Cellular Accumulation and Toxic Effects of Bile Acids in Cyclosporine A-Treated HepaRG Hepatocytes

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ABSTRACT

Alteration of bile acid (BA) profiles and secretion by cholestatic drugs represents a major clinical issue. Species differences exist in BA composition, synthesis, and regulation; however presently, there is no in vitro reproducible cell model to perform studies on BAs in humans. We have evaluated the capacity of the human HepaRG cell line to synthesize, conjugate, and secrete BAs, and analyzed changes in BA content and profile after cyclosporine A (CsA) treatment. Our data show that HepaRG cells produced normal BAs at daily levels comparable, though in different proportions, to those measured in primary human hepatocytes. A 4-h treatment with CsA led to BA accumulation and profile changes associated with occurrence of cholestatic features, while after 24 h BAs were decreased in cell layers and increased in media. The latter effects resulted from reduced function of BA uptake transporter (Na+-taurocholate cotransporting polypeptide), reduced expression of BA metabolizing enzymes, including cytochrome P4507A1, cytochrome P4508B1, and cytochrome P45027A1, and induction of alternative basolateral transporters. Noteworthy, HepaRG cells incubated in a 2% serum-supplemented medium showed dose-dependent accumulation of the cytotoxic BA lithocholic acid in a nonsulfoconjugated form associated with early inhibition of the canalicular transporter MRP2 and sulfotransferase 2A1. In summary, our data bring the first demonstration that an in vitro human liver cell line is able to produce and secrete BAs, and to accumulate endogenous BAs transiently, concomitantly to occurrence of various other cholestatic features following CsA treatment. Retention of the hydrophobic lithocholic acid supports its toxic role in drug-induced cholestasis. Overall, our results argue on the suitability of HepaRG cells for investigating mechanisms involved in the development of the disease.

Key words: cholestasis; primary bile acids; lithocholic acid; human hepatocytes; drug-induced liver injury

Bile acids (BAs) are the major organic solutes of bile. These amphiphilic molecules are involved in many different physiological processes; in particular they facilitate the intestinal absorption of fatsoluble compounds such as lipophilic vitamins and dietary lipids. Under physiological conditions, 70% of the human BA pool is composed of cholic acid (CA) and CA metabolites whereas 30% are constituted by chenodeoxycholic acid (CDCA) (Kullak-Ublick et al., 2004). The liver is the only organ that possesses the 14 enzymes required for de novo synthesis of these 2 primary BAs in humans. BAs are synthesized from cholesterol by well-differentiated and polarized hepatocytes via 2 pathways: the classic pathway which involves the cytochrome P450 enzymes cytochrome P4507A1
endogenous BAs has not been precisely investigated and no information exists regarding changes in endogenous BA profiles caused by cholestatic drugs.

The aims of this study were to profile and characterize endogenous BA synthesis and to analyze dose-dependent changes in BA content induced by the cholestatic drug cyclosporine A (CsA). Our data show the HepaRG cells are able to produce and following CsA treatment, to accumulate BAs.

MATERIALS AND METHODS

Reagents

CsA, methylthiobisoleterazolium (MTT), and 5(6)-carboxy-2',7'-dichlorofluorescein diacetate (CDF) were purchased from Sigma (St. Quentin Fallavier, France). [3H]-Taurocholic acid (TCA) ([3H]-TA) was from Perkin Elmer (Boston, Massachusetts). Specific antibodies against CYP7A1 were provided by Boster Biological Technology (Abingdon, UK), CYP27A1 and CYP8B1 from GeneTex Inc. (Alton Pkwy), sulfotransferase 2A1 (SULT2A1) from Promega (Madison, Wisconsin). Other chemicals were of reagent grade.

Cell Cultures

HepaRG cells were seeded at a density of 2.6 × 10⁴ cells/cm² in Williams’ E medium supplemented with 10% hyClone fetal bovine serum (HFS) (Thermo scientific), 100 IU/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 2 mM glutamine, and 50 μM hydrocortisone hemisuccinate. After 2 weeks, HepaRG cells were shifted to the same medium supplemented with 1.7% dimethyl sulfoxide for 2 more weeks in order to obtain confluent differentiated cultures with maximum functional activities. At this time, these cultures contained around the same number of hepatocyte-like and progenitors/primitive biliary-like cells (Cerec et al., 2007) and were ready to use.

Human hepatocytes were obtained from Biopredic International (St Gregoire, France). They were isolated by collagenase perfusion of histologically normal liver fragments from 2 adult donors (Guguen-Guillouzo et al., 1982). Donor 1 was a caucasian 40-year-old female with liver adenocarcinoma and donor 2 a caucasian 68-year-old male with hepatocarcinoma. Liver samples resected for hepatocyte isolation from both donors were histologically normal, and after seeding hepatocytes exhibited CYP activities in the range of those usually found. Primary cultures were obtained by hepatocyte seeding at a density of 1.5 × 10⁵ cells/cm² onto collagen-precocated plates in Williams’ E medium supplemented with 10% HFS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml insulin, 2 mM glutamine, and 1 μg/ml bovine serum albumin. The medium was discarded 12 h after cell seeding, and cultures were thereafter maintained in the same serum free-medium as for HepaRG cells. Supernatants were collected and media were renewed daily.

Measurement of Endogenous BA Content

Standard solutions. Standard stock solutions were prepared in methanol at a concentration of 1 mg ml⁻¹ and stored in a sealed container at –20°C. The stock solutions were pooled and diluted to obtain mixed calibration BA solutions. Standard solutions were available for 28 BAs ie, tauro ursodeoxycholic acid (TUDCA); tauro hyodeoxycholic acid (THDCA); tauro chenodeoxycholic acid (TCDCA); deoxycholic acid (DCA); tauro deoxycholic

(CYP7A1), cytochrome P4508B1 (CYP8B1), and mitochondrial cytochrome P45027A1 (CYP27A1) to produce ultimately primary BAs, CA, and CDCA. The alternative pathway is initiated by CYP27A1 and involves CYP7B1 and CYP39A1 leading predominantly to CDCA synthesis (Alnouti, 2009; Pikuleva and Waterman, 2013). Subsequently, BAs are conjugated with glycine and taurine, and may also be metabolized by different liver enzymes such as cytochrome P450s, glucuronosyl-and sulfo-transferases (Boyer, 2013; Chiang, 2009). BAs are excreted into bile canaliculi, in the human bile they are mostly conjugated with either glycine (75%) or taurine (25%). The most physiologically important canaliculal bile transporter, the bile salt export pump (BEFP), exports monovalent BAs and is responsible for bile salt-dependent flow whereas another canalicular transporter, the multidrug resistance-associated protein (MRP2), secretes divalent bile salts, glutathione, and its conjugates, generating therefore the major part of BA-independent bile flow (Siegel et al., 2007). An alternative efflux system represented by the 2 multidrug resistance-associated proteins (MRP), MRP3 and MRP4, is localized to the basolateral membrane domain and provides an alternative excretory route for bile constituents when their canalicular excretion is impaired (Trauner and Boyer, 2003). Around 95% of BA are recirculated through the so-called enterohepatic circulation (Dawson et al., 2009). Basolateral transporters, Na⁺--taurocholate cotransporting polypeptide (NTCP) and organic anion transporting polypeptides (OATPs), are primarily responsible for active hepatic uptake of BAs from blood (Boyer, 2013).

Intracellular accumulation of potentially toxic endogenous BAs can lead to intracellular cholestasis. In several population-based studies for drug-induced injuries (DILI), a cholestatic pattern has been found in 20%-40% of patients, and a mixed pattern in 12%-20% (Bjornsson and Jonasson, 2013). A major problem of drug-induced cholestasis is its low accurate prediction of risk. Many drugs are recognized to cause idiosyncratic cholestasis (Grattagliano et al., 2009; Morgan et al., 2013).

Because of their various physiological roles and their involvement in pathological processes BAs have been subject to increasing growing interest during the last years. Their use as natural drugs or as the basis of novel semisynthetic drugs is also encouraging (Mudaliar et al., 2013). However, since species differences exist in BA composition, synthesis, and regulation, it is critical to verify results from animal data on a suitable human model (Chiang, 2009). Human hepatocytes in primary culture have the capacity to synthesize, conjugate, and secrete the normal primary BAs, CA, and CDCA into the medium. However, these cells are of more limited life-span in vitro and exhibit large interdonor variability in various functions, including BA production (Ellis and Nilsson, 2010; Guguen-Guillouzo and Guillouzo, 2010).

Hepatocellular carcinoma cell line HepG2 synthesizes some BAs, but the levels are low and they are defective in oxidation of the side chain, conjugation, and transport (Eversen and Polokoff, 1986). The rat hepatoma–human fibroblast hybrid cell line WIF-B9 lacks the ability to further conjugate primary BAs (Monte et al., 2001). Contrary to other human liver cell lines HepaRG cells derived from a cholangiohepatocarcinoma express features of mature hepatocytes and are considered as a surrogate to primary human hepatocytes (PHHs) (Aninat et al., 2006; Antherieu et al., 2010). They are highly polarized cells with specialized apical and sinusoidal domains, are able to transport BAs (Bachour-El Azzi et al., 2015), and exhibit typical cholestatic features in response to treatment with cholestatic drugs (Antherieu et al., 2013; Sharanek et al., 2014). However, the ability of these cells to synthesize and conjugate
acid (TDCA); TCA; lithocholic acid (LCA); tauro lithocholic acid (TLCA)-3S; glycodeoxycholic acid (GCDCA)-3S; ursodeoxycholic acid (UDCA)-3S; CDCA-3S; DCA-3S; LCA-3S; CA-3S; TUDCA-3S; glyco ursodeoxycholic acid (GUDCA)-3S; GLCA; GUDCA; glycochenodeoxycholic acid (GCDCA); glycodeoxycholic acid (GDC); glycocholic acid (GCA); TLCA; UDCA; hyodeoxycholic acid; CDCA; CA; hyocholic acid; and α-muricholic acid. Among the 28 BAs, depending on culture conditions, up to 18 BAs were detected ie, TUDCA; TDCDA; TDCA; TCA; TLCA-3S; GLCA-3S; LCA-3S; GLCA; GUDCA; GCDCA; GDCA; GCA; TLCA; LCA; UDCA; CDCA; DCA; and CA.

Samples preparation and analysis. Both cells and media were collected from HepaRG cell cultures exposed to different serum concentrations for various time points. Similarly, samples from HepaRG cell cultures treated with CsA were also prepared. Before analysis, the samples were lyophilized, and then 1 ml of water was added to the dried samples, homogenized using a Polytron homogenizer for 30 s and clarified by centrifugation at 20000 x g for 20 min. The supernatant was collected and used for chromatography.

Real-Time Quantitative Polymerase Chain Reaction Analysis

Total RNA was extracted from 10^6 HepaRG cells with the SV total RNA isolation system (Promega). RNAs were reverse-transcribed into cDNA and real-time quantitative polymerase chain reaction (RT-qPCR) was performed using a SYBR Green mix. Primer sequences are listed in Supplementary Table S1. Primer sequences are listed in Supplementary Table S1.

Immunolabeling

Cells were washed with warm phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 20 min at 4°C, and then washed 3 times with cold PBS. After paraformaldehyde fixation, cells were permeabilized for 20 min with 0.3% Triton in PBS followed by 1 h incubation in PBS containing 1% bovine serum albumin and 5% normal donkey serum. Cells were then incubated overnight with primary antibodies directed against CYP7A1, CYP27A1, and MRP3, and washed with cold PBS before 2 h incubation with mouse or rabbit Alexa fluor 488 labeled secondary antibodies in the same buffer as described above. After washing with cold PBS, cells were incubated with Hoechst in PBS for 20 min for nuclei labeling. Immunofluorescence images were detected by Cellomics ArrayScan VTI HCS Reader (Thermo Scientific, New Hampshire) (Bachour-El Azzi et al., 2015).

Western Blotting

HepaRG cells and PHH were treated with 10 or 50 μM CsA for 24 or 48 h, washed with cold PBS, and finally resuspended in cell lysis buffer and a protease inhibitor cocktail (Roche, Mannheim, Germany). Aliquots containing equivalent total protein content, as determined by the Bradford procedure with bovine serum albumin as the standard, were subjected to sodium dodecyl sulfate/12% polyacrylamide gel electrophoresis, electrotransferred to Immobilon-P membranes, and incubated overnight with primary antibodies directed against CYP7A1, CYP27A1, and CYP8B1. After using a horseradish peroxidase conjugated anti-mouse/rabbit antibody (ThermoFisher Scientific, Waltham, Massachusetts), membranes were incubated with a chemiluminescence reagent (Millipore, Billerica) and bands were visualized and quantified by densitometry with Fusion-Capt software (Vilber Lourmat, Fusion FX7, France).

Carboxy Dichlorofluorescein Excretion

After 2 h exposure to CsA either in serum-free medium or 2% serum-supplemented medium cells were incubated for 20 min at 37°C with 3 μM 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDFDA), which is hydrolyzed by intracellular esterases to CDF, a substrate of MRP2. Then cells were washed with Williams’ E medium without phenol red and imaging was done using inverted microscope Zeiss Axiovert 200 M and AxioCam MRm (Sharanek et al., 2014).

Na^+–Dependent Taurocholic Cotransporting Polypeptide Activity

Activity of the Na^+-dependent taurocholic cotransporting polypeptide (NTCP) transporter was estimated through determination of sodium-dependent intracellular accumulation of the radio-labeled[^3H]-TA substrate. After treatment with CsA in serum-free medium or 2% serum-supplemented medium, cells were washed with standard buffer and incubated with 43.3 nM of radio-labeled TA for 30 min. Cells were then washed twice with standard buffer and lysed with 0.1N NaOH. Accumulation of radio-labeled substrate was determined through scintillation counting (Antherieu et al., 2013).

Data Analysis

The amount of individual BAs in each medium sample was estimated in pmol/ml (nM) and cell lysate samples were measured in pmol/well and scaled per mg of proteins. Total BA synthesis rate was calculated based on the amount of the BAs measured in cells + supernatant divided by time (in hours) and normalized per 10^6 hepatocytes. To avoid interference of bovine BAs, synthesis rates were calculated in serum-free medium and BAs retained in cells at T0 were subtracted; thus only de novo synthesized BAs were considered.

The basal primitive biliary cells did not express BAs metabolizing enzymes (Fig. 7), as well as BA transporters (Sharanek et al., 2014). Therefore, these cells were neglected for calculation of BA synthesis. Only HepaRG hepatocytes were considered and estimated to represent 50% of total cells in HepaRG cell cultures (Cerec et al., 2007). In the result section, total CA represented the sum of (unconjugated CA + TCA + GCA), whereas total CDCA represented the sum of (unconjugated CDCA + TCDA + GCDCA).
One-way ANOVA with Bonferroni’s multiple comparison test (GraphPad Prism 6.00) was performed to compare data. Each value corresponded to the mean ± standard error of mean (SEM) of at least 3 independent experiments. Data were considered significantly different when $p < 0.05$.

RESULTS

Time-Dependent BA Production by HepaRG Cells in Serum-Supplemented Medium

Previous studies on BA production by liver cells in vitro have frequently been carried out using a serum-supplemented medium. Because bovine serum contains BAs, we first analyzed their quantity and profiles in culture media before any incubation with the cells. BA profiles were determined in culture media supplemented with either 2% (S2) or 8% (S8) HPBS or without serum (SO) used as a control. While BAs were undetectable in SO medium they reached 365.3 ± 25 nM and 1456 ± 75.5 nM in S2 and S8 media, respectively. Since total and individual BA levels were around 4-fold higher in S8 than in S2 medium only the values found in S2 medium are described (Supplementary Fig. S1).

Both primary and secondary BAs were identified and found to represent 58% and 40% of total BAs, respectively. The predominant primary BA, CDCA, and its conjugates formed 48% of total BAs (189.7 ± 34 nM). The other primary BA, CA, and its conjugates, constituted 10% (21.9 ± 4 nM). DCA with its conjugates was the predominant secondary BA, reaching 34% of total BAs (159 ± 30 nM) whereas LCA with its conjugates represented 5% (16.8 ± 3.3 nM). UDCA was barely detected, not exceeding 2%. About 70%–80% BAs were conjugated; tauro- and glyco-conjugates were predominant and accounted for 40% and 32% of total BAs, respectively. Sulfo-conjugates were undetectable (Supplementary Fig. S1 and Table S2).

Since in normal culture conditions, HepaRG cells were incubated in the presence of serum (S8 medium) for 30 days, we first analyzed BA content retained in the cell layer during this culture period. At T0 (differentiated HepaRG cultures at day 30 of incubation in S8 medium), cells were rapidly washed with PBS and BA content was measured in cell extracts; it represented 40 ± 3 pmol/mg proteins. These intracellular BAs likely corresponded to both exogenous (bovine origin) and endogenous BAs (neosynthesis). Then, in a series of experiments HepaRG cells were washed with PBS and incubated in S2 medium for varying periods of time from 0 to 48 h and BA content was analyzed in cell extracts (cells + bile canaliculi) and media (supernatants) (Fig. 1A). Noticeably, total amounts of BAs (cell layer + supernatant per well = 3650 pmol) remained relatively unchanged between 0 and 48 h of incubation in S2 medium (Fig. 1A and B). Moreover, disposition of total BAs between cell layers and supernatants were also stable between 0 and 48 h. Indeed, HepaRG cell layers (cells + bile canaliculi) retained a constant amount of BAs in the range of 5 ± 10 pmol/mg of proteins (Fig. 1B), representing 5%–13% of total BAs (cell layer + supernatant) (Fig. 1C).

Even though total BA content in cells and medium did not change, important variations in BA profiles were evidenced with time in culture. Indeed, secondary BAs decreased gradually to 89 ± 12 nM, 75 ± 14 nM and traces in the medium after 2, 4, and 24 h of incubation, respectively, compared with 179 ± 33 nM in unexposed S2 medium (Fig. 1D). This decrease was due to a rapid decrease in both DCA and LCA content (Fig. 1E and F). In the cells only traces of bovine DCA and LCA representing 8 ± 4.6 and 0.3 ± 0.1 pmol/mg proteins, respectively, were observed after 2 h, and these secondary BAs became undetectable after 4 h of incubation (Fig. 1E and F).

In parallel, CA (TCA + GCA + unconjugated CA) increased in the supernatant to 53.6 ± 4.4, 63.6 ± 14, 98.5 ± 10 and 205 ± 4 nM after 2, 4, 24, and 48 h, respectively, compared with 21.9 ± 4 nM in nonincubated S2 medium (Fig. 2A). At T0 TCA was barely detectable (0.6 ± 0.3 nM) in S2 medium and present in low amounts in the cells (15.7 ± 1.3 pmol/mg of proteins). A fast and gradual increase of TCA to 38.4 ± 5, 88 ± 6, and 149 ± 5 nM occurred after 2, 24, and 48 h, respectively (Fig. 2B). No accumulation of TCA and GCA was detected in cell layers, indicating a rapid clearance of these BAs after synthesis. CDCA (TCDCA + GCDCA + unconjugated CDCA) was predominant at T0 in both media and cells, reaching 189.7 ± 34 nM and 243 ± 4.5 pmol/mg of proteins, respectively; it did not show any significant quantitative change in either cell layers or supernatants between 0 and 48 h (Fig. 2C). The increase in CA with no change in CDCA reflected a gradual increase in the CA/CDCA ratio; ie, 0.3, 0.4, 0.6, and 1.4 after 2, 4, 24, and 48 h, respectively, compared with a very low ratio (0.09) in nonexposed S2 medium (Fig. 2D).

Importantly, exogenous bovine BAs present in S2 medium underwent de novo tauro- and glyco-amination after exposure to HepaRG cells. Indeed, unconjugated BAs were undetectable after a 4 h exposure to HepaRG cells compared with 28% of total BAs in nonexposed medium (Fig. 2E) (Supplementary Table S2). In addition, sulfo-conjugates which were not detectable in S2 medium before incubation became detectable after 4 h of incubation.

Time-Dependent BA Production by HepaRG Cells in Serum-Free Medium

To better characterize their capacity to synthesize and conjugate BAs in the absence of serum, HepaRG cells were incubated in SO medium for various periods of time from 0 to 72 h without medium renewal, and BA content was analyzed in both cellular extracts and supernatants. Total BA amounts remained relatively unchanged in cell layers, representing 40 ± 4.6 pmol/mg of proteins between 0 and 48 h while it increased to 71 ± 6 pmol/mg of proteins after 72 h. Interestingly, BA content increased cumulatively in the medium reaching 54 ± 8, 80 ± 13, 111 ± 9.2, 276 ± 49, and 410 ± 1.5 nM after 2, 4, 24, 48, and 72 h, respectively, thereby reflecting the capacity of HepaRG cells to synthesize and secrete BAs (Fig. 3A). Only primary BAs were identified. CA (TCA + GCA + unconjugated CA) gradually increased in the medium from 28.5 ± 3 nM after 2 h to 150 ± 1.2 nM after 72 h (Fig. 3B). CDCA (TCDCA + GCDCA + unconjugated CA) increased to 25 ± 8 nM after 2 h and then remained relatively unchanged up to 24 h. However, later on an important increase was observed, reaching 162 ± 3 and 271 ± 1.8 nM after 48 and 72 h, respectively (Fig. 3C).

Total BAs synthesis rate was calculated per 106 HepaRG hepatocytes per hour after subtracting the initial amount of BAs retained in the cells at T0. Total BAs synthesis rate was high after 2 h (108 ± 16 pmol/106 hepatocytes/hour) and started to decrease after 4 h (85 ± 17 pmol/106 hepatocytes/hour) to become stable after 24, 48, and 72 h at 18.5 ± 3 pmol/106 hepatocytes/hour (Fig. 3E). Interestingly, the newly synthesized BAs were mostly found in their conjugated form: TCA and GCA for CA and GCDCA and TCDCA for CDCA. The prominent form of

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CA was TCA (70%–90% of total CA), whereas TCDCA and GCDCA were synthesized in equal percentages after the first 24 h. Then, the latter was the prominent form (70%–80% of total CDCA) after 48 and 72 h (Fig. 3D and F).

Comparative Time-Dependent BA Production by HepaRG Cells and PHHs

To analyze the synthetic capacity of BAs in HepaRG cells compared with PHH obtained from 2 donors; both cellular models
were incubated in S0 medium. BA profiles were analyzed every 24 h for 3–4 days and normalized per million hepatocytes. Three-fold differences were observed in total BAs produced by the 2 human hepatocyte populations, representing 167 and 456 pmol/10⁶ PHH for donors 1 and 2, respectively, at day 1 (Fig. 4A).

Interestingly, total BA production by HepaRG hepatocytes showed high interassay reproducibility and was in the same range as in PHH cultures with 316 ± 32 pmol/10⁶ hepatocytes/day. No major variation in the amount of synthesized BAs/10⁶ hepatocytes was then observed between days 1 and 3 or 4 of culture in the 2 models (Fig. 4A and B).
However, some differences in BA profiles were observed. CDCA represented 65% versus 35% for CA in HepaRG cell cultures whereas, by contrast, CA was the prominent BA produced by PHH representing 60%–80% versus 20%–40% for CDCA (Fig. 4C and D). Moreover, tauro- and glyco-conjugates represented 80% and 20% of total BAs, respectively, in HepaRG cells at day 1, and became in equal proportion at day 3. In PHH, tauro-conjugates that constituted 16% (donor 1) or 32% (donor 2) of total conjugates at day 1 were nearly undetectable at day 2; glyco-conjugates became paramount representing around

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**FIG. 3.** BA production and profiles in HepaRG cell cultures incubated in serum-free medium between 0 and 72 h without medium renewal. A, Total BA production. B, CA (TCA + GCA + unconjugated CA) production. C, CDCA (CDCA + GCDCA + unconjugated CDCA) production. D, TCA and GCA production. E, Total BA synthesis rate calculated as pmoles/10⁶ hepatocytes/hour. F, TCDCA and GCDCA production. Data are expressed as pmoles/mg of proteins (cell layers) or nM (supernatants). Values represent the mean ± SEM of duplicate measurements in 3 independent experiments. *p < .05 compared with the value at time 0 (T0) in media. #p < .05 compared with the value at time 0 (T0) in cells.
95% of the BA pool. Sulfo-conjugates did not exceed 5% (Fig. 4E and F).

**Effect of CsA on BA Profiles in HepaRG Cells**

BAs profiles were also analyzed after treatment of HepaRG cells with 10 and 50 μM CsA for 4 and 24 h in S0 and S2 media. Interestingly, a dose-dependent accumulation of total BAs in cell layers peaking at 44 ± 3 and 98 ± 15 pmol/mg of proteins was observed with 10 and 50 μM CsA after 4 h of treatment in S0 medium, respectively, compared with 10 ± 1.8 pmol/mg of proteins in untreated HepaRG cells (Fig. 5A). These BAs corresponded to a dose-dependent accumulation of conjugates of primary BAs, ie, 5.8- and 16-fold for TCA, 2.25- and 2.5-fold for TCDCA, and 3.2- and 5.7-fold GCDCA after treatment with 10 and 50 μM CsA, respectively. A parallel dose-dependent decrease in these individual BAs was found in the medium. Importantly, total BA amounts (supernatant + cells) remained nearly unchanged whatever the concentration of CsA after 4 h (Fig. 5A).

By contrast, intracellular BA accumulation was no longer observed after a 24 h CsA treatment; even more a dose-dependent decrease of total BAs in cell layers and a parallel increase in the supernatants were found. Indeed, after 24 h CsA at 50 μM caused a decrease in the total amount of BAs, particularly CA (TCA) and GCDCA (TCDCA in media and GCDCA in cells), reflecting an inhibition of synthesis of these primary BAs by CsA at high concentration (Fig. 5).

We further looked for whether bovine serum affected BA profiles in CsA-treated HepaRG cells. Surprisingly, unlike in cultures incubated in S0 medium a 4-h treatment did not cause BA intracellular accumulation in HepaRG cells cultured in S2.
FIG. 5. CsA effects on total BA disposition and profiles in HepaRG cell cultures incubated in serum-free medium. BAs were measured in medium (nM) and cells (pmol/mg of proteins) from cultures treated with 10 or 50μM CsA for 4 and 24 h and in corresponding untreated HepaRG cells and represented in (A) Total BAs, (B) TCA, (C) GCA, (D) GCDCDA, and (E) TCDCA. Data were normalized relative to the amount of proteins in each condition. Values represent the sum of mean ± SEM of duplicate measurements in 3 independent experiments, *p < 0.05 compared with values in supernatant of untreated cultures, #p < 0.05 compared with the values in cell layer of untreated cultures.
medium (Fig. 6A). However, marked changes were evidenced after a 24-h treatment; as in cultures incubated in S0 medium CsA caused a dose-dependent intracellular reduction and supernatant increase in total BA content. In addition, the proportion of primary/secondary BAs was modulated. At 50µM CsA prevented decrease of secondary BAs and increase of primary BAs as observed in corresponding untreated cultures between 0 and 24 h (Fig. 6B). Similarly, as observed in S0 cultures, neosynthesis of CA was inhibited by a 24-h treatment with 50µM CsA (Fig. 6C). Importantly, in 24-h CsA-treated cells DCA accumulated in the medium, reaching 28 ± 7.5 and 61 ± 9 nM with 10 and 50µM CsA, respectively, compared with 16 ± 4 nM in

FIG. 6. CsA effects on total BA disposition and profiles in HepaRG cells incubated in 2% bovine serum-supplemented medium. BAs were measured in medium (nM) and cells (pmol/mg of proteins) from cultures treated with 10 or 50µM CsA for 4 and 24 h and in corresponding untreated HepaRG cells and represented in (A) total BAs, (B) total secondary BAs, (C) CA, (D) DCA, and (E) LCA. Data were normalized relative to the amount of proteins in each condition. Values represent the sum of means ± SEM of duplicate measurements in 3 independent experiments, *p < .05 compared with values in supernatant of untreated cultures, #p < .05 compared with the values in cell layer of untreated cultures.
corresponding untreated cultures (Fig. 6D). LCA was only found in its unsulfated form after CsA treatment and accumulated in cell layers after 4 h (3\(\times\)1.8 and 10\(\times\)2 pmoles/mg of proteins), and after 24-h treatment (6.3\(\times\)0.8 and 20\(\times\)2.5 pmoles/mg of proteins) with 10 and 50 \(\mu\)M CsA, respectively, compared with undetectable amount in untreated cells either at 4 or 24 h (Fig. 6E).

**Effect of CsA on Metabolism, Conjugation, and Transport of BAs**

mRNA expression of genes responsible for BA synthesis by the classic (CYP7A1 and CYP8B1) and alternative (CYP27A1) pathways was investigated after treatment of HepaRG cells with 10 and 50 \(\mu\)M CsA for 4 and 24 h using RT-qPCR analysis. While after 4 h, CsA treatment had no significant effect on the expression of any of these enzymes, a 24-h treatment with 50 \(\mu\)M CsA inhibited CYP7A1 and CYP27A1 expression by 62% and 63%, respectively, and dramatically CYP8B1 expression by 96% (Supplementary Table S3).

Western blotting showed that CsA induced a dose-dependent decrease in CYP7A1, CYP27A1, and CYP8B1 protein content in HepaRG cells, slightly after 24 h and more intensely after 48 h (Supplementary Fig. S2). The 2 basolateral BA transporters, MRP3 and MRP4, were found to be deregulated after CsA treatment. Indeed, while after 4 h at either concentration CsA treatment did not show any significant change in gene expression of MRP3 and MRP4 after 24 h 10 and 50 \(\mu\)M CsA induced mRNA expression of MRP3 to 1.56\(\pm\)0.06- and 1.9\(\pm\)0.13-fold, respectively, and MRP4 to 1.71\(\pm\)0.14- and 2.9\(\pm\)0.2-fold, respectively. Interestingly, immunolocalization of MRP3 showed very intense labeling to the basolateral membrane of HepaRG cells after 4 and 24-h treatment with 10 and 50 \(\mu\)M CsA compared with the low intensity in their untreated counterparts (Fig. 7A).

In addition, using CDFDA, MRP2 activity was analyzed after 10 and 50 \(\mu\)M CsA treatment in S0 and S2 media. Fluorescent CDF was visualized in bile canaliculi of untreated HepaRG cells. However, 10 and 50 \(\mu\)M CsA inhibited canalicular excretion of CDF as early as 30 min, in both S0 and S2 media (Fig. 7B).

Gene expression of the 2 phase II conjugating enzymes, BA-CoA:amino acid N-acyltransferase (BAAT) and SULT2A1, was also analyzed. After 4 h only 50 \(\mu\)M CsA significantly inhibited expression of BAAT and SULT2A1 by 36% and 31%, respectively (Supplementary Table S3). A 24-h treatment with 10 and 50 \(\mu\)M CsA decreased BAAT expression by 42% and 84% and SULT2A1 by 34% and 39%, respectively. Noticeably, none of these tested genes was expressed in primitive biliary-like HepaRG cells at either mRNA or protein levels (Fig. 7C).

**FIG. 7.** Effects of CsA on MRP3 immunolabeling and MRP2 activity, and immunolabeling of some major BA metabolizing enzymes in HepaRG cell cultures. A, Representative immunofluorescence images of MRP3 localization in control and 10 and 50 \(\mu\)M CsA-treated HepaRG cells, for 4 and 24 h. B, HepaRG cells were treated with 10 or 50 \(\mu\)M CsA in S2 media for 30 and 60 minutes. MRP2 activity was estimated using CDF. C, Distribution of main BA metabolizing enzymes in HepaRG cells. Differentiated HepaRG cells were fixed and incubated with primary antibodies against SULT2A1, CYP7A1, CYP27A1, and CYP8B1. Labeling (white in black and white figure) is restricted to HepaRG hepatocytes (double arrows); no labeling is visible in the primitive biliary-like cell population (single arrow). Nuclei were labeled using Hoechst dye. Immunofluorescence images were obtained with a Cellomics ArrayScan VTI HCS Reader (ThermoScientific).
Cytotoxicity of BAs in HepaRG Cells

To better estimate the potential toxicity of BAs in CsA-treated cultures, cytotoxicity of individual BAs was evaluated using the MTT assay in the presence or absence of 10 or 50 μM CsA. A 24-h treatment with CA elicited no toxicity at up to 1 mM either alone or combined with 10 or 50 μM CsA. Similarly, no toxicity was observed with LCA up to 100 μM in untreated cultures, whereas when added with CsA at 10 and 50 μM, LCA at 50 μM become highly cytotoxic causing 34% and 60% cell death, respectively. DCA was less toxic than LCA causing 27% cell death only at 400 μM; however, this percentage rose to 50% and 68% when added with 10 and 50 μM CsA, respectively (Fig. 8).

DISCUSSION

Drug-induced intrahepatic cholestasis is characterized by intracellular hepatic accumulation of endogenous BAs which can cause toxicity through their detergent effects on cellular membranes, mitochondrial dysfunction, and ultimately cellular apoptosis or necrosis (Pauli-Magnus et al., 2005). Up to now, investigations on BA metabolism and homeostasis disturbances in humans have been hampered by the lack of a reproducible and easy to use in vitro human cell model. Furthermore, in vitro intracellular accumulation of endogenous BAs following treatment with cholestatic drugs has not been demonstrated. In this study, we show that the human HepaRG cell line synthesizes normal conjugated BAs, though at proportions different from human liver and human hepatocytes in primary culture, and that intracellular accumulation of BAs can be demonstrated following treatment with the cholestatic drug CsA. Contrary to HepG2 and other cell lines, PHH produce and excrete normal primary BAs but with a large up to 12-fold interdonor variability (Axelson et al., 2000; Ellis and Nilsson, 2010). In agreement, a 3-fold variation was found between the 2 human hepatocyte populations used in this study, demonstrating that HepaRG cells have a similar capacity to produce BA as PHH with a high interassay reproducibility.

Total BA production and profiles can greatly vary with experimental culture conditions. Previous studies have frequently been performed using either serum-supplemented or serum-free medium following previous incubation of liver cells in serum-supplemented medium. Although bovine serum is known to contain both primary and secondary BAs little information exists on their possible contamination of intracellular and medium pools of BAs and their influence on BA neosynthesis by liver cells after several hours of incubation. Our data showed that secondary BAs were detectable in HepaRG cell layers during the first hours and longer in the supernatants but become only barely detectable after 24 h, supporting our observation that no secondary BAs were identified in cultures...
incubated in serum-free medium when they were prepared from cells previously maintained for 48 h in serum-supplemented medium. Our data support and explain previous reports showing the presence of LCA or unidentified BAs in liver cell cultures incubated in serum-supplemented media (Axelson et al., 1991; Hoekstra et al., 2013).

Differences in synthesis of individual BAs were observed in HepaRG cells incubated in serum-supplemented and serum-free medium. Although both primary BAs CA and CDCA were found to be synthesized by HepaRG cells incubated in serum-free medium, only CA was increased in serum-supplemented medium; the lack of increase in CDCA could be explained by a feedback inhibition of its synthesis in the presence of high exogenous bovine CDCA. In support, CDCA was previously reported to be the strongest inhibitor of BA synthesis in PHH cultures (Ellis et al., 2003; Liu et al., 2014). Moreover, different time-dependent kinetics in total BA production were observed in HepaRG cells. While BA release in the medium was high during the first 4 h after medium renewal, it showed a lower and stable rate of synthesis between 24 and 72 h. Whether such a transient enhanced production of BAs during the first 4 h resulted from the absence of BAs in the medium after its renewal, and consequently an absence of feedback regulation, deserves further investigation.

The continuous BA production during a 72-h period following daily medium renewal supported in vitro maintenance of BA homeostasis and agreed with previous observations showing that differentiated HepaRG hepatocytes remain functionally relatively stable for many days at confluence (Josse et al., 2008). A similar observation was made with conventional primary hepatocytes. By contrast, a continuous time-dependent increase in PHH has been reported by other authors (Ellis et al., 1998). These discrepancies could be at least partly explained by the use of different experimental conditions. Indeed, it has been shown that addition of dexamethasone and the composition of the substratum can greatly influence the levels of BA production as well as the CA/CDCA ratio in PHH cultures. Noteworthy, the CA/CDCA ratio has been shown to vary from 1 to 0.2 (Ellis and Nilsson, 2010). Interdonor differences in BA synthesis capacity and time-dependent alterations in BA synthesizing enzyme activities could also explain such variations.

Like PHH, HepaRG cells were found to conjugate BAs with taurine and glycine. However, while glyco-conjugates increased from 80% to 95% between days 1 and 2 in PHH they did not exceed 50% in HepaRG cells. The higher percentage of tauro-conjugates in these latter could be explained by their tumor origin and proliferative potential in vitro. Indeed, if HepaRG cells express 81% to 92% of the genes active in PHH they also express an additional set of around 2900 genes usually expressed in cancerous and stem cells or related to the cell cycle (Rogue et al., 2012).

Daily production of BAs in the human liver is estimated to be around 0.35 mg/g liver (Ellis et al., 2003), corresponding to about 6000 pmol/g liver (Sohlensius-Sternbeck, 2006). Calculation of in vitro BA daily production led to 316 pmol/10^6 HepaRG hepatocytes and 167 (donor 1) or 456 (donor 2) pmol/10^6 PHH in HepaRG and PHH cultures, respectively, indicating that BA production in human liver is around 13-36-fold higher than in hepatocyte cultures. Our data are in agreement with previous studies on PHH in which about 220 and 1150 pmol/10^6 hepatocytes were produced daily during the first 2 days and after 4 days, respectively (Ellis et al., 1998). This demonstrated that cells maintained in vitro retained an active BA synthesis capacity.

To our best knowledge no study has reported yet intracellular accumulation of endogenous BAs in PHH cultures following treatment with a cholestatic drug (Enarsson et al., 2000; Marion et al., 2012). Recently, we reported occurrence of cholestatic features typified by early inhibition of efflux (BSEP) and uptake (NTCP) transporters associated with disruption of pericanalicular cytoskeletal F-actin distribution and constriction of bile canalicular structures within a 4-h treatment with CsA (Sharanek et al., 2014). Our present data clearly demonstrate that these CsA-induced cholestatic effects were associated with a dose-dependent intracellular accumulation of BAs when HepaRG cells were incubated in serum-free medium. However, intracellular accumulation of endogenous BAs was no longer evidenced after 24 h; even more a decrease in cell layers and parallel increase in the supernatant were observed. These major changes could have several nonexclusive explanations: first, CsA inhibited efflux and uptake of BAs (Sharanek et al., 2014); second, CsA is known as an inhibitor of BA synthesis by repressing transcription of the key genes involved in the first steps of BA synthesis, e.g., CYP7A1 and CYP27A1 (Axelson et al., 2000; Princen et al., 1991); accordingly, our data show that these 2 last genes as well as others, such as BAAT and SULT2A1, involved in conjugation activity, were strongly inhibited after a 24-h CsA treatment; third, the basolateral transporters MRP3 and MRP4 were increased supporting their role in BA excretion and therefore, their compensatory activity to supply canalicular transporters as previously suggested (Wagner et al., 2009; Zoliner et al., 2006).

Importantly, despite occurrence of all other cholestatic features total BAs did not accumulate in CsA-treated HepaRG cell cultures incubated in serum-supplemented medium, suggesting a fast induction of compensatory mechanisms in the presence of exogenous bovine BAs that could limit intracellular increase of BAs after CsA addition. Several nonexclusive arguments support such hypothesis. First, CsA-induced constriction of bile canalicular occurred around 1.5 h earlier in serum-supplemented than in serum-free cultures. Second, MRP3 was found to be highly localized to the basolateral membrane in the presence of serum compared with cultures deprived of serum (not shown). Third, inhibition of NTCP activity after CsA treatment occurred faster than in serum-free medium (Supplementary Fig. S3). Fourth, endogenous BA synthesis was likely inhibited by exogenous BAs present in serum-supplemented cultures. Altogether, these observations support a lower BA accumulation in CsA-treated HepaRG cell cultures in serum-supplemented medium.

Noteworthy, a dose-dependent accumulation of LCA present in S2 medium was demonstrated in cell layers after 4 and 24 h of incubation with CsA. LCA is normally sulfated conjugated before secretion in bile canalicular by MRP2 (Hofmann, 2004). Rapid inhibition of MRP2 and constriction of bile canalicular, as well as inhibition of SULT2A1 and sulfate esterification of LCA by CsA combined with the fact that sulfated BAs are better substrates for the basolateral transporters than unsulfated BAs (Alnouti, 2009; Hirohashi et al., 2000; Zelcer et al., 2003) could explain its intracellular accumulation in HepaRG cells treated with CsA, especially at 50 μM. However, since LCA is a metabolic substrate for CYP3A4 hydroxylation (Xie et al., 2001), its accumulation resulting from CYP3A4 inhibition by CsA (Amundsen et al., 2012) cannot be excluded. We also showed that LCA was the most hepatotoxic BA and that CsA strongly aggravated its toxicity after 1 addition at 50 μM or higher concentrations. If such toxic LCA concentrations cannot be achieved in vivo (Woolbright et al., 2015), it is noteworthy that lower LCA concentrations (≈10 μM)
were cytotoxic after 5 repeated daily CsA additions (data not shown). All these data lead to postulate that CsA-induced cholestasis and cytotoxicity could be, at least partly, mediated by LCA accumulation into liver cells. Noticeably, serum LCA was found to be dramatically elevated in cyclosporine-treated patients with hepatitis (Myara et al., 1996) and in intrahepatic cholestasis of pregnancy (Lucangioli et al., 2009) supporting the clinical relevance of our in vitro findings.

In summary, we demonstrated for the first time that a cholestatic drug could induce intracellular accumulation of BAs in human HepaRG hepatocytes, and that after a single treatment this effect was transient. Although CsA inhibited BA uptake and canalicular efflux as well as BA synthesis the cells remained able to evacuate accumulated intracellular BAs via their basolateral transporters. Moreover, our data favor the conclusion that HepaRG cells represent a unique reproducible cell model to analyze regulation of BAs and mechanisms involved in BA accumulation with cholestatic drugs after short and repeated treatments.

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

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