Modulation of Nuclear Steroid Receptors by Ursodeoxycholic Acid Inhibits TGF-β1-Induced E2F-1/p53-Mediated Apoptosis of Rat Hepatocytes[†]

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ABSTRACT: We have recently shown that both ursodeoxycholic acid (UDCA) and tauroursodeoxycholic acid (TUDCA) prevent transforming growth factor β1 (TGF-β1)-induced hepatocyte apoptosis by modulating the E2F-1/p53/Bax pathway. In addition, activation of glucocorticoid (GR) and mineralocorticoid receptors (MR) inhibits apoptosis in various systems. UDCA induces a ligand-independent activation of the GR, thus potentially regulating a number of targets. In this study, we investigated the role of GR and MR during TGF-β1-induced hepatocyte apoptosis, and identified additional antiapoptotic targets for UDCA. Our results showed that in primary hepatocytes, TGF-β1 induced 40−50% decreases in *gr* and *mr* mRNA expression ($p < 0.01$), together with up to 10-fold reductions in their protein levels ($p < 0.01$). Notably, pretreatment with UDCA resulted in a significant upregulation of nuclear steroid receptors ($p < 0.05$), which coincided with 2- and 3-fold increases in the level of GR and MR nuclear translocation, respectively, when compared with that of TGF-β1 alone ($p < 0.05$). Similarly, TUDCA induced GR and MR nuclear translocations ($p < 0.05$) and markedly prevented MR protein changes associated with TGF-β1 ($p < 0.05$) without affecting GR protein levels. Moreover, when interference RNA was used to inhibit GR and MR, UDCA no longer protected hepatocytes against TGF-β1-induced apoptosis. In fact, the protective effect of UDCA in TGF-β1-associated caspase activation decreased from 65 to <10% when GR or MR function was blocked. Finally, the TGF-β1-induced E2F-1/Mdm-2/p53 apoptotic pathway, normally inhibited by UDCA, was not regulated by the bile acid after GR or MR silencing. These results demonstrate that UDCA protects against apoptosis through an additional pathway that involves nuclear receptors GR and MR as key factors. Further, the E2F-1/Mdm-2/p53 apoptotic pathway appears to be a prime target for UDCA-induced steroid receptor activation.

Ursodeoxycholic acid (UDCA)[1] is used increasingly for the treatment of cholestatic liver diseases. This endogenous bile acid protects cholangiocytes against cytotoxicity of hydrophobic bile acids, stimulates hepatobiliary secretion, and inhibits apoptosis (*1*). It has been shown that UDCA plays a unique role in modulating the apoptotic threshold, in both hepatic and nonhepatic cells, by interfering with classic mitochondrial pathways (*2*−*4*). In fact, after several toxic stimuli, UDCA interrupts the apoptotic pathway by inhibiting mitochondrial membrane depolarization and channel formation, production of reactive oxygen species, release of cytochrome *c*, caspase activation, and cleavage of the nuclear enzyme poly(ADP-ribose) polymerase (*5*). In addition, we recently reported that UDCA interferes with alternate and upstream molecular targets such as the E2F-1/p53 apoptotic pathway (*6*).

Transforming growth factor β1 (TGF-β1) is a potent inducer of growth arrest and apoptosis in hepatic cells in culture and *in vivo* (*7*, *8*). Its apoptotic effect appears to be linked, in part, to decreased levels of phosphorylation and expression of the retinoblastoma protein (pRb), and subsequent E2F-1 transactivation (*9*, *10*). E2F-1 is the best-characterized member of the E2F family of transcription factors that regulates a number of genes involved in differentiation, development, and apoptosis (*11*, *12*). In fact, unbound E2F-1 was shown to modulate TGF-β1-induced apoptosis in hepatic cells (*6*, *10*, *13*). In addition, the ability of E2F-1 to promote apoptosis involves stabilization of tumor suppressor protein p53 via the transcription of $p14^{ARF}$, which markedly inhibits the p53 repressor Mdm-2 (*12*). The apoptotic targets of p53 include members of the Bcl-2 family, such as Bcl-2 and Bax (*14*−*16*), or the BH3-only proteins Noxa and Puma (*17*), and Apaf-1 (*18*), whose cellular balance determines the ultimate fate of the cell.

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[1] Abbreviations: FXR, farnesoid X-activated receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; NF-κB, nuclear factor κB; RT-PCR, reverse transcriptase polymerase chain reaction; SEM, standard error of the mean; TGF-β1, transforming growth factor β1; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid.

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Our studies suggest that UDCA inhibits the E2F-1/Mdm-2/p53 apoptotic pathway induced by TGF-β1, thus modulating the expression of Bcl-2 family members (*6*). However, it remains to be determined whether the activation of apoptosis by E2F-1 is modulated directly by UDCA or the result of an upstream effect(s) involving other molecular targets, such as nuclear receptors. In fact, UDCA is a cholesterol-derived molecule, suggesting a possible regulatory role for this bile acid in modulating classical nuclear steroid hormone receptors. A number of bile acids, including chenodeoxycholic, cholic, deoxycholic, and lithocholic acids, bind and inactivate the farnesoid X-activated receptor (FXR) in cultured cells (*19*−*21*). In contrast, UDCA does not activate FXR (*20*), but rather inhibits activation of this receptor by more hydrophobic bile acids (*22*). Further, it has been reported that UDCA modulates activation of the classic glucocorticoid receptor (GR), without eliciting its transactivational function (*23*). Rather, UDCA appears to interact with distinct regions of the ligand binding domain of GR, thus suppressing, for example, nuclear factor κB (NF-κB)-dependent transcription (*24*). Finally, dexamethasone, a strong GR activator, was shown to prolong cell viability, inhibit the development of apoptotic morphology, and stabilize the expression of procaspase-3 in both human and rat hepatocytes (*25*). The inhibitory effects of glucocorticoids against apoptosis have also been extended to TGF-β1-induced cell death in rat hepatoma cell lines (*26*). Interestingly, GR activation induces cell cycle arrest through distinct transcriptional regulatory mechanisms depending on the cell type (*27*), and increases the levels of antiapoptotic Bcl-2 family members (*26*, *28*, *29*).

GR is a member of the nuclear receptor superfamily and an important transcriptional regulator in diverse physiological functions such as control of embryonic development, cell differentiation, and metabolic homeostasis. The nuclear steroid receptors, in the absence of ligand binding, are transcriptionally inactive and located primarily in the cytoplasm by association with a variety of proteins. Thus, ligand stimulation of target cells leads to nuclear receptor dissociation and subsequent migration into the nucleus, where they can bind to chromatin-associated hormone response elements. This process is modulated *in vivo* by steroid or nonsteroidal compounds that interact directly with the receptor moiety or with its associated proteins (*30*, *31*). The heterogeneous function of nuclear receptors is affected by tissue-specific parameters, including alternative initiation sites within nuclear receptor genes, and different effects of comodulators. In this regard, the expression or attenuation of certain gene products by GR can be involved in either anti- or proapoptotic effects, depending on the cell type. At odds with its antiapoptotic effect in the liver, GR was shown to upregulate p53 and proapoptotic members of the Bcl-2 family in neuronal cells and leukemia cell lines, respectively (*32*−*34*). In contrast, it is generally recognized that another steroid receptor, the mineralocorticoid receptor (MR), has a predominantly antiapoptotic role, preventing GR-induced cell death in several neuronal systems (*32*, *35*−*38*). It is possible that the potential modulation of GR and MR by UDCA might also have diverse effects in different cell types.

We previously demonstrated that UDCA prevents TGF-β1-induced apoptosis by modulating the E2F-1/p53/Bax pathway, in part, through caspase-independent mechanisms. Here, we further explored the early molecular events linking nuclear steroid receptors and the protective role of UDCA during TGF-β1-induced apoptosis. Our results indicate that there is a differential regulatory action between UDCA and TGF-β1 on GR and MR. In addition, the E2F-1/Mdm-2/p53 cascade is a unique target for UDCA-induced nuclear steroid receptor activation.

MATERIALS AND METHODS

Hepatocyte Isolation and Cell Culture. Rat primary hepatocytes were isolated from male Sprague-Dawley rats (100−150 g) by collagenase perfusion as described previously (*39*). In brief, rats were anesthetized with phenobarbitol and the livers perfused with 0.05% collagenase. Hepatocyte suspensions were obtained by passing digested livers though 125 μm gauze and washing cells in William's E medium (Invitrogen Corp., Grand Island, NY) supplemented with 26 mM sodium bicarbonate, 23 mM HEPES, 0.01 unit/mL insulin, 2 mM L-glutamine, 10 nM dexamethasone, 5.5 mM glucose, 100 units/mL penicillin, 100 units/mL streptomycin, and 20% heat-inactivated (56 °C for 30 min) fetal bovine serum (FBS, Atlanta Biologicals Inc., Norcross, GA). Cell viability was determined by trypan blue exclusion and was typically 85−90%. After isolation, hepatocytes were resuspended in complete William's E medium and plated on Primaria tissue culture dishes (BD Biosciences, San Jose, CA) at a density of either 2.1×10^4 cells/cm^2 for transfection assays or 6.4×10^4 cells/cm^2 for all other experiments. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 for 6 h. Plates were then washed with medium to remove dead cells, and then incubated in William's E medium containing 10% heat-inactivated FBS.

Induction of Apoptosis. Freshly isolated hepatocytes were cultured for 6 h as described above, washed, and then incubated in 10% FBS William's E medium supplemented with either 100 μM UDCA or TUDCA (Sigma Chemical Co., St. Louis, MO), or no addition (control) for 12 h. Cells were then exposed to 1 nM recombinant human TGF-β1 (R&D Systems Inc., Minneapolis, MN) for 6, 12, 24, 36, or 48 h. Attached and floating cells were combined to extract cytosolic and total proteins for caspase activity and immunoblotting, respectively, and total RNA for RT-PCR.

Morphologic Evaluation of Apoptosis. Hoechst labeling of cells was used to detect apoptotic nuclei. In brief, the medium was gently removed at the indicated times with minimal detachment of the cells. The attached hepatocytes were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 10 min at room temperature, incubated with Hoechst dye 33258 (Sigma Chemical Co.) at 5 μg/mL in PBS for 5 min, washed with PBS, and mounted using PBS and glycerol (3:1, v/v). Fluorescent nuclei were scored blindly by laboratory personnel and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei showed noncondensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as nuclear fragmentation of condensed chromatin. Three random microscopic fields per sample of approximately 250 nuclei were counted and mean values expressed as the percentage of apoptotic nuclei.

Total, Nuclear, and Cytosolic Protein Extraction. For total protein extracts, cells were lysed in ice-cold buffer [10 mM

Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 1.5 mM KAc, 1% Nonidet P-40, 2 mM DTT, and protease inhibitor cocktail tablets (Complete, Roche Applied Science, Mannheim, Germany)] for 30 min and then homogenized with 20 strokes in a loose fitting Dounce. The lysate was centrifuged at 3160*g* for 10 min at 4 °C and the supernatant recovered. For nuclear and cytosolic extracts, cells were lysed with hypotonic buffer [10 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 1.5 mM KAc, 2 mM DTT, and protease inhibitors], homogenized with 20 strokes in a loose fitting Dounce, and centrifuged at 500*g* for 10 min at 4 °C. The cytosolic proteins were recovered in the supernatant, while the nuclear pellet was washed in buffer containing 10 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 0.25 M sucrose, 0.5% Triton X-100, and protease inhibitors, then resuspended, and sonicated in buffer containing 10 mM Tris-HCl (pH 7.6) and 0.25 M sucrose with protease inhibitors. Finally, the suspension was centrifuged through 0.88 M sucrose at 2000*g* for 20 min at 4 °C, and nuclear proteins were recovered in the supernatant. Lamin and keratin were used as markers for nuclear and cytosolic protein extraction, respectively.

Caspase Activation. Caspase activation was assessed in cytosolic protein extracts after cells had been harvested and homogenized in isolation buffer. General caspase-3-like activity was assayed by enzymatic cleavage of chromophore *p*-nitroanilide (pNA) from the substrate *N*-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA, Sigma Chemical Co.). The proteolytic reaction was carried out in isolation buffer containing 50 μg of cytosolic protein and 50 μM DEVD-pNA. The reaction mixtures were incubated at 37 °C for 1 h, and the formation of pNA was assessed at 405 nm using a 96-well plate reader.

Immunoblotting. Steady-state levels of E2F-1, p53, Mdm-2, GR, and MR proteins as well as nuclear receptor cellular distribution were determined by Western blot analysis. Briefly, 200 μg of total, cytosolic, or nuclear protein extracts was separated on an 8 or 12% SDS−polyacrylamide electrophoresis gel. Following electrophoretic transfer onto nitrocellulose membranes, immunoblots were incubated with 15% H_2O_2 for 15 min at room temperature. After being blocked with a 5% milk solution, the blots were incubated overnight at 4 °C with primary mouse monoclonal antibodies reactive to E2F-1, Mdm-2, and p53 or primary rabbit polyclonal antibodies to GR and MR (Santa Cruz Biotechnology, Santa Cruz, CA), and finally with secondary antibodies conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) for 3 h at room temperature. The membranes were processed for protein detection using Super Signal substrate (Pierce, Rockford, IL). Protein concentrations were determined using the Bio-Rad protein assay kit according to the manufacturer's specifications.

RNA Isolation and RT-PCR. Transcript expression of *gr* and *mr* was assessed by RT-PCR. Total RNA was extracted from rat primary hepatocytes using the TRIZOL reagent (Invitrogen Corp.). For RT-PCR, 5 μg of total RNA was reverse-transcribed using oligo(dT) (Integrated DNA Technologies Inc., Coralville, IA) and SuperScript II reverse transcriptase (Invitrogen Corp.). Specific oligonucleotide primer pairs were incubated with a cDNA template for PCR amplification using the Expand High Fidelity^{PLUS} PCR System from Roche Applied Science. The following sequences were used as primers: 5′-GCAGATTCCAAGCAGCAGAGG-3′ (*gr* sense), 5′-AGCCCAAGTCATTCCCCATC-3′ (*gr* antisense), 5′-CGCTCTATTACTCTACCCACCGTC-3′ (*mr* sense), 5′-GTGAAGAACGCTCCAAGGTCTG-3′ (*mr* antisense), 5′-CGTCTTCCCCTCCATCGTG-3′ (*β-actin* sense), and 5′-CCAGTTGGTTACAATGCCGTG-3′ (*β-actin* antisense). The product of the *β-actin* RNA was used as control.

Short Interference RNA and Transfection. Short Interference RNA (siRNA) sequences targeting *gr* (GenBank accession number NM012576) and *mr* (GenBank accession number M36074) mRNA corresponded to the coding regions of residues 129−149 (GGCCAAGGGAGGGGGAGCGTA) and 523−543 (GGCGCTGGAGTCAAGTGTCTC), respectively. The location of targeting sequences was 51 and 50 nucleotides downstream, relative to the first nucleotide of the start codon for *gr* and *mr*, respectively. The 23-nucleotide dsRNAs were prepared by annealing both forward and reverse sequences of GR and MR targeting regions: 5′-rGrGrCrCrArArGrGrGrArGrGrGrGrGrArGrCrGrUrATT-3′ (*gr* forward), 5′-rUrArCrGrCrUrCrCrCrCrCrUrCrCr-CrUrUrGrGrCrCTT-3′ (*gr* reverse), 5′-rGrGrCrGrCrUrGr-GrArGrUrCrArArGrUrGrUrCrUrCTT-3′ (*mr* forward), and 5′-rGrArGrArCrArCrUrUrGrArCrUrCrCrArGrCrGrCrCTT-3′ (*mr* reverse). A nonspecific duplex was used as a control (5′-rCrArGrUrGrGrArGrArUrCrArArCrGrUrGrCrArArGUU-3′), which did not significantly affect GR and MR mRNA and protein levels relative to the untransfected controls. Twelve hours after plating, hepatocytes at 40% confluence were transfected with ∼6 μg of siRNA using JetSI Transfection Reagent for siRNA (Qbiogene, Carlsbad, CA), according to the manufacturer's instructions. Four hours after the siRNA transfections, vehicle or either UDCA or TUDCA (100 μM) was added to the culture medium, and after an additional 12 h, 1 nM TGF-β1 was included. The cells were incubated with TGF-β1 for 24 h, after which all floating and attached cells were harvested for either RNA extraction or cytosolic and total protein extraction. Hoechst labeling of cells was used to detect apoptotic nuclei as described above. To assess gene silencing, transcript and protein levels of GR and MR were determined by RT-PCR and immunoblotting, respectively. Our results indicate that the silencing efficiency of the siRNAs for both nuclear receptors was ∼70% in UDCA-treated hepatocytes and 50% in untreated cells.

Densitometry and Statistical Analysis. The relative intensities of protein and nucleic acid bands were analyzed using the ImageMaster 1D Elite densitometric analysis program (Amersham Biosciences, Piscataway, NJ). Standard curves were run, and the data that were obtained were in the linear range of the curve. In addition, all values were normalized to their respective lane loading controls. Statistical analysis was performed using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA) for the ANOVA and Bonferroni's multiple-comparison tests. p values of <0.05 were considered statistically significant.

RESULTS

UDCA Prevents a TGF-β1-Induced Decrease in GR and MR Levels. It has been reported that glucocorticoids suppress TGF-β1-induced apoptosis in rat hepatoma cells (*26*). In addition, we have previously shown that UDCA inhibits

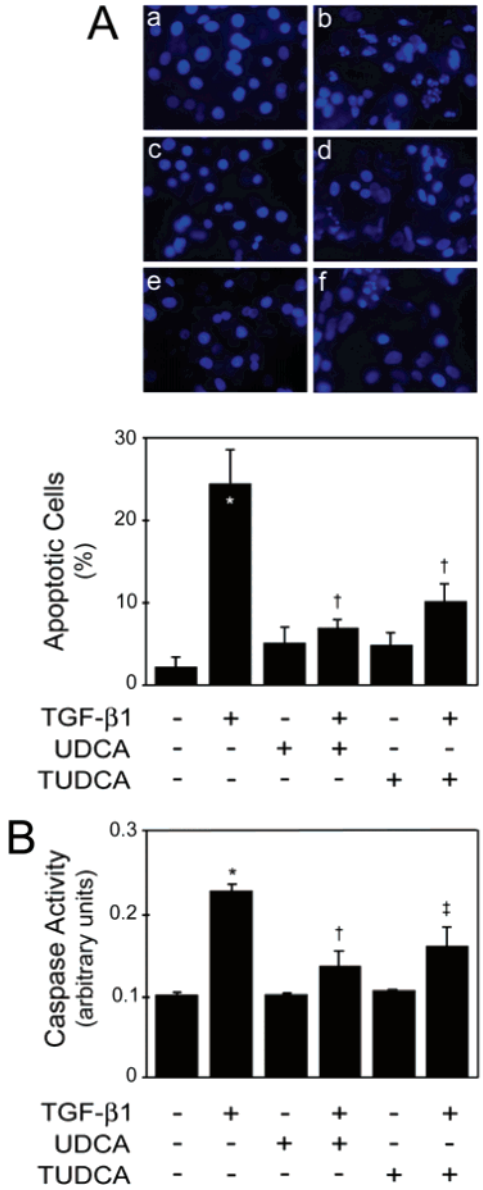


FIGURE 1: UDCA inhibits apoptosis induced by TGF-β1 in primary rat hepatocytes. Cells were incubated with 1 nM TGF-β1, or no addition (control), with or without 100 μM UDCA or TUDCA for 36 h. In coincubation experiments, hepatocytes were pretreated with either UDCA or TUDCA 12 h prior to incubation with TGF-β1. Cells were fixed and stained for morphological evaluation of apoptosis, and cytosolic proteins were extracted for caspase activity as described in Materials and Methods. (A) Fluorescent microscopy of Hoechst staining in control hepatocytes (a) and in cells exposed to either TGF-β1 (b), UDCA (c), TGF-β1 and UDCA (d), TUDCA (e), or TGF-β1 and TUDCA (f) for 36 h (top). Percent apoptosis in cells exposed to TGF-β1 for 36 h with or without bile acids (bottom). (B) DEVD-specific caspase activity in cytosolic fractions after incubation with TGF-β1 for 36 h with or without bile acids. The results are expressed as means ± SEM for at least three different experiments. An asterisk denotes a p value of <0.01 from control, a dagger a p value of <0.01, and a double dagger a p value of <0.05 from TGF-β1.

TGF-β1-induced cell death in human hepatoma HuH-7 cells and in primary rat hepatocytes (*3*, *5*), suggesting that GR could be an important regulatory factor targeted by UDCA. In this study, apoptosis was assessed by changes in nuclear morphology (Figure 1A) and caspase activity (Figure 1B). Significant levels of apoptosis existed in cultured primary rat hepatocytes after incubation with TGF-β1, with a maximum apoptotic response of >20% at 36 h ($p < 0.01$). UDCA and its taurine-conjugated derivative TUDCA protected against TGF-β1-induced nuclear fragmentation and caspase-3-like activation by 50−70% ($p < 0.01$). In addition, RT-PCR analysis showed a marked decrease in both *gr* and *mr* mRNA levels in primary rat hepatocytes incubated with TGF-β1 for 6, 12, and 24 h (Figure 2). In fact, *gr* transcript levels were reduced by 25% after 6 h with TGF-β1, and remained low throughout the time course ($p < 0.01$). The loss of *mr* mRNA was particularly significant at 24 h of TGF-β1 incubation ($p < 0.01$). Interestingly, UDCA alone markedly increased *gr* mRNA levels ($p < 0.05$), which resulted in a significant abrogation of TGF-β1-induced changes as early as 6 h ($p < 0.05$). TUDCA, in contrast, did not suppress the TGF-β1-induced *gr* mRNA alterations. Notably, *mr* transcript levels were extensively modulated by both bile acids. The level of *mr* mRNA was increased >5-fold with either UDCA or TUDCA compared to controls ($p < 0.05$). Further, both bile acids prevented the loss of *mr* mRNA induced by TGF-β1 ($p < 0.05$).

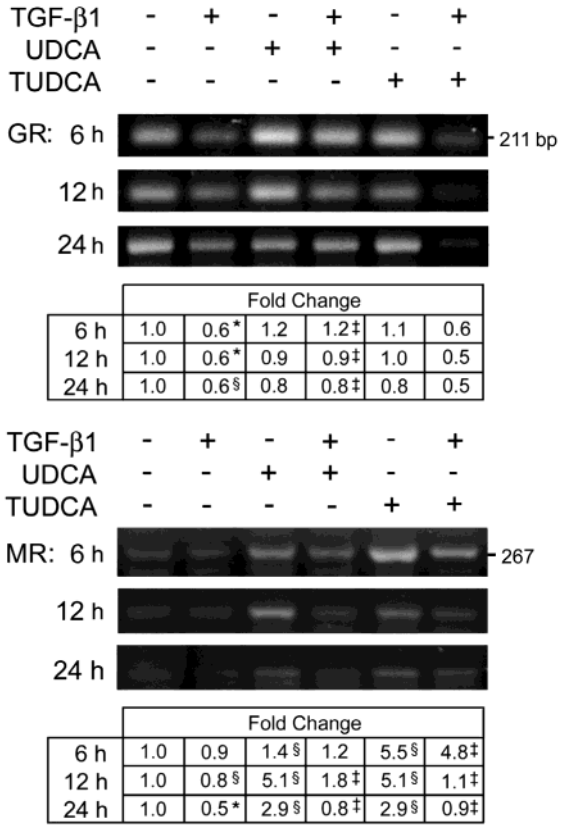
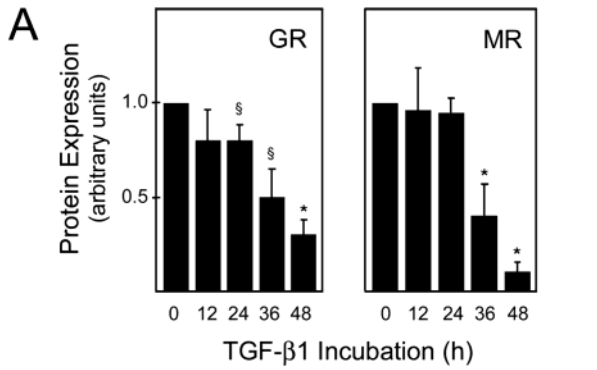


FIGURE 2: UDCA modulates *gr* and *mr* mRNA expression in primary rat hepatocytes. Cells were incubated with 1 nM TGF-β1, or no addition (control), with or without UDCA or TUDCA for 6, 12, and 24 h. In coincubation experiments, UDCA or TUDCA was added to hepatocytes 12 h prior to incubation with TGF-β1. Total mRNA was obtained for RT-PCR analysis as described in Materials and Methods. Representative RT-PCR of *gr* and *mr* and mean densitometry values of cells exposed to TGF-β1 with or without bile acids for at least five different experiments. All densitometry values were normalized to endogenous β-actin mRNA. An asterisk denotes a p value of <0.01 and a secant a p value of <0.05 from the respective control; a double dagger denotes a p value of <0.05 from TGF-β1.

The results from the semiquantitative RT-PCR were confirmed by Western blot analysis, with minor variances due possibly to post-transcriptional regulation. In fact, protein



B

TGF-β1	-	+	-	+	-	+
UDCA	-	-	+	+	-	-
TUDCA	-	-	-	-	+	+

GR: 12 h, 24 h, 36 h, 48 h — 95 kDa

	Fold Change					
12 h	1.0	0.8	1.2	1.0	0.9	0.9
24 h	1.0	0.8§	1.0	1.0‡	1.2	1.0
36 h	1.0	0.5§	1.0	0.7‡	1.0	0.7
48 h	1.0	0.3*	1.6§	0.5‡	2.2§	0.4

MR: 12 h, 24 h, 36 h, 48 h — 110

	Fold Change					
12 h	1.0	0.9	1.4	1.3	1.7§	1.4‡
24 h	1.0	0.9	1.5	1.2	1.5§	1.6‡
36 h	1.0	0.3*	1.4	0.6‡	1.2	0.5‡
48 h	1.0	0.1*	1.3	0.2	1.1	0.3‡

FIGURE 3: UDCA modulates GR and MR protein expression in primary rat hepatocytes. Cells were incubated with 1 nM TGF-β1, or no addition (control), with or without UDCA or TUDCA for 12, 24, 36, and 48 h. In coincubation experiments, UDCA or TUDCA was added to hepatocytes 12 h prior to incubation with TGF-β1. Total proteins were extracted for Western blot analysis as described in Materials and Methods. (A) Histograms of GR and MR expression in cells exposed to TGF-β1 alone. The results are expressed as means ± SEM arbitrary units for at least four different experiments. An asterisk denotes a p value of <0.01 and a secant a p value of <0.05 from the respective control. (B) Representative immunoblots of GR and MR and mean densitometry values of cells exposed to TGF-β1 with or without bile acids for at least five different experiments. All densitometry values were normalized to the endogenous β-actin protein. An asterisk denotes a p value of <0.01 and a secant a p value of <0.05 from the respective control; a double dagger denotes a p value of <0.05 from TGF-β1.

levels of GR and MR were markedly decreased after incubation with TGF-β1 (Figure 3A). At 48 h, TGF-β1 reduced GR and MR protein levels by 70 and 90%, respectively ($p < 0.001$). In addition, the changes associated with TGF-β1 were significantly abrogated by UDCA throughout the time course ($p < 0.05$), while TUDCA was markedly effective at preventing MR protein changes ($p < 0.05$) (Figure 3B). Thus, these data suggest that TGF-β1 downregulates both GR and MR expression during hepatocyte apoptosis, which in turn is significantly modulated by UDCA and/or TUDCA.

UDCA Induces GR and MR Nuclear Translocation. Since the activation of nuclear receptors requires translocation into the nucleus (*40*, *41*), we investigated the effect of TGF-β1, with or without bile acids, on the intracellular trafficking of GR and MR. Immunoblot analysis demonstrated that TGF-β1 does not induce GR or MR translocation to the nucleus; rather, both receptors are simultaneously reduced in the nucleus and cytoplasm as a result of a decreased level of protein production. In fact, TGF-β1 reduced GR nuclear levels ~30 and 60% at 24 and 36 h, respectively ($p < 0.001$) (Figure 4A). The decrease in the level of GR with TGF-β1 was accompanied by an overall decline in the level of nuclear MR ($p < 0.05$) (Figure 4B). Levels of cytosolic GR and MR were also decreased 30−70% in TGF-β1-treated cells compared with control hepatocytes ($p < 0.001$ and $p < 0.05$, respectively).

Interestingly, UDCA or TUDCA alone induced marked changes in nuclear translocation of both steroid receptors. In fact, UDCA almost doubled GR and MR nuclear levels at early and late time points ($p < 0.05$), respectively. In addition, MR seemed to be a preferential target for TUDCA alone, and its level increased ~3-fold in the nucleus of hepatocytes at 36 h compared with control cells ($p < 0.05$). The cytosolic levels of MR were not reduced with UDCA or TUDCA alone, suggesting that an increased level of MR *de novo* synthesis may have compensated for MR cytosolic levels after nuclear translocation (data not shown). Finally, coincubation with UDCA and TGF-β1 further enhanced the effect of the bile acid on the nuclear translocation of both steroid receptors ($p < 0.05$). Interestingly, in the presence of TGF-β1, pretreatment with TUDCA markedly induced GR nuclear translocation at 24 and 36 h, completely inhibiting the effect of TGF-β1 alone ($p < 0.05$ and $p < 0.01$, respectively). However, GR levels in the nucleus were not above control values, in part, because GR transcription and protein expression were not enhanced by TUDCA (Figures 2 and 3A). The protective effect of TUDCA was also clearly demonstrated for MR, particularly at 36 h. Nuclear levels increased >2-fold compared with that with TGF-β1 alone ($p < 0.05$). Thus, the induction of GR and MR by UDCA and/or TUDCA appears to result in active translocation of steroid receptors to the nucleus, suggesting that these molecules participate in the antiapoptotic effects of UDCA and TUDCA.

GR and MR Contribute to the Antiapoptotic Effect of UDCA. Although UDCA has been shown to activate GR in a human hepatoma cell line in the absence of ligands (*23*), the role of GR during the antiapoptotic function of UDCA remains unclear. In addition, our results suggested that UDCA is a more general modulator of GR and MR than TUDCA. Therefore, using synthetic siRNA, we performed post-transcriptional gene silencing experiments for both GR and MR, and evaluated the antiapoptotic function of UDCA in primary rat hepatocytes. Our results indicated that the decrease in the levels of GR and MR expression after transfection with the siRNAs was significant, particularly

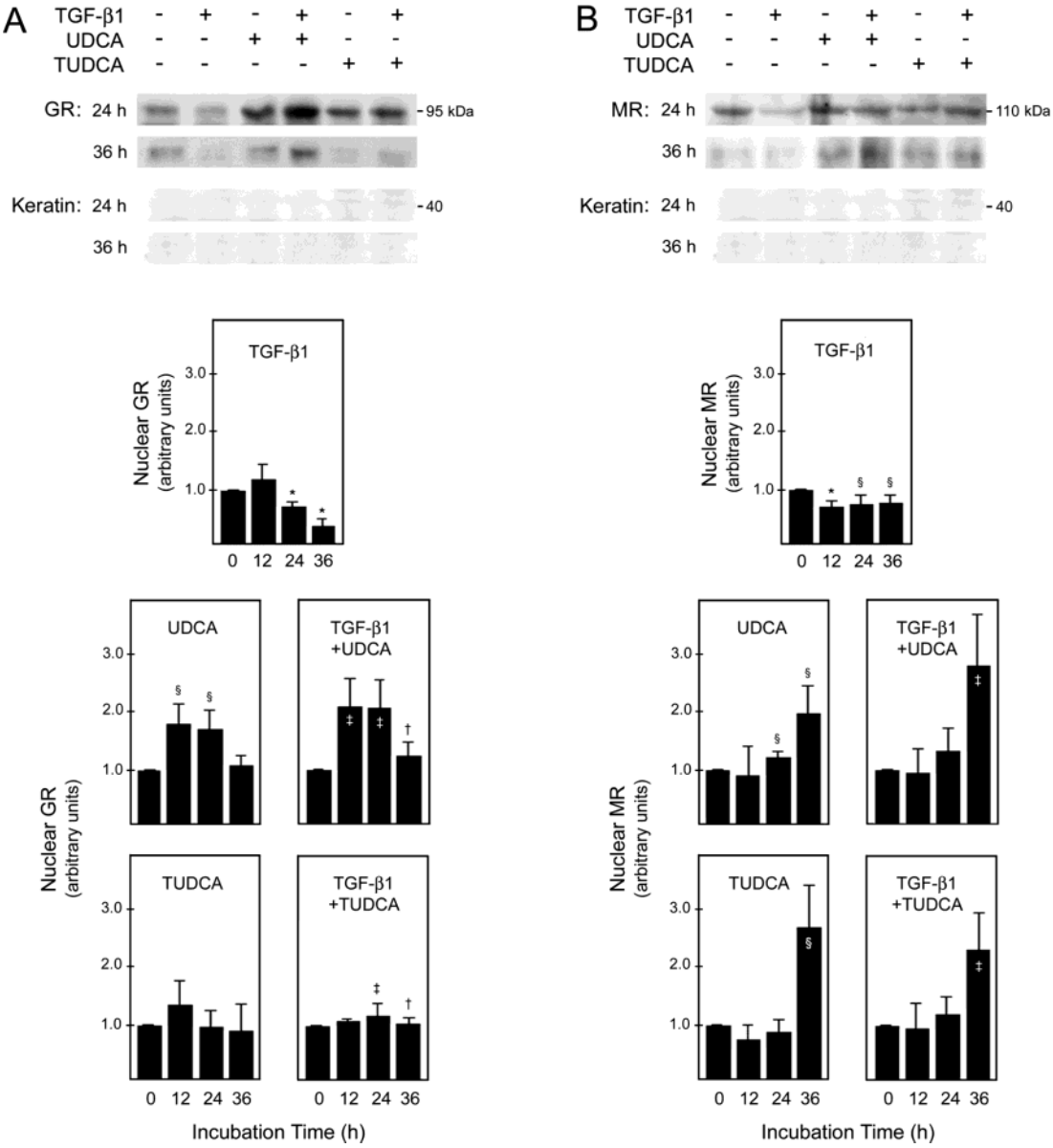


FIGURE 4: UDCA induces translocation of GR and MR to the nucleus. Cells were incubated with 1 nM TGF-β1, or no addition (control), with or without UDCA or TUDCA for 12, 24, and 36 h. In coincubation experiments, UDCA or TUDCA was added to hepatocytes 12 h prior to incubation with TGF-β1. Nuclear proteins were extracted for Western blot analysis as described in Materials and Methods. Immunoblots of GR (A) and MR (B) proteins (top) and the corresponding histograms (bottom) in cells exposed to TGF-β1 with or without bile acids, for the indicated times. The results are expressed as means ± SEM arbitrary units for at least four different experiments. Cytoplasmic contamination was determined by immunoblotting of keratin protein in nuclear extracts. An asterisk denotes a p value of <0.01 and a secant a p value of <0.05 from the respective control; a dagger denotes a p value of <0.01 and a double dagger a p value of <0.05 from TGF-β1.

in UDCA-treated hepatocytes (>70%, $p < 0.05$) in which both nuclear receptors were transcribed normally (Figure 5, top). GR silencing did not affect MR expression, or the reverse (data not shown). More importantly, GR- and MR-specific siRNA transfection completely abolished UDCA protection against TGF-β1-mediated apoptosis, as assessed by both nuclear morphology and caspase activation (Figure 5, bottom). In contrast, the nonspecific control duplex did not affect the antiapoptotic function of UDCA.

Activation of GR and MR by UDCA Targets the E2F-1/Mdm-2/p53 Apoptotic Pathway. It was previously demonstrated that glucocorticoids reduce the level of expression of the E2F-1 transcription factor involved in the G_1 to S phase transition of the cell cycle (*27*). In addition, the p53/Mdm-2 pathway is a downstream target of TGF-β1 via the E2F-1 transcription factor, which can stabilize p53 through inhibition of the Mdm-2 protein (*6*). Thus, we tested for an interaction between nuclear steroid receptors, and the E2F-1/Mdm-2/p53 apoptotic pathway triggered by TGF-β1 in primary rat hepatocytes. After transfection with either a control, GR-specific, or MR-specific siRNA, total protein extracts were prepared from primary rat hepatocytes incubated with TGF-β1 in the presence or absence of UDCA (Figure 6). Immunoblot analysis of E2F-1 expression in control siRNA transfected cells showed an ~60% increase in protein levels with TGF-β1 compared with untreated hepatocytes ($p < 0.05$). In control siRNA-transfected cells, incubation with UDCA alone slightly reduced E2F-1 levels,

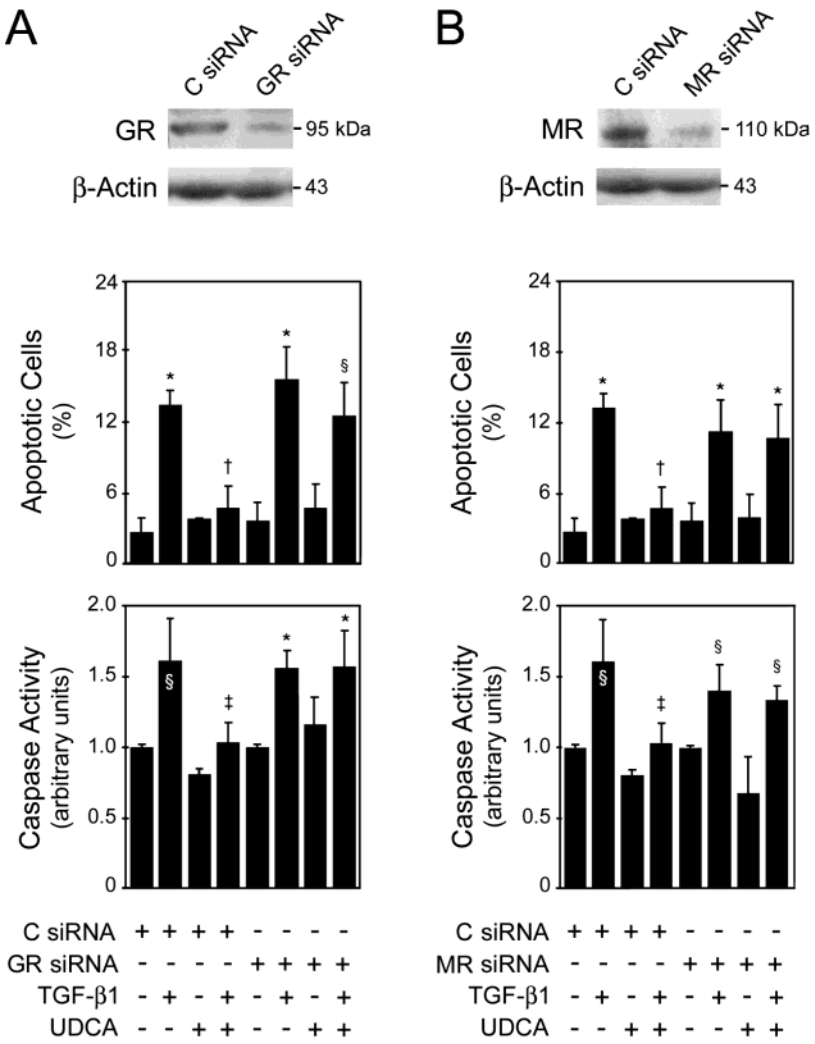


FIGURE 5: Antiapoptotic activity of UDCA is mediated via GR and MR in primary hepatocytes. Four hours after control, in GR or MR siRNA transfections, either vehicle or 100 μM UDCA was added to cells. After an additional 12 h, 1 nM TGF-β1 was included in the cultures for 24 h. Cells were fixed and stained for morphological evidence of apoptosis, and cytosolic proteins were extracted for caspase activity as described in Materials and Methods. (A) Representative immunoblot of GR in cells treated with UDCA and transfected with either control or GR siRNA (top). Percentage of apoptosis and DEVD-specific caspase activity in cells transfected with control or GR siRNA and exposed to TGF-β1 with or without UDCA (bottom). (B) Representative immunoblot of MR in cells treated with UDCA and transfected with either control or MR siRNA (top). Percentage of apoptosis and DEVD-specific caspase activity in cells transfected with control or MR siRNA and exposed to TGF-β1 with or without UDCA (bottom). The results are expressed as means ± SEM for at least three different experiments. β-Actin was used to control for lane loading. To assess mRNA silencing, both transcript and protein levels of GR and MR were quantified by RT-PCR and immunoblotting, respectively. An asterisk denotes a p value of <0.01 and a secant a p value of <0.05 from the respective control; a dagger denotes a p value of <0.01 and a double dagger a p value of <0.05 from cells transfected with GR or MR siRNA and exposed to TGF-β1 with or without UDCA. C denotes control.

while coincubation with TGF-β1 and UDCA completely inhibited the increase in E2F-1 levels ($p < 0.05$). In contrast, both GR and MR siRNAs abolished UDCA inhibitory effects on E2F-1 production in cells treated with either UDCA alone or TGF-β1 and UDCA.

The subsequent modulation of Mdm-2 and p53 expression was also evaluated in primary rat hepatocytes by immunoblot analysis. In control siRNA-transfected hepatocytes, the level of Mdm-2 was decreased ~50% ($p < 0.01$), which coincided with a >2-fold increase in p53 levels after TGF-β1 incubation. In addition, UDCA inhibited the observed decrease in Mdm-2 and increase in p53 with TGF-β1 ($p < 0.05$). GR and MR interference, in turn, caused a significant decrease in the protective activity of UDCA. In fact, both Mdm-2 and p53 were no longer modulated by UDCA with inhibition of GR and MR, thus confirming their potential role in the protective action of UDCA. Thus, the E2F-1/Mdm-2/p53 apoptotic pathway appears to be a unique target for UDCA-induced GR and MR activation.

DISCUSSION

Although UDCA increases the apoptotic threshold in many different cell types, the exact mechanisms involved remain unclear. We have previously reported that UDCA is a pleiotropic agent that prevents TGF-β1-induced hepatocyte apoptosis by both inhibiting the mitochondrial pathway of cell death (*3*, *5*) and modulating the E2F-1/Mdm-2/p53 pathway in part, without affecting caspase activity (*6*). The results presented here provide an extended mechanism of action for UDCA, showing that the steroid nuclear receptors GR and MR contribute to the protective effect of UDCA through the E2F-1/Mdm-2/p53 apoptotic pathway.

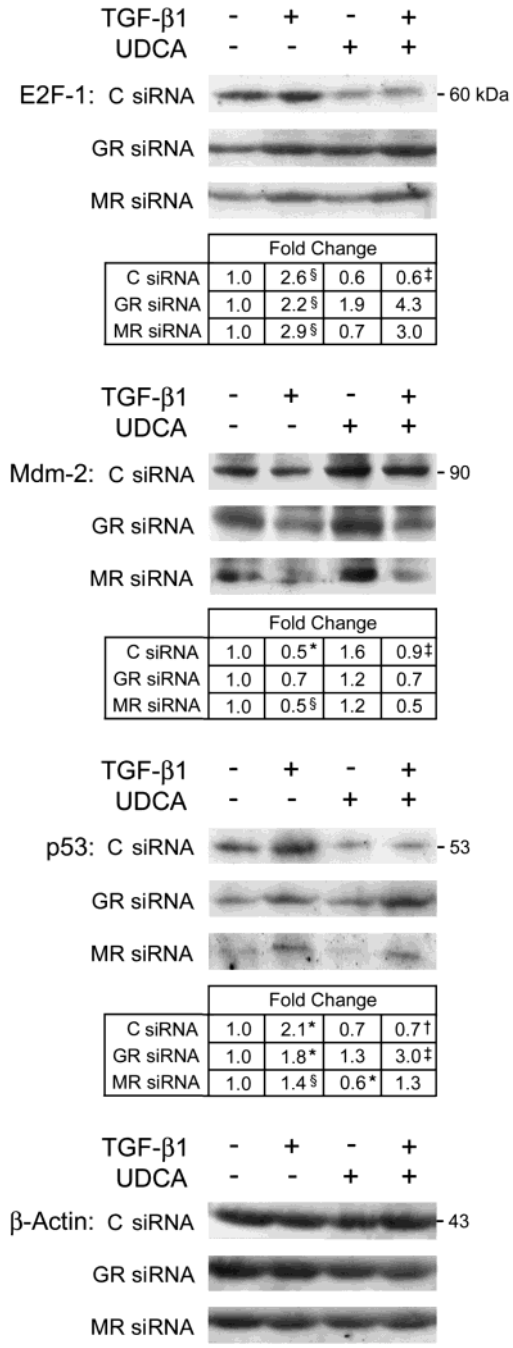


FIGURE 6: GR and MR contribute to UDCA-induced modulation of the E2F-1/Mdm-2/p53 pathway. Four hours after control, GR, or MR siRNA transfections, either vehicle or 100 μM UDCA was added to cells. After an additional 12 h, 1 nM TGF-β1 was included in the cultures for 24 h. Total proteins were extracted for Western blot analysis as described in Materials and Methods. Representative immunoblots of E2F-1, Mdm-2, and p53 proteins and mean densitometry values of cells transfected with either control, GR, or MR siRNA, and exposed to TGF-β1 with or without UDCA for at least three different experiments. β-Actin was used to control for lane loading. An asterisk denotes a p value of <0.01 and a secant a p value of <0.05 from the respective control; a dagger denotes a p value of <0.01 and a double dagger a p value of <0.05 from TGF-β1. C denotes control.

Recent studies have identified an increasing number of ligands for nuclear receptors, including certain bile acids (*19*−*21*). It has been demonstrated that UDCA activates GR, in the absence of ligand (*23*, *24*), suggesting a modulatory effect for this bile acid. Nevertheless, UDCA-induced GR activation has been correlated primarily with its anti-inflammatory properties, but not with UDCA's antiapoptotic function. In fact, nuclear receptors act as ligand-activated transcription factors that regulate expression of target genes to affect a variety of cellular processes, including apoptosis. It has been demonstrated that a number of apoptosis-related proteins such as p53, Bax, Bcl-2, Bcl-x_L, and the cellular inhibitor of apoptosis 2 (c-IAP2) are strongly regulated by nuclear steroid receptors in several cell types (*26*, *32*, *42*−*44*). Interestingly, the modulatory effect of nuclear receptors is cell-specific, often involving the expression and attenuation of the same gene between different tissues.

In this study, we demonstrated that significant degradation of GR and MR occurs during TGF-β1-induced hepatocyte apoptosis. In fact, the effect of TGF-β1 on GR is interesting in light of previous studies showing that activation of GR represses the effects of TGF-β1 in rat hepatoma cell lines (*26*, *45*, *46*). GR appears to modulate TGF-β1 effects by directly targeting the transcriptional activation function of Smad (*46*). It is now well-established that Smad plays a crucial role in transducing TGF-β1 signals from the cell membrane into the nucleus, where they orchestrate transcriptional responses (*47*, *48*). In addition, our results suggest a relevant function for another classic nuclear receptor during hepatocyte apoptosis, in that MR was significantly downregulated during TGF-β1 incubation. Although the antiapoptotic action of MR has been extensively described in neuronal cells (*32*, *35*−*38*), less is known about its role during hepatocyte apoptosis. Notably, UDCA inhibited TGF-β1-induced decreases in the level of GR and MR expression. Pretreatment with UDCA not only markedly upregulated both nuclear receptors but also resulted in significant increases in the extent of GR and MR nuclear translocation, when compared with that with TGF-β1 alone. In contrast, TUDCA modulated both MR and GR nuclear translocation, but only MR expression. Thus, taurine conjugation of UDCA significantly affects steroid receptor transcription and/or protein expression without altering nuclear translocation. In fact, prevention of TGF-β1-induced apoptosis by both bile acids appears to require nuclear translocation of the steroid receptors.

UDCA is a relatively hydrophilic molecule compared with other nuclear receptor ligands. Thus, it is postulated that UDCA initially interacts with the cell membrane and then modulates cytoplasmic events, one of which may include GR activation. In fact, it has been shown that UDCA neither specifically binds to GR nor directly interferes with dexamethasone and GR interaction (*23*), suggesting that UDCA dissociates receptor-associated proteins and GR via a second messenger signal. Several mechanisms have been proposed, including the activation of survival pathways and the regulation of calcium levels, which have already been described for certain bile acid molecules (*49*, *50*). However, UDCA appears also to act on a distinct region of the GR ligand binding domain, resulting in the loss of coactivator recruitment and differential regulation of gene expression by GR (*24*). It is interesting that UDCA was significantly concentrated in the nuclei of liver cells both in control rats and after UDCA feeding compared with the whole liver (*51*), suggesting that UDCA does function in the nucleus. In the study presented here, the association between upregulation, nuclear translocation, and the involvement of nuclear steroid

receptors in the antiapoptotic action of UDCA were confirmed by siRNA direct knockdown of GR and MR expression. Our results clearly showed that both receptors are involved in UDCA's antiapoptotic action, since GR and MR silencing resulted in the almost complete blockade of its protective effect.

To further investigate the role of GR and MR during apoptosis, and having shown that UDCA modulates molecular targets such as the E2F-1/Mdm-2/p53 pathway (*6*), we evaluated the involvement of steroid nuclear receptors on this apoptotic cascade. Transcription factor E2F-1 was found to be a potent target for the UDCA-induced GR and MR activation. Others have suggested that GR interferes with the expression of mitogenic factors, such as cyclins, CDKs, and E2F-1 (*27*). In addition, the subsequent increase in the level of Mdm-2 and the decrease in the level of p53 induced by UDCA were abrogated with GR and MR silencing, thus supporting the results on E2F-1 modulation. It is interesting that feeding rats with a combination diet containing deoxycholic acid and UDCA completely inhibited the drastic increase in the level of steady-state transcript expression for p53 and several cyclins associated with deoxycholic acid alone (*52*).

However, it remains to be determined whether Mdm-2 or p53 is directly modulated by steroid nuclear receptors, or a result of a downstream effect of E2F-1. We have recently shown that UDCA also prevents E2F-1-induced p53 and p53-associated Bax expression (*6*), suggesting that UDCA-induced GR and MR transactivation may have targets other than E2F-1. There is evidence that the antiapoptotic effect of glucocorticoids is mediated, in part, through modulation of NF-κB. In fact, GR activation was shown to prevent loss of NF-κB (*53*). Further, Bcl-x_L was markedly upregulated in liver from UDCA-fed rats (*54*). These results coupled with our previous study showing that UDCA inhibits TGF-β1-induced NF-κB degradation in primary rat hepatocytes (*6*) support the role of GR activation during UDCA protection. Finally, glucocorticoids are known to exert profound effects on mitochondrial membrane potential and function (*53*, *55*), and on promotion of fusion of mitochondria in certain cell types (*56*). It is conceivable that GR may be involved in the protective effect of UDCA on the mitochondrial membrane. Nevertheless, it remains to be determined if any of these pleiotropic molecular actions of GR contribute to UDCA protection against apoptosis.

Collectively, our studies identify a mechanistic antiapoptotic function for GR and MR in hepatocytes, and further expand the role of UDCA in cytoprotection. The results demonstrate that UDCA requires both GR and MR to protect hepatocytes from undergoing apoptosis with TGF-β1 stimulation. In addition, the E2F-1/Mdm-2/p53 apoptotic pathway appears to be a prime target for UDCA-induced GR and MR activation. Finally, it is important to identify other molecules involved in the activation of nuclear steroid receptors by UDCA in an effort to develop novel therapeutic interventions for apoptosis in liver diseases.

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