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Conjugated Bile Acids Regulate Hepatocyte Glycogen Synthase Activity In Vitro and In Vivo via $G\alpha_i$ Signaling

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ABSTRACT

The regulation of glycogen synthase activity by bile acids in primary hepatocytes and in the intact liver was investigated. Bile acids (deoxycholic acid, DCA; taurocholic acid, TCA) activated AKT and glycogen synthase (GS) in primary rat hepatocytes. Incubation with a phosphatidyl inositol-3 kinase inhibitor or expression of dominant-negative AKT in primary rat hepatocytes abolished activation of AKT and GS by DCA and TCA. TCA, but not DCA, activated $G\alpha_i$ proteins in primary rat hepatocytes. Treatment of cells with pertussis toxin or expression of dominant-negative $G\alpha_i$ blocked TCA-induced activation of AKT and of GS but did not alter AKT or GS activation caused by DCA. TCA caused activation of AKT and GS in intact rat liver. Expression of dominant-negative $G\alpha_i$ reduced TCA-induced activation of AKT and of GS in intact rat liver. Together, our findings demonstrate that bile acids are physiological regulators of glycogen synthase in rat liver and that conjugated bile acids use a $G\alpha_i$-coupled G protein-coupled receptor to regulate GS activity in vitro and in vivo.

Bile acids are detergent molecules, synthesized from cholesterol in the liver, actively secreted into bile, stored in the gallbladder, and released into the gut upon feeding. Bile acids, after feeding, re-enter the liver via the portal vein together with digested nutrients and are recirculated back into the gallbladder for use during the next feeding cycle (Holt, 1972; Benage and O'Connor, 1990; Roberts et al., 2002). The levels of individual bile acids and their conjugation status to the amino acids glycine or taurine change between individuals based on diet and age (Hardison, 1970; Trautwein et al., 1993): persons eating a meat diet tend to have more taurine-conjugated bile acids in their bile acid pool than those eating a vegetarian diet. When retained within the liver because of impaired secretion into the bile canaliculi, individual bile acids are also known to have hepatocellular toxicity both in vivo and in vitro (Poupon et al., 2000).

We have reported that treatment of primary rodent and human hepatocytes with bile acids caused activation of ERBB1 (the epidermal growth factor receptor) and the insulin receptor, which were responsible for activation of the ERK1/2 and PI3K-AKT pathways (Qiao et al., 2001b, 2002a,b; Han et al., 2004; Dent et al., 2005a,b). Several other groups have also discovered that bile acids can activate ERBB1, the membrane-associated tyrosine kinase SRC, and the fatty acid synthase receptor (Qiao et al., 2001a; Werneburg et al., 2003; Yoon et al., 2004; Reinehr et al., 2005a,b). Based on the observation that bile acids activated the PI3K-AKT pathway and that PI3K-AKT signaling has been linked to the regulation of glycogen synthase (GS) in insulin-responsive tissues, we then demonstrated that bile acids activated GS in primary hepatocytes and that bile acid-induced activation of PI3K was causal in GS activation (Cohen et al., 1997; Cohen, 1999; Han et al., 2004).

In some cell types, it has been noted that activation of ERBB1 in response to growth factor stimulation occurs via a

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ABBREVIATIONS: ERK, extracellular signal-regulated kinase; PI3K, phosphatidyl inositol-3 kinase; GS, glycogen synthase; DCA, deoxycholic acid; TCA, taurocholic acid; GPCR, G protein-coupled receptor; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; CDCA, chenodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; TDCA, taurodeoxycholic acid; GDCA, glycodeoxycholic acid; GSK3, glycogen synthase kinase 3.

circuitous route, via the actions of paracrine ligands or more directly through the actions of nonreceptor tyrosine kinases (El-Shewy et al., 2004; Hagan et al., 2004; Fischer et al., 2006; Shah et al., 2006). Based on these observations we subsequently performed more detailed analyses of how bile acids activated ERBB1 and the insulin receptor in primary hepatocytes; we discovered that bile acid-induced activation of receptor tyrosine kinases was dependent on the generation of reactive oxygen species (Fang et al., 2004). In addition to reactive oxygen species signaling, we noted that taurine and glycine-conjugated bile acids, but not unconjugated bile acids, stimulated ERBB1 and insulin receptor tyrosine kinase activity and the activity of AKT via a $G\alpha_i$-coupled, G protein-coupled receptor (GPCR)-dependent mechanism (Dent et al., 2005a). Several studies have shown that established GPCR ligands can cause activation of receptor tyrosine kinases and intracellular signaling pathways in primary hepatocytes (Melien et al., 1998, 2000).

The present study was designed, initially, to determine whether conjugated bile acids activate GS in primary cultures of hepatocytes via an AKT- and $G\alpha_i$-coupled GPCR-dependent mechanism. Based on the discovery that bile acids caused in vitro activation of hepatocyte GS via PI3 kinase/AKT/GSK3 signaling, we then determined whether bile acids cause GS activation in the intact liver and whether this occurred via a $G\alpha_i$-coupled GPCR-dependent mechanism.

Materials and Methods

Materials

All bile acids were obtained from Sigma Chemical (St. Louis, MO). Phospho-/total-ERK1/2, Phospho-/total-GSK3, anti-Ser473 AKT, anti-Thr308 AKT, and total AKT were purchased from Cell Signaling Technology (Danvers, MA). All of the secondary antibodies (anti-rabbit-HRP, anti-mouse-HRP, and anti-goat-HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The PI3 kinase inhibitor (LY294002) was supplied by Calbiochem (San Diego, CA) as powder, dissolved in sterile dimethyl sulfoxide, and stored frozen under light-protected conditions at −80°C. Enhanced chemiluminescence (ECL) kits were purchased from Amersham (Little Chalfont, Buckinghamshire, UK) and NEN Life Science Products (Boston, MA). Trypsin-EDTA, Williams medium E, and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). Other reagents were as described elsewhere (Qiao et al., 2001b, 2002a,b; Han et al., 2004; Dent et al., 2005a,b).

Methods

Primary Culture of Rodent Hepatocytes. Hepatocytes were isolated from adult male Sprague-Dawley rats by the two-step collagenase perfusion technique. The freshly isolated hepatocytes were plated on rat-tail collagen (Invitrogen)-coated plate at a density of 2×10^5 cells/well and cultured in Williams medium E supplemented with 250 nM insulin, 0.1 nM dexamethasone, 1 nM thyroxine, and 100 μg/ml penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. The initial medium change was performed 3 h after cell seeding to minimize the contamination of dead or mechanically damaged cells.

Poly(L-Lysine) Adenoviral Vectors: Generation and Infection In Vitro. A psoralen-treated replication-defective adenovirus was conjugated to poly(L-lysine) and a cDNA plasmid construct to express a dominant-negative $G\alpha_{i1}$, as described in Dent et al. (2005a,b). Hepatocytes were transfected/infected with adenovirus at an approximate multiplicity of infection of 250 in vitro. Cells were further incubated for 24 h to ensure adequate expression of the transduced gene product.

Recombinant Adenoviral Vectors: Generation and Infection In Vitro. Hepatocytes were infected with control (null vector) recombinant adenovirus or with a recombinant adenovirus to express dominant-negative AKT in vitro as described previously (Qiao et al., 2003) at a multiplicity of infection of 30.

Cell Treatments, SDS-PAGE, and Western Blot Analysis. Cells were treated with either pertussis toxin (300 ng/ml) or vehicle PBS diluent 16 or 6 h as indicated before bile acid addition. Cells were then exposed to deoxycholic acid (DCA)/taurocholic acid (TCA) (100 μM) or water diluent as indicated. Water diluent or treatment of hepatocytes with CHAPS did not alter the activation of signaling pathways, in agreement with Qiao et al. (2001b; data not shown). For SDS-PAGE and immunoblotting, at various time points after the indicated treatment, hepatocytes were lysed in either a nondenaturing lysis buffer and were prepared for immunoprecipitation or in whole-cell lysis buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, and 0.02% bromphenol blue), and the samples were boiled for 30 min. After immunoprecipitation, samples were boiled in whole-cell lysis buffer. The boiled samples were loaded onto 7 to 10% SDS-PAGE, and electrophoresis was run overnight. Proteins were electrophoretically transferred onto 0.22 μm of nitrocellulose and immunoblotted with various primary antibodies against different proteins. All immunoblots were visualized by ECL. For presentation, immunoblots were digitally scanned at 600 dpi using Adobe PhotoShop CS (Adobe Systems, Mountain View, CA), and their color was removed and figures were generated in PowerPoint software (Microsoft Corp., Redmond, WA).

Long-Term Bile Fistula Rats and Intraduodenal Infusion of Bile Acids. Adult male Sprague-Dawley rats weighing between 280 and 350 g were housed under controlled lighting conditions on a natural light/dark cycle (6:00 AM to 6:00 PM light phase). Groups of age- and weight-matched animals were used in all experiments, as described in Heuman et al. (1989). In brief, the animals were weighed and kept under isoflurane anesthesia during the whole surgical procedure. Through an upper midline incision (laparotomy), the common bile duct was exposed, ligated close to the duodenum, and cannulated with silastic tubing (internal diameter, 0.020; outer diameter, 0.037 inches), allowing the bile to flow freely. A polyethylene infusion cannula (PE50) was placed in the duodenum via a gastric puncture and connected to a syringe pump (Harvard Apparatus, Holliston, MA). The surgical wound was sealed with staples. Both the infusion and the bile fistula cannulas were tunneled subcutaneously to the back of the neck and brought out of the animal via a flexible spring harness sutured to the skin overlying the occiput, allowing rats free movement and access to food and water. All animals received a continuous intraduodenal infusion of a glucose electrolyte solution [5% (w/v) glucose, 50 mM NaCl, 3 mM KCl and 15 mM $NaHCO_3$] throughout the experiment at a rate of 1.05 ml/h.

To inhibit the putative conjugated bile acid-activated G-protein coupled receptor, 100 μg of dominant-negative $G\alpha_i$ plasmid was diluted in 150 μl of saline and injected directly into four sites of the exposed liver lobes using a 26-gauge needle (Kuemmerle et al., 2000) immediately after laparotomy/surgery and insertion of the fistula. Negative control rats were injected in an identical manner with 100 μg of PCDNA3.1 plasmid. Forty-eight hours after plasmid injections, TCA (Calbiochem, San Diego, CA) was added to the intraduodenal infusate at a concentration calculated to produce a constant rate of 36 μmol/h/100 g of rat for 1.5 h. At the indicated times after infusion, rats were anesthetized and killed humanely by exsanguination. Livers were removed from treated and control rats and frozen immediately in liquid nitrogen. Glycogen synthase assays were performed essentially as described previously (Lazar et al., 1995; Liu and Brautigan, 2000; Van Horn et al., 2001). For analysis, livers were homogenized with lysis buffer [50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 100 mM NaCl, 50 mM NaF, 1 μM microcystin-LR, 1% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 40 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone, and 40 μg/ml *N*-tosyl-L-lysine chloromethyl ketone) and centrifuged at 10,000 rpm. Fifty microliters of

the supernatant fluid (100–200 μg of protein) was added to an equal volume of GS assay buffer [50 mM Tris/HCl pH 7.8, 10 mM EDTA, 50 mM NaF, 1 μM Microcystin-LR containing UDP-[^{14}C] glucose (0.5 μCi/mmol), 15 mg/ml glycogen, and ± 10 mM glucose-6-phosphate]. After 15 min of incubation at 37°C, tubes were then chilled for 15 min on ice, after which the entire tube contents were spotted onto Whatman GF/A 2.4-cm filter papers (Whatman, Maidstone, UK). Spotted filter papers were immediately immersed in 25 ml of 70% (v/v) ethanol (4°C) and washed twice for 30 min each time. Filter papers were air-dried; radioactivity incorporated into glycogen was determined by liquid scintillation spectrometry.

AKT Kinase Activity Measurement. AKT was immunoprecipitated from bile acid-treated hepatocytes using established procedures (Dent et al., 2005a). Immunoprecipitates were suspended in a final volume of 50 μl of 25 mM β-glycerophosphate, pH 7.4, 1 mM sodium orthovanadate containing 0.2 mM [γ-^{32}P]ATP (2000 cpm/pmol), 1 μmol/liter Microcystin-LR containing 10 mg/ml RRGRPRTSSFAEG for AKT assays, which initiated reactions, and then incubated at 37°C. After 20 min, 40 μl of the reaction mixtures was spotted onto 2-cm circles of P81 phosphocellulose paper (Whatman) and immediately placed into 180 mM phosphoric acid. Papers were washed four times (10 min each) with phosphoric acid and once with acetone, and ^{32}P incorporation into peptide substrate was quantified by liquid scintillation spectroscopy. Preimmune controls were performed to ensure that phosphorylation was dependent on specific immunoprecipitation of AKT.

Identification of Receptor-Activated G-Proteins by Guanosine 5′-*O*-(3-[^{35}S]Thio)triphosphate Binding Assay. Cells were homogenized in 20 mM HEPES, pH 7.4, containing 2 mM $MgCl_2$, 1 mM EDTA, and 2 mM 1,4-dithiothreitol. The homogenate was centrifuged at 30,000*g* for 30 min at 4°C, and the membranes were solubilized at 4°C in 20 mM HEPES, pH 7.4, buffer containing 0.5% CHAPS. The cell membranes were incubated with 100 nM guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate in a solution containing 10

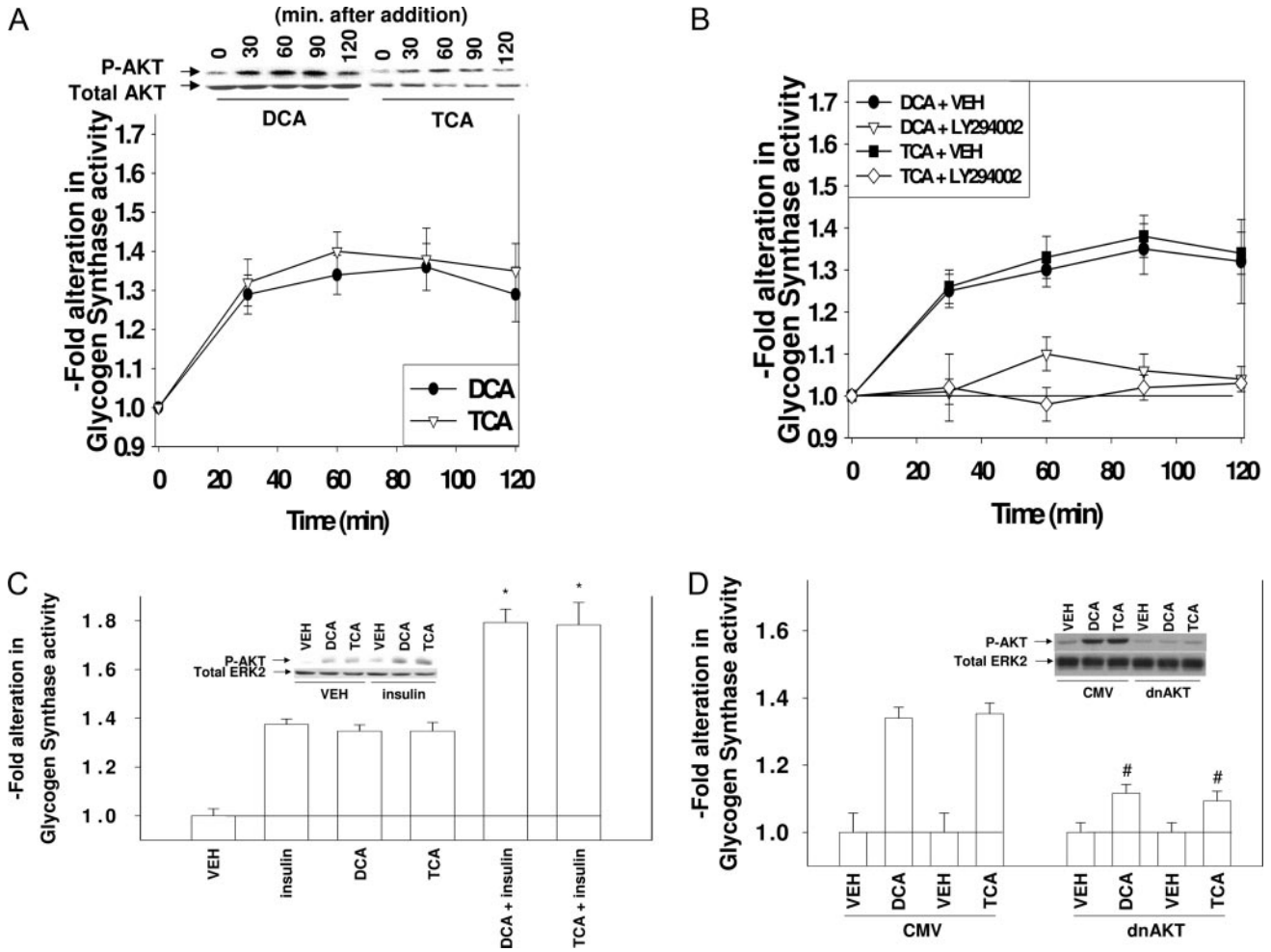

Fig. 1. Bile acids activate glycogen synthase in an AKT-dependent fashion. A, primary rat hepatocytes were treated with DCA or TCA (100 μM), and the activity of glycogen synthase was measured 0 to 120 min after treatment (n = 3, ± S.E.M.). Inset, primary rat hepatocytes were treated with DCA or TCA (100 μM), and phosphorylation of AKT (S473) and total protein levels of AKT were measured by immunoblotting 0 to 120 min after treatment. A representative experiment (n = 5) is shown with an exposure time of 1 min. B, primary rat hepatocytes were pretreated with vehicle (VEH, DMSO) or with LY294002 (10 μM) 30 min before being treated with DCA or TCA (100 μM). The activity of glycogen synthase was measured 0 to 120 min after bile acid treatment (n = 3, ± S.E.M.). C, primary rat hepatocytes were treated with insulin (50 nM), with DCA or TCA (100 μM), or with insulin and bile acid, and the activity of glycogen synthase measured 30 min after treatment (n = 3, ± S.E.M.). Inset, primary hepatocytes were treated with insulin, DCA, or TCA, and phosphorylation of AKT (Ser473) and total protein levels of ERK2 were measured by immunoblotting 30 min after treatment. A representative experiment (n = 3) is shown with an exposure time of 20 s. D, primary rat hepatocytes were infected with a null control virus or a recombinant adenovirus at a multiplicity of infection of 30 to express dominant-negative AKT. Twenty-four hours after infection, cells were treated with DCA or TCA (100 μM), and 30 min after bile acid treatment, cells were isolated, and the activity of glycogen synthase was measured (n = 3, ± S.E.M.; #, $p < 0.05$ less than vector control-infected cells). Inset, primary hepatocytes infected with control virus or with a virus to express dominant-negative AKT were treated with DCA or TCA, and phosphorylation of AKT (Ser473) and total protein levels of ERK2 were measured by immunoblotting 30 min after treatment. A representative experiment (n = 3) is shown with an exposure time of 1 min.

mM HEPES, pH 7.4, 0.1 mM EDTA, and 10 mM $MgCl_2$ for 20 min at 37°C in the presence or absence of bile acid agonist. The reaction was stopped with 10 volumes of 100 mM Tris-HCl, pH 8.0, containing 10 mM $MgCl_2$, 100 mM NaCl, and 20 μmol/l GTP. The membranes were incubated for 2 h on ice in wells precoated with specific antibodies to $G_{i1\alpha}$, $G_{i2\alpha}$, and $G_{i3\alpha}$. The wells were washed with phosphate buffer containing 0.05% Tween 20, and the radioactivity from each well was counted by liquid scintillation spectrometry. Data are presented as the total amount of binding for $G_{i1\alpha}$, $G_{i2\alpha}$, and $G_{i3\alpha}$.

Data Analysis. Comparison of the effects of various treatments was performed using one-way analysis of variance and a two-tailed *t* test. Differences with a *p* value of <0.05 were considered statistically significant. Experiments shown are the means of multiple individual points (± S.E.M.).

Results

Initial studies examined the activation of AKT and GS by the bile acids DCA and TCA. DCA and TCA activated AKT and GS in rat hepatocytes (Fig. 1A). In general agreement with prior studies using insulin, treatment of hepatocytes with a PI3K inhibitor blocked DCA- and TCA-induced activation of GS (Fig. 1B) (Han et al., 2004). Treatment of hepatocytes with insulin plus either TCA or DCA exhibited additive levels of AKT, and of GS, activation 30 min after combined exposure (Fig. 1C). Inhibition of AKT phosphorylation in primary rat hepatocytes, by expression of a domi-

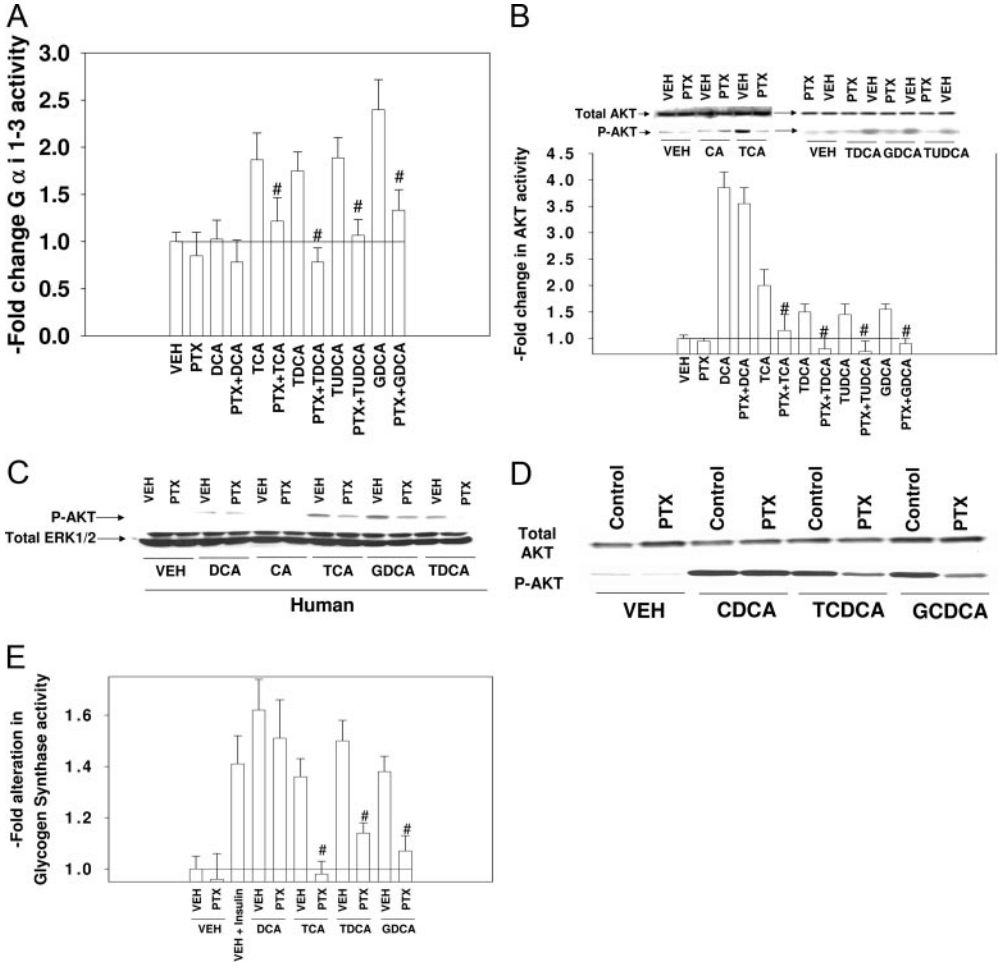

Fig. 2. Conjugated bile acids activate AKT and glycogen synthase in a pertussis toxin-dependent fashion. A, primary rat hepatocytes were treated 4 h after plating with PBS vehicle (VEH) or with pertussis toxin (PTX, 300 ng/ml) for 16 h. Hepatocyte membranes were isolated and treated with water control or with DCA, TCA, TDCA, TUDCA, and GDCA (100 μM each). Cells were isolated 10 min after treatment and were prepared for the determination of GTP loading (G protein activity) for $G\alpha_i$ 1 to 3. Data are the means of three values per experiment from three independent experiments ± S.E.M.; #, $p < 0.05$ less than PTX-treated cells. B, primary rat hepatocytes were treated 4 h after plating with PBS vehicle (VEH) or with pertussis toxin (PTX, 300 ng/ml) 16 h before bile acid exposure. Cells were then treated with the indicated bile acids DCA, TDCA, TCA, and TUDCA (100 μM each). Cells were isolated 20 min after treatment, and AKT was isolated by immunoprecipitation. Immune complex kinase assays to determine AKT activity (using a synthetic peptide) were performed as described under *Materials and Methods*. Data are mean ± S.E.M. ($n = 4$); #, $p < 0.05$ less than control-treated cells. Inset, the phosphorylation of AKT (Ser473) in primary rat hepatocytes pretreated with vehicle or PTX and then treated with bile acids (CA, TCA, TDCA, GDCA, and TUDCA) was determined by immunoblotting after SDS-PAGE. C, primary human hepatocytes were treated 4 h after plating with PBS vehicle (VEH) or with pertussis toxin (PTX, 300 ng/ml) 16h before bile acid exposure. Cells were then treated with DCA, TDCA, GDCA, CA, and TCA (100 μM each). Cells were isolated 20 min after treatment and prepared for SDS-PAGE, and immunoblotting was performed to determine the phosphorylation of AKT (Ser473). Data are from a representative experiment ($n = 3$). D, primary rat hepatocytes were treated 4 h after plating with PBS vehicle (VEH) or with pertussis toxin (PTX, 300 ng/ml) 16 h before bile acid exposure. Cells were then treated with CDCA, taurochenodeoxycholic acid, and glycochenodeoxycholic acid (100 μM each). Cells were isolated 20 min after treatment and prepared for SDS-PAGE, and immunoblotting was performed to determine the phosphorylation of AKT (Ser473). Data are from a representative experiment ($n = 3$). E, primary rat hepatocytes were treated 4 h after plating with PBS vehicle (VEH) or with pertussis toxin (PTX, 300 ng/ml) 16 h before bile acid exposure. Cells were then treated with the indicated bile acids DCA, TDCA, TCA, GDCA, and TDCA (100 μM, each). Cells were isolated 30 min after treatment, and the activity of glycogen synthase was measured ($n = 5$, ± S.E.M.; #, $p < 0.05$ less than control treated cells).

nant-negative AKT protein, suppressed DCA- or TCA-induced activation of GS (Fig. 1D).

The molecular mechanisms by which DCA and TCA activated AKT and GS in primary hepatocytes was investigated in further detail. Conjugated but not unconjugated bile acids activated a $G\alpha_i$-coupled GPCR in primary rat hepatocytes, as judged by a pertussis toxin-dependent increase in $G\alpha_i$ 1 to 3 activity (Fig. 2A). Pretreatment of rat hepatocytes with pertussis toxin abolished TCA- but not DCA-induced activation of AKT catalytic activity, which was in general agreement with data examining AKT S473 phosphorylation (Fig. 2B and inset). Pretreatment of primary human hepatocytes with pertussis toxin also significantly reduced TCA-induced activation of AKT (Fig. 2C). Pertussis toxin treatment did not alter AKT activation induced by CDCA but suppressed AKT activation induced by conjugated forms of CDCA, taurochenodeoxycholic acid, and glycochenodeoxycholic acid, arguing that the pertussis toxin-dependent effects could be generalized to multiple bile acids (Fig. 2D). Pretreatment of primary rat hepatocytes with pertussis toxin reduced activation of GS by TCA but not by DCA (Fig. 2E).

To confirm our findings with pertussis toxin using a molecular tool, we performed identical studies expressing a dominant-negative form of $G\alpha_i$ 1 in primary rat hepatocytes. Expression of dominant-negative $G\alpha_i$ 1 did not alter DCA-induced activation of AKT but abolished TCA-induced AKT activity (Fig. 3A). In general agreement with our pertussis toxin data in Fig. 2, expression of dominant-negative $G\alpha_i$ 1 abolished TCA-induced GS activity and caused a surprising nonsignificant (trend) increase in DCA-induced GS activity (Fig. 3C). Thus, conjugated bile acids, (e.g., TCA) but not unconjugated bile acids (e.g., DCA) promote a $G\alpha_i$-dependent activation of AKT in primary hepatocytes that is, in turn, causal in the activation of hepatocyte glycogen synthase activity in vitro.

Based on our in vitro findings, we next determined in the intact liver performing a laparotomy and using a bile fistula model system, with intraduodenal cannulas, whether TCA activated AKT and GS in the intact liver. Infusion of TCA into the duodenum of Sprague-Dawley rats rapidly activated AKT in the liver, which correlated with increased GSK3 phosphorylation (Fig. 4A). Infusion of TCA into the duodenum also rapidly activated GS in the liver (Fig. 4A). To determine in the intact liver whether TCA signaled via a GPCR to activate AKT and GS, we injected the liver with either a control vector plasmid or a plasmid to express dominant-negative $G\alpha_i$ 1 and then treated animals 48 h after plasmid injection with TCA. The liver and kidneys have been shown competent to take up naked plasmid injected directly into these tissues (Kuemmerle et al., 2000). Injection of vector control plasmid did not alter TCA-induced activation of AKT or GS in the liver (Fig. 4B). Expression of dominant-negative $G\alpha_i$ inhibited TCA-induced activation of both AKT and GS in rat liver (Fig. 4B). Together, the findings in Fig. 4 together with those in Figs. 1 through 3 demonstrate that bile acids, when infused into the duodenum and subsequently absorbed into the blood and hence to the liver, cause activation of AKT and its downstream target GS in the intact rat liver and that conjugated bile acids activate AKT in the intact liver via a $G\alpha_i$-coupled GPCR.

Discussion

Previous studies by this group have linked bile acid-induced ERBB1 and insulin receptor activation to enhanced ERK and AKT signaling, leading to a cytoprotective response versus bile acid-induced fatty acid synthase receptor/caspase activation (Qiao et al., 2001b; Dent et al., 2005b). In addition, these studies also demonstrated that bile acid-induced activation of the PI3K pathway caused activation of GS in vitro and that conjugated bile acids caused $G\alpha_i$-dependent activation of the PI3K-AKT pathway in vitro (Han et al., 2004; Dent et al., 2005a). The present studies were designed to determine whether conjugated bile acids activate GS in primary cultures of hepatocytes via an AKT- and $G\alpha_i$-coupled GPCR-dependent mechanism and whether this occurred via the same mechanism in the intact liver.

Treatment of primary rat hepatocytes with either DCA or TCA caused activation of AKT and GS. The relative amount of GS activation caused by DCA or TCA was very similar to that caused by treatment with insulin, and both TCA and DCA interacted with insulin to further enhance AKT and GS activation. Pretreatment of hepatocytes with a specific $G\alpha_i$ inhibitory enzyme, pertussis toxin, or expression of dominant-negative $G\alpha_i$ suppressed TCA-induced activation of AKT and GS but had no inhibitory effect on DCA-induced activation of AKT and GS. TCA also activated AKT in pri-

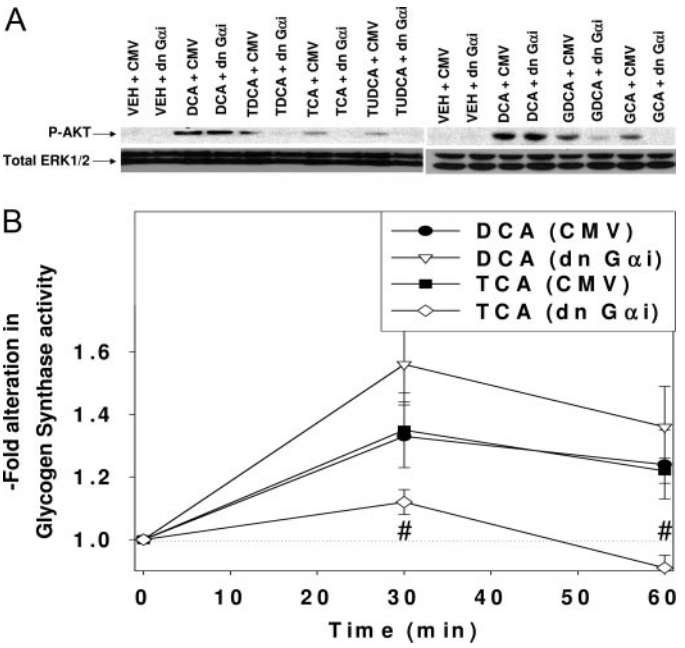

Fig. 3. Conjugated bile acids activate AKT and glycogen synthase in vitro in a $G\alpha_i$-dependent fashion. A, primary rat hepatocytes were infected 4 h after plating with poly(L-lysine)-conjugated adenoviruses to express either a control plasmid (CMV) or a plasmid to express dominant-negative $G\alpha_i$ 1 (dn Gαi). Twenty-four hours after infection, infected hepatocytes were treated with water (control) or with the bile acids DCA, TDCA, TCA, GDCA, GCA, and TUDCA (100 μM each). Cells were isolated 20 min after treatment and were prepared for SDS-PAGE, and immunoblotting was performed to determine the phosphorylation of ERK1/2 and AKT (Ser473). Data are from a representative experiment ($n = 4$). B, primary rat hepatocytes were infected 4 h after plating with poly(L-lysine)-conjugated adenoviruses to express either a control plasmid (CMV) or a plasmid to express dominant-negative $G\alpha_{i1}$ (dn Gαi). Twenty-four hours after infection, infected hepatocytes were treated with water (control) or with the bile acids DCA or TCA (100 μM each). Cells were isolated 30 and 60 min after treatment, and the activity of glycogen synthase was measured ($n = 5$, ± S.E.M.; #, $p < 0.05$ less than control infected cells).

mary human hepatocytes in a $G\alpha_i$-dependent fashion. In general agreement with the concept that TCA activated AKT and GS via a GPCR, TCA increased the activity of $G\alpha_i$ subunits in a pertussis toxin-dependent fashion. Infusion of TCA into rat duodenum promoted rapid (30-min) activation of AKT in the liver, which correlated with increased GSK3 phosphorylation and with activation of GS. Expression of dominant-negative $G\alpha_i$ in the liver suppressed TCA-induced activation of AKT and GS. Together, our in vitro and in vivo data demonstrate that conjugated bile acids, via a $G\alpha_i$-coupled GPCR, promote activation of AKT, which is causal in increased GS activity.

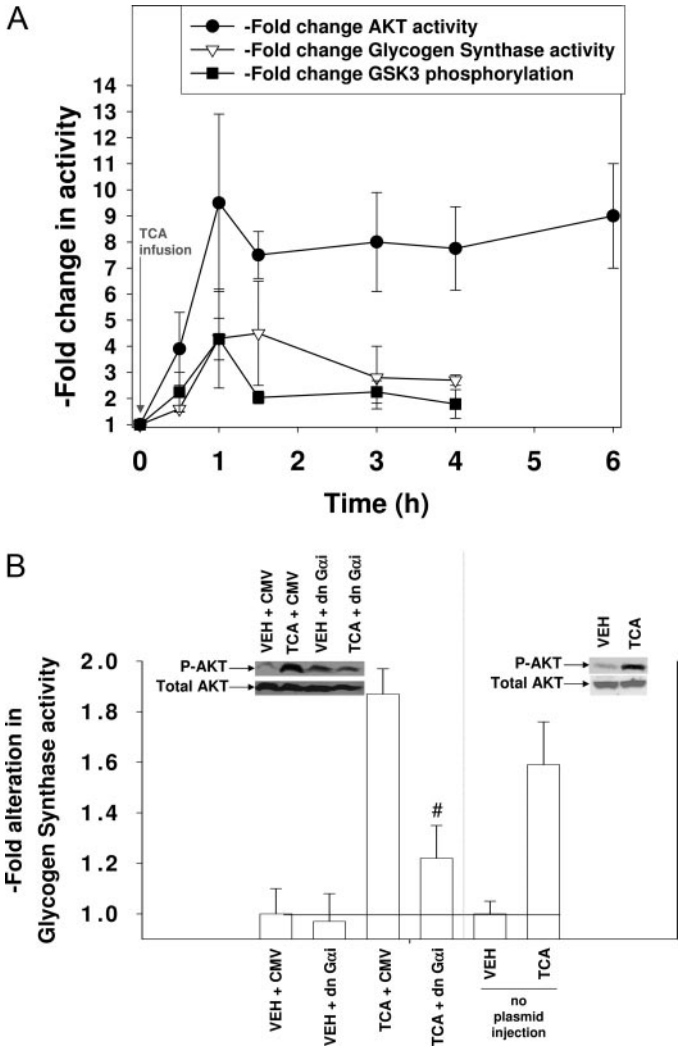

Fig. 4. Conjugated bile acids activate AKT and glycogen synthase in vivo in a $G\alpha_i$-dependent fashion. A, bile fistulas and intraduodenal cannulas were placed into the duodena of rats, and 48 h after surgery, vehicle or TCA was infused into the duodenum of each rat, as described under Methods. At the indicated times after infusion (0–6 h), the livers of each animal were isolated and snap-frozen. Portions of the frozen liver were taken, ground to powder, and lysed to permit the determination of AKT activity after immunoprecipitation of AKT (± S.E.M., $n = 4$), the determination of GSK3 phosphorylation after SDS-PAGE and immunoblotting of GSK3 S9 phosphorylation ($n = 4$); and the determination of glycogen synthase activity (± S.E.M., $n = 5$). Infusion of PBS did not alter the phosphorylation of GSK3 and did not alter glycogen synthase activity (data not shown). B, bile fistulas and intraduodenal cannulas were placed into the duodena of rats and the livers of animals infused via the portal vein with plasmids: control empty vector plasmid (CMV) or a plasmid to express dominant-negative $G\alpha_i$. Forty-eight hours after surgery, vehicle or TCA was infused into the duodenum of each rat, as described under Methods. Sixty minutes after infusion, the livers of each animal were isolated and snap-frozen. Portions of the frozen liver were taken, ground to powder, and lysed to permit the determination of AKT phosphorylation after SDS-PAGE and immunoblotting of AKT Ser473 phosphorylation (inset, $n = 3$) and the determination of glycogen synthase activity (± S.E.M., $n = 3$), #, $p < 0.05$ less than control infected cells. Infusion of PBS did not alter the phosphorylation of GSK3 and did not alter glycogen synthase activity (data not shown).

Two GPCRs have been reported in the literature as receptors for conjugated and, to a lesser extent, unconjugated bile acids: muscarinic family receptors, and the orphan receptor TGR5, with neither report using primary hepatocytes in their studies (Cheng et al., 2002a,b; Raufman et al., 2002; Kawamata et al., 2003; Raufman et al., 2003; Katsuma et al., 2005). The liver is known to only express high levels of the muscarinic M3 receptor, and this was stated to be in hepatocyte progenitor cells not adult hepatocytes (Cassiman et al., 2002). In addition, the M3 receptor is reported to be coupled to G_q, and the TGR5 receptor was noted to be G_s-coupled, which would tend to negate both of these GPCRs as part of our G_i-dependent response. Neither inclusion of atropine nor use of M3 receptor −/− hepatocytes modified the activation of AKT by conjugated bile acids compared with wild-type cells (data not shown). Using reverse transcription-polymerase chain reaction, we were unable to detect expression of the TGR5 receptor in either human or rodent hepatocytes (data not shown). Thus, the identity of the novel bile acid-responsive GPCR is presently unknown, and its characterization will require studies beyond the scope of this article.

Insulin has been known for many decades to promote the storage of glucose as glycogen in tissues, with the liver playing a key role in regulating glucose homeostasis (Kanzaki and Pessin, 2001; Roach, 2002). Studies over the last 10 years have linked insulin receptor signaling to GS and to the activation of the PI3 kinase/AKT/GSK3 pathway, with phosphorylation and inactivation of GSK3 by AKT resulting in decreased phosphorylation of sites 3a/b/c in GS, which in turn leads to dephosphorylation and activation of GS (Cohen, 1999). Based on the ability of bile acids and insulin to cooperate in enhancing GS activation in vitro and on our in vivo data demonstrating that bile acids activate GS in the intact liver, it is possible that bile acids may aid the liver in the storage of glucose after feeding. As digested food enters the liver via the portal vein, the pancreas releases insulin, which can promote glucose storage in the liver. Bile acids such as DCA and TCA also re-enter the liver with the digested food. Our findings suggest that bile acids may be able to assist insulin as regulatory molecules in the control of plasma glucose-homeostatic control by the liver and may represent an additional regulatory component within the hepatic portion of the Cori cycle in vivo.

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