Original Paper

Tauroursodeoxycholate Protects Rat Hepatocytes from Bile Acid-Induced Apoptosis via β_1 -Integrin- and Protein Kinase A-Dependent Mechanisms

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Key Words

TUDC • Integrin • GCDC • CD95 • JNK • cAMP • MKP-1 • Apoptosis

Abstract

Background/Aims: Ursodeoxycholic acid, which *in vivo* is rapidly converted into its taurine conjugate, is frequently used for the treatment of cholestatic liver disease. Apart from its choleric effects, tauroursodeoxycholate (TUDC) can protect hepatocytes from bile acid-induced apoptosis, but the mechanisms underlying its anti-apoptotic effects are poorly understood. **Methods:** These mechanisms were investigated in perfused rat liver and isolated rat hepatocytes. **Results:** It was found that TUDC inhibited the glycochenodeoxycholate (GCDC)-induced activation of the CD95 death receptor at the level of association between CD95 and the epidermal growth factor receptor. This was due to a rapid TUDC-induced β_1 -integrin-dependent cyclic AMP (cAMP) signal with induction of the dual specificity mitogen-activated protein (MAP) kinase phosphatase 1 (MKP-1), which prevented GCDC-induced phosphorylation of mitogen-activated protein kinase kinase 4 (MKK4) and c-jun-NH₂-terminal kinase (JNK) activation. Furthermore, TUDC induced a protein kinase A (PKA)-mediated serine/threonine phosphorylation of the CD95, which was recently identified as an internalization signal for CD95. Furthermore, TUDC inhibited GCDC-induced CD95 targeting to the plasma membrane in a β_1 -integrin- and PKA-dependent manner. In line with this, the β_1 -integrin siRNA knockdown in sodium taurocholate cotransporting polypeptide (Ntcp)-transfected HepG2 cells abolished the protective effect of TUDC against GCDC-induced apoptosis. **Conclusion:** TUDC exerts its anti-apoptotic effect via a β_1 -integrin-mediated formation of cAMP, which prevents CD95 activation by hydrophobic bile acids at the levels of JNK activation and CD95 serine/threonine phosphorylation.

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Introduction

The hydrophilic bile acid ursodeoxycholic acid (UDCA) has choleric as well as cytoprotective and immunomodulatory properties [1-3]. UDCA is *in vivo* rapidly converted into its taurine conjugate, tauroursodeoxycholate (TUDC) [4]. TUDC was shown to inhibit CD95 (Fas, apoptosis antigen-1)-dependent apoptosis, which is induced by hydrophobic bile acids, however, the underlying mechanism remained unclear. Recently, β_1 -integrins were identified as sensors for TUDC in the liver [5, 6], and molecular dynamics simulation studies showed that TUDC directly interacts with $\alpha_5\beta_1$ -integrins resulting in activation of β_1 -integrin and the initiation of the downstream integrin signaling [6]. Such downstream signaling events include activation of c-Src and focal adhesion kinase, which lead to an activation of the epidermal growth factor receptor (EGFR) and induction of hepatocyte proliferation. Furthermore, TUDC-induced integrin activation triggers the activation of the extracellular signal-regulated kinases-1/-2 (Erks) and p38 mitogen-activated protein kinase (p38^{MAPK}) downstream, which are required to induce TUDC-induced choleresis and inhibition of proteolysis [5-8].

Cytotoxic bile acids such as tauroolithocholylsulfate or glycochenodeoxycholate (GCDC) have previously been shown to induce hepatocyte apoptosis through a ligand-independent activation of CD95 death receptor pathway [9-12]. Here, activation of CD95 starts inside the hepatocyte and is triggered by a bile salt-induced activation of NADPH oxidase [12-14]. The resulting formation of reactive oxygen species (ROS) leads to an activation of c-Jun-NH₂-terminal kinase (JNK) and of the Src kinase family member Yes, which allows for transactivation of the EGFR [15-17]. The JNK signal is required in order to allow for an association of activated EGFR with CD95, which becomes tyrosine-phosphorylated by the EGFR kinase activity [15, 16, 18]. CD95 tyrosine phosphorylation is required for CD95 oligomerization [19] and trafficking of the CD95/EGFR complex to the plasma membrane, where formation of the death-inducing signaling complex (DISC), i.e. recruitment of Fas-associated death domain (FADD) and caspase 8 occurs [15, 16, 18]. TUDC was shown to protect against bile salt-induced apoptosis and a modulation of the mitochondrial membrane permeability by TUDC [20] and activation of survival pathways, such as p38^{MAPK}, Erks and phosphatidylinositol 3-kinase (PI3-K) were suggested to contribute to this cytoprotective effects [21, 22]. However, TUDC prevents CD95 trafficking to the plasma membrane by toxic bile acids indicating that it interferes with CD95 dependent signaling towards apoptosis at a step prior to caspase 8 activation [12].

The present study was undertaken to elucidate the molecular mechanisms that underlie the anti-apoptotic effects of TUDC against GCDC-induced apoptosis in rat liver. It is shown that TUDC inhibits GCDC-induced apoptosis via an intrahepatocytic activation of $\alpha_5\beta_1$ -integrins and formation of cAMP, which triggers a PKA-dependent serine/threonine phosphorylation of the CD95, which was identified as an internalization signal for CD95 [23]. Furthermore, TUDC largely prevented GCDC-induced JNK activation through upregulation of the dual specificity mitogen-activated protein (MAP) kinase phosphatase 1 (MKP-1), a transcriptionally regulated immediate early gene-encoded enzyme. This prevented GCDC-induced CD95/EGFR association and activation of the CD95-dependent pro-apoptotic signaling.

Materials and Methods

Materials

The materials used were purchased as follows: William's Medium E was from Biochrom (Berlin, Germany), FBS (fetal bovine serum), penicillin/streptomycin and H89 dihydrochloride were purchased from Tocris/Biozol (Eching, Germany). Collagenase, insulin, Dibutyryl-cAMP sodium salt (Db-cAMP), 3-Isobutyl-1-methylxanthine (IBMX), GCDC and TUDC were from Sigma Aldrich (Munich, Germany). ProLong® Gold Antifade reagent with DAPI (4', 6-diamidino-2-phenylindole, dihydrochloride), 5-(and 6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate (CM-H₂DCFDA), Lipofectamine 2000, G418 geneticin and Dulbecco's

modified Eagle's medium Nutrimix F12 were from Life Technologies GmbH (Darmstadt, Germany), Wortmannin, NSC 150117, H-Gly-Arg-Gly-Asp-Ser-Pro-OH (*GRGDSP*) and H-Gly-Arg-Ala-Asp-Ser-Pro-OH (*GRADSP*) from Merck-Millipore (Darmstadt, Germany), horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG from Bio-Rad Laboratories (Munich, Germany) and Dako (Hamburg, Germany). The antibodies used were purchased as follows: antibodies recognizing caspase 8, FADD, p47^{phox}, CD95 (IP) and EGFR were from Santa Cruz Biotechnology (Heidelberg, Germany), Yes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), MKP-1, phospho-tyrosine and -threonine from Merck-Millipore (Darmstadt, Germany), phospho-JNK-1/-2 and CD95 (WB) from Life Technologies GmbH (Darmstadt, Germany), JNK-1/-2 from BD Bioscience (Heidelberg, Germany), cleaved caspase 3, phospho-Src family, MKK4 from Cell Signaling Technology, Inc. (Danvers, USA), γ -tubulin from Sigma Aldrich (Munich, Germany), β_1 -integrin from Biocat (Heidelberg, Germany) and phospho-serine from Enzo Life Sciences (Lörrach, Germany). All other chemicals were from Merck-Millipore (Darmstadt, Germany), at the highest quality available.

Preparation and culture of primary rat hepatocytes

Hepatocytes were isolated from livers of male Wistar rats (160-180g) by a collagenase perfusion technique in an adapted version as described previously [24]. Animals were fed ad libitum with a standard diet. Aliquots of rat hepatocytes were plated on collagen-coated culture plates and maintained in bicarbonate-buffered Krebs-Henseleit medium (115mmol/l NaCl, 25mmol/l NaHCO₃, 5.9mmol/l KCl, 1.18mmol/l MgCl₂, 1.23mmol/l NaH₂PO₄, 1.2mmol/l Na₂SO₄, 1.25mmol/l CaCl₂), supplemented with 6mmol/l glucose in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. After 2h, the medium was removed and the cells were washed twice. Subsequently the culture was continued for 24h in William's Medium E, supplemented with 2mmol/l glutamine, 100nmol/l insulin, 100U/ml penicillin, 0.1mg/ml streptomycin, 100nmol/l dexamethasone and 5% FBS. After 24h experimental treatments were performed using William's Medium E that contained 2mmol/l glutamine, 100nmol/l dexamethasone. The viability of hepatocytes was more than 95% as assessed by trypan blue exclusion. The experiments were approved by the responsible local authorities.

Rat liver perfusion

Livers from male Wistar rats (140-160g) were perfused as described previously [25] in a non-recirculating manner. As a perfusion medium the bicarbonate-buffered Krebs-Henseleit saline plus L-lactate (2.1mmol/l) and pyruvate (0.3mmol/l) gassed with 5% CO₂ and 95% O₂ at 37°C was used. The addition of bile salts and inhibitors to influent perfusate was made by dissolution into the Krebs-Henseleit buffer. Viability of the perfused livers was assessed by measuring lactate dehydrogenase leakage into the perfusate. The portal pressure, the effluent K⁺ concentration and pH were continuously monitored. Ligation and excision of liver lobes was performed in a way that kept portal pressure constant, i.e. the perfusion flow was adjusted to maintain portal pressure constant. The experiments were approved by the responsible local authorities.

Generation of stably expressing FLAG-Ntcp-EGFP cells, transfection and culture

FLAG-Ntcp-EGFP was transfected into HepG2 cells by using Lipofectamine 2000 (Life Technologies, Darmstadt, Germany) according to the manufacturer's guidelines. Stable cell lines were established with 0.5% of geneticin as selection agent. HepG2 cells stably expressing FLAG-Ntcp-EGFP cells were cultured in 6-well culture plates in Dulbecco's modified Eagle's medium Nutrimix F12 supplemented with 5% FBS and 0.5% geneticin until subconfluence.

siRNA knockdown of β_1 -integrin

Small interfering RNA (siRNA) was prepared by Qiagen (HP GenomeWide siRNA, Qiagen, Hilden, Germany) targeting the β_1 -integrin sequence 5'-AAA AGT CTT GGA ACA GAT CTG-3'. Cells were washed thrice with serum-free Dulbecco's modified Eagle's medium Nutrimix F12 plus 0.5% geneticin followed by siRNA transfection using HiPerFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instruction.

Immunoblot analysis

At the end of the incubation period, the medium was removed and the cells were immediately lysed at 4°C by using a lysis buffer containing 20mmol/l Tris-HCl (pH 7.4), 140mmol/l NaCl, 10mmol/l NaF, 10mmol/l

sodium pyrophosphate, 1% (v/v) Triton X-100, 1mmol/l EDTA, 1mmol/l EGTA, 1mmol/l sodium vanadate, 20mmol/l β -glycerophosphate, and protease inhibitor cocktail. The lysates were kept on ice for 10min and then centrifuged at 8000rpm for 8min at 4°C, and aliquots of the supernatant were taken for protein determination using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). Equal amounts of protein were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes using a semidry transfer apparatus (GE Healthcare, Freiburg, Germany). Membranes were blocked for 30min in 5% (w/v) bovine serum albumin containing 20mmol/l Tris (pH 7.5), 150mmol/l NaCl, and 0.1% Tween 20 (TBS-T) and exposed to primary antibodies overnight at 4°C. After washing with TBS-T and incubation at room temperature for 2h with horseradish peroxidase-coupled anti-mouse or anti-rabbit IgG antibody, respectively (all diluted 1:10000), the immunoblots were washed extensively and bands were visualized using the FluorChem E detection instrument from ProteinSimple (Santa Clara, CA). Semi-quantitative evaluation was carried out by densitometry using the Alpha View image acquisition and analysis software from ProteinSimple.

Immunoprecipitation

Liver samples were harvested in lysis buffer containing 136mmol/l NaCl, 20mmol/l Tris HCl, 10% (v/v) glycerol, 2mmol/l EDTA, 50mmol/l β -glycerophosphate, 20mmol/l sodium pyrophosphate, 0.2mmol/l Pefablock, 5mg/l aprotinin, 5mg/l leupeptin, 4mmol/l benzamidine, 1mmol/l sodium vanadate, supplemented with 1% (v/v) Triton X 100. The protein amount was determined as described above. Samples containing equal protein amounts were incubated for 2h at 4°C with an anti-CD95 or anti-EGFR antibody to immunoprecipitate CD95 or EGFR, respectively. Then protein A-/G-agarose (Santa Cruz Biotechnology, Heidelberg, Germany) was added and incubated at 4°C overnight. Immunoprecipitates (IPs) were washed 3-times with lysis buffer supplemented with 0.1% (v/v) Triton X-100 and then transferred to Western blot analysis as described above. The anti-phospho-tyrosine, -serine and -threonine antibody was used to detect activating phosphorylation of CD95 or EGFR (exclusively anti-phospho-tyrosine) in the respective IPs. Caspase 8 and FADD antibodies were used to detect association to CD95.

Immunofluorescence staining

To detect CD95 translocation, isolated hepatocytes were cultured for 24h on collagen-coated glass coverslips in 24-well culture plates. After treatment, cells were fixed using paraformaldehyde (4% (w/v), 20min, 4°C), permeabilized using Triton X-100 (0.1% (v/v), 2min, 4°C) and blocked with FBS (5% (w/v), 30min, room temperature). Then cells were exposed to a rabbit anti-CD95 antibody and a mouse anti-Na⁺/K⁺-ATPase antibody (1:200 in PBS, overnight, 4°C), washed off, and stained with an anti-mouse-FITC and an anti-rabbit Cy3-conjugated antibody (1:500 in PBS, 2h, room temperature). Following immuno-fluorescence staining, samples were covered with ProLong® Gold Antifade reagent with DAPI and CD95, respectively Na⁺/K⁺-ATPase localization was visualized by confocal laser scanning microscopy using LSM510 META (Zeiss, Oberkochen, Germany).

Detection of ROS

Hepatocytes were seeded on collagen-coated 6-well culture plates (BD Falcon, Heidelberg, Germany) and cultured for 24h. Detection of ROS was performed as described before [26].

Assessment of apoptosis

Apoptosis was detected using a terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labelling (TUNEL) assay (In Situ Cell Death Detection Kit; Roche Applied Science, Mannheim, Germany). Therefore, primary rat hepatocytes were cultured on collagen-coated glass coverslips in 24-well culture plates for up to 18h. The number of apoptotic cells was determined by counting the percentage of fluorescein-positive cells. At least 300 cells from five different cell preparations each were counted for each condition. Cells were visualized on an LSM510 META laser scanning microscope (Zeiss, Oberkochen, Germany).

cAMP assay

Primary rat hepatocytes were grown on 6-well culture plates, stimulated for the time points indicated and then lysed with lysis buffer. Rat liver lobes were immediately frozen in liquid nitrogen and were pulverized to a fine powder under liquid nitrogen. After evaporation of the liquid nitrogen, the frozen liver

tissue was weighted and homogenized in lysis buffer. cAMP-assay was conducted using the manufacturer's instruction (Sigma Aldrich, Munich, Germany). Forskolin was used as a positive control (data not shown).

Realtime-PCR analysis

Total RNA was isolated using the RNAeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturers' protocol. RNA was quantified using NanoDrop1000 System (Thermo Scientific, Wilmington, USA) and first strand cDNA was synthesized from RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Gene expression levels were quantified using SensiMix SYBR No-ROX Kit (Bioline, Luckenwalde, Germany) on a TOptical cyler (Biometra, Göttingen, Germany). Hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) was used as reference gene for the normalization of the results obtained by the $2(-\Delta\Delta Ct)$ method. PCR-primer sequences: MKP-1 5'-CGTAGTGCTGCTGGACGAAC-3' and 5'-GAAGCCGAAAACGCTTCATA-3'; HPRT1 5'-TGCTCGAGATGTCATGAAGGA-3' and 5'-CAGAGGGCCACAATGTGATG-3'.

Statistical analysis

Results from at least three independent experiments are expressed as mean values $\pm$ standard error of the mean (SEM). For each experimental treatment and time point analysed, a separate control experiment was carried out. Differences between experimental groups were analysed by student's t test, one-way analysis of variance following Dunnett's multiple comparison post hoc test or two-way analysis of variance following Bonferroni's multiple comparison post hoc test where appropriate (GraphPad Prism; GraphPad, La Jolla, USA; Microsoft Excel for Windows, Redmond, USA). $p < 0.05$ was considered statistically significant.

Results

Inhibition of bile acid-induced apoptosis and caspase activation by TUDC

In line with previous data [27], addition of GCDC (50 μ mol/l) induced hepatocyte apoptosis, as evidenced by caspase 3 cleavage (Fig. 1A) and TUNEL staining (Fig. 1B). Addition of TUDC (100 μ mol/l) prevented caspase 3 cleavage (Fig. 1A) and strongly blunted GCDC-induced apoptosis, as assessed by TUNEL staining (Fig. 1B). The protective effect of TUDC on GCDC-induced TUNEL-reactivity (Fig. 1B) was blunted in presence of the integrin-antagonistic hexapeptide *GRGDSP* peptide (100 μ mol/l), but not by the inactive control hexapeptide *GRADSP* (100 μ mol/l). These findings suggest that the protective effect of TUDC on GCDC-induced apoptosis is mediated at least in part through activation of β_1 -integrins. Interestingly, also the PKA-inhibitor H89 (10 μ mol/l) counteracted the effect of TUDC on GCDC-induced apoptosis (Fig. 1B). The important role of β_1 -integrin in mediating the anti-apoptotic effect of TUDC was also substantiated in experiments with Ntcp-transfected HepG2 cells after β_1 -integrin knockdown using an siRNA approach (Fig. 1C,D). β_1 -integrin knockdown was performed by specific siRNA (100 nmol/l) as described in the Materials and Methods section and as described previously [6]. Parallel experiments were carried out using nonsense siRNA as a control. A significant β_1 -integrin knockdown was achieved after 72h of siRNA treatment, whereas no change in β_1 -integrin expression occurred in cells treated with nonsense siRNA (Fig. 1C). β_1 -integrin knockdown completely abolished the protective effect of TUDC (20 μ mol/l) on GCDC (100 μ mol/l)-induced apoptosis in these cells, as assessed by TUNEL staining (Fig. 1D).

TUDC inhibits GCDC-induced activation of CD95 at the level of CD95/EGFR association

In line with previous data [17], GCDC (50 μ mol/l) induced a rapid serine phosphorylation of p47^{phox} (Fig. 2A) and increased carboxy-H₂-DCFDA fluorescence by 2.06 ± 0.21 -fold within 15min (data not shown). GCDC induced within 1min the activation of the Src kinase family member Yes, followed by an association of Yes with EGFR and subsequent EGFR tyrosine phosphorylation (Fig. 2A). The activation of the CD95 system with respect to EGFR/CD95 association, CD95 tyrosine phosphorylation and DISC formation, i.e. association of FADD and caspase 8 to the death receptor became detectable after 30-60min (Fig. 2A). Coadministration of TUDC (100 μ mol/l) together with GCDC (50 μ mol/l) had no effect on

Fig. 1. Inhibition of bile acid-induced caspase 3 activation and apoptosis by TUDC. (A) Primary rat hepatocytes were cultured for 24h and thereafter stimulated with GCDC (50 μ mol/l), TUDC (100 μ mol/l) or the combination of both for up to 120min. Samples were taken at the time points indicated. Cleaved caspase 3 was analysed by Western blot and subsequent densitometric analysis. Representative immunoblots are shown. The values from densitometric analyses of at least 4 independent experiments were normalized to the level of γ -tubulin and expressed as the mean-fold increase over control $\pm$ SEM. For the individual time points control was set to 1. Closed black squares, GCDC; closed grey squares, TUDC+GCDC; open squares, TUDC. *, $p < 0.05$ statistical significance compared with the unstimulated control; #, indicates a significant inhibition of GCDC-induced caspase 3 cleavage by TUDC ($p < 0.05$). (B) Primary rat hepatocytes were cultured for 24h and thereafter stimulated with GCDC (50 μ mol/l), TUDC (100 μ mol/l) or a combination of both for 18h and the number of apoptotic cells was examined by TUNEL staining. When indicated H89 (10 μ mol/l), GRADSP or GRGDSP (each 100 μ mol/l) were added 30min prior to addition of bile salts. Statistical analyses of at least 5 independent experiments for each condition are shown. ^a The asterisk indicates the statistical significance compared to the unstimulated control ($p < 0.05$), ^b indicates the significant inhibition of GCDC-induced TUNEL-reactivity by TUDC ($p < 0.05$), ^c indicates the significant inhibition of the TUDC-induced inhibition of TUNEL-reactivity by H89 ($p < 0.05$), ^d indicates the statistical significance of the GRGDSP effect compared to GRADSP ($p < 0.05$). (C) Ntcp-transfected HepG2 cells were treated with either nonsense or β_1 -integrin (ITGB1) siRNA (100nmol/l each) for 72h and underwent thereafter analysis for β_1 -integrin expression by Western blot. γ -Tubulin served as a loading control. A significant β_1 -integrin knockdown was achieved after 72h of siRNA treatment, whereas no significant change in β_1 -integrin expression occurred in cells treated with nonsense siRNA. (D) Ntcp-transfected HepG2 cells were treated with either nonsense or β_1 -integrin (ITGB1) siRNA (100nmol/l each) for 72h and then stimulated with GCDC (100 μ mol/l), TUDC (20 μ mol/l) or the combination of both for 18h and the number of apoptotic cells was examined by TUNEL staining. Data represent means $\pm$ SEM ($n=3$). *, $p < 0.05$ statistically significantly different from the unstimulated control; # significant inhibition of GCDC-induced TUNEL-reactivity by TUDC ($p < 0.05$).

Fig. 2. Inhibition of GCDC-induced activation of the CD95 system by TUDC. (A) Rat hepatocytes were cultured for 24h and subsequently stimulated with GCDC (50 μ mol/l), TUDC (100 μ mol/l) or the combination of both for up to 60min. Samples were taken at the time points indicated. Phosphorylation of p47^{phox} was analysed by immunoblotting. Yes, EGFR and CD95 were immunoprecipitated and activating Yes phosphorylation (Yes-Y⁴¹⁸-P), EGFR-tyrosine phosphorylation (P-EGFR-Y), CD95-tyrosine phosphorylation (P-CD95-Y) or EGFR/CD95, caspase 8/CD95 and FADD/CD95 association (i.e. DISC formation) were analysed by Western blot using specific antibodies. Total p47^{phox}, Yes, EGFR and CD95 served as respective loading controls. Representative immunoblots from 3 independent experiments are depicted. (B) Rat hepatocytes were cultured for 24h and subsequently stimulated with GCDC (50 μ mol/l) or GCDC plus TUDC (100 μ mol/l) for up to 60min. GRADSP respectively GRGDSP (each 100 μ mol/l) were added 30min prior to the addition of bile salts. Samples were taken at the time points indicated. EGFR and CD95 were immunoprecipitated and activating CD95-tyrosine phosphorylation (P-CD95-Y), EGFR/CD95, caspase 8/CD95 and FADD/CD95 association were analysed by Western blotting using specific antibodies. Total CD95 served as respective loading control. Representative immunoblots of at least 6 independent experiments are depicted. Relative protein expression following GCDC treatment at t=30min and t=60min, respectively was set to 100%. White bars, GCDC; light grey bars, GRADSP+TUDC+GCDC; dark grey bars, GRGDSP+TUDC+GCDC. *, $p < 0.05$ statistical significance compared to GCDC treatment; #, $p < 0.05$ statistical significance between GRADSP+TUDC+GCDC and GRGDSP+TUDC+GCDC; n.s., no statistical significance between GCDC and GRGDSP+TUDC+GCDC ($p > 0.05$).

GCDC-induced activation of p47^{phox} and on the GCDC-induced oxidative stress response (2.10 $\pm$ 0.25-fold; data not shown) and did not prevent Yes/EGFR association and the Yes-

Fig. 3. Inhibition of TUDC-induced CD95 internalization. Primary rat hepatocytes were cultured on glass coverslips for 24h, stimulated with GCDC (50 μ mol/l), TUDC (100 μ mol/l) or the combination of both for 90min and immunostained for CD95 and Na⁺/K⁺-ATPase expression. H89 (10 μ mol/L), GRADSP or GRGDSP (100 μ mol/l, each) were instituted 30min prior to addition of GCDC and TUDC. CD95 translocation was visualized by confocal laser scanning microscopy. The scale bar corresponds to 20 μ m. Representative pictures of 3 independent experiments are depicted. (B) Colocalizing pixels were quantified by calculation of the weighted colocalization coefficient using LSM510 Meta 4.2 Software (Zeiss, Jena, Germany). *, $p < 0.05$ denotes statistical significance.

dependent phosphorylation of the EGFR (Fig. 2A). However, TUDC largely prevented the association between EGFR and CD95, subsequent CD95 tyrosine phosphorylation and DISC formation (Fig. 2A). Immunoprecipitation studies revealed that TUDC (100 μ mol/l) itself had no effect on the activation of p47^{phox}, Yes and the CD95 system (Fig. 2A). Indeed, TUDC led to phosphorylation of the EGFR, in line with its β_1 -integrin activating potency [5]. Compared to the GCDC-induced EGFR activation, the TUDC-induced activation was weaker and delayed (Fig. 2A). The integrin-inhibitory peptide GRGDSP (100 μ mol/l) attenuated the TUDC-induced inhibition of EGFR/CD95 association and CD95 activation (Fig. 2B). Also the TUDC-induced inhibition of DISC formation was largely prevented by the RGD-peptide (Fig. 2B). The control peptide GRADSP had no effect on TUDC-induced inhibition of EGFR/CD95 association, CD95 tyrosine phosphorylation and caspase 8 and FADD association with CD95 (Fig. 2B).

The subcellular localization of CD95 was studied by immunofluorescence analysis in primary rat hepatocytes. CD95 and Na⁺/K⁺-ATPase (plasma membrane marker protein) colocalizing pixels were quantified by cal-

Fig. 4. Inhibition of GCDC-induced activation of JNK-1/-2 by TUDC. (A) Primary hepatocytes were cultured for 24h and thereafter stimulated with GCDC (50 μ mol/l), TUDC (100 μ mol/l) or the combination of both for up to 60min. Samples were taken at the time points indicated. Phosphorylation of JNK-1/-2 was analysed by Western blot using specific antibodies and subsequent densitometric analysis. Total JNK-1/-2 served as respective loading control. For the individual time points control was set to 1. Closed black squares, GCDC; closed grey squares, TUDC+GCDC; open squares, TUDC. Data represent the mean $\pm$ SEM of at least 10 independent experiments. *, $p < 0.05$ statistical significance compared to the unstimulated control. #, $p < 0.05$ statistical significance between GCDC and TUDC+GCDC. (B) Parenchymal cells from rat liver were stimulated with GCDC (50 μ mol/l), TUDC (100 μ mol/l) or the combination of GCDC+TUDC for up to 60min. When indicated, cells were pre-treated with H89 (10 μ mol/l), *GRADSP*, *GRGDSP* (100 μ mol/l each) or NSC 150117 (5 μ mol/l). (C) In another set of experiments rat livers were perfused with GCDC (20 μ mol/l), TUDC (20 μ mol/l) or the combination of GCDC+TUDC for up to 60min. H89 (2 μ mol/l), *GRADSP* and *GRGDSP* (10 μ mol/l each) were instituted 30min prior to addition of TUDC and GCDC. (B, C) Phosphorylation of JNK-1/-2 was analysed by Western blot using specific antibodies and subsequent densitometric analysis. Total JNK-1/-2 served as respective loading control. For the individual time points control was set to 1. Data represent the mean $\pm$ SEM of at least 3 independent experiment. ^a The asterisk indicates the statistical significance compared to the unstimulated control ($p < 0.05$), ^b indicates the significant inhibition of GCDC-induced JNK activation by TUDC ($p < 0.05$), ^c indicates the significant inhibition of the TUDC-induced inhibition of JNK phosphorylation by the respective inhibitor ($p < 0.05$), ^d indicates the statistical significance of the *GRGDSP* effect compared to *GRADSP* ($p < 0.05$).

Fig. 5. Inhibition of GCDC-induced activation of MKK4 by TUDC. Primary rat hepatocytes were cultured for 24h and thereafter stimulated with GCDC (50 μ mol/l), TUDC (100 μ mol/l) or the combination of both for up to 60min. When indicated H89 (10 μ mol/l) was instituted 30min prior to addition of the bile salts. Samples were taken at the time points indicated. Phosphorylation of MKK4 was analysed by Western blotting using specific antibodies and subsequent densitometric analysis. GAPDH served as a loading control. For the individual time points control was set to 1. Closed black squares, GCDC; closed grey squares, TUDC+GCDC; open squares, TUDC; open triangle, H89+TUDC+GCDC. Data represent the mean $\pm$ SEM of at least 5 independent experiments. *, $p < 0.05$ statistical significance compared to the unstimulated control. #, $p < 0.05$ statistical significance between GCDC and TUDC+GCDC, or TUDC+GCDC and H89+TUDC+GCDC, respectively.

culation of the weighted colocalization coefficient according to a preset threshold. As shown in Fig. 3A, GCDC (50 μ mol/l) triggered within 90min an enhancement of CD95 immunoreactivity at the plasma membrane. Under these condition the number of colocalizing CD95- and Na⁺/K⁺-ATPase-positive pixels in the plasma membrane was significantly increased (Fig. 3B), in line with the reported translocation of the CD95 to the plasma membrane following CD95 activation [18]. Coadministration of TUDC (100 μ mol/l) and GCDC (50 μ mol/l) prevented the translocation of CD95 to the plasma membrane (Fig. 3). TUDC itself had no effect on receptor trafficking (Fig. 3). H89 (10 μ mol/l) and *GRGDSP* (100 μ mol/l) counteracted the TUDC-induced inhibition of CD95 trafficking to the plasma membrane (Fig. 3). No significant effect was observed after treatment with the non-antagonistic *RAD*-peptide (100 μ mol/l; Fig. 3B). Localization of the plasma membrane protein Na⁺/K⁺-ATPase was unaffected under these conditions (Fig. 3A).

TUDC prevents the GCDC-induced JNK-1/-2- and MKK4/7-activation

As shown previously [12], GCDC (50 μ mol/l) caused a rapid and sustained activation of JNK-1/-2 in primary hepatocytes (Fig. 4). As shown above, TUDC inhibited the GCDC-induced CD95 activation process at the level of EGFR/CD95 association (Fig. 2), i.e. a step which is known to be triggered by JNK [15, 16, 18].

As shown in Figure 4B, GCDC-induced JNK-1/-2 activation was strongly blunted by TUDC (100 μ mol/l, primary hepatocytes and 20 μ mol/l, perfused liver) in a *GRGDSP* (100 μ mol/l, primary hepatocytes and 10 μ mol/l, perfused liver)- H89 (10 μ mol/l, primary hepatocytes and 2 μ mol/l, perfused liver)- and NSC 150117 (5 μ mol/l, primary hepatocytes)

Fig. 6. TUDC triggers the formation of cAMP. (A) Rat livers were perfused with IBMX (50 μ mol/l) a phosphodiesterase inhibitor alone or the combination of IBMX and TUDC (100 μ mol/l) for the time period indicated. (B) Primary rat hepatocytes were cultured for 24h and then exposed to IBMX (500 μ mol/l) or the combination of IBMX and TUDC (100 μ mol/l) for the time period indicated. When indicated *GRADSP* and *GRGDSP* (100 μ mol/l each) were instituted 30min prior to addition of TUDC and IBMX. (A,B) cAMP levels of at least 3 independent experiments were expressed as the mean-fold increase over control (set to 1) $\pm$ SEM. * indicates the statistical significance compared to the respective control ($p < 0.05$), # indicates the statistical significance of the *GRGDSP* effect compared to *GRADSP* ($p < 0.05$). White columns, IBMX; grey columns, IBMX+TUDC.

[28]-sensitive way (Fig. 4B,C), suggesting that TUDC inhibits GCDC-triggered JNK activation via an integrin-, PKA and MKP-1-dependent mechanism. These observations were made in both, isolated rat hepatocytes and isolated perfused rat liver (Fig. 4B,C). In line with this, also cAMP (10 μ mol/l) ameliorated the GCDC-induced JNK-1/-2 activation in a H89-sensitive way (data not shown). TUDC (Fig. 4B,C) and cAMP (data not shown) by themselves had no significant effects on JNK-1/-2 phosphorylation.

Wortmannin (50nmol/l) did not affect the TUDC-induced inhibition of GCDC-induced JNK-1/-2 activation (data not shown); indicating that inhibition of GCDC signaling towards JNKs by TUDC occurred in a PI3-K-independent way. The inhibitors, i.e. H89, *GRGDSP*, *GRADSP*, wortmannin and NSC 150117 by themselves had no effect on JNK-1/-2 activation within 60min (data not shown).

GCDC (50 μ mol/l) induced the phosphorylation of the dual-specificity MAPK kinase, MKK4, which is upstream of JNK-1/-2 activation (Fig. 5). Coadministration of TUDC (100 μ mol/l) prevented the activating effect of GCDC on MKK4-phosphorylation in a H89-sensitive manner (Fig. 5). Likewise, a significant MKK7 activation by GCDC was also detectable, albeit to a smaller extent (data not shown).

TUDC triggers the formation of cAMP

As shown in Fig. 6A, TUDC (100 μ mol/l) led to a rapid increase of intracellular cAMP levels in perfused rat liver. Likewise, exposure of primary rat hepatocytes to TUDC (100 μ mol/l) also increased hepatocellular cAMP levels (Fig. 6B). IBMX (3-isobutyl-1-methylxanthine), a phosphodiesterase inhibitor was required in these experiments in order to pick up the increase in cAMP levels. Addition of IBMX in the absence of bile acids also increase cAMP lev-

Fig. 7. Effect of TUDC on CD95 serine/ threonine phosphorylation. (A) Primary rat hepatocytes were cultured for 24h and thereafter stimulated with TUDC (100μmol/l) for up to 120min. When indicated H89 (10μmol/l), *GRADSP* and *GRGDSP* (100μmol/l each) were instituted 30min prior to addition of TUDC. (B) Rat livers were perfused with TUDC (100μmol/l) for up to 120min. When indicated H89 (2μmol/l), *GRADSP* and *GRGDSP* (10μmol/l each) were instituted 30min prior to addition of TUDC. (A,B) CD95 were immunoprecipitated and analysed by Western blotting using specific antibodies against P-Ser and P-Thr. Total CD95 served as respective loading control. The values from densitometric analyses were normalized to the level of CD95 and expressed as the mean-fold increase over control $\pm$ SEM. For the individual time points control was set to 1. Data represent the mean of at least 3 independent experiments. * The asterisk indicates the statistical significance compared to the unstimulated control ($p < 0.05$), # indicates the significant inhibition of TUDC-induced CD95 phosphorylation by H89 or by the RGD peptide ($p < 0.05$), $^{\$}$ indicates the statistical significance of the *GRGDSP* effect compared to *GRADSP* ($p < 0.05$).

els in the perfused rat liver and in primary hepatocytes, but this did not reach significance (Fig. 6).

The TUDC-induced cAMP formation was significantly inhibited in presence of the integrin-antagonistic hexapeptide *GRGDSP* (100μmol/l), but was unaffected by the inactive control peptide *GRADSP* (100μmol/l) (Fig. 6B).

Fig. 8. Effect of TUDC on MKP-1-induction. (A) Primary rat hepatocytes were cultured for 24h and thereafter stimulated with GCDC (50 μ mol/l), TUDC (100 μ mol/l) or the combination of both for 15min (A; mRNA) or 30min (B; protein). Db-cAMP (100 μ mol/l) served as a positive control. RNA was extracted and MKP-1 mRNA expression levels were analysed by realtime-PCR. MKP-1 mRNA expression levels are given relative to the control. Data represent the mean $\pm$ SEM of 5 independent experiments *, $p < 0.05$ statistical significance compared to the unstimulated control. (C) When indicated, cells were pre-treated with H89 (10 μ mol/l), GRADSP or GRGDSP (100 μ mol/l each) for 30min. (B, C) MKP-1 protein expression was analysed by Western blotting and subsequent densitometric analysis. γ -tubulin served as a loading control. MKP-1 expression under control condition was set to 1. Data represent the mean $\pm$ SEM of at least 3 independent experiments. * indicates the statistical significance compared to the unstimulated control ($p < 0.05$), # indicates the significant inhibition by H89 or by the RGD peptide ($p < 0.05$), § indicates the statistical significance of the GRGDSP effect compared to GRADSP ($p < 0.05$). (D) Rat livers were perfused with GCDC (20 μ mol/l), TUDC (100 μ mol/l) or the combination of both for 15min. RNA was extracted and MKP-1 mRNA expression levels were analysed by realtime-PCR. MKP-1 mRNA expression levels are given relative to the control. Data represent the mean $\pm$ SEM of 4 independent

experiments *, $p < 0.05$ statistical significance compared to the unstimulated control. (E) Rat livers were perfused with GCDC (20 $\mu\text{mol/l}$), TUDC (100 $\mu\text{mol/l}$) or the combination of both for 30 min. MKP-1 protein expression was analysed by Western blot and subsequent densitometric analysis. γ -Tubulin served as a loading control. MKP-1 expression under control condition was set to 1. Data represent means $\pm$ SEM of at least 3 independent experiments. *, $p < 0.05$ statistically significant compared to the unstimulated control.

Because cAMP was recently shown to induce serine/threonine phosphorylation of the CD95 [23], the effect of TUDC on CD95 phosphorylation was studied. Phosphorylation of CD95 on serine/threonine residues acts as a signal for CD95 internalization and inhibition of apoptosis [23], whereas phosphorylation of the CD95 tyrosine residues within the death domain is associated with CD95 oligomerization, DISC formation and induction of apoptosis [15, 17-19]. In the perfused rat liver (Fig. 7B), as well as in primary hepatocytes (Fig. 7A), TUDC (100 $\mu\text{mol/l}$) triggered a rapid and sustained CD95 serine/threonine phosphorylation, which was sensitive to inhibition by H89 (2 $\mu\text{mol/l}$, perfused liver and 10 $\mu\text{mol/l}$, primary hepatocytes), indicating an involvement of PKA (Fig. 7). The TUDC-induced CD95 serine/threonine phosphorylation was largely abolished in presence of the β_1 -integrin antagonistic peptide *GRGDSP*, whereas *GRADSP* (each 10 $\mu\text{mol/l}$, perfused liver and each 100 $\mu\text{mol/l}$, primary hepatocytes) had no effect (Fig. 7).

TUDC induces mitogen-activated protein kinase phosphatase-1 (MKP-1) expression

MKP-1 mRNA abundance was already increased after 15 min of incubation with TUDC (100 $\mu\text{mol/l}$) and TUDC plus GCDC (20 $\mu\text{mol/l}$ in perfused liver; 50 $\mu\text{mol/l}$ in primary hepatocytes) (Fig. 8). No significant increase in MKP-1 mRNA was observed after treatment with GCDC alone (Fig. 8). Db-cAMP (10 $\mu\text{mol/l}$) was used as a positive control (Fig. 8A). MKP-1 protein in primary hepatocytes and perfused liver was upregulated within 30 min after stimulation with TUDC (100 $\mu\text{mol/l}$) and TUDC plus GCDC (20 $\mu\text{mol/l}$ in perfused liver; 50 $\mu\text{mol/l}$ in primary hepatocytes) (Fig. 8). A mild upregulation of MKP-1 protein was also observed in response to GCDC alone (Fig. 8). The TUDC-induced attenuation of MKP-1 expression was abolished in presence of H89 (10 $\mu\text{mol/l}$) and *GRGDSP* (100 $\mu\text{mol/l}$), suggestive for an integrin-dependent cAMP formation as upstream event (Fig. 8C). Similar findings were obtained when TUDC was added together with GCDC, whereas GCDC alone led to an only mild increase of MKP-1 expression, which was insensitive to H89 and the RGD peptide (Fig. 8C).

Discussion

TUDC is known to inhibit the GCDC-induced hepatocyte apoptosis [12, 22, 29-31], however, the underlying molecular mechanisms remained poorly defined. The present study shows that the anti-apoptotic action of TUDC occurs at the level of CD95 activation and involves (i) a β_1 -integrin-dependent rapid generation of cAMP and a PKA-dependent induction of MKP-1, which inhibits GCDC-induced JNK activation and subsequent EGFR/CD95 association and (ii) a β_1 -integrin- and PKA-dependent serine/threonine phosphorylation of the CD95, which has previously been shown to trigger CD95 internalization [23]. TUDC did not affect the GCDC-induced oxidative stress response and the downstream Yes-dependent EGFR activation, but prevented EGFR/CD95 association, and DISC formation in a β_1 -integrin and PKA-dependent way.

Prevention of GCDC-induced apoptosis by TUDC was not due to a competition of both bile salts via Ntcp-mediated entry, because β_1 -integrin knockdown strongly blunted the protective effect of TUDC (Fig. 1D). Furthermore, inhibitors of the integrin-signaling such as *GRGDSP* largely prevented TUDC-induced inhibition of GCDC-triggered apoptosis (Fig. 1B). Apoptosis induction by hydrophobic bile acids involves CD95 trafficking to the plasma membrane [10, 12], which was inhibited by TUDC in a *GRGDSP* and H89-sensitive manner (Fig. 3). TUDC prevented the GCDC-induced JNK activation at least during the first 60 min of

GCDC addition (Fig. 4). This is explained by a TUDC-activated integrin/PKA/MKP-1 signaling pathway, because inhibition of this pathway counteracted the inhibitory effect of TUDC on GCDC-induced JNK activation (Fig. 4). JNKs are activated via the sequential activation of protein kinases that includes the two dual-specificity MKK4 and MKK7 [32, 33]. Both MKKs are rapidly phosphorylated in response to GCDC in a TUDC-sensitive way (Fig. 5).

Previous data have shown that cAMP can protect against bile-acid induced apoptosis [27, 34-36]. cAMP did not prevent the bile acid-induced oxidative stress response and EGFR/CD95 association, however abolished EGFR activation and subsequent CD95 tyrosine phosphorylation, CD95 membrane trafficking, and DISC formation in a H89-sensitive way [23]. This observation differs from the present study which showed that TUDC does not affect GCDC-induced EGFR activation, but inhibits EGFR/CD95 association (Fig. 2), whereas cAMP already inhibited GCDC-induced EGFR activation [23]. This difference is most likely explained by the fact that TUDC not only produces cAMP (Fig. 6), but also triggers EGFR activation in a β_1 -integrin-dependent way by itself [6]. However, like TUDC, cAMP abolished the GCDC-induced JNK-activation in a PKA-dependent way (Fig. 4). Inhibition of PI3-K by wortmannin did not significantly affect the TUDC-mediated inhibition of the GCDC-induced JNK activation (data not shown), indicating that PI3-K survival pathways, as suggested previously [33, 37] are not involved.

The mechanisms underlying TUDC-induced formation of cAMP are incompletely understood. TUDC-induced cAMP formation is small and its detection requires inhibition of phosphodiesterases by IBMX. This may explain why others failed to detect cAMP generation in response to TUDC [38]. TUDC-induced cAMP formation was found in both, isolated hepatocytes and perfused rat liver (Fig. 6), suggesting that liver parenchymal cells are the primary source of cAMP formation. Most importantly, TUDC-induced cAMP formation was sensitive to inhibition of β_1 -integrin by the *GRGDSP* hexapeptide, but not the control peptide *GRADSP*, suggesting that cAMP formation is a downstream event of TUDC-induced integrin activation (Fig. 6B). A β_1 -integrin-triggered cAMP formation and protein kinase A activation has also been reported in HT-1080 human fibrosarcoma cells [39] and MDA-MB-435 human breast carcinoma cells [40]. TUDC [41, 42] as well as integrin ligation [43] were reported to increase intracellular free Ca^{2+} levels, which could activate Ca^{2+} -sensitive adenylate cyclase isoforms, as described in mouse parotid acinar cells [44]. Such a mechanism, however, would be difficult to reconcile with the RGD-peptide sensitivity of TUDC-induced cAMP formation. Adenylyl cyclases, however, are more typically activated as a result of the heterotrimeric $G_{\alpha s}$ protein coupling to G-protein-coupled receptors on the cytoplasmic side of the plasma membrane [45] and a recent study in platelets showed that $G_{\alpha_{13}}$ can directly bind to the β_1 -integrin cytoplasmic domain [46]. Therefore, we speculate that TUDC-induced β_1 -integrin activation may trigger coupling with $G\alpha$ proteins, and that this interaction may stimulate the cAMP/PKA pathway.

cAMP was shown to induce MKP-1 expression in primary rat hepatocytes by a PKA-dependent mechanism [47]. The induction of this transcriptionally regulated immediate early gene-encoded phosphatase contributes to inactivation of MAP kinases by dephosphorylating the MAP kinases on the Thr/Tyr residues critical for their activation [48]. TUDC triggers the transient expression of MKP-1 (Fig. 8) by an integrin/PKA-dependent mechanism (Fig. 8C) and prevents GCDC-induced JNK activation (Fig. 4). In the absence of this JNK signal the GCDC-triggered CD95 activation process is arrested at the level of EGFR/CD95 association [15, 16, 18]. In line with this, MKP-1 induction by cAMP also prevented apoptosis in Rat-1 and NIH/3T3 cells through inhibition of JNK activation [49].

Taken together, the present study shows that the inhibition of bile acid-induced apoptosis by TUDC involves an integrin/PKA/MKP-1-dependent JNK-inhibition, which prevents EGFR/CD95 association and an integrin/PKA-dependent internalization of the CD95 due to serine/threonine phosphorylation of this death receptor. Interestingly, the TUDC-induced β_1 -integrin activation was shown to occur inside the hepatocyte [6]. Physiological ligands of intracellular integrins are not yet known, however, their identification may offer new aspects for hepatoprotection.

Abbreviations

CD95 (Fas, apoptosis antigen-1); CM-H₂DCFDA (5-(and 6)-chloromethyl-2',7' dichlorodihydrofluorescein diacetate); GAPDH (glyceraldehyde 3-phosphate dehydrogenase); DAPI (4', 6-diamidino-2-phenylindole, dihydrochloride); Db-cAMP (dibutyryl-cyclic adenosine mono-phosphate); DISC (death inducing signaling complex); EGFR (epidermal growth factor receptor); Erk (extracellular regulated kinases); FADD (Fas-associated death domain); FBS (fetal bovine serum); GCDC (glycochenodeoxycholate); HPRT (hypoxanthine-guanine phosphoribosyltransferase); IBMX (3-Isobutyl-1-methylxanthine); IP (immunoprecipitation); ITGB1 siRNA (β_1 -integrin small interfering RNA); JNK (c-Jun-NH₂-terminal kinase); MAP (mitogen-activated protein); MKK (mitogen-activated protein kinase kinase); MKP-1 (mitogen-activated protein kinase phosphatase-1); Ntcp (sodium taurocholate cotransporting polypeptide); p38^{MAPK} (p38 mitogen-activated protein kinase); PI3-K (phosphatidyl-inositide 3-kinase); PKA (protein kinase A); ROS (reactive oxygen species); SEM (standard error of the mean); TUDC (tauroursodeoxycholate); TUNEL (terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labelling); UDCA (ursodeoxycholic acid).

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Disclosure Statement

The authors have nothing to disclose.

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