepG2-Ntcp cells were treated with dilutent or TLCA (20 μmol/L) in the absence and presence of curcumin at the indicated concentrations for 4 h. Apoptosis was quantified by measuring capase-3/7 activity. Results are expressed as means ± SD of three independent experiments (*$p < 0.01$ vs. TLCA).

we show distinct and differential effects of the bile acids TLCA, TCA and TUDCA on the expression of c-fos and JunB and on AP-1 transcriptional activity in a human hepatoma cell line. The hydrophobic proapoptotic bile acid TLCA significantly increased c-fos and JunB mRNA levels and AP-1 acitvity in HepG2-Ntcp cells, while none of these responses were triggered by the hydrophilic bile acids TUDCA and TCA. However, only TUDCA inhibited transcriptional activity of AP-1 induced by TLCA, suggesting that reversal of AP-1 activation may be one mechanism by which TUDCA exerts its antiapoptotic effects.

Hydrophobic bile acids induce apoptosis *in vivo* and in both primary rat hepatocytes and human hepatoma cell lines involving different mechanisms, such as activation of the death-receptors Fas and TRAIL, mitochondrial dysfunction, generation of oxidative stress and stimulation of c-Jun-N-terminal kinase (JNK)/activator protein-1 (AP-1)-signaling pathway [5,6,19–23]. In cultured rat hepatocytes, taurolithocholic acid-3 sulfate induced prolonged JNK activation and apoptosis by mediating ligand-independent CD95 (Fas) trafficking to the membrane [5]. In cultures of human colon cancer cell lines, deoxycholic acid, chenodeoxycholic acid or lithocholic acid activated the transcription factor AP-1, possibly through activation of both extracellular signal-related kinase (ERK) and members of the protein kinase C (PKC) family [24,25]. Recently, AP-1 was identified as a downstream target of glycochenodeoxycholic acid-induced apoptosis in human hepatoma cells and inhibition of AP-1 by curcumin decreased apoptosis by this bile acid [10].

Regulation of AP-1 activity is complex and involves transcription of genes encoding AP-1 subunits, modulation of stability of their mRNAs, dimer composition and interaction with specific protein kinases and transcriptional coactivators [26,27]. Several proteins of the Jun, Fos, ATF and JDP protein family can form dimers that bind to the AP-1 site. These proteins, however, have considerably different transactivation potentials [26], which are further modulated by phosphorylation at specific sites. In the current study, TLCA increased levels of c-Fos and JunB mRNA and induced JNK phosphorylation in liver cells. Moreover, TLCA increased AP-1 transcriptional activity as shown in the luciferase reporter gene assays.

UDCA can block apoptosis both *in vitro* in rat liver and human hepatocytes and *in vivo* by interrupting classic pathways of apoptosis [12,13,28–30]. In human hepatocytes, apoptosis induced by toxic bile acids, ethanol, transforming growth factor-β1 (TGF-β1), anti-Fas antibodies and okadaic acid could all be inhibited by UDCA coadministration [13]. However, the mechanisms underlying the protective antiapoptotic effects of UDCA are complex and involve different molecular pathways. UDCA reduces mitochondrial membrane permeability transition (MPT) and prevents translocation of the proapoptotic protein Bax to mitochondria, mitochondrial cytochrome c release and downstream caspase activation through both direct and indirect pathways [6,13,31,32]. More recently, UDCA was shown to inhibit apoptosis by modulating gene expression of the E2F-1/Mdm-2/p53/Bax apoptotic pathway [33,34]. Apoptosis can not only be inhibited by blocking proapoptotic pathways, but also by activation of intracellular survival signals. Indeed, UDCA was shown to activate the phosphatidylinositol 3-kinase and the MAPK survival pathways [35]. TUDCA inhibited TLCA-induced AP-1 transcriptional activity and TLCA-induced apoptosis in the present study. Together with the finding that inhibition of AP-1 by curcumin resulted in hepatocyte survival, these data suggest that reversal of AP-1 activation may be relevant for the antiapoptotic effect of TUDCA. Interestingly, UDCA suppressed DCA-induced apoptosis as well as AP-1 DNA binding and transactivation activity in human colon cancer cells, that was mediated in part through suppression of EGFR/Raf-1/ERK signaling [36].

In conclusion, the data presented in the current study suggest that TLCA-induced apoptosis seems to be mediated by the transcription factor AP-1 and that TUDCA decreases bile acid-induced apoptosis by reversal of AP-1 transcriptonal activation. Further studies should identify the precise molecular mechanism by which TUDCA interacts with AP-1 in an effort to direct novel therapies at the AP-1 signaling pathway for liver diseases with deregulated apoptosis.

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