Bile acid synthesis. VI. Regulation of cholesterol 7α-hydroxylase by taurocholate and mevalonate

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Abstract Taurocholate, a relatively hydrophobic bile salt, is a potent down-regulator of HMG-CoA reductase and cholesterol 7α-hydroxylase (C7αH), the rate-determining enzymes of the cholesterol and bile acid biosynthetic pathways, respectively. Inhibition of cholesterol synthesis with a bolus dose of mevinolin (lovastatin) a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, profoundly decreases the specific activity of cholesterol 7α-hydroxylase and rate of bile acid synthesis in rats with complete biliary diversion. It is therefore conceivable that taurocholate may suppress cholesterol 7α-hydroxylase primarily by down-regulating the activity of HMG-CoA reductase. To test this hypothesis, taurocholate was coinfused simultaneously to rats with chronic bile fistula with mevalonate (administered as mevalonolactone), an intermediate in the cholesterol biosynthetic pathway. Mevalonolactone was administered to provide a constant supply of newly synthesized cholesterol to cholesterol 7α-hydroxylase, in order to overcome any inhibitory effect of taurocholate on HMG-Coa reductase. Infusions were started 72 h after biliary diversion, and carried out for an additional 48 h. Complete biliary diversion resulted in an increase in C7αH specific activity (510%), protein mass (550%), steady-state mRNA levels (1430%), and transcriptional activities (330%) as compared to control rats with intact enterohepatic circulations. When rats with biliary diversion were infused intraduodenally with taurocholate, the specific activities of HMG-CoA reductase and cholesterol 7α-hydroxylase activities decreased by 75% ($P < 0.001$) and 73% ($P < 0.001$), respectively. Cholesterol 7α-hydroxylase mass, mRNA, and transcriptional activity decreased after intraduodenal infusion of taurocholate to levels similar to those of rats with an intact enterohepatic circulation. The combination of constant infusion of mevalonate and taurocholate failed to reverse the inhibitory effects of taurocholate on cholesterol 7α-hydroxylase activity, mRNA levels, and in vitro transcriptional rates. These data provide evidence that taurocholate represses cholesterol 7α-hydroxylase at the level of gene transcription, and not via down-regulation of HMG-CoA reductase. Infusion of mevalonate alone to biliary diverted rats did not alter cholesterol 7α-hydroxylase activity or mRNA levels, while leading to a 57% decrease in C7αH gene transcription. This latter finding suggests that mevalonate or its metabolites may be capable of stabilizing C7αH mRNA levels while down-regulating transcriptional activity.—**Pandak, W. M., Z. R. Vlahcevic, J. Y. L. Chiang, D. M. Heuman, and P. B. Hylemon.** Bile acid synthesis. VI. Regulation of cholesterol 7α-hydroxylase by taurocholate and mevalonate. *J. Lipid Res.* 1992. **33:** 659–668.

Supplementary key words cholesterol 7α-hydroxylase • liver • HMG-CoA reductase • bile acids

Cholesterol 7α-hydroxylase, a cytochrome P-450 enzyme located in the hepatocyte smooth endoplasmic reticulum, is the initial as well as the rate-determining enzyme of bile acid synthesis (1–4). According to current concepts, hydrophobic bile salts returning to the liver via the portal vein regulate their own synthesis by repressing cholesterol 7α-hydroxylase activity by negative bile acid feedback control (5–8). Until recently, our understanding of the regulation of cholesterol 7α-hydroxylase has been derived primarily from determination of cholesterol 7α-hydroxylase specific activity under various physiologic and experimental conditions. The molecular basis by which bile salts affect cholesterol 7α-hydroxylase was not known. Purification of cholesterol 7α-hydroxylase and cloning and sequencing of its cDNA have provided the tools with which to study the regulation of this key enzyme at the molecular level by bile salts and other regulatory agents (9–11). Cholesterol 7α-hydroxylase activity, protein mass, and mRNA levels have now been shown to increase in response to partial and complete biliary diversion (9–15); conversely, continuous intraduodenal infusion of taurocholate in rats with chronic bile fistula leads to a decrease in cholesterol 7α-hydroxylase specific activity, protein mass, mRNA levels, and transcriptional activity. These findings provided direct evidence that taurocholate, a

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HPLC, high performance liquid chromatography; CBD, chronic biliary diversion.

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relatively hydrophobic bile salt, regulates cholesterol 7α-hydroxylase predominantly at the level of gene transcription (15). However, the mechanism of repression of the cholesterol 7α-hydroxylase gene by other hydrophobic bile salts is still not known.

Heuman et. al. (6) have previously demonstrated that relatively hydrophobic bile salts (taurine and glycine conjugates of cholic, chenodeoxycholic and deoxycholic) suppress 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and cholesterol 7α-hydroxylase to a similar extent in rats with chronic bile fistula and in rats with intact enterohepatic circulation. Hydrophilic bile salts had no effect on either of the two enzymes. Recently, it was demonstrated that administration of a single dose of mevinolin, a competitive inhibitor of HMG-CoA reductase, to rats with complete biliary diversion resulted in a down-regulation of cholesterol 7α-hydroxylase activity and suppression of bile acid synthesis. Both of these effects were reversed by a continuous infusion of mevalonate (16). A quantitatively similar down-regulation of cholesterol 7α-hydroxylase activity was observed in rats with an intact enterohepatic circulation following a single dose of mevinolin (M. P. Jones, W. M. Pandak, P. B. Hylemon, J. Y. L. Chiang, and Z. R. Vlahcevic, unpublished results). Therefore, the possibility could not be excluded that the inhibitory effect of taurocholate and other hydrophobic bile salts on cholesterol 7α-hydroxylase might occur as a result of an inhibitory effect on HMG-CoA reductase accompanied by a reduction in the supply of newly synthesized cholesterol.

In the present study, we examined this hypothesis by determining the effects of taurocholate on cholesterol 7α-hydroxylase specific activity, protein mass, mRNA levels, and transcriptional activity in the presence and absence of a continuous infusion of mevalonate, an intermediate in the cholesterol biosynthesis pathway beyond the HMG-CoA reductase step. More specifically, our purpose was to determine whether a constant intravenous infusion of mevalonate, by maintaining the rate of cholesterol synthesis, could prevent the down-regulation of cholesterol 7α-hydroxylase observed during continuous intraduodenal infusion of taurocholate. In addition, we studied the molecular basis by which cholesterol 7α-hydroxylase is regulated following infusion of taurocholate, mevalonate, and a combination of both.

METHODS AND MATERIALS

Materials

Dithiothreitol, EDTA, proteinase K, ribonuclease A, NADP, NADPH, isocitrate dehydrogenase, Na-isocitrate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 3-hydroxy-3-methylglutaryl coenzyme A were obtained from Sigma Chemical Company. Taurocholic acid and cholesterol oxidase were purchased from Calbiochem. Goat anti-rabbit IgG alkaline phosphatase conjugate, 5-romo-4-chloro-3-indolyphosphate and nitro blue tetrazolium were obtained from Bio-Rad. The nick translation kit was obtained from Bethesda Research Laboratories and guanidine thiocyanate was purchased from Fluka (Ronkonkoma, NY). Deoxyribonuclease I was obtained from Worthington, and Sephadex G 50, fine, DNA grade was purchased from Pharmacia. [α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P]GTP (800 Ci/mmol), [4-^{14}C]cholesterol (59.4 mCi/mmol), DL-[^{3}H]3-hydroxymethyl-3-glutaryl coenzyme A (57.6 mCi/mmol), and DL[^{3}H]mevalonate (30 Ci/mmol) were all purchased from New England Nuclear. All other reagents were of the highest quality available. Silica gel thin-layer chromatography plates were obtained from Fisher Scientific. Intramedic polyethylene tubing (P-50) and Dow Corning silastic tubing were obtained from American Scientific.

Chronic biliary diverted (CBD) rat model

Male Sprague-Dawley rats (Charles River, Cambridge, MA) weighing between 250 and 350 g were housed under controlled lighting conditions on a natural light–dark cycle (0600–1800 light phase). Groups of age- and weight-matched animals were used in all experiments. With the rats under brief methoxyflurane anesthesia, the creation of biliary fistulas and placement of intraduodenal cannulas were carried out as previously described (6). After surgery, animals were placed in individual metabolic cages with free access to water and laboratory chow (Purina lab chow 50-01). All animals received continuous intraduodenal infusion of glucose-electrolyte replacement solution (6). Diverted bile was collected in timed increments throughout the course of the experiment. After 72 h of chronic biliary diversion, taurocholate (36 μmol/h per 100 g) and/or mevalonolactone (mevalonate in its lactone form, 180 μmol/h) were infused for 48 h intraduodenally or intravenously, respectively. The rate of mevalonate infusion was sufficient to provide 30 μmol/h of newly synthesized cholesterol; an amount more than sufficient to replace calculated losses of cholesterol and/or suppression of newly synthesized cholesterol under the experimental conditions used. Infusions were timed so that animals could be killed between 9 and 10 AM. At the conclusion of the experiment, livers were harvested and four 1-g pieces were removed. Two 1-g pieces of liver were used to extract total RNA, and two to isolate nuclei. The remainder of the liver was used in the preparation of microsomes as previously described (7).

Assays for HMG-CoA reductase and cholesterol 7α-hydroxylase activity

Microsomal HMG-CoA reductase specific activity was assayed by the method of Whitehead et al. (17). The specific activity of cholesterol 7α-hydroxylase was determined in microsomes using a high performance liquid

chromatography (HPLC) assay procedure as described previously by us (18).

Western blot analysis of cholesterol 7α-hydroxylase

Immunoblot analysis of cholesterol 7α-hydroxylase was performed as recently described (12, 15). Under these experimental conditions, the amount of purified cytochrome P-450 cholesterol 7α-hydroxylase protein gave a linear densitometric response between 10 and 200 ng.

RNA preparation

The livers were removed and two 1-g pieces were taken from different lobes for isolation of RNA. Isolation of total and poly(A) RNA was previously described in detail (15).

Quantitation of cholesterol 7α-hydroxylase mRNA

Poly(A) RNA was diluted in 20 × SSC, 7.4% formaldehyde, and applied to a nitrocellulose membrane using a Hybri-Dot Manifold from BRL. The membrane was then baked 1 h at 80°C in a vacuum oven. The membrane was prehybridized in 4 × SSC, 1% SDS, 5 × Denhardt's, 0.1 mg/ml salmon sperm DNA, and 50% formamide for 2 h at 42°C and hybridized with *Eco*R1-*Acc*1 fragment of plasmid pBSK 7α6 which contains a cholesterol 7α-hydroxylase cDNA (9) that was labeled with [α-^{32}P]dCTP using a BRL nick translation kit. After hybridization with plasmid pBSK7α6, the membrane was washed three times in boiling water for 5 min in order to remove the cholesterol 7α-hydroxylase cDNA probe and to prepare the membrane for rehybridization with labeled plasmid pRSA and with p1B15 which served as internal controls. Rat albumin and cyclophilin cDNA insert of plasmid pRSA 57 and p1B15, respectively, were then labeled with [α^{32}P]dCTP and used to probe the same membrane under the same hybridization conditions. The membrane was air-dried and exposed to X-ray film at −70°C. The film was then developed and radioactivity levels were quantitated using a Shimadzu laser densitometer.

The absorbencies for hybridization to pBSK 7α6 as standardized to albumin pRSA 57 were determined for each condition, and the resulting indices were compared to the control animals.

Northern blot hybridization

Poly A-RNA was size-fractionated by electrophoresis in 1% agarose gel containing 7% formaldehyde. Northern blotting was then performed as previously described (15).

Nuclear run-on studies

Nuclear run-on studies were carried out as previously described (15). In brief, after isolation of nuclei, they were frozen at −70°C. After thawing, several nucleotides were added (1.25 mM ATP, 1.25 mM CTP, 1.25 mM UTP, 5 mM DTT, 0.2 units/μl of RNasin, and 30 μl [α-^{32}P] and GTP 800 Ci/mmol, 10 mCi/ml). They were incubated for 30 min at 30°C and the reaction was stopped by the addition of 24 μg of DNase I. After 5 min at 30°C, 200 μg of proteinase K in 0.5 M Tris-HCl, pH 7.4, 5% SDS, 0.125 M EDTA was added and the mixture was incubated 45 min at 42°C. This solution was then extracted with 1 ml of phenol–chloroform–isoamyl alcohol 25:24:1(v/v/v) and the interface was re-extracted with 1 ml of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA (TE buffer). The combined aqueous phases were precipitated with 1/10 volume 3 M sodium acetate, pH 5.0, and 2.5 volumes ethanol using a dry ice–ethanol bath for 30 min. The RNA was recovered by centrifugation at 10,000 *g* for 30 min and resuspended in 100 μl of TE buffer. This was then applied to a spin-column made from a 1-ml syringe and Sephadex G-50, fine, DNA grade. The column was centrifuged for 2 min at 200 *g* and washed twice with 100 μl TE buffer. The RNA was mildly digested by adding 1/4 volume of 1 M NaOH on ice for 5 min and neutralized by adding a volume of 1 M HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid). This was precipitated with 2.5 volumes of ethanol at −20°C overnight.

Nitrocellulose strips containing spots of dilutions of pBSK 7α6 and pRSA 57 were placed in small heat-sealable bags with 4 ml of 10 mM TES, pH 7.4, 10 mM EDTA, 0.2% SDS, 0.3 M NaCl, and 1 × Denhardt's (hybridization solution). They were prehybridized for at least 2 h at 65°C. The RNA was recovered by centrifugation at 4°C and resuspended in 100 μl 10 mM TES, pH 7.4, 10 mM EDTA, 0.2% SDS. An aliquot was counted, the samples were standardized to the lowest counts, and the appropriate volume was added to fresh hybridization solution in each bag. Hybridization was performed at 65°C for 44 h. The strips were rinsed 5 min at 65°C in 2 × SSC and washed 2 times for 1 h at 65°C in 2 × SSC. They were then incubated at 37°C for 30 min. in 40 ml of 2 × SSC containing 40 μg of RNase A, washed 1 h at 37°C in 2 × SSC and then air-dried. They were exposed to X-ray film for 3–10 days. Specific transcriptional activity of cholesterol 7α-hydroxylase was expressed relative to albumin. The relative amounts of cholesterol 7α-hydroxylase and albumin mRNA transcripts were quantitated by laser densitometry, and the ratios of the two RNAs were determined.

Microsomal cholesterol

Microsomal free cholesterol was extracted with acetone–ethanol and quantified after digitonide precipitation by the method of Abell et al. (19).

Statistical analysis

Statistical differences between groups were tested by means of Student's *t* test for unequal samples.

RESULTS

Effect of chronic biliary diversion, taurocholate infusion and/or mevalonolactone infusion on the specific activities of HMG-CoA reductase and of cholesterol 7α-hydroxylase

After chronic biliary diversion (**Fig. 1A and B**) a 4.8-fold increase ($P < 0.001$) in the specific activity of HMG-CoA reductase and a 5.1-fold increase ($P < 0.001$) in cholesterol 7α-hydroxylase specific activity were observed as compared to rats with intact enterohepatic circulation. When rats with biliary diversion were infused intraduodenally with taurocholate, HMG-CoA reductase (Fig. 1A) and cholesterol 7α-hydroxylase specific activities (Fig. 1B) decreased by 75% ($P < 0.001$) and 73% ($P < 0.001$), respectively, from the control bile fistula rats. Intravenous infusion of mevalonolactone led to an 83% ($P < 0.001$) reduction in HMG-CoA reductase specific activity as compared to chronic biliary diverted controls. Specific activity of cholesterol 7α-hydroxylase after intravenous infusion of mevalonolactone was not significantly different from controls. Simultaneous infusion of mevalonolactone plus taurocholate to rats with chronic biliary diversion led to an additive decrease in HMG-CoA reductase specific activity ($P < 0.01$) as compared to mevalonolactone or taurocholate alone (Fig. 1A). Intravenous infusion of mevalonolactone in addition to intraduodenal infusion of taurocholate did not prevent the down-regulation of cholesterol 7α-hydroxylase specific activity induced by taurocholate alone (Fig. 1B). However, cholesterol 7α-hydroxylase specific activity was significantly higher (1.5-fold) in the group infused with taurocholate plus mevalonolactone as compared to those infused with taurocholate alone ($P < 0.03$).

Cholesterol 7α-hydroxylase specific activity was measured simultaneously in the same microsomes by reverse phase HPLC using both endogenous and exogenous [4-^{14}C]cholesterol as substrate. Both determinations demonstrate similar changes in the same directions (data not shown). However, whenever mevalonolactone was infused, specific activity of cholesterol 7α-hydroxylase, determined by using exogenous cholesterol as substrate (isotope incorporation), was approximately 50% of that determined using endogenous cholesterol as substrate. These observations suggest that the isotope incorporation method for determining cholesterol 7α-hydroxylase activity may underestimate specific activity when a surplus of newly synthesized cholesterol is present.

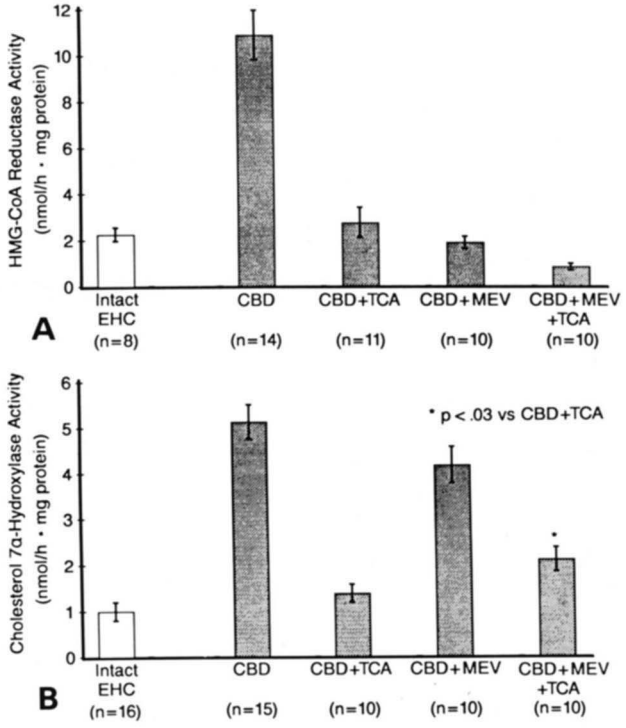

Fig. 1. Effect of chronic biliary diversion, intraduodenal infusion of taurocholate and/or intravenous infusion of mevalonolactone on the specific activities of HMG-CoA reductase and cholesterol 7α-hydroxylase. Hepatic microsomes were prepared from rats with an intact enterohepatic circulation (Intact EHC), from rats after chronic biliary diversion (CBD) alone, and from chronic biliary diverted rats infused for 48 h with taurocholate (CBD+TCA), with mevalonolactone (CBD+MEV), or both (CBD+MEV+TCA). Values shown are the mean ± SE. Fig. 1A demonstrates the specific activity of HMG-CoA reductase as determined by isotope incorporation. Fig. 1B demonstrates cholesterol 7α-hydroxylase specific activity as determined using a high performance liquid chromatography assay.

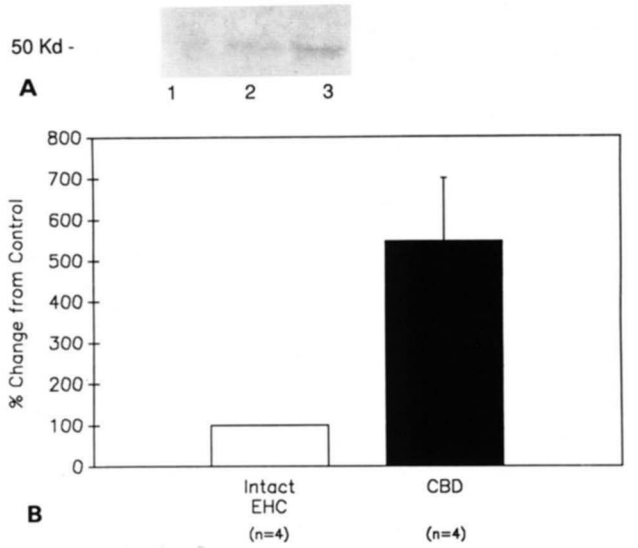

Fig. 2. Effect of chronic biliary diversion on cholesterol 7α-hydroxylase protein mass. A: Representative Western immunoblot of microsomes (20 μg/lane) prepared from the livers of a rat with an intact enterohepatic circulation (lane 2) versus that following chronic biliary diversion (CBD) (lane 3). Lane 1 represents the molecular weight marker. B: Cumulative data expressed as % change from intact enterohepatic circulation levels demonstrating relative amounts of immunoreactive cholesterol 7α-hydroxylase protein in the livers of rats with intact enterohepatic circulation (Intact EHC) versus that following CBD. Values shown are expressed as the mean ± SE.

Effect of chronic biliary diversion, taurocholate infusion and/or mevalonolactone infusion on cholesterol 7α-hydroxylase mass

The relative mass of cholesterol 7α-hydroxylase was determined using a Western immunoblot technique which gives a linear response to pure cholesterol 7α-hydroxylase protein mass from 10 to 200 ng (15). A representative Western immunoblot of cholesterol 7α-hydroxylase in rats with intact enterohepatic circulation and rats with CBD is shown in **Fig. 2A**. After chronic biliary diversion (120 h), there was a 5.5-fold increase in enzyme mass as compared to nonsurgical control rats with an intact enterohepatic circulation (Fig. 2B).

Fig. 3A also demonstrates a representative Western immunoblot analysis using specific antibodies against cholesterol 7α-hydroxylase. When rats with CBD were infused with taurocholate and taurocholate plus mevalonolactone, the cholesterol 7α-hydroxylase mass decreased by 59% and 48%, respectively ($P < 0.001$) (Fig. 3B). As with specific activity, cholesterol 7α-hydroxylase mass was greater (1.3-fold) in the group receiving mevalonolactone plus taurocholate as compared to taurocholate alone (Fig. 3B). This difference, however, did not reach statistical significance.

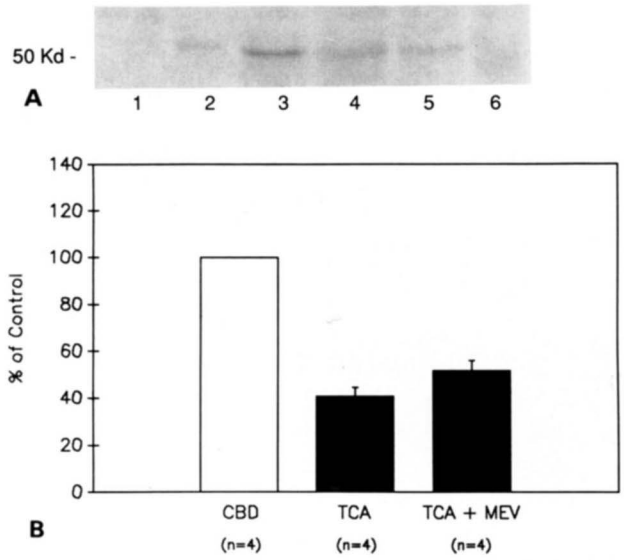

Fig. 3. Effect of 48 h infusion of taurocholate and/or mevalonolactone on cholesterol 7α-hydroxylase protein mass in chronic biliary diverted rats. A: Representative Western immunoblot of microsomes (20 μg/lane) prepared from livers of chronic biliary diverted rats (CBD) infused for 48 h intraduodenally with taurocholate (CBD+TCA) or with the combined infusion of intravenous mevalonolactone (MEV) and intraduodenal taurocholate (CBD+MEV+TCA). Lane 1, negative control (bacterial protein); lane 2, pure cholesterol 7α-hydroxylase (60 ng/lane); lane 3, CBD; lane 4, CBD+TCA; lane 5, CBD+MEV+TCA; lane 6, molecular weight marker. B: Cumulative data expressed as % of CBD level, demonstrating relative amounts of immunoreactive cholesterol 7α-hydroxylase protein in livers of chronic biliary diverted rats (CBD) infused for 48 h with taurocholate (TCA) or mevalonolactone plus taurocholate (MEV+TCA). Values shown are expressed as the mean ± SE.

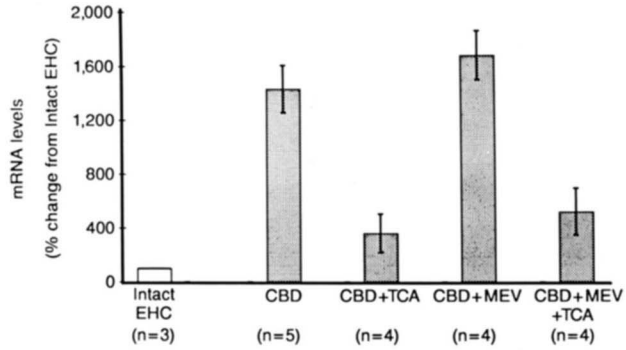

Fig. 4. Effect of chronic biliary diversion, taurocholate infusion and/or mevalonolactone infusion on cholesterol 7α-hydroxylase steady state mRNA levels. Polyadenylated RNA isolated from livers of animals with intact enterohepatic circulations (Intact EHC), with chronic biliary diversion (CBD), and with chronic biliary diversion infused for 48 h with taurocholate (CBD+TCA), with mevalonolactone (CBD+MEV), or both (CBD+MEV+TCA). The figure summarizes data obtained by dot-blot hybridization of cholesterol 7α-hydroxylase mRNA (see Methods for details). Values shown represent the mean ± SE. Note: for one blot the same intact EHC rat served as control for more than one group of biliary diverted rats.

Effect of chronic biliary diversion, taurocholate infusion and/or mevalonolactone infusion on cholesterol 7α-hydroxylase steady state mRNA levels

Steady-state mRNA levels were quantitated using poly(A) RNA (0.6–10 μg) blotted onto nitrocellulose membranes as described under Methods. After biliary diversion (120 h), cholesterol 7α-hydroxylase mRNA levels detected in the liver increased 14.3-fold ($P < 0.003$) as compared to rats with an intact enterohepatic circulation **(Fig. 4)**. Intraduodenal infusion of taurocholate led to a 75% ($P < 0.002$) decrease in mRNA levels as compared to rats with biliary diversion. Mevalonolactone infusion did not affect mRNA levels as compared to rats with bile fistula. Moreover, simultaneous infusion of mevalonolactone and taurocholate did not prevent a decrease in steady-state mRNA levels observed with taurocholate alone. As with specific activity and enzyme mass, mRNA levels were greater (1.4-fold) in the group receiving mevalonolactone plus taurocholate as compared to taurocholate alone. This difference did not reach statistical significance. Ten μg of poly(A) RNA from the each of the five experimental conditions was then subjected to Northern blot analysis. Changes in cholesterol 7α-hydroxylase mRNA levels similar to those observed with dot-blot analysis were found (data not shown).

Rats have been shown to exhibit a diurnal variation in cholesterol 7α-hydroxylase specific activity (5). Recently, Noshiro, Nishimoto, and Okuda (20) demonstrated a similar diurnal variation in cholesterol 7α-hydroxylase mRNA levels both in control and cholestyramine-fed

animals. In these studies, mRNA levels in cholestyramine-treated animals were found to be at their highest when the rats were killed at midnight. At this time in the rat's diurnal cycle, changes in specific activity and mRNA levels correlated well, being only about 3- to 4-fold higher than their controls. However, at 10 AM, at the nadir in the diurnal cycle for cholesterol 7α-hydroxylase, increases over appropriate control values in mRNA levels for cholestyramine-fed rats were elevated out of proportion to increases in specific activity. These findings are quantitatively similar to the increase in cholesterol 7α-hydroxylase mRNA levels seen in our studies in rats with chronic biliary diversion killed at the same time in the diurnal cycle (i.e., 10 AM).

As described in the Methods section, the absorbencies for hybridization to pBSK 7α6 as compared to albumin pRSA 57 were determined for each condition, and the resulting indices were compared to the control animals. In order to ascertain that albumin metabolism was not altered in bile fistula rats, we used cyclophilin as a second standard. Cyclophilin is a nonspecific cytosolic protein that binds cyclosporine. It has been used previously as an internal standard by others (21). **Fig. 5** shows the representative dot-blot hybridization of cholesterol 7α-hydroxylase (panel A), albumin (panel B), and cyclophilin (panel C). The data show that for albumin (panel B) and cyclophilin (panel C), the mRNA levels did not change, suggesting that either of the two could be used as internal standards under the experimental conditions used. Thus, the observed changes in mRNA levels reflect changes in cholesterol 7α-hydroxylase mRNA levels.

Effect of chronic biliary diversion, taurocholate infusion and/or mevalonolactone infusion on cholesterol 7α-hydroxylase in vivo transcriptional activities

The data in **Fig. 6** illustrate cholesterol 7α-hydroxylase transcriptional activity as determined in rat liver nuclei after complete biliary diversion (120 h), and after complete biliary diversion plus intraduodenal infusion of taurocholate and/or intravenous infusion of mevalonolactone, as compared to nonsurgical controls. The ratio of transcriptional activity of the cholesterol 7α-hydroxylase gene to that of the albumin gene increased 3.3-fold ($P < 0.01$) after biliary diversion. Intraduodenal infusion of taurocholate led to a 44% decrease in transcriptional activity as compared to controls. Infusion of mevalonolactone alone or mevalonolactone plus taurocholate both led to a 57% decrease in transcriptional activity as compared to chronic biliary diverted controls ($P < 0.03$). This decrease did not differ significantly from that observed with taurocholate infusion alone.

Effect of chronic biliary diversion, taurocholate infusion and/or mevalonolactone infusion on microsomal cholesterol content in the chronic biliary diverted rat

Microsomal cholesterol (μmol/mg protein) in chronic bile fistula rats did not change significantly after taurocholate infusion (**Fig. 7**). Infusion of mevalonolactone, whether alone or in combination with taurocholate, led to a small (288%) but significant increase in microsomal cholesterol as compared to chronic biliary diverted controls.

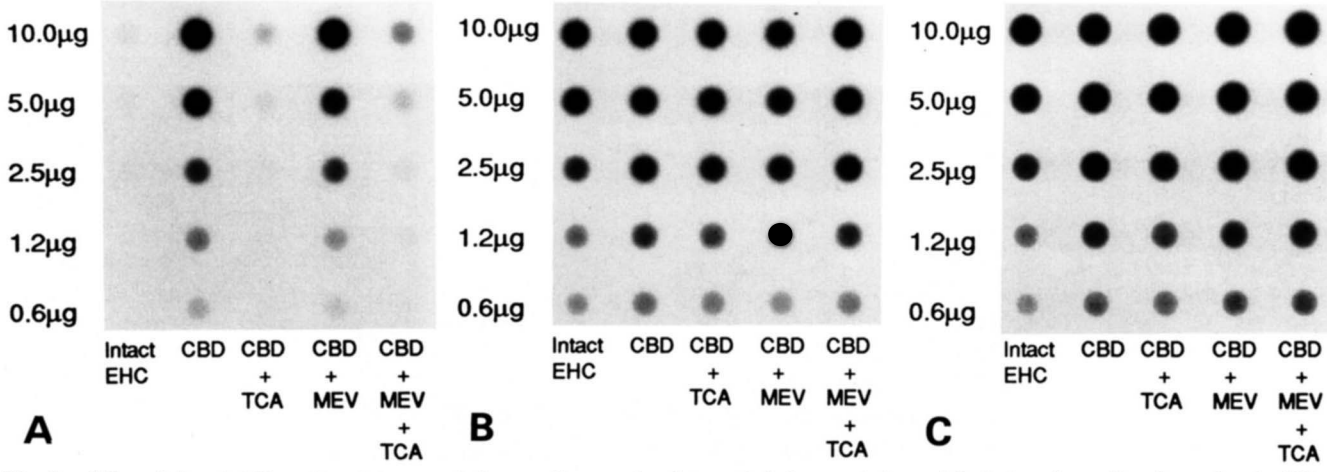

Fig. 5. Effect of chronic biliary diversion, taurocholate, and/or mevalonolactone infusion on cholesterol 7α-hydroxylase, albumin, and cyclophilin steady state mRNA levels as determined by dot-blot hybridization. Polyadenylated RNA was isolated from livers of animals with intact enterohepatic circulation (Intact EHC), with chronic biliary diversion (CBD), and with chronic biliary diversion infused for 48 h with taurocholate (CBD+TCA), with mevalonolactone (CBD+MEV), or both (CBD+MEV+TCA). Shown are representative dot-blot hybridizations of (A) cholesterol 7α-hydroxylase, (B) albumin, and (C) cyclophilin.

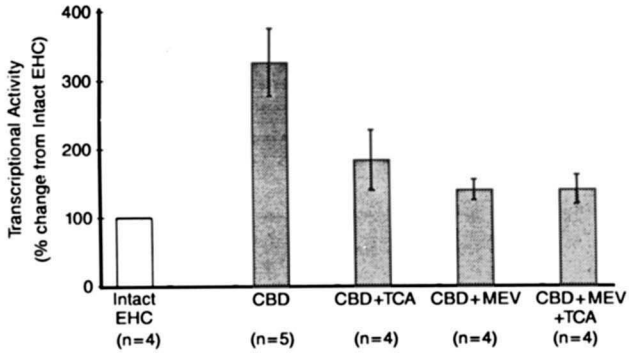

Fig. 6. Effect of chronic biliary diversion, taurocholate infusion and/or mevalonolactone infusion on cholesterol 7α-hydroxylase transcriptional activity as determined in isolated hepatic nuclei. Nuclei were isolated from the livers of rats with intact enterohepatic circulations (Intact EHC), with chronic biliary diversion (CBD), and with chronic biliary diversion infused for 48 h with taurocholate (CBD+TCA), with mevalonolactone (CBD+MEV), or both (CBD+MEV+TCA). Nascent RNA transcripts were allowed to elongate in the presence of [α-^{32}P]GTP. ^{32}P-labeled RNA was then isolated and hybridized to cDNA from cholesterol 7α-hydroxylase or albumin as described in Methods. Relative amounts of RNA hybridizing to probe were then quantified by laser densitometer. Summarization of data is displayed in graphic form expressed as the % change in activity from animals with an intact enterohepatic circulation (Intact EHC). Values shown are the mean ± SE.

DISCUSSION

The regulation of cholesterol 7α-hydroxylase has been in the process of re-evaluation for some time. The original and widely accepted concept of negative bile acid feedback control was recently questioned by Davis et al. (22) and others (23) who failed to observe inhibition of bile acid synthesis in cultured rat hepatocytes even after addition of supraphysiologic concentration of bile salts. These observations lead to the hypothesis that a major factor in regulation of cholesterol 7α-hydroxylase may be the availability of cholesterol substrate. Björkhem and Åkerlund (24) and Einarsson, Reihnér, and Björkhem (25) subsequently reported that in rats and humans with intact enterohepatic circulation, cholesterol 7α-hydroxylase is saturated, a finding not consistent with the role of cholesterol substrate as a sole regulator of this enzyme. Failure to elicit negative bile acid feedback control in cultured rat hepatocytes probably occurred as a result of very low levels of cholesterol 7α-hydroxylase observed after the plating of hepatocytes (23). Interestingly, in pig hepatocytes the suppression of bile acid synthesis by bile acids could be demonstrated (26). In order to clarify these inconsistencies, Heuman et al. (6–8) initiated a series of in vivo studies in rats with intact enterohepatic circulation and with chronic bile fistula. They confirmed the original concept of negative bile acid feedback control and, in addition, provided evidence that only hydrophobic bile salts down-regulate cholesterol 7α-hydroxylase; hydrophilic bile salts had no effect on either of the two enzymes. Later, Pandak et al. (15), using specific antibody to cholesterol 7α-hydroxylase and cDNA probe, demonstrated that taurocholate effects cholesterol 7α-hydroxylase at the level of gene transcription. Recent preliminary evidence from our laboratories suggests that other hydrophobic bile salts also affect cholesterol 7α-hydroxylase at the level of gene transcription (W. M. Pandak, Z. R. Vlahcevic, D. M. Heuman, P. B. Hylemon, and J. Y. L. Chiang, unpublished observations).

A number of questions regarding the regulation of cholesterol 7α-hydroxylase by bile salts remain unanswered. Specifically, it is not certain whether taurocholate, a powerful down-regulator of HMG-CoA reductase, affects cholesterol 7α-hydroxylase directly or indirectly. Several lines of evidence suggest that cholesterol 7α-hydroxylase is indirectly regulated by taurocholate. *a*) Hydrophobic bile salts down-regulate HMG-CoA reductase and cholesterol 7α-hydroxylase by roughly similar proportions (8); *b*) newly synthesized cholesterol appears to be a preferred substrate for cholesterol 7α-hydroxylase (27, 28); and *c*) in short-term experiments in bile fistula rats and rats with intact enterohepatic circulation, inhibition of HMG-CoA reductase results in a prompt and profound down-regulation of cholesterol 7α-hydroxylase (16, M. P. Jones, W. M. Pandak, P. B. Hylemon, J. Y. L. Chiang, and Z. R. Vlahcevic, unpublished results). The down-regulation of cholesterol 7α-hydroxylase in these later experiments could be prevented by a constant infusion of mevalonate, suggesting that the newly synthesized cholesterol plays an important role in the regulation of cholesterol 7α-hydroxylase (16). The purpose of the present investigation was to define whether taurocholate affects cholesterol 7α-hydroxylase primarily via suppression of HMG-CoA reductase. The results obtained in these studies provide strong evidence that taurocholate suppresses cholesterol

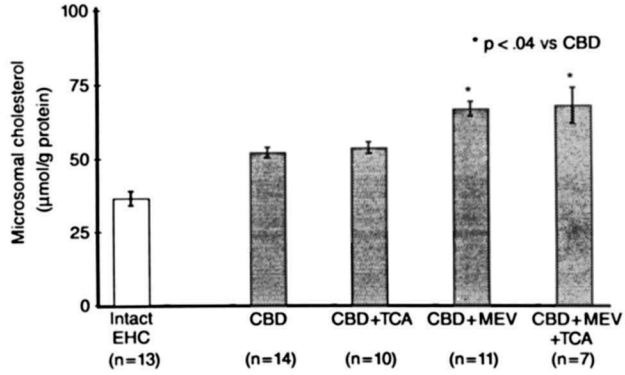

Fig. 7. Effect of chronic biliary divertion, taurocholate infusion and/or mevalonolactone infusion on microsomal cholesterol. The relative concentration of microsomal cholesterol (as determined by the method of Abell et al. (19) in hepatic microsomes prepared from rats with an intact enterohepatic circulation (Intact EHC), with chronic biliary diversion (CBD), and with chronic biliary diversion infused for 48 h with taurocholate (CBD+TCA), with mevalonolactone (CBD+MEV), or both (CBD+MEV+TCA). Values shown are the mean ± SE.

7α-hydroxylase directly and at the level of gene transcription. The failure of a continuous infusion of mevalonolactone to prevent down-regulation of cholesterol 7α-hydroxylase by taurocholate suggests that the primary mode of action of taurocholate on cholesterol 7α-hydroxylase is not due to its inhibitory effect on HMG-CoA reductase. Furthermore, our data suggest that the transcriptional regulation of cholesterol 7α-hydroxylase by taurocholate occurs predominantly via a mechanism which appears to be independent of availability of newly synthesized cholesterol.

Whether taurocholate is the regulator itself, or whether it leads to down-regulation of cholesterol 7α-hydroxylase via interaction with other putative regulatory agents, remains to be determined. Jelinek et al. (21) have identified what may represent a DNA sequence in the promoter region of cholesterol 7α-hydroxylase gene that could interact with bile acids. Inasmuch as no cytosolic bile acid binding protein with characteristics of a steroid hormone receptor was identified and no good evidence exists that bile salts enter the nucleus, an indirect mechanism of regulation of cholesterol 7α-hydroxylase by bile acids appears plausible (29). For example, hydrophobic bile salts could repress cholesterol 7α-hydroxylase by displacing hormones such as thyroxine and dexamethasone from their cytosolic receptors. These two hormones have been shown to have a stimulatory effect on cholesterol 7α-hydroxylase (30, 31).

While the infusion of mevalonolactone failed to prevent the down-regulation of cholesterol 7α-hydroxylase by taurocholate, a small, but significant, increase in enzyme activity was observed in the mevalonolactone plus taurocholate group as compared to the group infused with taurocholate alone. Although not statistically significant, similar increases in protein mass and mRNA levels were also apparent in this group as compared to taurocholate alone. In light of our previous observations demonstrating an increase in cholesterol 7α-hydroxylase specific activity and mRNA levels after cholesterol feeding, we believe that the modest increases in these parameters after mevalonate infusion represent a real phenomenon that could be due to the stimulatory effects of newly synthesized cholesterol on cholesterol 7α-hydroxylase (15).

The role of cholesterol in the regulation of cholesterol 7α-hydroxylase is also controversial. Einarsson, Åkerlund, and Björkhem (32) found no correlation between the concentration of free microsomal cholesterol and the activity of cholesterol 7α-hydroxylase. Björkhem et al. (24) provided evidence that substrate saturation and levels of HMG-CoA reductase are not major regulators of cholesterol 7α-hydroxylase activity. In contrast, Straka et al. (33) provided evidence that addition of cholesterol to microsomes in form of liposomes stimulated cholesterol 7α-hydroxylase. In the most recent studies by Pandak et al. (15), cholesterol feeding (2 weeks) has been shown to result in a marked increase of cholesterol 7α-hydroxylase activity, mass, mRNA, and in vitro transcriptional activity, suggesting that cholesterol regulates cholesterol 7α-hydroxylase at the level of gene transcription.

A surprising finding in the present study was that infusion of mevalonolactone, an intermediate in the cholesterol biosynthesis pathway which is eventually converted to cholesterol, led to a marked decrease in cholesterol 7α-hydroxylase transcriptional activity in the face of no change in the steady-state mRNA levels (Figs. 4 and 6). The most likely explanation for this unexpected finding is that mevalonate and/or its metabolites may be capable of down-regulating cholesterol 7α-hydroxylase transcriptional activity, while stabilizing mRNA levels. Currently, very little is known about factors that control cholesterol 7α-hydroxylase mRNA stabilization and turnover. Accurate determination of transcriptional activity requires the comparison of the newly transcribed mRNA to a standard, i.e., albumin in this study. Therefore, one possible explanation for the suppressed transcriptional activity after mevalonolactone infusion is that albumin transcription might have been altered by creation of bile fistula, infusion of mevalonolactone, or both. In order to exclude this possibility, we used cyclophilin as another standard. Cyclophilin is a nonspecific cytosolic protein that binds cyclosporine. It was previously used as a reliable standard by others (21). As we have shown in this study, cyclophilin and albumin were not altered under any of the experimental conditions, strongly suggesting that the effects of mevalonate on cholesterol 7α-hydroxylase are not artifactual (Figs. 5B and C). In addition, preliminary findings of a separate study demonstrate similar decreases in cholesterol 7α-hydroxylase transcription rates in the presence of increased specific activity, protein mass, and mRNA levels in rats with intact enterohepatic circulations after intravenous mevalonolactone infusion (M. P. Jones, W. M. Pandak, P. B. Hylemon, J. Y. L. Chiang, and Z. R. Vlahcevic, unpublished results). It is therefore plausible that mevalonate, and/or its nonsterol or sterol metabolites are involved in both the transcriptional and post-transcriptional control of cholesterol 7α-hydroxylase. A precedent for this hypothesis can be found in the regulatory scheme proposed for HMG-CoA reductase, which appears to be regulated by both sterol and nonsterol metabolites of mevalonate at the transcriptional and post-transcriptional levels (34). The physiologic significance of this mechanism of control of cholesterol 7α-hydroxylase is uncertain. One could speculate that, in the presence of excess supply of mevalonate and/or its metabolites, a decrease in HMG-CoA reductase activity coupled with stabilization of cholesterol 7α-hydroxylase mRNA levels could represent an efficient way of maintaining hepatic cholesterol homeostasis.

The infusion of mevalonolactone alone to chronic biliary diverted rats was unable to further up-regulate

cholesterol 7α-hydroxylase specific activity and mRNA levels. This finding is in contrast to that observed by Sundseth and Waxman (35) in rats with an intact enterohepatic circulation in which continuous infusion of mevalonolactone for 3 h led to a 2.5-fold increase in cholesterol 7α-hydroxylase specific activity and a 3.6-fold increase in mRNA levels. Under similar experimental conditions, we have also demonstrated increases in cholesterol 7α-hydroxylase specific activity and steady-state mRNA levels after mevalonolactone infusion in the intact rat (M. P. Jones, W. M. Pandak, P. B. Hylemon, J. Y. L. Chiang, and Z. R. Vlahcevic, unpublished results). It therefore appears that in the rats with maximally stimulated bile acid synthesis, further up-regulation of cholesterol 7α-hydroxylase specific activity and mRNA levels by mevalonolactone is not possible.

Our data on the down-regulatory effects of continuous infusion of mevalonate on HMG-CoA reductase activity are consistent with the results of in vitro studies (34). It has been previously observed that a nonsterol mevalonate-derived effector is involved in the down-regulation of HMG-CoA reductase (36, 37). Evidence now suggests that the observed down-regulation of the enzyme is the result of diverting a sufficient amount of mevalonate into a nonsterol regulatory product which leads to the accelerated degradation of HMG-CoA reductase (34, 38). In addition to the down-regulation of HMG-CoA reductase induced by mevalonolactone infusion, coinfusion of mevalonolactone with taurocholate appears to have had an additive effect on the down-regulation of HMG-CoA reductase activity suggesting different modes of regulation by the two compounds. This down-regulation of HMG-CoA reductase by taurocholate has been postulated to be the result of increased intestinal cholesterol absorption induced by taurocholate acting at the level of gene transcription (39).

In summary, continuous supply of newly synthesized cholesterol, mevalonate, or metabolic products of mevalonate did not prevent down-regulation of cholesterol 7α-hydroxylase after infusion of taurocholate. This effect is independent of cholesterol availability, and is mediated through a decrease in gene transcriptional activity. Mevalonate and/or mevalonate metabolites appear to exert a regulatory influence on cholesterol 7α-hydroxylase both at the transcriptional and post-transcriptional level. It appears that the control of cholesterol 7α-hydroxylase may be a complex process involving a number of regulators. Further studies are needed to define multiple levels of regulation of cholesterol 7α-hydroxylase by different regulatory compounds.

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