Bile Acids Induce Hepatic Stellate Cell Proliferation via Activation of the Epidermal Growth Factor Receptor

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Background & Aims: **Hepatic stellate cell (HSC) proliferation is a key event in the development of liver fibrosis. In many liver diseases, HSCs are exposed to inflammatory cytokines, reactive oxygen species, and bile acids. Although inflammatory cytokines and reactive oxygen species are known to promote proliferation of HSCs, nothing is known about the effects of bile acids on HSC proliferation or apoptosis. The aim of this study was to investigate the effects of bile acids on HSC proliferation.** ***Methods:*** **HSCs were exposed to bile acids with different hydrophobicity (5–200 μmol/L). HSC proliferation and cell cycle–related events were assessed by bromodeoxyuridine incorporation, cell counting and proliferating cell nuclear antigen and cyclin E expression, apoptosis by caspase-3 activity assay, immunocytochemistry for active caspase-3 and acridine orange staining, and activation of signal transduction pathways by Western blot using phospho-specific antibodies. Uptake of bile acids was investigated using fluorescent bile acids.** ***Results:*** **All bile acids, at concentrations >25 μmol/L, induce a 2.5- to 3-fold increase in HSC proliferation via activation of the epidermal growth factor receptor. Bile acid–induced proliferation is mediated by activation of a protein kinase C/extracellular signal–regulated kinase/p70^{S6K}–dependent pathway. Bile acids did not induce apoptosis in HSCs. HSCs do not take up fluorescent bile acids and do not express the bile acid importer ntcp.** ***Conclusions:*** **Bile acids at levels reached in cholestatic conditions are an independent profibrogenic factor. Bile acids induce HSC proliferation via the activation of the epidermal growth factor receptor, whereas HSCs are protected against bile acid–induced apoptosis by excluding bile acids.**

A common feature of chronic cholestatic liver disease is the activation and proliferation of hepatic stellate cells (HSCs), resulting in fibrosis and eventually cirrhosis. The exact initiating triggers of HSC activation remain elusive, although cytokines, growth factors such as transforming growth factor (TGF)-β, and exposure to reactive oxygen species and lipid peroxidation products are involved.[1] A hallmark of chronic cholestatic liver diseases is the accumulation of bile acids in the liver. Some bile acids, such as glycochenodeoxycholic acid (GCDCA), are potent inducers of apoptosis in hepatocytes and cholangiocytes.[2] However, nothing is known about the effects of bile acids on HSCs. Only one study examined the effect of bile acids on intracellular signal transduction pathways and observed that bile acids activate the extracellular signal–regulated kinase (ERK) and protein kinase C (PKC) signal transduction pathways in HSCs, but this study did not investigate the effects of bile acids on HSC proliferation or apoptosis.[3] On the other hand, it has been shown that bile acids can induce phosphorylation of intracellular signaling pathways,[4,5] which could play a key role in regulating HSC proliferation and resistance to apoptosis. Compared with our knowledge of the effects of bile acids on hepatocytes, the lack of data on effects of bile acids on HSCs is very surprising. In chronic cholestatic liver diseases, hepatocytes are lost due to necrotic and apoptotic cell death, whereas HSCs become activated and start to proliferate.[1] This difference in response of hepatocytes and HSCs makes it very relevant to study the actions of bile acids on HSCs. Therefore, the aim of our study was to establish whether bile acids modulate HSC proliferation and apoptosis. We show that, in contrast to their effects on hepatocytes, bile acids stimulate HSC proliferation by activation of the epidermal growth factor receptor

Abbreviations used in this paper: BrdU, bromodeoxyuridine; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal–regulated kinase; GCDCA, glycochenodeoxycholic acid; HSC, hepatic stellate cell; JNK, c-Jun N-terminal kinase; MEK, mitogen-activated protein kinase kinase; MMP, matrix metalloprotease; mTOR, mammalian target of rapamycin; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; SMA, smooth muscle actin; TGF, transforming growth factor.

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(EGFR) and that this bile acid–induced proliferation is dependent on intact ERK and PKC signaling pathways in HSCs.

This study represents the first demonstration that bile acids, at the same concentrations commonly observed during chronic cholestasis, are able to activate specific intracellular pathways leading to HSC proliferation. Bile acids are not taken up by HSCs, thus preventing bile acid–induced apoptosis.

Materials and Methods

Materials

Culture media were from Gibco (Grand Island, NY). Nycodenz was from Life Technologies (Milan, Italy). Kinase inhibitors were from Calbiochem (La Jolla, CA). Nitrocellulose membranes were from Schleicher & Schuell (Dassell, Germany). Mouse monoclonal anti–α-smooth muscle actin (SMA), anti–proliferating cell nuclear antigen (PCNA), and peroxidase-conjugated rabbit anti-mouse immunoglobulin G were from Dako (Glostrup, Denmark). Antibodies against the diphosphorylated form of ERK1/2 (pERK), against the Thr-308 phosphorylated form of Akt (pAkt), against the phosphorylated form of EGFR (pEGFR), against the phosphorylated form of c-Jun N-terminal kinase (JNK), and against $p70^{S6K}$, ERK1 and ERK2, EGFR, and cyclin E were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against the Ser-241 phosphorylated form of PDK-1 (pPDK1) and against the Ser-2448 phosphorylated form of the mammalian target of rapamycin (pmTOR) were from Cell Signaling Technology, Inc (Beverly, MA). The neutralizing anti-EGFR antibody was from Santa Cruz Biotechnology. The neutralizing anti–TGF-α antibody was from Oncogene Research Products (San Diego, CA). The matrix metalloprotease (MMP) inhibitor GM6001 was from Chemicon International (Temecula, CA). The antibody against active caspase-3 was from Cell Signaling Technologies (Beverly, MA). Annexin-V/biotin was from Roche (Mannheim, Germany). β-muricholic and β-tauromuricholic acid were kindly provided by Professor T. Iida (Nihon University, Tokyo, Japan). All other reagents were from Sigma Chemical Co (Milan, Italy).

Animals

Pathogen-free male CD Sprague–Dawley rats (220–250 g) were used. Animals were housed under standard laboratory conditions with free access to standard laboratory chow diet and water. Each experiment was performed following the guidelines of the local committee for care and use of laboratory animals in Ancona and Groningen.

HSC Isolation and Culture

HSCs were isolated by the pronase and collagenase method and then subcultured as previously described.[6]

Experimental Design

Passaged[1–3] activated HSCs were used until the monolayers were 75%–80% confluent. The cell cultures were made quiescent by 48 hours of incubation in serum- and insulin-free Iscove's medium followed by incubation with different bile acids for indicated time periods in the presence or absence of different inhibitors: calphostin C (PKC inhibitor), 500 nmol/L[7]; wortmannin and LY294002 (phosphatidylinositol 3-kinase [PI3K] inhibitors), 100 nmol/L and 5 μmol/L, respectively[8,9]; PD98059 and U0126 (mitogen-activated protein kinase kinase [MEK] inhibitors), 50 μmol/L and 10 μmol/L, respectively[10,11]; rapamycin ($p70^{S6K}$ inhibitor), 2 ng/mL[12]; AG1478 (EGFR inhibitor), 10 μmol/L[13]; neutralizing anti-EGFR antibody, 10 mg/mL[14]; neutralizing anti–TGF-α antibody, 2 μg/mL[15]; GM6001 (MMP inhibitor), 25 μmol/L[16]; *N*-acetylcysteine (antioxidant), 30 mmol/L. None of these compounds were toxic to HSCs, as determined by the trypan blue exclusion test (data not shown).

Determination of HSC Proliferation

HSCs were incubated in serum- and insulin-free Iscove's medium for 48 hours. After this time, the medium was removed and cells were incubated in the same medium containing the indicated bile acids for an additional 24 hours. In parallel experiments, cells were also preincubated (30 minutes) with the different kinase inhibitors. Bromodeoxyuridine (BrdU) incorporation into newly synthesized DNA of replicating cells was measured using an enzyme-linked immunoassay kit (Roche, Monza, Italy) according to the manufacturer's instructions. Data were normalized on cell number measured by microscopic counting.

For PCNA analysis, cells were incubated with different bile acids for the indicated period and Western blot was performed as previously described.[17] The intensity of the bands was determined by scanning video densitometry using the ChemiI-imager 4000 low-light imaging system (Alpha Innotech, San Leandro, CA). In parallel experiments, resolved proteins were also incubated with antibodies against cyclin E (1:1000 final dilution).

In parallel experiments, cell proliferation was performed on H&E-stained cells after 48 hours of incubation with the indicated bile acids. Cell counting was performed by using a computerized image analysis system connected to an Olympus microscope (Olympus Vanox AHBT3; Olympus Optical Co Ltd, Tokyo, Japan) as previously described.[18] Cells were expressed as number per square millimeter.

Western Blot and Kinase Activation Assay

Cells were scraped and homogenized in ice-cold buffer (pH 7.4) consisting of 50 mmol/L Tris, 150 mmol/L KCl, 1% Triton X-100, 1 mmol/L EDTA, 5 mmol/L *N*-ethylmaleimide, and 0.2 mmol/L phenylmethylsulfonyl-fluoride. Kinase phosphorylation was evaluated by Western blot as previously described.[19] Briefly, cell extracts (50 μg/lane) were boiled for 3 minutes in Laemmli sample buffer, separated by gel electrophoresis, and transferred to nitrocellulose. Nitrocellulose

sheets were then incubated with antibodies against pERK (pERK) (1:5000 final dilution), ERK1 and ERK2 (1:1000 final dilution), pAkt (1:1000 final dilution), pJNK (1:4000 final dilution), pEGFR (1:3000 final dilution), α-SMA (1:1000 final dilution), $p70^{S6K}$ (1:1000 final dilution), and EGFR (1:400 final dilution). For pERK, pAkt, pEGFR, EGFR, pJNK, and α-SMA, the antigen-antibody complexes were visualized by using the enhanced chemiluminescence detection system as recommended by the manufacturer (Amersham, Arlington Heights, IL). For ERK1, ERK2, and $p70^{S6K}$, specific protein bands were detected colorimetrically using 7 mg nitroblue tetrazolium and 3.5 mg 5-bromo-4-chloro-3-indolyl phosphate as alkaline phosphatase substrates, dissolved in 10% diethanolamine buffer (pH 9.8).

Caspase-3 Assay

Caspase-3 enzyme activity was assayed using a caspase-3 activity kit with fluorometric detection (Promega, Leiden, The Netherlands) according to the manufacturer's instructions and, as previously described for hepatocytes,[20] 20 μg of protein was used. Cell lysates of hepatocytes exposed for 4 hours to 50 μmol/L GCDCA were used as positive control.

(Immuno)Cytochemistry for Active Caspase-3, Annexin V, and Nuclear Morphology

Analysis of active caspase-3 was performed on HSCs cultured on coverslips. Coverslips were washed in phosphate-buffered saline and fixed in 4% paraformaldehyde for 10 minutes followed by incubation in 1% Triton X-100 for 5 minutes. Before adding primary antibodies, cells were washed twice with phosphate-buffered saline. Antibody against active caspase-3 was used at a dilution of 1:50 for 1 hour. Goat anti-rabbit antibody coupled to fluorescein isothiocyanate (Molecular Probes, Leiden, The Netherlands) was added at a dilution of 1:600 for 45 minutes. Slides were evaluated on a Leica (Rijswijk, The Netherlands) confocal laser scanning microscope. Nuclear morphology was evaluated using acridine orange as fluorescent dye. Annexin V staining was performed as previously reported by our group.[21]

Uptake of Fluorescent Bile Acids

Stellate cells on glass cover slides were incubated with cholyl lysyl fluorescein and lithocholyl lysyl fluorescein at final concentrations of 2 μmol/L at 37°C. After 1 hour of incubation, slides were washed 3 times with phosphate-buffered saline and examined under a Leica confocal laser scanning microscope. Early culture primary rat hepatocytes and HepG2 cells stably transfected with the bile acid importer ntcp were used as positive control.

Reverse-Transcription Polymerase Chain Reaction Assays for ntcp, Collagen Type I, TGF-β, and Glyceraldehyde-3-Phosphate Dehydrogenase

RNA isolation and complementary DNA synthesis were performed exactly as described previously.[18] Primer sequences and polymerase chain reaction (PCR) cycle characteristics for ntcp, collagen type I, and glyceraldehyde-3-phosphate dehydrogenase have been described previously.[20,22] PCR for TGF-β was performed using 28 cycles with the following primers: TGF-β sense primer, 5′-CTGTCCAAACTAAGGCTCGC-3′; TGF-β antisense primer, 5′-CGTCAAAAGACAGCCACTCA-3′.

Results

Effect of Bile Acids on HSC Proliferation, Cyclin Expression, Collagen Type I, and TGF-β Messenger RNA Levels

To evaluate the effect of different bile acids on HSC proliferation, cells were serum and insulin starved for 48 hours and then incubated with different concentrations of cholic acid, GCDCA, and tauroursodeoxycholic acid. These bile acids exerted no effect at 5 μmol/L. HSC proliferation was significantly increased at 25 μmol/L and was 2.5- and 3-fold the control values at 50 and 200 μmol/L, respectively (Figure 1*A*).

To confirm that the increased BrdU incorporation was indicative of DNA synthesis, we evaluated the expression of a second proliferation marker (PCNA). When HSCs were incubated with the different bile acids (200 μmol/L), PCNA expression peaked at 30 minutes and was maintained up to 24 hours, while no increase was observed at 10 and 20 minutes (Figure 1*B*). A similar pattern of increased PCNA expression up to 24 hours was observed in HSCs incubated with medium containing 10% serum used as a positive control (data not shown).

HSC incubation with bile acids was also associated with an increase in cyclin E expression at 30 minutes and 1 hour that decreased at 6 and 12 hours. A representative blot of cyclin E expression in HSCs incubated with GCDCA is shown in Figure 1*C*.

The increase in BrdU incorporation and PCNA and cyclin E expression was accompanied by a significant increase in the total number of cells after 48 hours of incubation with the different bile acids starting at 25 μmol/L (Figure 2).

No toxic effects were observed, as determined by the trypan blue exclusion test (data not shown). Bile acids did not change the expression of collagen type I and TGF-β messenger RNA in HSCs (data not shown).

Effect of Bile Acids on ERK1/2, PI3K, and $P70^{S6K}$ Activation

Western blot analysis was performed to evaluate the effect of bile acids on specific intracellular signaling pathways (Figure 3). After 10 minutes of incubation, bile acid–exposed HSCs were shown to have a noticeable decrease in the mobility pattern of the 70-kilodalton

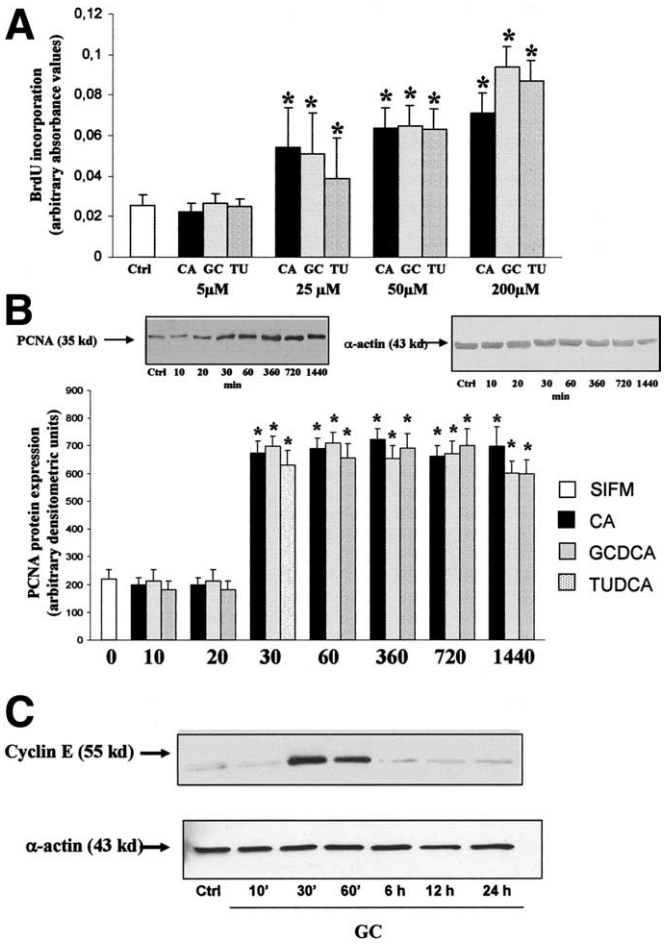

Figure 1. Effect of cholic acid (CA), GCDCA (GC), and tauroursodeoxycholic acid (TUDCA) on (*A*) BrdU incorporation and (*B*) PCNA and (*C*) cyclin E expression. (*A*) Proliferation of HSCs was evaluated by enzyme-linked immunosorbent assay measuring BrdU incorporation in the nuclei of S-phase cells. Cells were cultured for 48 hours in serum- and insulin-free medium and then incubated in the same medium with the indicated concentrations of bile acids for an additional 24 hours. (*B*) PCNA expression. Cells were cultured for 48 hours in serum- and insulin-free medium and then incubated in the same medium with bile acids (200 μmol/L) for the indicated period. Cells were then lysed and Western blot for PCNA was performed as indicated in Materials and Methods. The membranes were then stripped and reprobed for α-SMA expression that, as a marker of activated HSCs, was used to demonstrate equal loading. The intensity of the bands is expressed as arbitrary densitometric units. A representative Western blot of PCNA and α-SMA expression in HSCs incubated with GC is shown (*inset*). (*C*) Cells were incubated with bile acids for the indicated period and then treated as in *B*. A representative Western blot of cyclin E expression in HSCs incubated with GC is shown. Data are expressed as mean ± SD. **P* < .05 vs controls.

protein triplet (Figure 3*A*), as compared with the control untreated sample, indicative of $p70^{S6K}$ phosphorylation and activation.[23] In addition, all bile acids tested induced an increase in the total amount of pERK (Figure 3*A*), indicating ERK1/2 activation.[24] No effect on the total amount of ERK1 (data not shown) and ERK2 (Figure 3*A*) was observed.

On the other hand, no evidence of Thr308 phosphorylation of Akt, a downstream component of the PI3K pathway,[25] or JNK1/2 was observed after incubation with bile acids. Platelet-derived growth factor (25 ng/mL) and culture medium containing 10% serum were used as a positive control (Figure 3*B*).

Activation of intracellular signaling pathways in HSCs incubated with bile acids (50 μmol/L) was studied in time-course experiments. The reduced gel mobility of the 70-kilodalton trimer indicative of $p70^{S6K}$ phosphorylation was maintained up to 24 hours. On the other hand, the increase in ERK1/2 phosphorylation observed at 10 minutes returned to control levels at 30 minutes and increased again at 60 minutes and 6 hours. No differences in ERK1/2 phosphorylation compared with control cells were observed after 12 and 24 hours of incubation. No evidence of Akt or JNK phosphorylation was observed up to 24 hours of incubation with bile acids. A representative Western blot of HSCs incubated with GCDCA for 24 hours is shown in Figure 4*A* and *B*.

PI3K and mitogen-activated protein kinase are activated in cells stimulated by bile acids.[26] Further experiments were then performed to confirm the lack of activation of the PI3K pathway in our experimental model. Epidermal growth factor (EGF; 10 ng/mL) was used as a positive control and induced phosphorylation of true mediators of PI3K signal, such as PDK1 (at 10 minutes) and Akt (at 10 and 30 minutes) (Figure 5*A*). mTOR was also phosphorylated by EGF (Figure 5*A*). On the other hand, when HSCs were stimulated by bile acids, only mTOR phosphorylation was observed. A representative Western blot of HSCs incubated with GCDCA for 24 hours is shown in Figure 5*A* and *B*.

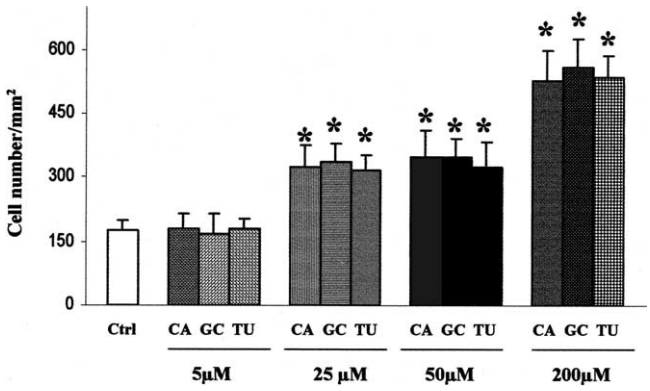

Figure 2. Effect of cholic acid (CA), GCDCA (GC), and tauroursodeoxycholic acid (TU) on cell number. Cells grown on glass coverslips were cultured for 48 hours in serum- and insulin-free medium and then incubated in the same medium with the indicated concentrations of bile acids for an additional 48 hours. Cell number was evaluated on H&E-stained cells, counted by using a computerized image analysis system, and expressed as cells per square millimeter. Data are expressed as mean ± SD. **P* < .05 vs controls.

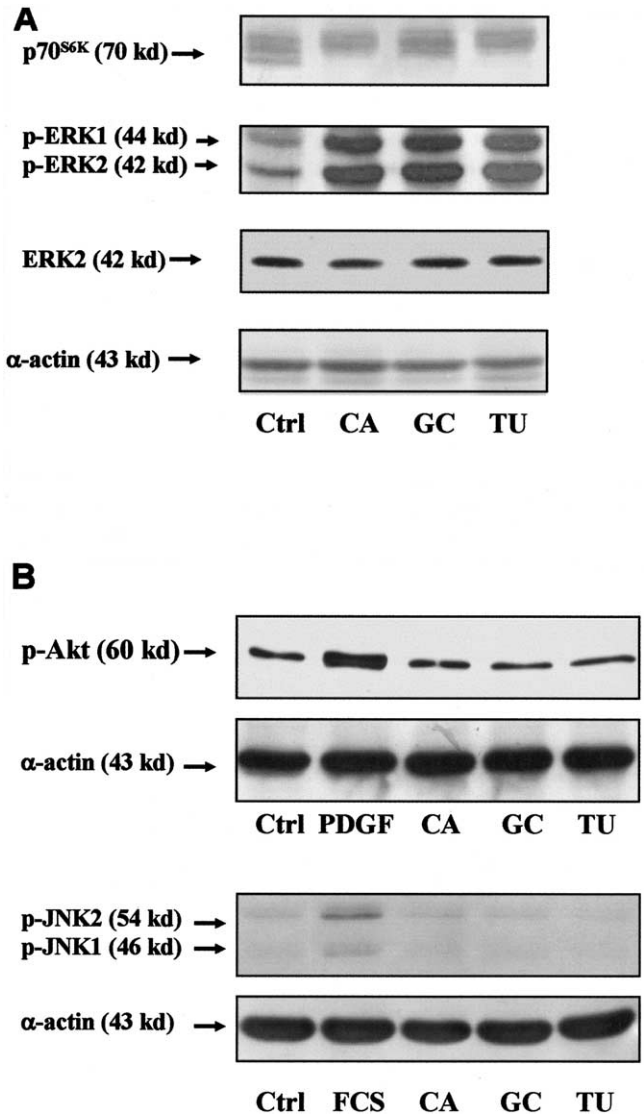

Figure 3. Effect of cholic acid (CA), GCDCA (GC), and tauroursodeoxycholic acid (TU) (50 μmol/L) on (*A*) $p70^{S6K}$ and ERK1/2 and (*B*) Akt and JNK1/2 phosphorylation. Cells were cultured for 48 hours in serum- and insulin-free medium and then incubated in the same medium with bile acids for 10 minutes. In *B,* platelet-derived growth factor (25 ng/mL) and 10% serum (fetal calf serum) were used as a positive control. Cell lysates (50 μg/lane) were separated by electrophoresis, transferred to nitrocellulose, and then incubated with specific antibodies. α-SMA expression, as a marker of activated HSCs, was used to show equal loading. Molecular weight markers are indicated on the left of each panel.

To finally confirm the effect of bile acids on intracellular signaling pathways, the effect of β-muricholic acid (Figure 6) and β-tauromuricholic acid (data not shown) on ERK phosphorylation was evaluated. While no differences compared with controls were observed at 5 μmol/L, increased ERK phosphorylation was observed starting from 25 μmol/L and peaking at 200 μmol/L for both bile acids. These data thus indicate that, under cholestatic conditions, bile acids are able to stimulate intracellular signaling kinase phosphorylation in activated HSCs and that this effect is not dependent on their hydrophobicity.

It has been previously shown that ERK1/2 and $p70^{S6K}$ are important elements in signal transduction pathways, which can be differentially regulated by upstream components. ERK1/2 and $p70^{S6K}$ are involved in mediating the effect of mitogenic extracellular stimuli in HSCs.[1] To study more specifically the signal transduction pathways triggered by bile acids in rat HSCs, the effect of various kinase inhibitors on ERK1/2 and $p70^{S6K}$ activation was evaluated. Because it has been reported that bile acids activate PKC in rat HSCs,[3] we first tested the effect of the PKC inhibitor calphostin C. As shown in Figure 6, calphostin C completely abolished a bile acid–induced increase in ERK1/2 and $p70^{S6K}$ phosphorylation.

To test whether bile acid–mediated $p70^{S6K}$ activation is exerted through ERK1/2, we studied the effect of the MEK inhibitors PD98059 and U0126 in con-

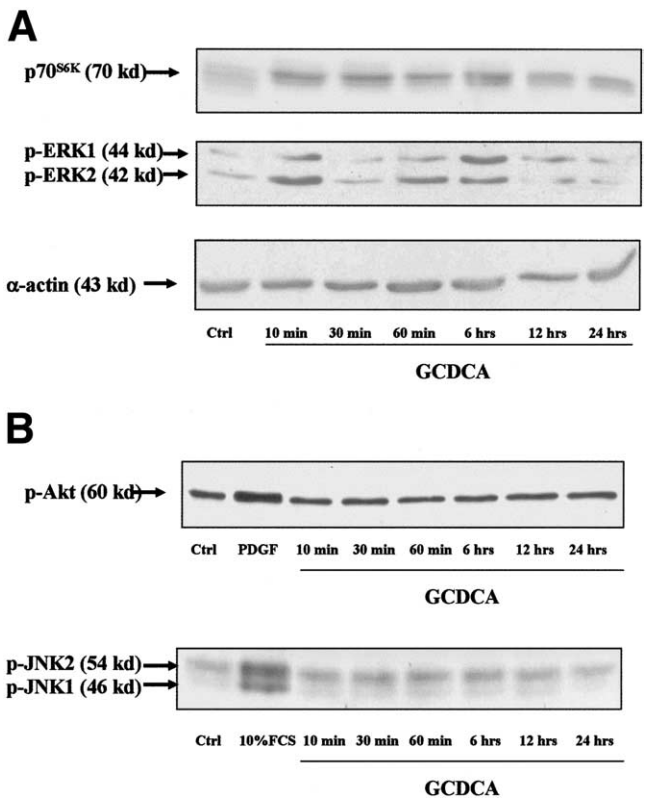

Figure 4. Time-course experiment on the effect of GCDCA (50 μmol/L) on (*A*) $p70^{S6K}$ and ERK1/2 and (*B*) Akt and JNK1/2 phosphorylation. Cells were cultured for 48 hours in serum- and insulin-free medium and then incubated in the same medium with bile acids for 10 minutes. In *B,* platelet-derived growth factor (25 ng/mL) and 10% serum (fetal calf serum) were used as a positive control. Cell lysates (50 μg/lane) were separated by electrophoresis, transferred to nitrocellulose, and then incubated with specific antibodies. α-SMA expression, as a marker of activated HSCs, was used to show equal loading. Molecular weight markers are indicated on the left of each panel.

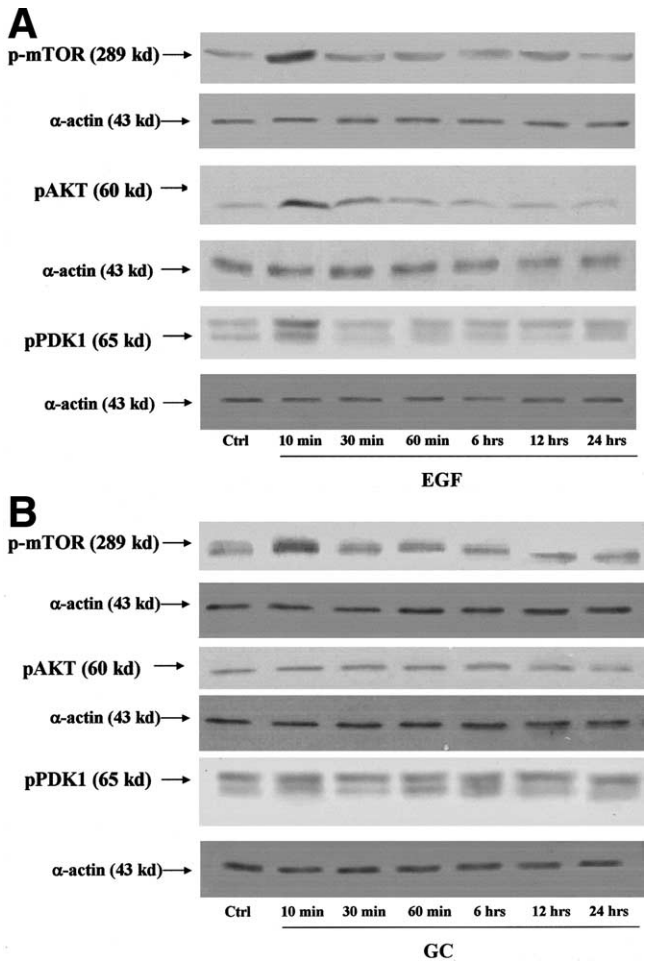

Figure 5. Time-course experiment on the effect of (*A*) EGF (10 ng/mL) and (*B*) GCDCA (GC; 50 μmol/L) on mTOR, Akt, and PDK1 phosphorylation. Cells were cultured for 48 hours in serum- and insulin-free medium and then incubated in the same medium with EGF or GCDCA for 10 minutes. Cell lysates (50 μg/lane) were separated by electrophoresis, transferred to nitrocellulose, and then incubated with specific antibodies. α-SMA expression, as a marker of activated HSCs, was used to show equal loading. Molecular weight markers are indicated on the left of each panel.

trol and bile acid–treated HSCs. Both PD98059 (Figure 7) and U0126 (data not shown) inhibited the increase in bile acid–induced phosphorylation of $p70^{S6K}$ and ERK1/2. These results suggest that $p70^{S6K}$ may be a downstream component of the ERK1/2 pathway in bile acid–treated HSCs. To further explore this possibility, the effect of the $p70^{S6K}$ inhibitor rapamycin was tested. As shown in Figure 7, rapamycin significantly inhibits $p70^{S6K}$ in bile acid–treated HSCs. In contrast to this result, and as shown in Figure 7, incubation with rapamycin fails to prevent the increase in ERK1/2 phosphorylation induced by bile acids, thus confirming that $p70^{S6K}$ represents a downstream component in this signaling pathway.

Effect of Kinase Inhibitors on Bile Acid–Induced HSC Proliferation

Different intracellular pathways activated in bile acid–induced HSCs could selectively stimulate cell DNA synthesis. The effect of different kinase inhibitors on cyclin E expression was tested. Calphostin C, PD98059, and rapamycin completely inhibited the increase in cyclin E expression at 1 hour, indicating a direct relationship with the PKC/ERK/$p70^{S6K}$ pathway (Figure 8*A*).

In addition, the PKC inhibitor calphostin C significantly decreased bile acid–induced HSC proliferation to control levels. Similar results were obtained using the MEK antagonists PD98059 and U0126 and the $p70^{S6K}$ inhibitor rapamycin. No effects on cell proliferation were observed when HSCs were incubated with calphostin C, PD98059, or rapamycin alone (Figure 8*B*).

Mechanism of Activation of Kinase Phosphorylation and Proliferation in Bile Acid–Stimulated HSCs

It has been shown previously that ligand-independent activation of EGFR may account, at least in part, for the effect of bile acids on cell signaling.[4,5] HSC incubation with bile acids increased phosphorylation of EGFR, as shown by Western blot (Figure 9*A*, upper blot). HSCs incubated with EGF (10 ng/mL) were used as a positive control. HSC incubation with the EGFR inhibitor AG1478 abolished EGFR phosphorylation in HSCs incubated with either EGF or bile acids (Figure 9*A,* upper blot). No EGFR phosphorylation was observed when HSCs were incubated with the antibiotic fusidic acid (10 μmol/L)[27] (Figure 9*B,* upper blot). Bile acid–induced EGFR phosphorylation can occur in a ligand-independent manner or through TGF-α membrane release by MMP followed by its interaction with EGFR.[4,28] To elucidate this in our in vitro model, HSCs were incu-

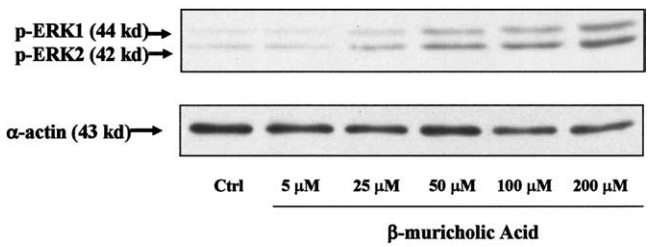

Figure 6. Effect of β-muricholic acid on ERK phosphorylation. Cells were cultured for 48 hours in serum- and insulin-free medium and then incubated in the same medium with β-muricholic acid (5–200 μmol/L) for 10 minutes. Cell lysates (50 μg/lane) were separated by electrophoresis, transferred to nitrocellulose, and then incubated with specific antibodies. α-SMA expression, as a marker of activated HSCs, was used to show equal loading. Molecular weight markers are indicated on the left of each panel.

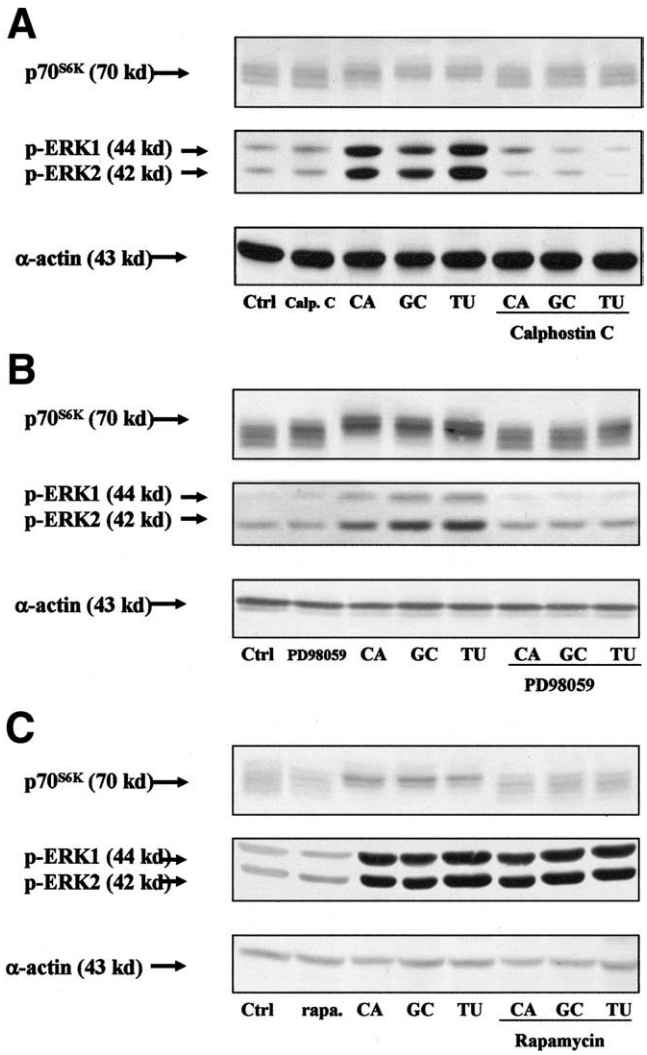

Figure 7. Effect of (*A*) calphostin C (500 nmol/L), (*B*) PD98059 (50 μmol/L), and (*C*) rapamycin (2 ng/mL) on $p70^{S6K}$ and ERK1/2 phosphorylation. Cells were cultured for 48 hours in serum- and insulin-free medium and then incubated in the same medium with bile acids for 10 minutes in the presence or absence of the indicated kinase inhibitor (30-minute preincubation). Cell lysates (50 μg/lane) were separated by electrophoresis, transferred to nitrocellulose, and then incubated with specific antibodies. α-SMA expression, as a marker of activated HSCs, was used to show equal loading. Molecular weight markers are indicated on the left of each panel.

bated with bile acids in the presence or absence of an EGFR-neutralizing antibody, of a TGF-α–neutralizing antibody, and of the MMP inhibitor GM6001. Bile acid–induced EGFR phosphorylation was ligand independent, because no effects were observed when the neutralizing antibodies or GM6001 were added in the culture medium (Figure 9*C*).

It has also been reported that EGFR phosphorylation by bile acids can be mediated by reactive oxygen species formation, which trigger EGFR phosphorylation.[29] EGFR phosphorylation was completely inhibited in bile acid–stimulated HSCs when cells were preincubated with the antioxidant *N*-acetylcysteine (Figure 9*D*).

To confirm the relationship between EGFR phosphorylation and intracellular signaling activation in bile acid–stimulated HSCs, ERK phosphorylation was evaluated in the presence of the EGFR inhibitor AG1478. While bile acids were able to stimulate ERK1/2 phosphorylation, no ERK1/2 activation was observed in the presence of AG1478 when HSCs were incubated with bile acids (Figure 9*E,* upper blot).

To further elucidate the relationship between a ligand-independent EGFR phosphorylation and mitogen-activated protein kinase activation, HSCs were incubated with bile acids in the presence or absence of an EGFR-

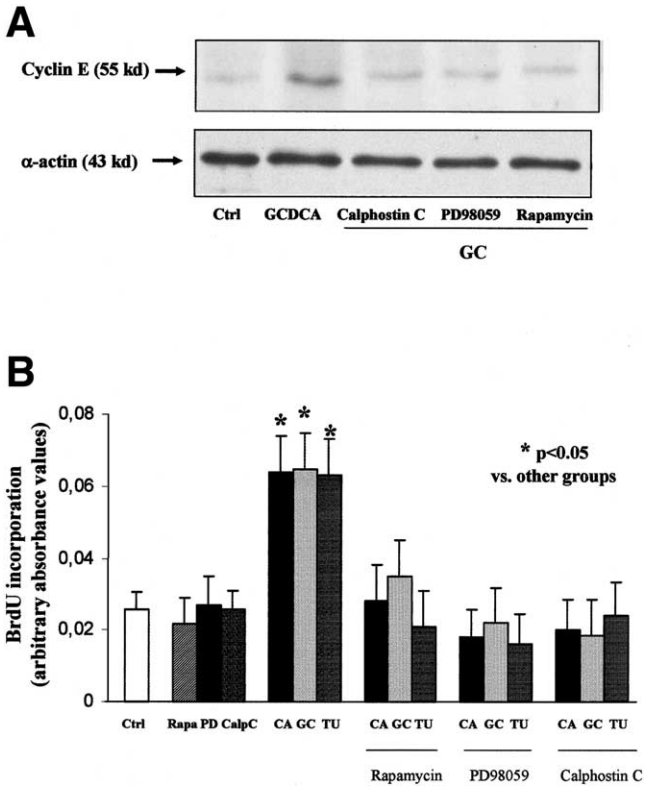

Figure 8. Effect of calphostin C (500 nmol/L), PD98059 (50 μmol/L), and rapamycin (2 ng/mL) on bile acid–induced (*A*) cyclin E expression and (*B*) HSC proliferation. (*A*) Cells were cultured for 48 hours in serum- and insulin-free medium and then incubated in the same medium with GCDCA for 60 minutes in the presence or absence of the indicated kinase inhibitor (30-minute preincubation). Cell lysates (50 μg/lane) were separated by electrophoresis, transferred to nitrocellulose, and then incubated with the specific antibody. α-SMA expression, as a marker of activated HSCs, was used to show equal loading. Molecular weight markers are indicated on the left of each panel. (*B*) For cell proliferation, cells were cultured for 48 hours in serum- and insulin-free medium and then incubated in the same medium with bile acids (50 μmol/L) for an additional 24 hours in the presence or absence of the indicated kinase inhibitors (30-minute preincubation). Proliferation of HSCs was evaluated by enzyme-linked immunosorbent assay measuring BrdU incorporation in the nuclei of S-phase cells. $*P < .05$ vs all other groups.

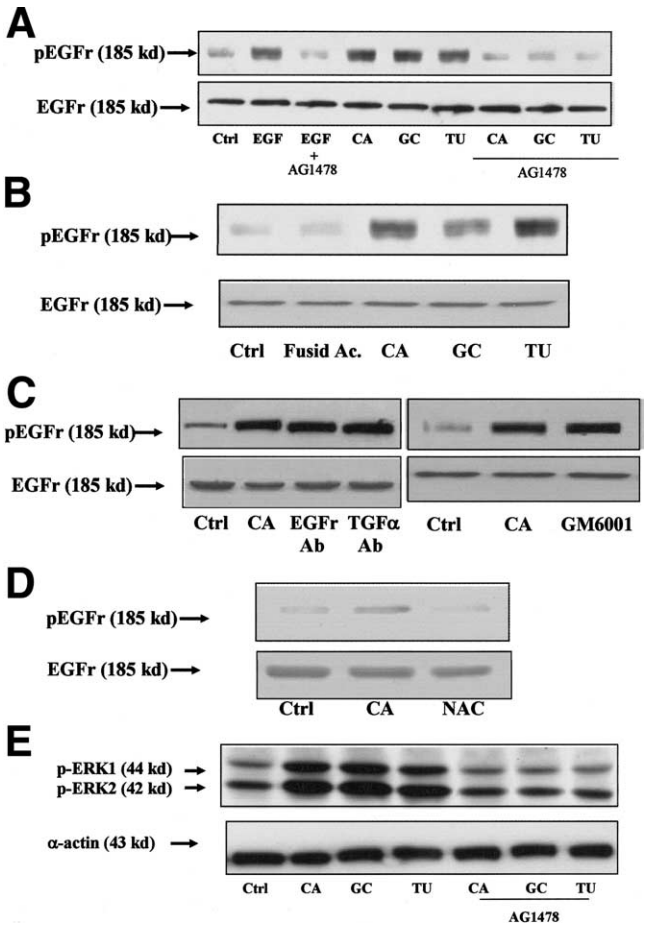

Figure 9. (*A–D*) Effect of bile acids on EGFR phosphorylation and (*E*) effect of EGFR phosphorylation inhibition on ERK activation. For evaluation of EGFR phosphorylation, cells were cultured for 48 hours in serum- and insulin-free medium and then incubated in the same medium with EGF (10 ng/mL) or (*A–E*) bile acids (50 μmol/L) or (*B*) fusidic acid (10 μmol/L) for 10 minutes. In parallel experiments, cells were also preincubated (30 minutes to 2 hours) with the EGFR inhibitor AG1478 (10 μmol/L), with neutralizing antibodies for EGFR (10 μg/mL) or for TGF-α (2 μg/mL), or with the antioxidant *N*-acetylcysteine (30 mmol/L). Finally, cells were also preincubated (16 hours) with the MMP inhibitor GM6001 (25 μmol/L) dissolved in dimethyl sulfoxide. In this experiment, all cells received the same amount of dimethyl sulfoxide. Cell lysates (50 μg/lane) were separated by electrophoresis, transferred to nitrocellulose, and then incubated with the specific antibody. Membranes were then stripped and incubated with antibodies against EGFR or α-SMA to show equal loading. Molecular weight markers are indicated on the left of each panel.

neutralizing antibody, a TGF-α–neutralizing antibody, the MMP inhibitor GM6001, and the EGFR inhibitor AG1478. ERK phosphorylation was evaluated at 10 minutes and 6 hours after bile acid incubation (Figure 10). No effects were observed on ERK phosphorylation by neutralizing antibodies or GM6001 at either 10 minutes or 6 hours (Figure 10*A–C*). On the other hand, both AG1478 and *N*-acetylcysteine completely inhibited ERK phosphorylation at 10 minutes and 6 hours (Figure 10*D* and data not shown).

To test the role of EGFR activation in bile acid–stimulated HSC proliferation, HSCs were incubated with different bile acids or EGF in the presence or absence of the EGFR inhibitor AG1478. Bile acids and EGF stimulated HSC proliferation similarly to about 2.5-fold the control level, and this effect was completely abolished by the EGFR antagonist AG1478 (Figure 11). Finally, AG1478 also blocked the increase in cyclin E expression in HSCs incubated with bile acids for 1 hour (data not shown).

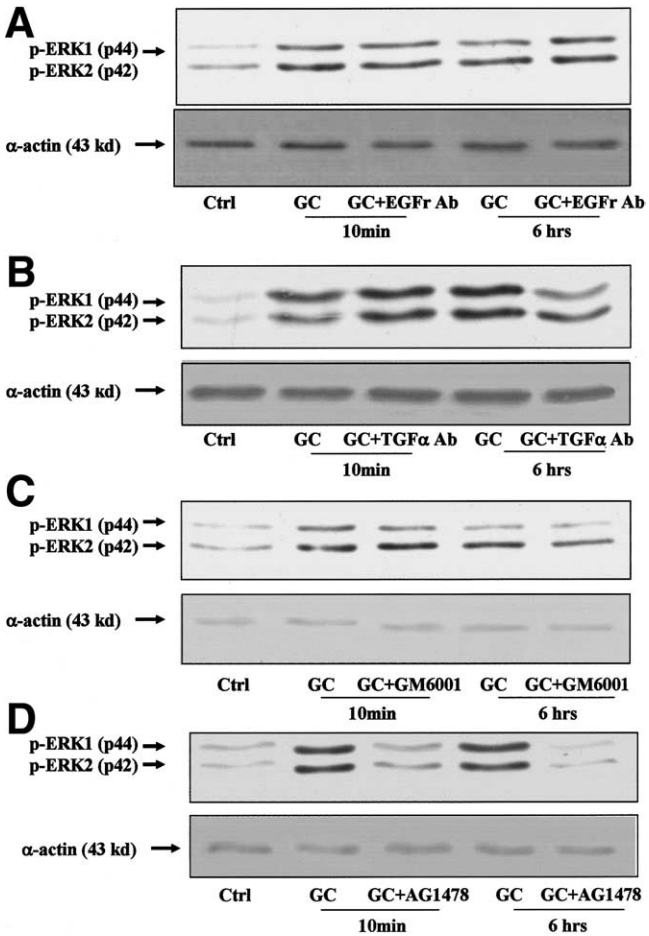

Figure 10. Effect of different inhibitors on bile acid–induced ERK phosphorylation. Cells were cultured for 48 hours in serum- and insulin-free medium and then incubated in the same medium with bile acids (50 μmol/L) for 10 minutes and 6 hours in the presence or absence (30 minutes to 2 hours) of the neutralizing antibodies for (*A*) EGFR (10 μg/mL) or (*B*) TGF-α (2 μg/mL) (30 minutes), (*C*) of the MMP inhibitor GM6001 (25 μmol/L) (16 hours), or (*D*) of the EGFR inhibitor AG1478 (10 μmol/L) (30 minutes). Cell lysates (50 μg/lane) were separated by electrophoresis, transferred to nitrocellulose, and then incubated with the specific antibody. Membranes were then stripped and incubated with antibodies against α-SMA to show equal loading. Molecular weight markers are indicated on the left of each panel.

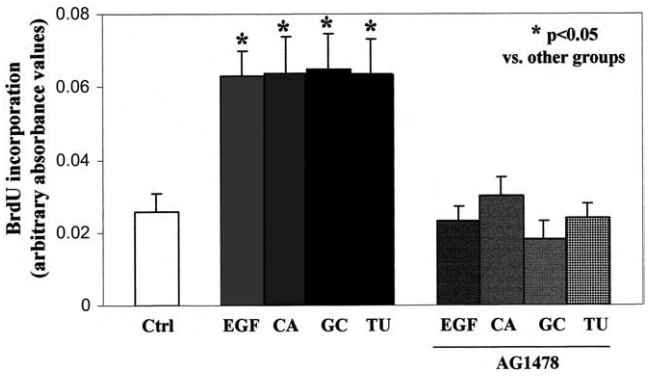

Figure 11. Effect of the EGFR inhibitor AG1478 (10 μmol/L) on bile acid–induced HSC proliferation. EGF-induced HSC proliferation serves as control for AG1478. For cell proliferation, cells were cultured for 48 hours in serum- and insulin-free medium and then incubated in the same medium with EGF (10 ng/mL) or bile acids (50 μmol/L) for an additional 24 hours in the presence or absence of the indicated kinase inhibitors (30-minute preincubation). Proliferation of HSCs was evaluated by enzyme-linked immunosorbent assay measuring BrdU incorporation in the nuclei of S-phase cells. $*P < .05$ vs all other groups.

Effects of Bile Acids and Kinase Inhibitors on HSC Apoptosis

Bile acids induce massive apoptosis in cultured hepatocytes. Therefore, the effect of various bile acids on HSC apoptosis was examined. Neither GCDCA (Figure 12), a bile acid that induces apoptosis in hepatocytes, nor cholic acid (data not shown) induced caspase-3 activity in HSCs. Similar findings were observed when the number of apoptotic cells was measured by annexin V staining or staining for activated caspase-3. Next, the role of various intracellular pathways in the protection against bile acid–induced apoptosis was examined. As shown in Figure 12, addition of the PKC inhibitor calphostin or the MEK antagonist PD98059 slightly increased both basal and bile acid–induced caspase-3 activation, although the increase was very low and the observed levels are >50-fold lower than in GCDCA-treated hepatocytes. A more than 2-fold increase in the number of annexin V–positive HSCs was observed when cells were incubated with calphostin or PD98059 in the absence and presence of bile acids. HSC incubation with rapamycin did not exert any effect on caspase-3 activity and annexin V staining (data not shown). Immunocytochemistry for active caspase-3 and nuclear staining with acridine orange revealed <4% apoptotic cells in HSC cultures treated with calphostin or PD98059. No evidence of positive immunostaining or nuclear alterations was observed in control or bile acid–incubated HSCs without calphostin or PD98059.

Expression of Bile Acid Importers and Uptake of Fluorescent Bile Acids

Using reverse-transcription PCR, we did not detect ntcp expression in HSCs, whereas freshly isolated hepatocytes clearly expressed ntcp (data not shown). The organic anion transporters oatp1 and oatp2 were not expressed in HSCs, and only a very low expression of oatp3 was detected by reverse-transcription PCR (Figure 13). HSCs did not take up fluorescent bile acids, whereas HepG2 cells stably transfected with the bile acid importer ntcp and early primary cultures of rat hepatocytes showed significant accumulation of fluorescent bile acids in bile canalicular structures (Figure 13).

Discussion

In many liver diseases, the various liver cell types are exposed to increased levels of inflammatory cytokines, reactive oxygen species, and bile acids. Although the effect of cytokines and reactive oxygen species on HSC activation and proliferation and matrix production is well documented, the effect of bile acids on HSCs has hardly been addressed. Only one study has described that

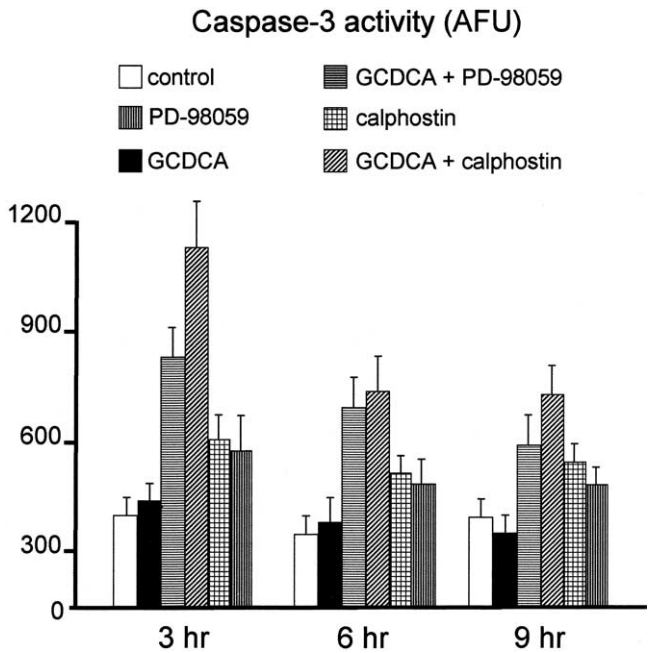

Figure 12. Effect of calphostin C (500 nmol/L) and PD98059 (50 μmol/L) on GCDCA-induced (50 μmol/L) apoptosis. Apoptosis was determined using the caspase-3 activity assay. As positive control, cell lysates from primary cultures of rat hepatocytes exposed to 50 μmol/L GCDCA for 4 hours were used. The increase observed with calphostin C at 3 hours and PD98059 at 6 hours and 9 hours in the presence and absence of GCDCA is statistically significant compared with control and GCDCA alone (Mann–Whitney test; $P < .05$). Positive control values for hepatocytes exposed to 50 μmol/L GCDCA for 4 hours are between 40,000 and 60,000 arbitrary fluorescence units (AFU).

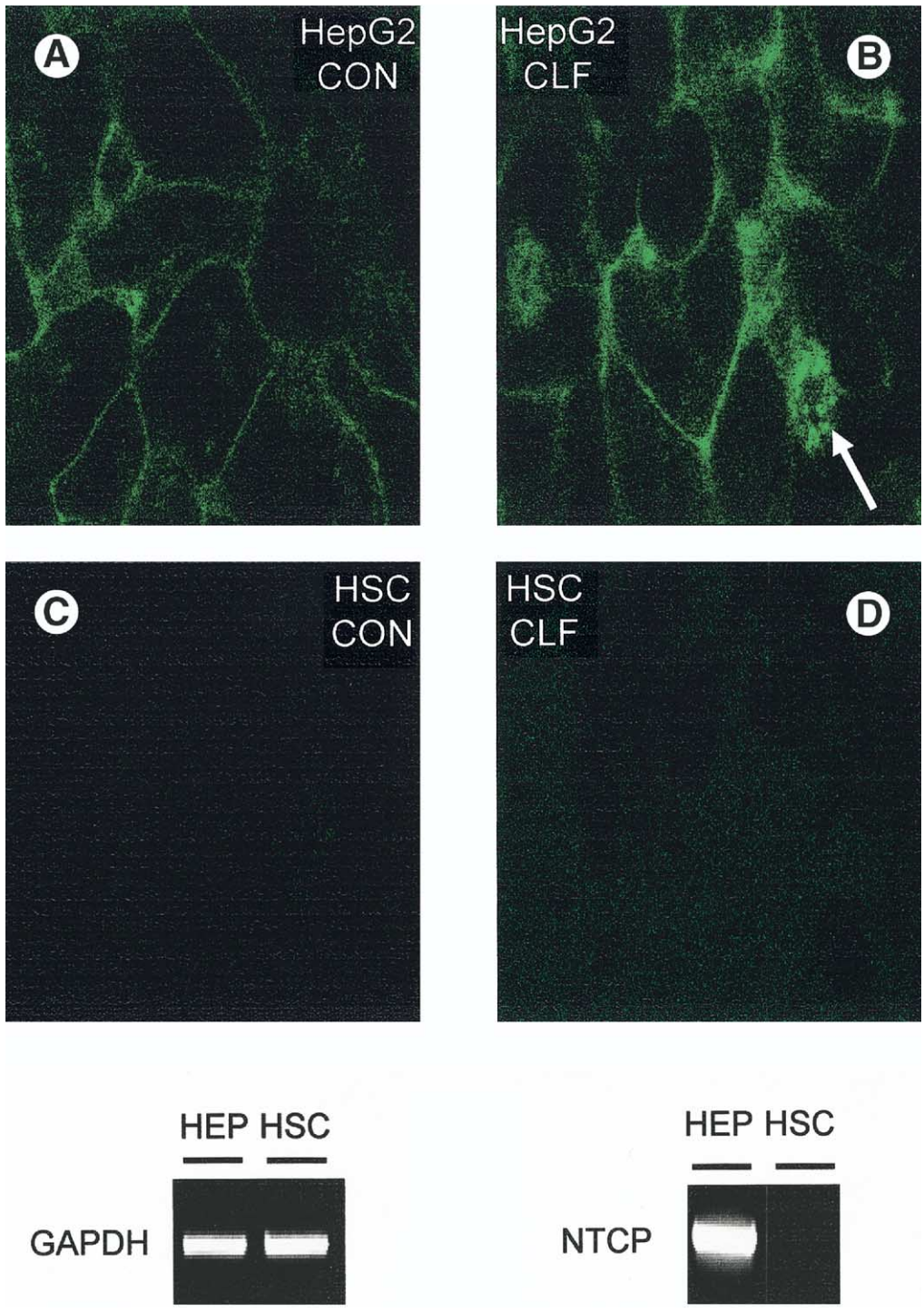

Figure 13. Lack of ntcp expression, determined by reverse-transcription PCR, in HSCs. In contrast, freshly isolated hepatocytes express ntcp. Uptake of the fluorescent bile acid cholyl lysyl fluorescein (CLF) is absent in HSCs (*D*), whereas HepG2 cells, stably transfected with an ntcp construct, take up CLF in bile canalicular-like structures (*B*). The membranous fluorescence observed in control HepG2.ntcp cells (*A*) is due to the fact that the ntcp construct contained a GFP sequence, facilitating the localization of the ntcp protein in the transfected HepG2 cells. HSCs incubated without fluorescent bile acids show no fluorescence (*C*). Identical results were obtained with the fluorescent bile acid litocholyl lysyl fluorescein (LLF).

bile acids activate the ERK and PKC pathway in HSCs, but this study did not address the consequences of this activation for HSC behavior.[3] This is surprising, because the effects of bile acids on hepatocytes are very well documented. Many bile acids (eg, GCDCA) induce apoptosis in hepatocytes.[2] Our study investigates the effect of bile acids on HSCs and shows that bile acids induce proliferation of activated HSCs. Bile acids did not change the expression of collagen type I and TGF-β messenger RNA in HSCs, indicating that the effect of bile acids was restricted to proliferation. This is the first time this effect is documented, and it identifies elevated bile acid levels as a potential profibrogenic factor.

Despite the lack of effect on collagen type I and TGF-β messenger RNA levels, bile acids were able to induce HSC proliferation, which represents a main event

during liver fibrogenesis.[1] To demonstrate this, we provide in this study data showing that bile acids increase the entrance of the cell in the S phase of the cell cycle by evaluating BrdU incorporation into the nuclei and the expression of PCNA. Furthermore, and in support, we show that bile acids increase the amount of cyclin E. Cyclin E is expressed during a definite window at the G_1-S transition and promotes the cell entry into the S phase.[30] This progression through the cell cycle was accompanied by a corresponding increase in the total number of cells when HSCs were incubated with bile acids for 48 hours. It should be noted that the concentrations of bile acids necessary to induce HSC proliferation are well above the levels in normal, noncholestatic conditions, as shown by the lack of bile acid–induced HSC proliferation at bile acid concentrations of 5 μmol/L. Therefore, in normal noncholestatic conditions, bile acids will not induce HSC proliferation.

We have recently shown that after bile duct ligation in vivo, ERK1/2 and $p70^{S6K}$ phosphorylation occurs during the process of HSC activation.[31] Following stimulation with bile acids in vitro, we observed activation of ERK1/2 and $p70^{S6K}$, which are important elements in mediating the effect of mitogenic extracellular stimuli in HSCs.[1] In accordance with Qiao et al using bile acid–stimulated hepatocytes,[4] we observed a prolonged ERK phosphorylation when HSCs were exposed to bile acids. In agreement with a previous observation,[3] we also found that PKC represents an upstream component able to activate ERK1/2 and, in our hands, $p70^{S6K}$. In time-course experiments, $p70^{S6K}$ phosphorylation was maintained up to 24 hours in our hands. This is not surprising because 12-*O*-tetradecanoylphorbol-13-acetate–treated adult cardiocytes show a similar time course in $p70^{S6K}$ protein phosphorylation that is PKC dependent and PI3K independent, as in our experimental model.[32] On the other hand, no phosphorylation of Akt and JNK was observed after HSC incubation with bile acids, indicating that one specific member of the mitogen-activated protein kinase family was induced in our experimental conditions. Several reports also indicate a close link between ERK/$p70^{S6K}$ and cyclin E expression.[33–36] Using specific kinase inhibitors, we were able to show that the PKC/ERK/$p70^{S6K}$ pathway regulates proliferation and cyclin E expression of bile acid–stimulated HSCs, providing reasonable targets to modify this process, leading to hepatic fibrosis in chronic cholestasis.

It has been reported that bile acids can activate other intracellular signaling pathways in hepatocytes or in a hepatoma cell line, such as JNK or PI3K.[26,29,37] In our in vitro experimental model, no activation of effector molecules responsible for transducing the PI3K signal was observed. To study this, we evaluated phosphorylation of PDK1, which by itself activates Akt by phosphorylation on Thr-308.[38] While platelet-derived growth factor and EGF, used as positive control, were able to induce PDK1 and Akt phosphorylation, no effects were observed when HSCs were incubated with the different bile acids. On the other hand, both EGF and bile acids were able to induce mTOR phosphorylation on Ser-2448, thus raising the possibility that mTOR activation by bile acids is mediated through mitogen-activated protein kinase. Although we did not test the effect of PD98059 on mTOR phosphorylation, it has been reported that several agonists such as amino acids or 12-*O*-tetradecanoylphorbol-13-acetate can activate mTOR/$p70^{S6K}$ by a PI3K-independent mechanism and that this happens through an ERK-dependent pathway in the latter case.[39,40] This hypothesis was also confirmed in our hands, where rapamycin was able to completely block the increase in BrdU incorporation induced by bile acids in HSCs.

Despite intensive investigations, the molecular events by which bile acids activate intracellular signal transduction pathways remain poorly understood. This effect was not dependent on the bile acid hydrophobicity, because it was observed also by incubating HSCs with both β-muricholic acid and β-tauromuricholic acid. Muricholic acid, as well as cholic acid, is present in the bile of normal rats, and it becomes the most abundant bile acid after bile duct ligation.[41] The effect of these bile acids on ERK phosphorylation and HSC proliferation became evident at bile acid concentrations >25 μmol/L and was not observed at physiologic concentrations of 5 μmol/L, indicating that a cholestatic condition is needed to stimulate activated HSCs. Recently it has been shown that bile acids cause ligand-dependent and ligand-independent activation of EGFR in hepatocytes and cholangiocytes and that this is associated with intracellular signaling activation and resistance to apoptosis.[4,5,28,29,37,42,43] In our study, we have observed EGFR phosphorylation following HSC incubation with bile acids, but not with the antibiotic fusidic acid, and a reduction of bile acid–induced proliferation and ERK1/2 phosphorylation when a specific inhibitor of EGFR (AG1478) was used. Thus, it can be speculated that bile acids activate the PKC/ERK/$p70^{S6K}$ pathway and induce HSC proliferation through EGFR. EGFR can also be activated by different ligands, and Werneburg et al showed that, in cholangiocytes, this occurs through TGF-α release by the cell membrane and that this effect is mediated by MMPs.[28] Recently, Reinehr et al[29] failed to show reduced EGFR phosphorylation in bile acid–stimulated hepatocytes after AG1478 exposure. On

the other hand, in agreement with Qiao et al,[4] bile acid–induced EGFR phosphorylation in HSCs appeared to occur in a ligand-independent manner because no effects were observed by incubating cells with neutralizing antibodies or with the MMP inhibitor GM6001. A plausible mechanism relating bile acids to EGFR activation could be represented by oxidative stress. It is known that bile salts can induce oxidative stress, and a link between reactive oxygen species formation and EGFR phosphorylation in bile acid–stimulated hepatocytes has been recently shown.[29,42–44] Although we did not determine the extent of reactive oxygen species formation, the antioxidant *N*-acetylcysteine was able to block EGFR and ERK phosphorylation in bile acid–stimulated HSCs. We also observed reduced EGFR phosphorylation when HSCs were incubated with bile acids in the presence of AG1478. Therefore, our data underline the role of the intrinsic protein tyrosine kinase activity of the receptor in mediating EGFR phosphorylation in this experimental model, possibly associated with a reduction in membrane-bound protein tyrosine phosphatase activity as reported by others and mediated by oxidative stress.[45] Furthermore, in HSCs, fusidic acid failed to activate EGFR, confirming that the effects of bile acids on HSCs are independent of their uptake into the cell. A possible interpretation of this phenomenon is that bile acids are able to activate EGFR in HSCs in a ligand-independent manner by inducing reactive oxygen species formation within the cell membrane, thus presumably inducing membrane perturbation, as previously shown for other experimental conditions.[46,47] The observation that AG1478 blocked EGFR and ERK phosphorylation, and BrdU incorporation in S-phase nuclei, confirms the role of EGFR in mediating intracellular signaling activation leading to cell proliferation in bile acid–incubated HSCs.

Bile acids do not induce apoptosis in activated HSCs. The reason for this resistance against bile acid–induced apoptosis, compared with hepatocytes, is the lack of bile acid uptake by HSCs. In contrast to hepatocytes, HSCs do not take up fluorescent bile acids and lack the important bile acid importer ntcp. Uptake of bile acids via oatp isoforms is also unlikely because HSCs lack oatp1 and oatp2 and expression of oatp3 is extremely low. We have recently shown that bile acid–induced apoptosis in primary rat hepatocytes and in HepG2 hepatoma cells is dependent on the ntcp-mediated uptake of bile acids.[48] Intracellular pathways involved in bile acid–induced HSC proliferation are often also involved in the protection against apoptosis. Indeed, inhibition of the PKC and ERK pathways slightly increased basal and bile acid–induced caspase-3 activation in activated HSCs. However, the percentage of apoptotic active caspase-3–positive cells in HSC cultures exposed to inhibitors of PKC and ERK was very limited and the significance of this increase in apoptosis remains to be established. Rapamycin (a $p70^{s6K}$ inhibitor) did not increase HSC susceptibility to bile acid–induced apoptosis. Although apparently surprising, this is in agreement with Coutant et al, who recently showed that the ERK/$p70^{s6K}$pathway mediates hepatocyte proliferation, whereas survival signals are only dependent on ERK activation, indicating that a specific stimulus regulates the growth-related response by acting on distinct steps.[49]

In conclusion, we have shown that bile acids induce HSC proliferation via activation of EGFR. HSCs appear to be protected from bile acid–induced apoptosis by the lack of bile acid uptake. Our results indicate that elevated bile acid levels in cholestasis are an independent profibrogenic factor.

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