Bile acid stimulation of early growth response gene and mitogen-activated protein kinase is protein kinase C-dependent

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Hepatic stellate cells are exposed to elevated bile acid levels during hepatic injury and fibrogenesis. Upon activation, the stellate cell becomes a major effector cell during the development of hepatic fibrosis and cirrhosis. Bile acids may function as co-stimulatory signalling molecules. This hypothesis was tested *in vitro* using rat-derived hepatic stellate cells. Bile acids were studied at concentrations that occur during cirrhosis *in vivo*.

Conjugated and unconjugated bile acids rapidly induced *egr* and *fos* gene expression as well as cytoplasmic mitogen-activated protein kinase (MAPK) activation. Protein kinase C was required for both *egr* induction and MAPK activation. These studies imply that bile acids could contribute to the perpetuation of hepatic fibrosis by helping to keep the stellate cell in an activated state.

INTRODUCTION

Bile acids have long been implicated in the development of colon cancer and cholestatic liver disease [1–5]. However, their molecular mechanisms of action remain poorly understood. Extensive studies of the physicochemical properties of various bile acids have distinguished hydrophobic bile acids (e.g. lithocholic acid, chenodeoxycholic acid and cholic acid) and more water-soluble conjugates of those bile acids (e.g. taurocholic acid) [3,5]. Under normal physiological conditions, 90–95% of bile acids are conjugated with glycine or taurine [3,5]. Through a complex enterohepatic circulation, primary bile acids (cholic acid and chenodeoxycholic acid) are transformed into secondary bile acids (e.g. lithocholic acid and deoxycholic acid) [3,5]. Alterations in the metabolism of bile acids occur during cholestatic liver disease [5]. This leads to increased concentrations of the conjugated bile acids as well as the more membrane-damaging hydrophobic bile acids (e.g. lithocholic acid) [3,5].

Previous studies have indirectly suggested that bile acids could play a role in intracellular signalling. Studies involving intestinal cells as well as hepatocytes suggest that protein kinase C (PKC) may be stimulated by bile acids [1,4]. However, many of the former assays utilized millimolar concentrations of bile acid which are likely to have detergent-like effects, and therefore the results are difficult to interpret [1,4]. Physiologically relevant micromolar (< 50 μ M) concentrations appear to stimulate hepatocyte PKC whereas supraphysiological (100–200 μ M) concentrations may induce abnormal HLA gene expression during short-term hepatocyte culture [6,7]. It is unclear whether these effects on hepatocytes involve the recently described plasma-membrane bile acid transporters [8–12]. Receptor saturation of these transporters has been reported to occur at these same micromolar concentrations (< 50 μ M) [8–12]. The hepatic sinusoidal stellate cell (previously referred to as the Ito cell or lipocyte) may be exposed to the same elevated bile acid levels as the adjacent hepatocyte during periods of hepatic cholestasis and liver injury [3,5]. Since the latter often results in the activation of the stellate cell into a proliferating myofibroblast, bile acids could play a co-stimulatory role as signalling molecules [13].

The current study examined the capacity of bile acids to stimulate hepatic stellate cells in culture. This cell culture model

was previously utilized to demonstrate the preferred signalling cascade induced by 1,25-dihydroxyvitamin D₃, another lipid co-stimulant of stellate cells [14,15]. These studies found that 1,25-dihydroxyvitamin D₃ acutely activates Raf kinase and mitogen-activated protein kinase (MAPK) and the transcription of the early growth response gene (*egr*), an immediate early gene [14,15]. Although numerous reports have linked the cytoplasmic kinases, Raf and MAPK, to gene transcription, the agonists involved generally utilize known plasma-membrane tyrosine kinase or GTP-binding proteins as the initial upstream receptor [16,17]. In contrast, vitamin D is not known to interact initially with either receptor subtype [18]. The previously mentioned study found that PKC was required as the more proximal upstream signalling molecule for 1,25-dihydroxyvitamin D₃ [14,15]. In addition, dominant negative gene transfection studies confirmed that, although both Raf kinase and MAPK were activated, only MAPK was required for the induction of *egr* gene transcription by 1,25-dihydroxyvitamin D₃ [14]. This is consistent with many subsequent reports of upstream pathways that are parallel to Raf which may also lead to MAPK activation (e.g. via *mos*) [16,17].

The current study demonstrates that physiologically relevant concentrations of both conjugated and unconjugated hydrophilic bile acids are capable of inducing immediate early gene expression with kinetics similar to other agonists [e.g. platelet-derived growth factor (PDGF)] that utilize well-established tyrosine kinase-dominated pathways. In addition, these same bile acids rapidly stimulate MAPK. As demonstrated for 1,25-dihydroxyvitamin D₃, bile acid activation of MAPK and the induction of nuclear *egr* requires PKC α and/or δ . A potential bile acid co-stimulatory activation cascade is discussed.

MATERIALS AND METHODS

Chemicals

PDGF-BB was obtained from Gibco-BRL. Bile acids and octyl β -D-glucopyranoside (Sigma, St. Louis, MO, U.S.A.) were prepared fresh before use or were stored as aqueous or ethanol stock solutions at 4 °C for less than 1 week. The role of PKC was

Abbreviations used: *egr*, early growth response gene; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; PDGF, platelet-derived growth factor; MBP, myelin basic protein; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MBP, myelin basic protein.

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assessed by pretreating cells for 24 h with phorbol 12-myristate 13-acetate (PMA; 1 μ M, Sigma) or for 1 h with the specific PKC inhibitor GF-109203X (LC Laboratories, Woburn, MA, U.S.A.). Control cultures treated with equivalent concentrations of ethanol or DMSO vehicle (final concn. < 1 %) were indistinguishable from untreated cells.

Cell culture

Hepatic stellate cells were isolated from Sprague–Dawley male rats by previously described methods and subcultured on tissue-culture flasks precoated with type-I calf collagen [19]. Experimental manipulations were performed with cells at passage 2–6 maintained on 75 cm² precoated plates.

PKC abundance

Stellate cell cultures were pretreated with 1 μ M PMA as previously described and then the washed cell layers were harvested for Western blotting as previously described [15,19]. Aliquots of equivalent protein concentrations were resolved by SDS/PAGE (8 % gel) and then electroblotted on to poly(vinylidene difluoride) membranes (Immobilon P; Millipore, Bedford, MA, U.S.A.). The blots were then blocked as previously described and probed (4 °C for 18 h or room temperature for 1 h) with PKC isoform-specific antibodies [PKC α (Upstate Biotech Inc., Lake Placid, NY, U.S.A.); PKC δ and ϵ (Santa Cruz Biotech, Santa Cruz, CA, U.S.A.)] [15,19]. The blots were then washed and probed with the appropriate peroxidase-conjugated secondary antibodies (Amersham) followed by the Amersham enhanced chemiluminescence method. Autoradiograms were developed using Kodak XAR-5 film and intensifying screens as needed.

Activation of MAPK and Raf kinase

Ito cells maintained in media with 0.4 % fetal calf serum were treated with bile acids for 2–10 min. p44^{MAPK} was immunoprecipitated [anti-(ERK 1) polyclonal antibody (Santa Cruz Biotech)] from cell lysates as described previously [14,19]. After three washes in lysis buffer and two in kinase buffer (10 mM Hepes, pH 7.4, 5 mM MgCl₂, 1 mM MnCl₂, 10 mM *p*-nitrophenyl phosphate), the immune complexes were incubated in kinase buffer (50 μ l/sample) containing myelin basic protein (MBP; 10 μ g/reaction), 25 μ M ATP and [³²P]ATP (2 μ Ci) (3 min; 30 °C). The eluted proteins were resolved by SDS/PAGE on a 14 % gel and then fixed, dehydrated in acetone, dried, exposed for autoradiography and quantified as previously described [14,19]. Raf kinase was assessed after immunoprecipitation with a Raf monoclonal antibody as previously described [15]. All experiments were repeated two to three times with similar results.

Measurement of *egr* and *fos* mRNA

The relative transcript abundance of *egr* and *fos* was assessed by Northern blotting using the Chomczynski method of RNA extraction as previously described [14,19]. The probes used included an *egr* and *fos* cDNA (kindly provided by Dr. V. Sukhatme, Beth Israel Hospital, Boston, MA, U.S.A.) [14,19]. Signal intensities were normalized to RNA abundance using an 18S ribosomal RNA probe as previously described [14,19]. Representative gels are shown for each measurement. All experiments were repeated three times with similar results.

RESULTS AND DISCUSSION

Bile acids stimulate immediate early gene expression

Cholic acid represents a major class of hydrophobic bile acids which increases in concentration during liver injury [3,5]. This bile acid was therefore evaluated over a wide range of concentrations and time points to determine if stellate cells are responsive to bile acid. For comparison, the cells were stimulated in parallel with PDGF, a well-described stellate cell mitogen known to activate a classic tyrosine kinase cascade and acutely stimulate immediate early gene expression [13–17]. As shown in Figures 1(A) and 1(B), stellate cells responded to cholic acid exposure (25–200 μ M) with the rapid induction of *egr* and *fos* expression. The kinetics of *fos* and *egr* induction were identical with those of PDGF. The rapidity (see below as well) of this response suggests that the *de novo* production of an intermediary protein is unlikely to mediate the cholic acid effect. The dose range and response threshold suggest the possibility that a receptor or transporter could be involved. A similar dose range was reported for hepatocyte responses [6,7]. Although a transporter could be involved, the classic liver bile acid transporters have been reported exclusively in the hepatocyte to date [8–12]. In addition, studies on the transporters have not revealed that they contain any intrinsic tyrosine kinase or GTP-binding protein activity [8–12]. To explore the possibility that the response was specific for cholic acid, stellate cells were exposed to several other major bile acids which are believed to play significant roles during liver disease. All of the bile acids were studied at a concentration of 50 μ M and at the 30 min time points, as these appeared to be the optimal conditions for cholic acid stimulation. As shown in Figure 2(A), chenodeoxycholic acid stimulates *egr* comparably with cholic acid. After normalization for differences in RNA loading (by comparing each lane with the abundance of the 18 S ribosomal band), ursodeoxycholic acid caused a reduced induction of *egr* compared with cholic acid. This may be the result of different mechanisms of action for ursodeoxycholic acid and cholic acid (see below). All stimulants are shown compared with PDGF,

Figure 1 Induction of *egr* and *fos* by cholic acid

Cultured stellate cells were maintained in media containing 0.4 % fetal calf serum for 48 h and then stimulated with 10 ng/ml PDGF or 50–200 μ M cholic acid (A) or 25 μ M cholic acid (B) for the indicated time points. The relative abundance of the *egr* and/or *fos* mRNA transcript is shown. Comparable RNA loading was confirmed by reprobing the blots with an 18 S ribosomal probe as shown. The kinetics of induction were similar to those of PDGF-induced *egr* and *fos*, which are shown for comparison.

Figure 2 Induction of *egr* by conjugated and unconjugated bile acids

(A) As described in Figure 1, stellate cells were stimulated with 50 μ M chenodeoxycholic acid (CN), lithocholic acid (Li) or ursodeoxycholic acid (Ur) for 30 min and then the abundance of the *egr* transcript was measured. For comparison, parallel cells were stimulated with either cholic acid (CH) or PDGF (P). Comparable RNA loading was confirmed by reprobing the blot with an 18 S ribosomal probe as shown. (B) Various concentrations of lithocholic acid (Li) were used for stimulation (30 min), and then *egr* transcript abundance was measured, as in (A).

which was a more potent stimulant of *egr*. Taurocholic acid, a major conjugated bile acid, also stimulated *egr* comparably with cholic acid (results not shown).

Since conjugated bile acids are unlikely to cross plasma membranes by passive diffusion, these observations further suggest that some form of carrier (?receptor)-mediated transport could be involved during this response [1–5,6–12]. It was also

Figure 3 Requirement of PKC α and/or PKC δ for cholic acid induction of *egr*

(A) Stellate cells were pretreated with 1 μ M PMA (TPA) (or diluent) to down-regulate PKC and then stimulated with 50 μ M cholic acid (CH) as described in Figure 1. Abundance of *egr* transcript was then measured and relative RNA loading confirmed by reprobing with the 18 S ribosomal probe. (B) Stellate cells were pretreated with GF-109203X (GF-pre; a PKC-specific inhibitor) for 1 h, and then stimulated with cholic acid (CH), and *egr* transcript abundance measured, as in (A). (C) The effects of pretreatment were assessed on the major PKC isoforms of the stellate cell as indicated. Lanes 1, — PMA; lanes 2, + PMA. Molecular masses are given in kDa. Western blotting and reprobing of the same membrane revealed that only PKC α and PKC δ were down-regulated by PMA treatment, whereas PKC ϵ was insensitive to this condition. Similar observations were made with several stellate cell lysates and additional Western blots.

Figure 4 Activation of MAPK by cholic acid

Cultured stellate cells were maintained in media containing 0.4% fetal calf serum for 48 h and then stimulated with 50 μ M cholic acid as indicated for 2 or 10 min or PDGF (10 ng/ml; 10 min). Equivalent cell lysates (600 μ g/plate) were then immunoprecipitated with an ERK 1 polyclonal antibody and an immune complex kinase assay performed with MBP as the phosphoacceptor protein. The phosphorylated MBP was then resolved by SDS/PAGE on a 14% gel, and the gel exposed for autoradiography. The 32 P-labelled band is shown.

found that lithocholic acid markedly stimulated *egr* (Figures 2A and 2B). However, lithocholic acid is a potent hydrophobic bile acid and its greater potency may be related to the ease with which it could be transported (or diffused) across the plasma membrane. The possibility that lithocholic acid may stimulate *egr* by a different mechanism was further substantiated by the observation (Figure 2B) that doses as low as 5 μ M were capable of stimulating *egr*. This was in marked contrast with the less hydrophobic bile acids, which lost most of their efficacy for cytoplasmic signalling at concentrations below 25 μ M (see below).

Induction of *egr* is PKC-dependent

As previously mentioned, the only cytoplasmic signalling molecule that has been shown to respond to bile acids is PKC [1,4,7]. Our previous studies with 1,25-dihydroxyvitamin D₃ demonstrated that stimulation of *egr* can be initiated by a lipid via a non-tyrosine kinase receptor pathway with PKC as a major proximal signalling molecule [14,15]. Stellate cells were therefore pretreated with 1 μ M PMA, the same concentration used to show the PKC-dependence of the 1,25-dihydroxyvitamin D₃ response [14,15]. This concentration of PMA for 24 h down-regulates many PKC isoforms [14,15]. As shown in Figure 3(A), this pretreatment abolished *egr* induction by cholic acid. This concentration caused no alterations in cell morphology or uptake of [3 H]thymidine (results not shown). The control cells were pretreated with comparable concentrations of DMSO diluent. The relative selectivity of this pretreatment is shown in the Western blot in Figure 3(C). It is seen that the major PKC isoforms of the stellate cell are α , δ and ϵ , as previously noted by other workers [20]. PMA pretreatment down-regulates only α and δ , with no effect on the ϵ isoform. This is consistent with previous studies in other cell types [21]. Therefore these observations suggest that the proximal mediator of the cholic acid effect is likely to be PKC α or δ . In subsequent studies, we confirmed these findings by observing that pretreatment of stellate cells with the selective PKC inhibitor GF-109203X (2 μ M) for 1 h also abolished the capacity of cholic acid to induce *egr* (Figure 3B).

Bile acids stimulate MAPK

The downstream signalling cascade which follows PKC activation is complex and varies with different agonists and different cell types [16,17,21]. Since cytoplasmic MAPK played a central role in our previous PKC-dependent lipid-agonist-induced pathway, we evaluated the capacity of cholic acid to activate MAPK using an immune complex kinase assay [14]. This assay is specific for the ERK 1 isoform of MAPK [14,19]. As shown in Figure 4, cholic acid at 50 μ M caused a rapid 3–4-fold activation of

Figure 5 Requirement for PKC for cholic acid activation of MAPK and demonstration that conjugated bile acids also stimulate MAPK

Stellate cells were treated with 50 μ M cholic acid (CH), taurocholic acid (TC) or lithocholic acid (Li) for 10 min. As indicated, some cells were pre-treated with the specific PKC inhibitor GF-109203X (GF-pre) for 45 min and then stimulated with 50 μ M cholic acid. For comparison, parallel cells were also stimulated with PDGF (P) (10 ng/ml). MAPK activity was then assessed as described in Figure 4.

Figure 6 Bile acid concentration versus MAPK stimulation

Bile acids (cholic, ursodeoxycholic and lithocholic) at various concentrations (for 10 min) were compared with respect to MAPK stimulation, as in Figure 4. Each data point represents duplicate determination (mean $\pm$ variation). The phosphorylated MBP band was then excised from the gel and counted in a scintillation counter with the data expressed as MAPK in c.p.m. units. Symbols: ■, cholic acid; ◆, ursodeoxycholic acid; ○, lithocholic acid.

MAPK comparable with that induced by 1,25-dihydroxyvitamin D_3 and similar to the kinetics previously described for other agonists [14,19]. The upstream agonists which lead to MAPK activation are numerous, and several parallel pathways may exist for the same agonist [16,17]. One potential major pathway that leads to MAPK activation is via Raf kinase [16,17]. Raf in turn leads to MAPK activation by activating MEK, the immediate upstream kinase responsible for MAPK phosphorylation [16,17]. We confirmed that cholic acid (50 μ M) has the capacity to activate Raf kinase using a Raf immune complex kinase assay with kinase-deficient MEK as the reporter (results not shown) [15].

As observed for *egr* induction, the activation of MAPK was not limited to cholic acid; both taurocholic acid and lithocholic acid activated this enzyme (Figure 5). As previously, lithocholic acid appeared to be more potent than the other equimolar bile acid concentrations (Figure 6). Lithocholic acid caused MAPK activation at 2.5 and 5 μ M whereas cholic acid caused moderate MAPK activation at 12.5 μ M. Cholic acid approached the same degree of MAPK stimulation as lithocholic acid at the 25 μ M concentration. In addition, it is unlikely that the bile acids 'non-specifically' induce MAPK in stellate cells because (i) the related ursodeoxycholic acid had no significant effect on MAPK

activation at any concentration tested (Figure 6) and (ii) the non-ionic non-cytolytic membrane detergent octyl β -D-glucopyranoside (0.05 % for 10 min) had no effect on MAPK activation (MAPK in unstimulated control, 594 ± 74 c.p.m.; MAPK in detergent, 319 ± 145 c.p.m.; mean $\pm$ S.D., $n = 3$) [22]. Furthermore, the bile acid effect may have some cell-specificity because treatment of unrelated 3T3 fibroblasts with high concentrations of lithocholic acid (25 μ M for 10 min) caused no detectable MAPK-induced MBP phosphorylation, as detected by autoradiography. These cumulative studies suggest that bile acids activate the major cytoplasmic signalling kinases of the stellate cell which are felt to regulate many plasma-membrane receptor-driven cascades associated with tyrosine kinase or GTP-binding protein receptors [16,17].

MAPK stimulation is PKC-dependent

As our previous studies implied that the 1,25-dihydroxyvitamin D_3 lipid agonist required PKC to activate MAPK, we decided to determine if this is a generalizable phenomenon for the other lipid stimulants. We also needed to determine whether the PKC-dependence of cholic acid-induced *egr* applied to MAPK as well. Therefore stellate cells were pretreated with the PKC inhibitor GF-109203X (2 μ M) and then stimulated with cholic acid for 10 min (Figure 5). This pretreatment abolished the ability of cholic acid to stimulate MAPK. This inhibitor abolishes responsiveness to phorbol esters in numerous cell systems and displays a high degree of specificity for PKC inhibition [23,24].

In conclusion, these studies demonstrate that a variety of bile acids with differing physicochemical properties can stimulate stellate cells in culture and induce two major components of the activation cascade, MAPK and immediate early gene expression. It is possible that plasma-membrane receptor/transporters mediate some of these effects. Simple diffusion could partly explain the response to cholic acid. Previous studies have found that urea can induce *egr* expression in renal mesangial cells, a cell type similar to hepatic stellate cells [25]. In that system, alterations in osmolarity may play a role but no plasma-membrane receptor was identified [25]. However, conjugated bile acids are unlikely to diffuse passively across the plasma membrane and therefore a different mechanism is required to explain the capacity of taurocholic acid to induce the cascade. In addition, the more hydrophobic bile acid, lithocholic acid, may induce the cascade at relatively low concentrations because of its membrane-diffusing properties.

These studies suggest a generalized cascade that follows bile acid exposure and the likely entry of the various bile acids into the cell. This leads to immediate early gene activation through a pathway that involves both PKC and MAPK activation. Although the most proximal components of this cascade may vary between the different bile acids, future studies will be needed to identify the role of any plasma-membrane bile acid receptor/transporter in these responses. In this regard, recent studies suggest that a multispecific organic anion transporter that is capable of transporting conjugated bile acids may have a more ubiquitous distribution than the previously described hepatocyte taurocholic acid transporter [8–12]. The current studies demonstrate that bile acids can initiate a PKC–MAPK signalling cascade. Therefore the elevated bile acid levels which are a frequent counterpart of liver injury and hepatic fibrogenesis could play a co-stimulatory role with respect to stellate cell activation *in vivo*.

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