Potency of Individual Bile Acids to Regulate Bile Acid Synthesis and Transport Genes in Primary Human Hepatocyte Cultures

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The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.

ABSTRACT

Bile acids (BAs) are known to regulate their own homeostasis, but the potency of individual bile acids is not known. This study examined the effects of cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA) on expression of BA synthesis and transport genes in human primary hepatocyte cultures. Hepatocytes were treated with the individual BAs at 10, 30, and 100μM for 48 h, and RNA was extracted for real-time PCR analysis. For the classic pathway of BA synthesis, BAs except for UDCA markedly suppressed CYP7A1 (70–95%), the rate-limiting enzyme of bile acid synthesis, but only moderately (35%) down-regulated CYP8B1 at a high concentration of 100μM. BAs had minimal effects on mRNA of two enzymes of the alternative pathway of BA synthesis, namely CYP27A1 and CYP7B1. BAs increased the two major target genes of the farnesoid X receptor (FXR), namely the small heterodimer partner (SHP) by fourfold, and markedly induced fibroblast growth factor 19 (FGF19) over 100-fold. The BA uptake transporter Na+-taurocholate co-transporting polypeptide was unaffected, whereas the efflux transporter bile salt export pump was increased 15-fold and OSTα/β were increased 10–100-fold by BAs. The expression of the organic anion transporting polypeptide 1B3 (OATP1B3; sixfold), ATP-binding cassette (ABC) transporter G5 (ABCG5; sixfold), multidrug associated protein-2 (MRP2; twofold), and MRP3 (threefold) were also increased, albeit to lesser degrees. In general, CDCA was the most potent and effective BA in regulating these genes important for BA homeostasis, whereas DCA and CA were intermediate, LCA the least, and UDCA ineffective.

Key words: Human primary hepatocyte cultures; bile acids; CYP7A1; FGF19; BSEP; OSTα/β

ABBREVIATIONS

CYP Cytochrome P450
FXR farnesoid X receptor
SHP small heterodimer partner
CA cholic acid
CDCA chenodeoxycholic acid
DCA deoxycholic acid
LCA lithocholic acid
UDCA ursodeoxycholic acid
FGF19 fibrosis growth factor 19
NTCP Na+-taurocholate co-transporting polypeptide
OATP1B3 organic anion-transporting polypeptide 1B3
BSEP bile salt export pump
OSTα/β organic solute transporter α/β
MRP multidrug-resistant protein

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Bile acids (BAs) are physiological detergents that regulate bile flow and facilitate the intestinal absorption of lipids, nutrients, and lipid-soluble vitamins (Chiang, 2009). For the BA-biosynthetic enzymes in liver, cholesterol 7α-hydroxylase (CYP7A1) is the rate-limiting enzyme in the classic pathway of BA synthesis. In humans, further hydroxylation at the 12 position of the steroid nucleus by CYP8B1 leads to one of the primary BAs, cholic acid (CA); chenodeoxycholic acid (CDCA) is another primary BA (Vlahcevic et al., 1999). In addition, derivatives of cholesterol, namely the oxysterols, can also be catabolized to BAs through another BA synthetic pathway, often referred to as the alternative pathway. Two critical enzymes responsible for the synthesis of BAs in the alternative pathway are CYP27A1 and CYP7B1 (Chiang, 2009; Russell, 2009; Vlahcevic et al., 1999).

In humans, the primary BAs are CA and CDCA. The two major secondary BAs, namely deoxycholic acid (DCA) and lithocholic acid (LCA), are formed by 7-dehydroxylation of CA and CDCA, respectively, by intestinal bacteria (Zhang and Klaassen, 2010). In addition, derivatives of secondary BAs, namely deoxycholic acid (DCA) and lithocholic acid (LCA), are formed by 7-dehydroxylation of CA and CDCA, respectively, by intestinal bacteria (Zhang and Klaassen, 2010). In addition, derivatives of secondary BAs, namely deoxycholic acid (DCA) and lithocholic acid (LCA), are formed by 7-dehydroxylation of CA and CDCA, respectively, by intestinal bacteria (Zhang and Klaassen, 2010). In addition, derivatives of secondary BAs, namely deoxycholic acid (DCA) and lithocholic acid (LCA), are formed by 7-dehydroxylation of CA and CDCA, respectively, by intestinal bacteria (Zhang and Klaassen, 2010). In addition, derivatives of secondary BAs, namely deoxycholic acid (DCA) and lithocholic acid (LCA), are formed by 7-dehydroxylation of CA and CDCA, respectively, by intestinal bacteria (Zhang and Klaassen, 2010). In addition, derivatives of secondary BAs, namely deoxycholic acid (DCA) and lithocholic acid (LCA), are formed by 7-dehydroxylation of CA and CDCA, respectively, by intestinal bacteria (Zhang and Klaassen, 2010).

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Materials and methods

Human primary hepatocyte cultures. Human primary hepatocyte cultures were obtained from the Liver Tissue Cell Distribution System of the National Institute of Diabetes and Digestive and Kidney Diseases (LTCDS; N01-DK-7-004, HHSN267200700004C, S. Strom, University of Pittsburgh, PA). The hepatocytes were from six donors: HH1850, HH1849, HH1649, HH1479, HH1426, and HH1425. Hepatocytes were maintained in William E medium supplemented with 10% fetal bovine serum, antibiotics, and hepatocyte growth supplements, and cultured in 5% CO2 and 50% humidity for 48 h before addition of BAs.

BA treatments. CA, CDCA, DCA, LCA, and UDCA were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO), and the 10, 30, and 100 mM stock solutions were prepared in dimethyl sulfoxide (DMSO), and added to cultures to the final concentrations of 10, 30, and 100 μM. The BA's concentration selection was based on CDCA (1–100 μM) used in human hepatocytes (Song et al., 2009). The final DMSO concentration in the BA-treated groups and DMSO control group was 0.1%. Forty-eight hours after cell treatment, cells were harvested in RNA-Bee (Tel-Test Inc., Friendswood, TX) and frozen at −80°C prior to experiments.

RNA isolation. Total RNA was isolated using RNA-Bee reagent according to the manufacturer's instructions. RNA quantity was determined by the 260/280 ratio (>1.8) and quality by formaldehyde-agarose gel electrophoresis for visualization of 18S and 28S rRNA bands after ethidium bromide staining. Total RNA was diluted with diethyl pyrocarbonate-treated deionized water to a concentration of 100 ng/μl.

Real-time RT-PCR analysis. Total RNA was purified with RNeasy columns (Qiagen, Valencia, CA) and reverse transcribed with Multiscript reverse transcriptase using a High Capacity RT kit from Applied Biosystems (Foster City, CA). Primers were designed with Primer3 software (version 4), and are listed in Supplementary table 1. The Power SYBR Green Master Mix (Applied Biosystems) was used for real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis. Differences in gene expression between groups were calculated using cycle threshold (Ct) values, which were normalized with glyceraldehyde 3-
**RESULTS**

Human hepatocyte cultures were obtained from six donors over a three-year period. Although there were significant individual variations in response to BA treatments, the trend of alterations was similar. The data obtained from each donor was considered as $n = 1$. For the expression of key genes (CYP7A1, FXR, SHP, FGF19, BSEP, OSTα, and OSTβ), all six donors were examined; for other genes, 3–5 donors were used.

**Effect of Various BAs on BA Synthesis Genes**

The mRNA expression of genes responsible for BA synthesis by the classic (CYP7A1 and CYP8B1) and alternative (CYP27A1 and CYP7B1) pathways is shown in Figures 1 and 2. CA, DCA, CDCA, and LCA dramatically and dose-dependently decreased the expression of CYP7A1, the rate-limiting enzyme for BA biosynthesis (Chiang, 2009). The four BAs dose-dependently decreased CYP7A1 mRNA 75–90%, but UDCA had no effect on CYP7A1 mRNA levels (Fig. 1). CDCA and DCA were more effective than CA and LCA in decreasing CYP7A1 at 10μM. In comparison, CYP8B1 mRNA was decreased only at the high concentration of 100μM of CA, CDCA, and DCA (Fig. 2). Regarding genes encoding the alternative pathway of BA biosynthesis (CYP27A1 and CYP7B1), the BAs had little or no effect; only the highest concentration of LCA decreased the mRNA of CYP27A1, and DCA at the highest concentration decreased CYP7B1 (Fig. 2).

**Effect of Various BAs on FXR-SHP and FXR-FGF19 Pathways**

BAs are physiological ligands of FXR, a nuclear receptor (Parks et al., 1999), and TGR5, a membrane receptor (Pols et al., 2011; Sato et al., 2008). The effects of BAs on the mRNA levels of FXR and TGR5 are shown in Figure 3. CA, CDCA, DCA, and LCA increased the mRNA of FXR, but no dose-response was evident. At the highest dose (100μM), the expression of FXR was not induced by the BAs. UDCA was ineffective in increasing the mRNA of FXR. Similarly, BAs, except for UDCA, increased mRNA levels of the BA sensor TGR5 2–3-fold, without clear dose-responses (Fig. 3).

Both FXR-SHP and FXR-FGF19 pathways are major BA homeostasis regulators (Chiang, 2009). In contrast to modest induction of FXR and TGR5, BAs markedly induced small heterodimer partner (SHP) (up to fourfold) and FGF19 (up to 400-fold), with clear dose-responses (Fig. 4). All BAs except UDCA increased the mRNA expression of SHP, and CDCA was the most effective, followed closely by CA and DCA. In comparison, all BAs increased the mRNA of FGF19. For example, CDCA increased FGF19 17-fold at 10μM, and 400-fold at 100μM. Even UDCA increased FGF19 by sixfold at 100μM. The rank orders of FGF19 induction were CDCA $\approx$ CA $>$ DCA $\approx$ LCA $>$ UDCA.

**Effects of BAs on BA Efflux Transporters**

BSEP is a major BA efflux transporter responsible for the efflux of BAs into bile canaliculi, and thus for the prevention of cellu-
FIG. 3. Effects of BAs on the mRNA levels of BA sensors FXR and TGR5. Human primary hepatocyte cultures were treated with individual BAs at concentrations of 10, 30, and 100 μM for 48 h, and the expression of FXR and TGR5 was determined by real-time RT-PCR analysis. Data are mean ± SEM (n = 6). *Significantly different from DMSO-treated controls, p < 0.05.

FIG. 4. Effects of BAs on the mRNA levels of SHP and FGF19. Human primary hepatocyte cultures were treated with individual BAs at concentrations of 10, 30, and 100 μM for 48 h, and the expression of SHP and FGF19 was determined by real-time RT-PCR analysis. Data are mean ± SEM (n = 6).

FIG. 5. Effects of BAs on mRNA levels of BSEP. Human primary hepatocyte cultures were treated with individual BAs at concentrations of 10, 30, and 100 μM for 48 h, and the expression of BSEP was determined by real-time RT-PCR analysis. Data are mean ± SEM (n = 6). *Significantly different from DMSO-treated controls, p < 0.05.

Effects of BA Treatments on OATP1B3 and MRP3 mRNA Expression
Both OATP1B1 and OATP1B3 are responsible for hepatic uptake of xenobiotics (Klaassen and Aleksunes, 2010), and Oatp1b2-null mice are deficient in hepatic uptake of unconjugated BAs (Csanky et al., 2011). In human hepatocytes, OATP1B3 is a transporter of BAs (Briz et al., 2006; Mahagita et al., 2007). In the present study, BAs, except for UDCA, increased mRNA expression of OATP1B3 3–6-fold (Fig. 7). CDCA was the most effective, CA and DCA intermediate, followed by LCA. UDCA did not increase mRNA levels of OATP1B3; instead, it slightly decreased OATP1B3 expression by 25–40%.

MRP3 is a transporter that effluxes BAs from liver into blood (Klaassen and Aleksunes, 2010). In the present study, all BAs increased mRNA expression of MRP3 in a concentration-dependent manner (Fig. 7). Again, CA was the most effective, DCA intermediate, followed by LCA. UDCA did not increase mRNA levels of MRP3; instead, it slightly decreased MRP3 expression by 25–40%.

Effects of BA Treatments on ABCG5, MRP2, and NTCP mRNA Expression
The ABCG5 and ABCG8 genes encode half-transporter proteins that heterodimerize to form a transporter for efflux of plant sterols and BAs (Dieter et al., 2004; Klaassen and Aleksunes, 2010). All BAs except UDCA increased the expression of the efflux transporter ABCG5 by 2–3-fold, and CDCA was the most effective (Fig. 8). The effects of BA treatments on hepatic efflux accumulation and toxicity (Marion et al., 2012; Ogimura et al., 2011). The expression of BSEP was increased 10–20-fold by all BAs except for UDCA (Fig. 5). CDCA was most effective in induction of BSEP at 100 μM, followed by CA. Compared with CDCA and CA, both LCA and DCA were more effective at 30 μM, but were less effective at 100 μM. UDCA was ineffective in induction of BSEP at all concentrations.

OSTα and OSTβ form heterodimers for the efflux of BAs from the liver back into the circulation (Ballatori et al., 2005). Treatment with CDCA, CA, LCA, and DCA increased the mRNA of OSTα 3–10-fold, and CDCA was the most effective (Fig. 6). UDCA was ineffective in induction of OSTα at any concentration. In comparison, CA and DCA at 100 μM markedly increased OSTβ 30-fold, DCA and CA increased OSTβ 40–50-fold, and LCA 13-fold. UDCA at the highest concentration of 100 μM increased OSTβ fourfold (Fig. 6).
FIG. 6. Effects of BAs on mRNA levels of OSTα and OSTβ. Human primary hepatocyte cultures were treated with individual BAs at concentrations of 10, 30, and 100 μM for 48 h, and the expression of OSTα and OSTβ was determined by real-time RT-PCR analysis. Data are mean ± SEM (n = 5).

transporter MRP2 are shown in the middle panel of Figure 8. Only the primary BAs (CA and CDCA) moderately increased mRNA expression of MRP2 at high concentrations (30–100 μM) by about threefold, whereas DCA, LCA, and UDCA were ineffective. NTCP is thought to be the main uptake transporter in the liver for conjugated BAs (Csanaky et al., 2011). The mRNA expression of NTCP was slightly decreased at a high concentration (100 μM) of CDCA and CA, and was not significantly altered by any concentration of DCA, LCA, or UDCA (Fig. 8).

Hierarchical Clustering Dendrogram
Patterns of expression of mRNAs in response to BA treatment of human hepatocytes are shown in Figure 9. The 20 selected genes are important in BA biosynthesis (CYP7A1, CYP8B1, CYP27A1, and CYP7B1), BA homeostasis (FXR, SHP, FGFr19, and TGR5), BA efflux (BSEP, OSTα, OSTβ, and MRP3), and other transporters (OATP1B3, ABCG5, MRP2, and NTCP). BAs are known to decrease BA synthesis and as noted in the top panel of Figure 9, DCA was the most potent and effective BA in decreasing the mRNA of CYP7A1, 27A1, and 8B1. Following DCA in potency and effectiveness was CA, followed by CA and LCA, and UDCA was relatively ineffective. The bottom portion of the figure indicates the mRNA of genes that were increased by BAs. It has been shown previously that BAs increase SHP and FGFr19 (Inagaki et al., 2005), and CDCA was the most potent and effective in increasing these two genes, as well as BSEP, OSTβ, ABCG5, and MRP3. Whereas CDCA was the most potent and effective in increasing the mRNA of these genes, it was followed by DCA and CA, and UDCA was relatively ineffective in altering the expression of any of these genes.

DISCUSSION
The present study demonstrates that treatment of primary human hepatocytes with individual BAs produces significant changes in mRNA expression of genes encoding BA synthesis, BA signaling, and BA transport. The primary purpose of this study is to simultaneously compare five different BAs, i.e., the primary BAs (CA and CDCA) and secondary BAs (DCA and LCA), and the therapeutic UDCA in altering the expression of these genes. Previous studies have shown that using cells derived from a monkey kidney (CV-1), FXR was activated the most by CDCA, and was followed by DCA > LCA > CA (Makishima et al., 1999; Parks et al., 1999).

CYP7A1 is the rate-limiting enzyme of BA synthesis. In the present study, CYP7A1 is markedly repressed by BA treatments of human hepatocytes. The present results (Fig. 1) are consistent with other reports that CDCA reduces CYP7A1 in human primary hepatocytes and in human cell lines (Han and Chiang, 2009, 2010; Li et al., 2006, 2011; Owsley and Chiang, 2003; Song et al., 2007, 2009). What is novel in the present study is that the CDCA reduction of CYP7A1 is simultaneously compared with the ability of CA, DCA, LCA, and UDCA at the same concentrations in six human donor livers to decrease CYP7A1. The results demon-
determined by real-time RT-PCR analysis. Data are mean ± SEM (with increase in BA concentration (Fig. 9), however the decrease in ABCG5, MRP2, and NTCP is ineffective. Thus, individual BAs vary in their capability to decrease the mRNA of CYP7A1.

FIG. 8. Effects of BAs on mRNA levels of ABCG5, MRP2, and NTCP. Human primary hepatocyte cultures were treated with individual BAs at concentrations of 10, 30, and 100 μM for 48 h, and the expression of ABCG5, MRP2, and NTCP was determined by real-time RT-PCR analysis. Data are mean ± SEM (n = 3–4). *Significantly different from DMSO-treated controls, p < 0.05.

strate that CDCA and DCA are very effective in suppression of CYP7A1, whereas CA and LCA have moderate effects, and UDCA is ineffective. Thus, individual BAs vary in their capability to decrease the mRNA of CYP7A1.

Other enzymes involved in BA synthesis, namely CYP27A1 and CYP8B1, were also categorized as mRNAs that decreased with increase in BA concentration (Fig. 9), however the decrease in expression of these two enzymes was much less than CYP7A1.

Whereas BAs can decrease the mRNA of their synthesis genes, they can also enhance the mRNA of some BA transporter genes. The primary transporter responsible for bile salt secretion into bile is the bile salt export pump (BSEP, ABCB11), a member of the ATP-binding cassette (ABC) superfamily, which is located at the bile canalicular domain of hepatocytes (Lam et al., 2010). Several in vitro studies have shown that CDCA up-regulates BSEP mRNA in HepG2 cells (Plas et al., 2002), and in human hepatocytes (Song et al., 2007). The present study extends these studies and demonstrates that BSEP is also induced by CA, DCA, CDCA, and LCA, but not UDCA. BSEP inhibitors increase cellular BA concentrations when exposed to BA mixtures in human and rat hepatocytes, and this inhibition is considered as a mechanism of drug-induced hepatotoxicity (Marion et al., 2012; Oginura et al., 2011). Thus, increases in BSEP in human hepatocytes can be envisioned as an adaptive mechanism to reduce cellular BA burden to avoid BA toxicity.

Ostα/Ostβ heterodimers efflux BAs from the liver back into the blood (Ballatori et al., 2005; Boyer et al., 2006). The mRNA expression of the two genes is increased in patients with PBC, as well as in bile-duct ligated (BDL) rats (Boyer et al., 2006). In FXR-null mice, the mRNA expression of Ostα and Ostβ after BDL did not increase, suggesting that the induction of Ostα/Ostβ by BAs is FXR dependent (Boyer et al., 2006). The present data demonstrate that CDCA is the most effective inducer of Ostα (up to 10-fold) and Ostβ (up to 100-fold), followed by DCA and CA, and LCA being less effective, and UDCA the least effective. Drugs that inhibit BA transport in human hepatocytes also result in BA accumulation in rats, which corresponds to increased hepatotoxicity (Kostrubsky et al., 2003). A better understanding of BA regulation of transporters may aid the development of a therapeutic strategy for cholestasis.

MRP3/ABCC3 is also located on the sinusoidal membrane of hepatocytes, and it transports BAs out of hepatocytes back into the blood. Increased MRP3 is thought to be beneficial for elimination of increased BAs in hepatocytes during cholestasis (Klaassen and Slitt, 2005). Thus, the induction of the major BA efflux transporters (MRP2, MRP3, Ostα/Ostβ) by BAs may help to prevent BA-induced hepatotoxicity.

The major role of ABCG5 is to transport plant sterols out of cells (Dieter et al., 2004; Klaassen and Aleksunes, 2010). Feeding mice 1% CDCA increases hepatic expression of Abcg5/Abcg8 expression twofold (Dieter et al., 2004). Feeding mice BAs is likely to result in an accumulation of cholesterol and oxysterols, which are known activators of liver X receptor (LXR). The activation of LXR by oxysterols is known to up-regulate Abcg5/Abcg8 (Repa et al., 2002). In the present study with human hepatocytes, high concentrations (30–100 μM) of CDCA, CA, DCA, and LCA increased ABCG5 3–5-fold. Therefore, BA-induced increase in ABCG5 mRNA expression may contribute to increased cholesterol efflux out of hepatocytes.

NTCP/SLC10A1 is considered the main transporter for uptake of conjugated BA into hepatocytes (Klaassen and Aleksunes, 2010). Down-regulation of Ntcp mRNA has been observed in several cholestatic animal models, including BDL rats (decreased 60%) (Slitt et al., 2007). However, whether this is due directly to BAs or hepatotoxicity is not known. In the present study, BAs have no appreciable effects on Ntcp mRNA expression in cultured human hepatocytes. Only CDCA and CA at the highest concentration decreased Ntcp mRNA levels.

The nuclear BA receptor FXR plays a central role in the feedback repression of CYP7A1 through the FXR-SHP (Chiang, 2009) and the FXR-FGF19 signaling pathways (Holt et al., 2003; Song et al., 2009), and is thought also to be important in the up-regulation of BA transporters. In the present study, the FXR target gene SHP was most effectively induced by CDCA, intermediate by CA and DCA, least by LCA, and not by UDCA. The ability of BAs to induce FGF19, the other major target gene of FXR, was led by CDCA and CA as the most effective, DCA and LCA intermediate, and DCA had no effect. Thus, the ability of individual BAs to activate FXR in cultured liver cells is quite different than reported previously using a kidney cell line where LCA was the second most effective BA to activate FXR, and CA was least effective (Makishima et al., 1999; Parks et al., 1999). This difference might be due to marked differences in the transport of BAs by the liver and kidney. It should be noted that the magnitude of FGF19 mRNA induction (up to 400-fold) is much higher than for SHP (fourfold). It was first shown that mouse Fgf15 (human homolog FGF19) was synthesized by the terminal small intestine and delivered to the liver to decrease BA synthesis (Inagaki et al., 2005). However, it was later shown (Song et al., 2009) that human hepatocytes synthesize FGF19, whereas it appears mouse liver does not synthesize Fgf15. In mice, it is thought that Fgf15 is more important than SHP down-regulation of BA synthesis (Kong et al., 2012; Song et al., 2009).
This study demonstrated that human primary hepatocyte cultures are a good model to study the potency of individual BA to regulate gene expression in human hepatocytes. The results obtained, together with our recent in vivo study in rats using these five individual BAs (Song et al., 2011), would greatly add to our understanding of physiological and toxicological effects of these five individual BAs, especially for CDCA, a primary BA with most potent effects on human hepatocytes (Song et al., 2009).

In summary, both the FXR-SHP and FXR-FGF19 signaling pathways are activated in cultured human hepatocytes by treatments with primary and secondary BAs. The classic pathway of (CYP7A1) BA synthesis is depressed more than the alternative pathway (CYP27A1). BA treatments dramatically activated the FXR-FGF19 pathway (100-fold), along with activation of the FXR-SHP pathway (fourfold). Notably, BA treatment of human hepatocytes also increased BA efflux transporters, namely BSEP, OSTa, and OSTb. Among the BAs investigated, in general, CDCA is the most potent and effective, DCA and CA intermediate, LCA the least, whereas UDCA is generally ineffective in modulating these genes that regulate BA homeostasis.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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