Bile acids (BAs) are natural detergents involved in cholesterol homeostasis. These acids are formed from cholesterol in the liver, and their synthesis represents an important pathway for cholesterol elimination from the body. In humans, the BA pool is composed mainly of the primary cholic acid (CA) and chenodeoxycholic acid (CDCA), the secondary lithocholic acid and deoxycholic acid (DCA), and the 6α-hydroxylated hyocholic acid (HCA) and hyodeoxycholic acid (HDCA). BAs sustain a strong enterohepatic recirculation, through which they are excreted from the liver into the bile, stored in the gallbladder, and secreted in the intestine, where they serve as natural detergents for dietary lipid absorption. Approximately 95% of BAs are absorbed in the intestine and return to the liver through the portal vein. BAs are cytotoxic at high concentrations, and their accumulation in liver cells leads to oxidative stress, apoptosis, and subsequent damage to the liver parenchyma. Such features are characteristic of cholestatic phenomena, in which a reduction of the bile flow limits BA elimination from hepatocytes. A reduction of BA hepatic levels is therefore an important goal for anticholestatic strategies, particularly for the treatment of chronic conditions such as primary biliary cirrhosis (PBC) or primary sclerosing cholangitis, two autoimmune hepatobiliary diseases.

Glucuronidation, catalyzed by uridine 5′-diphospho-glucuronosyltransferase (UGT) enzymes, detoxifies cholestatic bile acids (BAs). We aimed to (i) characterize the circulating BA-glucuronide (BA-G) pool composition in humans, (ii) determine how sex and UGT polymorphisms influence this composition, and (iii) analyze the effects of the lipid-lowering drug fenofibrate on the circulating BA-G profile in 300 volunteers and 5 cholestatic patients. Eleven BA-Gs were determined in pre- and postfenofibrate samples. Men exhibited higher BA-G concentrations, and various genotype/BA-G associations were discovered in relevant UGT genes. The chenodeoxycholic acid-3G (CDCA-3G) concentration was associated with the UGT2B7 802C>T polymorphism. Glucuronidation assays confirmed the predominant role of UGT2B7 and UGT1A4 in CDCA-3G formation. Fenofibrate exposure increased the serum levels of five BA-G species, including CDCA-3G, and upregulated expression of UGT1A4, but not UGT2B7, in hepatic cells. This study demonstrated that fenofibrate stimulates BA glucuronidation in humans and thus reduces BA toxicity in the liver.
our understanding of their contribution to BA detoxification in clinics is currently limited. Indeed, BA-Gs are rarely investigated in humans, and very little is known about the circulating BA-G pool composition.

Among the newly identified therapeutic approaches for chronic cholestasis, the cholesterol-lowering fibrates (fenofibrate, bezafibrate, and clofibrate) improve liver biochemistries in PBC and patients with primary sclerosing cholangitis.8–13 These beneficial effects were associated with classical fibrate actions on lipoprotein metabolism and anti-inflammatory processes (reviewed in ref. 14). However, as pharmacological activators for the peroxisome proliferator–activated receptor-α (PPAR-α), these drugs also control BA metabolism (reviewed in ref. 15). PPAR-α is a ligand-activated transcription factor controlling the expression of target genes through binding to their regulatory regions as a heterodimer with the retinoid X receptor.15 For example, upon fibrate activation, PPAR-α binds to and stimulates the expression of the genes encoding the two BA-conjugating UGT1A3 and UGT2B4 enzymes in human liver cells and animal models.16–18 On the other hand, we recently observed that fenofibrate reduces circulating BA concentrations in noncholestatic volunteers.2 The current study aimed at investigating the possibility that such a reduction actually reflects an increased BA glucuronidation. For this purpose, the profile of circulating BA-Gs was established and compared in pre- and postfenofibrate sera from 150 male and 150 female volunteers. Similar analyses were also conducted in a pilot study comprising five fenofibrate-treated PBC patients. How the noncholestatic BA-G profile and its response to fenofibrate are affected by gender or nonsynonymous mutations in BA-conjugating UGT genes were also investigated. These analyses evidenced the need for additional in vitro and ex vivo experiments aimed at establishing the relative contribution of human UGTs for the hepatic formation of abundant BA-G species and at measuring the reactivity of their functional variants. Finally, whether PPAR-α also controls the expression of these newly identified BA-conjugating UGTs was investigated in hepatic cells.

RESULTS

Serum profile of BA glucuronides, and its modulation by sex and genetic determinants

The composition of the serum BA glucuronide pool is illustrated in Table 1. The species distribution was HCA-6G>CDCA-3G>HCA-6G>DCA-3G>HCA-24G=CDCA-24G=LCA-3G>LCA-24G=HCA-24G=CDCA-24G=HDCA-24G=CDCA-3G. The hydroxyl-linked glucuronidated acids (i.e., -3G and -6G) represented 96.5% of the circulating BA-G pool; and the four most abundant conjugates (HCA-6G, CDCA-3G, HDCA-6G, and DCA-3G) are the most abundant species; and (iii) serum levels of selected BA-G species are significantly linked to nonsynonymous mutations in genes encoding BA-conjugating UGTs.

UGT1A4 and UGT2B7 catalyze hepatic formation of CDCA-3G

Until now, CDCA-3G was considered a minor CDCA metabolite owing to its limited production observed in vitro with human liver extracts.17 Consequently, the mechanisms governing its formation received little attention.17 In this context, an in vitro screening was performed with human liver microsomes and recombinant UGTs (Figure 2). As previously reported,17 the formation of CDCA-3G was detected in the presence of liver microsomes but at an 8.3-fold lower level than the production of CDCA-24G in the same assay (Figure 2a). Assays with UGT bacculosomes revealed the predominant contribution of the UGT1A4 and UGT2B7 isoforms in producing CDCA-3G (Figure 2b). To determine whether frequent (≥1%) nonsynonymous mutations in the coding region of these enzymes affect their ability to convert CDCA into CDCA-3G,25,26 additional assays were performed with microsomes from HK293 cells stably expressing the wild-type (WT) (R11P241.48) or variant UGT1A4 T24, V48, and W11 proteins, as well as the WT H268 or Y268 UGT2B7 enzymes (Figure 2c–f).25,26 Activity values were adjusted for UGT1A4 or UGT2B7 protein content as determined by western blot (Figure 2c,d).26 All UGT1A4 variants exhibited a similar CDCA-3G formation (Figure 2e), whereas the UGT2B7 Y268 protein was 1.3-fold more active than the H268 allozyme (P < 0.05) (Figure 2f).
Table 1  Gender differences in the baseline profile of serum bile acid glucuronides in the GOLDN population

|                    | Men + women (n = 300) | Men (n = 150) | Women (n = 150) |
|--------------------|-----------------------|---------------|-----------------|
| **Serum levels**   |                       |               |                 |
| CDCA-3G            | 59.6 ± 4.2            | 72.7 ± 7.3    | 46.4 ± 3.9***   |
| CDCA-24G           | 0.8 ± 0.1             | 0.9 ± 0.1     | 0.7 ± 0.1       |
| CA-24G             | 1.9 ± 0.2             | 2.1 ± 0.3     | 1.6 ± 0.1       |
| LCA-3G             | 2.0 ± 0.1             | 2.3 ± 0.2     | 1.6 ± 0.1*      |
| LCA-24G            | 1.2 ± 0.1             | 1.3 ± 0.1     | 1.1 ± 0.1       |
| DCA-3G             | 24.3 ± 1.6            | 27.9 ± 2.6    | 20.6 ± 1.7      |
| DCA-24G            | 0.9 ± 0.1             | 0.9 ± 0.1     | 0.8 ± 0.1       |
| HDCA-6G            | 36.7 ± 2.1            | 42.6 ± 3.5    | 30.7 ± 2.1*     |
| HDCA-24G           | 0.7 ± 0.1             | 0.7 ± 0.1     | 0.7 ± 0.1       |
| HCA-6G             | 63.5 ± 8.6            | 81.9 ± 16.2   | 45.1 ± 5.3***   |
| HCA-24G            | 0.9 ± 0.1             | 1.0 ± 0.1     | 0.8 ± 0.1       |
| **Total**          | 192.3 ± 13.1          | 234.4 ± 24.9  | 150.1 ± 8.8***  |
| **Metabolic ratios** |                      |               |                 |
| CDCA-3G/CDCA       | 0.60 ± 0.05           | 0.58 ± 0.07   | 0.63 ± 0.07     |
| CDCA-24G/CDCA      | 0.01 ± 0.00           | 0.01 ± 0.00   | 0.02 ± 0.01     |
| CA-24G/CA          | 0.16 ± 0.04           | 0.23 ± 0.08   | 0.09 ± 0.02     |
| LCA-3G/LCA         | 0.12 ± 0.01           | 0.14 ± 0.02   | 0.11 ± 0.01     |
| LCA-24G/LCA        | 0.08 ± 0.01           | 0.08 ± 0.01   | 0.09 ± 0.01     |
| DCA-3G/DCA         | 0.08 ± 0.01           | 0.10 ± 0.01   | 0.07 ± 0.01     |
| DCA-24G/DCA        | 0.01 ± 0.00           | 0.01 ± 0.00   | 0.01 ± 0.00     |
| HDCA-6G/HDCA       | 0.98 ± 0.06           | 1.06 ± 0.09   | 0.91 ± 0.09*    |
| HDCA-24G/HDCA      | 0.02 ± 0.01           | 0.03 ± 0.01   | 0.02 ± 0.01     |
| HCA-6G/HCA         | 8.90 ± 0.69           | 9.02 ± 0.93   | 8.89 ± 0.89     |
| HCA-24G/HCA        | 0.19 ± 0.03           | 0.18 ± 0.04   | 0.19 ± 0.03     |

Bile acid-glucuronide concentrations are expressed in nmol/l. Values represent the mean ± SEM. The metabolic ratio for each species was calculated as the ratio of glucuronide to unconjugated precursor (Supplementary Information S4 online). Statistically significant differences between male and female serum samples were determined by the rank-sum Wilcoxon/Mann–Whitney test: *P < 0.05; ***P < 0.001.

CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; G, glucuronide; GOLDN, Genetics of Lipid Lowering Drugs and Diet Network; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; Total, sum of all bile acid glucuronides.

Figure 1  Effects of the (a,b) UGT1A3 31T>C, (c) UGT2B7 802C>T, and (d) UGT2B4 1374T>A polymorphisms on baseline serum levels of (a) LCA-24G, (b) total bile acid-24 glucuronides, (c) CDCA-3G, and (d) HDCA-24G. Bile acid glucuronide levels were determined through LC–MS/MS analyses as described in the Methods section and Supplementary Information S1 online. Genomic DNA was isolated from peripheral blood leukocytes and genotyped for the (a,b) UGT1A3 31T>C (n = 227 donors), (c) UGT2B7 802C>T (n = 142 donors), and (d) UGT2B4 1374T>A (n = 197 donors) mutations. All values represent the mean ± SEM. A repeated analysis of variance model was used to evaluate the effect of each genotype: *P < 0.05; **P < 0.01. All analyses were adjusted for the effects of age and sex. Only significant associations are shown; the complete results are provided in Supplementary Information S5–S7 online. CDCA, chenodeoxycholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; LC–MS/MS, liquid chromatography–tandem mass spectrometry; UGT, uridine 5′-diphospho-glucuronosyltransferase.


**Figure 2** UGT1A4 and UGT2B7 catalyze the conversion of CDCA into CDCA-3G. (a) Ten micrograms of human liver microsomes were incubated in the presence of CDCA, and the quantification of the two CDCA glucuronides was achieved by LC–MS/MS. (b) The formation of CDCA-3G by UGT bacculosomes (10 µg) was analyzed by LC–MS/MS. (c,d) The UGT protein contents in microsomes from (c) UGT1A4- or (d) UGT2B7-HEK293 cell clones were compared by western blot experiments using the (c) anti-UGT1A (1:2,000) or the (d) anti-UGT2B (1:2000) antibodies. The equal loading of each lane was ensured by hybridizing with an anticalnexin antibody (1:5,000; bottom panels). (e,f) The formation of CDCA-3G by microsomes (10 µg) from HK293 cells overexpressing (e) each of the four UGT1A4 (WT, T24, V48, and W11) or (f) the two UGT2B7 (WT and Y268) allele variants was quantified by LC–MS/MS and normalized according to the UGT protein level in the microsomal extract as determined by western blot (c,d: WT proteins were arbitrarily set at 1). All assays were performed for 1 h at 37 °C in the presence of 100 µmol/l CDCA and 2 mmol/l of the cosubstrate uridine 5′-diphospho-glucuronic acid. Data represent the mean ± SD. The statistical significance of differences in glucuronidation activity between WT and UGt variants was determined through the nonparametric Student t-test (*P < 0.05). CDCA, chenodeoxycholic acid; LC–MS/MS, liquid chromatography–tandem mass spectrometry; UGT, uridine 5′-diphospho-glucuronosyltransferase; WT, wild-type.

These *in vitro* analyses identify UGT1A4 and UGT2B7 as major enzymes for hepatic CDCA-3G formation.

**Fenofibrate causes an accumulation of selected BA glucuronide species in sera from noncholestatic and PBC donors**

Treatment with fenofibrate resulted in a significant increase (P < 0.001) of the total glucuronide concentration in donors in the GOLDN (Genetics of Lipid Lowering Drugs and Diet Network) study (Figure 3a). Such induction reflected the 88% increase in HDCA-6G levels (P < 0.001) but also the accumulation of HDCA-24G (P < 0.001), HCA-6G (P < 0.001), HCA-24G (P < 0.001), and CA-24G (P < 0.001) (Figure 3b–d). LCA-3G (−10%) was the only species negatively affected by fenofibrate (Figure 3d; P < 0.01). Other species (CDCA-3 and 24G, DCA-3 and 24G, and LCA-24G) were not significantly altered, although CDCA-3G tended to increase (+13%; P = 0.07, Figure 3b). The unique difference in the response to fenofibrate between men and women corresponded to the LCA-3G reduction, which reached statistical significance only in men (Supplementary Information S8 online). Significant interactions were observed for the UGT2B7H268Y genotype: CDCA-3G and CA-24G levels were significantly increased by fenofibrate in samples from C/C donors, whereas carriers of the C/T genotype remained unresponsive (Supplementary Information S8 online).

In these donors, fenofibrate also significantly increased the relative abundance of BA-G within the serum BA pool (Figure 3e, P < 0.001), as well as increasing the MRs for CDCA-3G (P < 0.001), DCA-3G (P < 0.001), DCA-24G (P < 0.05), LCA-24G (P < 0.05), HDCA-6G (P < 0.001), HDCA-24G (P < 0.001), and HCA-6G (P < 0.01) (Figure 3f–i). The most impressive changes were observed for HDCA-6G and HDCA-24G (Figure 3f,j). Interestingly, the fenofibrate-dependent changes in LCA-3G and HCA-24G species (Figure 3c,d) did not result in significant modulation of their MRs (Figure 3h). In the same vein, not only did the increased concentration of circulating CA-24G not lead to an increase in its MR, but this ratio was even significantly reduced (Figure 3h, P < 0.01). Finally, MRs for DCA-24G and HCA-6G were differentially affected in men and women (Supplementary Information S8 online).

To investigate whether fenofibrate also affects the circulating BA-G profile in cholestatic patients, a pilot study was performed with samples from five fenofibrate-supplemented PBC patients (Supplementary Information S9 online; Figure 4). The great interindividual variability and the small population...
size drastically reduced the statistical significance of the following observations. Nevertheless, it is remarkable that fenofibrate exerted effects similar to those in noncholestatic donors: four of five donors exhibited increases in the total BA-G concentration (Figure 4a). Circulating CDCA-3G (Figure 4b), HDCA-6G (Figure 4c), and HCA-6G (Figure 4d) levels were increased after fenofibrate exposure but only in three, three, and two donors, respectively. These results demonstrate that fenofibrate increases serum BA-G in healthy donors and PBC patients.

**PPAR-α activation upregulates UGT1A4 but not UGT2B7 expression in human hepatic cells**

We next investigated whether hepatic expression of known (UGT2B4, UGT2B7, and UGT1A3) or newly identified (UGT1A4) BA-conjugating enzymes were affected by the PPAR-α agonists fenofibrate and Wy14,643 (Figure 5).

According to previous reports,16,17 both activators upregulated UGT1A3 and UGT2B4 mRNA expression in human hepatocytes and hepatoma HepG2 cells (Figure 5a–c). Interestingly, the two CDCA-3G–forming enzymes were differentially affected: UGT1A4 was induced in fenofibrate- and Wy14,643-treated hepatocytes, whereas UGT2B7 mRNA levels remained unaffected (Figure 5a,b). The dose- and time-dependent, as well the transcriptional and direct nature of the Wy14,643-dependent UGT1A4 activation, was confirmed in dose–response (Figure 5c) and time course experiments (Figure 5d) and by the concomitant use of inhibitors for gene transcription (actinomycin D) or mRNA translation (cycloheximide) (Figure 5e). According to previous reports,17,27 glucuronidation assays performed with human hepatocytes exposed or not exposed to Wy14,643 (75 µmol/l) resulted in a twofold increased CDCA-24G production (Figure 5f). A similar increase was observed...
with CDCA-3G formation; however, the glucuronide productions of control and treated cells were below the limit of quantification of the liquid chromatography–tandem mass spectrometry (LC–MS/MS) method, which is also in accordance with previous studies reporting the predominant conversion of CDCA into its acyl glucuronide conjugates in human liver cells.17,27

These observations demonstrate that PPAR-α activators stimulate the expression of three of the four human BA-glucuronidating enzymes.

**BA glucuronides are less cytotoxic than their unconjugated precursors in HepG2 cells**

Because some glucuronides, such as LCA-3G, can exert toxic and/or cholestatic effects,28 we next investigated whether the abundant CDCA-3G and the potentially toxic LCA-3G affect hepatoma HepG2 cells’ viability (Figure 5g,h). As compared with their unconjugated precursors used at the same dose (100 µmol/l), LCA-3G and CDCA-3G failed to cause any alteration of cell viability in MTS reduction assays (Figure 5g). When living cells were discriminated by fluorescence-activated cell sorting, the two glucuronides also revealed less cytotoxicity than their precursors (Figure 5h).

**DISCUSSION**

This study provides the first comprehensive analysis of the BA-glucuronide profile in human sera and its response to fenofibrate.

Together four species—HCA-6G, HDCA-6G, CDCA-3G, and DCA-3G—represent 95 and 89% of all the BA-G pool in normal and PBC sera, respectively. HDCA-6G and HCA-6G are efficiently formed by the human liver,15,30 and these species were expected to be abundant in human serum. By contrast, CDCA-3G and DCA-3G are minimally produced in vitro.13,17,30 The major products in glucuronidation assays correspond to CDCA-24G and DCA-24G,15,17,30 two C24 acylglucuronides detected at very low serum concentrations in the current study. Such a paradoxical situation was foretold by Hofmann,31 who proposed that the C24 carboxyl group of BA molecules is preferentially used for glycine or taurine conjugation in vivo, thus precluding its use for glucuronidation.17,29,31 It is also possible, however, that the C24 BA-G formed in the human liver cannot be efficiently detected in the blood as a result of extensive degradation. Acyl glucuronide conjugates are unstable at physiological pH, and their abundance is usually underestimated.32–34 Accordingly, we previously observed that incubation of C24 BA-G in neutral to basic solutions for 2 h results in a strong reduction of the glucuronide concentrations.30 Whether acyl-BA-Gs are underestimated due to degradation or their low levels reflect a reduced formation remains to be clarified.

Women exhibit lower CDCA-3G, HDCA-6G, and HCA-6G levels than men and consequently exhibit a lower total BA-G concentration. This observation is consistent with the previous report that BA species such as CDCA and HCA are more abundant in men than in women.2 Therefore, the higher CDCA-3G and HCA-6G concentrations may reflect only greater availability of these acids for glucuronidation in the male liver. By contrast, HDCA concentrations were previously found to be identical in male and female sera from the GOLDN study,2 suggesting that conversion of HDCA into this glucuronide derivative is favored in men. Accordingly, the MRs for CDCA-3G and HCA-6G were not influenced by gender, and men also exhibited a higher HDCA-6G/HDCA ratio than women. Gender-related differences in glucuronidation rates have often been reported and appear to be dependent on gender-specific growth and sex hormone secretions.35

The UGT1A3 31T>C polymorphism influences baseline carboxyl-linked BA-G levels, thus confirming in vivo the previous in vitro characterization of UGT1A3 as a specialized enzyme for acyl glucuronidation of BAs.17,29,36–38 More surprising was the significant association between the UGT2B4 1374T>A polymorphism and HDCA-24G concentration. Indeed, this enzyme is by far more efficient in converting HDCA into HDCA-6G than into HDCA-24G.15,23,37,39,40 Interestingly, HDCA-6 and HDCA-6G concentrations may reflect only greater availability of these acids for glucuronidation in the male liver. By contrast, HDCA concentrations were previously found to be identical in male and female sera from the GOLDN study,2 suggesting that conversion of HDCA into this glucuronide derivative is favored in men. Accordingly, the MRs for CDCA-3G and HCA-6G were not influenced by gender, and men also exhibited a higher HDCA-6G/HDCA ratio than women. Gender-related differences in glucuronidation rates have often been reported and appear to be dependent on gender-specific growth and sex hormone secretions.35

The UGT1A3 31T>C polymorphism influences baseline carboxyl-linked BA-G levels, thus confirming in vivo the previous in vitro characterization of UGT1A3 as a specialized enzyme for acyl glucuronidation of BAs.17,29,36–38 More surprising was the significant association between the UGT2B4 1374T>A polymorphism and HDCA-24G concentration. Indeed, this enzyme is by far more efficient in converting HDCA into HDCA-6G than into HDCA-24G.15,23,37,39,40 Interestingly, HDCA-6 and HDCA-6G levels sustain a similar accumulation in donors carrying the A1374 allele, but only the HDCA-24G variation
reaches statistical significance. It is therefore likely that the functional impact of the resulting UGT2B4 ΔE386E amino acid change reaches statistical significance only for low-efficiency catalytic reactions. On the other hand, CDCA-3G levels were significantly associated with the UGT2B7 802C>T polymorphism, which suggested an important role for this enzyme in converting CDCA into CDCA-3G. This was further confirmed through the in vitro screening experiments. Indeed, among the recombinant UGTs tested, only UGT2B7 and UGT1A4 were efficient in producing CDCA-3G. Our data support complementary roles for these two isoforms, with UGT2B7 being predominant for baseline CDCA-3G levels and UGT1A4 possibly being responsible for increased production of CDCA-3G upon fenofibrate exposure. Indeed, in vitro assays confirmed the functional impact of the UGT2B7 802C>T mutation on CDCA-3G formation, for which none of the common UGT1A4 variants tested exhibited altered activity. By contrast, the improved UGT1A4 expression in PPAR-α–activated hepatic cells identifies this UGT1A enzyme as responsible for the increased CDCA-3G/CDCA ratio observed in postfenofibrate serum samples (Figure 6).

In the same vein, although both UGT2B4 and UGT2B7 efficiently convert HDCA and HCA into 6-glucuronide conjugates in vitro, the increased HDCA-6 and HCA-6G concentrations in postfenofibrate sera probably reflect the PPAR-α–dependent activation of UGT2B4 expression (Figure 6).

The present study also evidences BA glucuronidation as a deeply fenofibrate-regulated process in humans. Indeed, among the 11 BA-G species analyzed, 5 were significantly increased in posttreatment samples, and 6 exhibited higher MR values. Within the 28 total BA species analyzed to date...
Figure 6 Proposed mechanism of action for the fenofibrate-dependent stimulation of BA detoxification in the cholestatic liver. In cholestatic liver cells, fenofibrate activates the nuclear receptor PPAR-α, which then forms an active heterodimer with its partner RXR and activates the regulatory regions of several target genes, including the BA-conjugating enzymes UGT2B4, UGT1A3, and UGT1A4 and the glucuronide efflux transporter, MRP3 (refs. 16–18,41,42; Figure 5). By contrast, the fenofibrate-activated receptor does not bind to or activate the UGT2B7 gene. The increased UGT1A3 (ref. 17; Figure 5), UGT1A4 (Figure 5), and UGT2B4 (ref. 16; Figure 5) enzymes conjugate the accumulating bile acids that cannot be excreted in the bile canaliculus due to bile flow impairment. The resulting bile acid glucuronides are then secreted into the blood through the increased MRP3 protein (refs. 41–43) at the basolateral membrane of the cells. Glucuronides accumulate in the blood (Figure 3 and Table 1), before being removed by the kidneys and definitively eliminated in the urine (refs. 7,45). Thus, fenofibrate stimulates a complete detoxification process that reduces the burden of toxic acids in the liver, an effect that may participate in the reported improvement of liver functioning in treated cholestatic patients (refs. 8,11–13). BA, bile acid; MRP, multidrug resistance–associated protein; PPAR-α, peroxisome proliferator–activated receptor-α; RXR, retinoid X receptor; UGT, uridine 5′-diphospho-glucuronosyltransferase.

(i.e., 17 nonglucuronides\(^2\) + 11 glucuronides), the most impressive change caused by fenofibrate corresponds to the 88% increase in HDCA-6G levels. Such a change illustrates the efficacy of fenofibrate to stimulate BA glucuronidation (Figure 6), an effect further confirmed by the strong induction of UGT2B4, UGT1A3, and UGT1A4 mRNA expression in PPAR-α–activated cells. Beyond UGTs, PPAR-α also upregulates the multidrug resistance–associated protein–3 gene.\(^41,42\) Multidrug resistance–associated protein–3 is an efflux transporter involved in the basolateral secretion of glucuronide derivatives from hepatocytes.\(^33,44\) Thus, not only does PPAR-α activation stimulate formation of BA-Gs, but it also stimulates their excretion into the systemic circulation, where their concentration is significantly increased as evidenced in the current study (Figure 6). Circulating BA-Gs are then removed by the kidneys and definitively eliminated in the urine.\(^7,45\) Therefore, by activating the PPAR-α–dependent regulation of hepatic glucuronidation, fenofibrate stimulates a complete detoxification process, allowing BA excretion in the urine (Figure 6). Such a mechanism is of particular clinical relevance in the context of chronic cholestatic liver diseases, where BAs accumulate to toxic levels in hepatocytes as a result of an impaired biliary excretion\(^1\) (Figure 6). A series of small clinical trials recently revealed that fibrates such as fenofibrate improve liver biochemistries in PBC and patients with primary sclerosing cholangitis.\(^8–13,46\) Our results suggest that this improvement reflects an increased BA glucuronidation and detoxification, in addition to the previously proposed anti-inflammatory and lipid-lowering mechanisms.\(^14\) This idea is supported by results from the pilot study involving five fenofibrate-treated PBC patients, in whom the drug also led to an increase of serum BA-G levels. However, additional studies with larger PBC populations are required to validate the results from this pilot study and, more important, to establish the amplitude of BA-G modulation by fenofibrate in a cholestatic context.

In conclusion, these results provide the first clinical evidence that fenofibrate stimulates BA glucuronidation in humans. This effect reflects a PPAR-α–dependent activation of BA-conjugating UGTs in liver cells and reduces BA toxicity in hepatocytes.

METHODS

Materials are detailed in Supplementary Information S1 online.

Subjects. The noncholestatic population for this study consisted of 300 Caucasian participants (150 men and 150 women) from the GOLDN study. The study design, eligibility criteria, population demographics, and laboratory characteristics have been extensively described previously\(^2\) and are summarized in Supplementary Information S2 online. Briefly, participants were given 160 mg of fenofibrate per day (TriCor; Abbott Laboratories, Chicago, IL).\(^2–47\) A 12-h fasting blood sample was drawn before and after the fenofibrate treatment period, and serum was isolated and frozen at −80 °C until subsequent analyses.\(^2\)

The PBC samples were from patients attending the Liver Clinics at the University of Florida (Gainesville, FL) and at the Mayo Clinic (Rochester, MN).\(^12\) The population of the study consisted of 20 patients, but stored serum was available from only 5 of these patients (2 men and 3 women) for further BA-G measurement. The study design, eligibility criteria, population demographics, and laboratory characteristics have been described previously\(^12\) and are summarized in Supplementary Information S3 online. Briefly, patients with incomplete response to ursodeoxycholic acid were given 160 mg/day of the insoluble drug delivery microparticle fenofibrate (Triglide; Sciele Pharma, Atlanta, GA) for 48 weeks, in addition to their usual dosages of daily ursodeoxycholic acid.\(^12\) A 12-h
fasting blood sample was drawn at entry and at the end of the study, and serum was isolated and frozen at −80 °C until subsequent analyses. The protocols were approved by the institutional review boards at the University of Minnesota (Minneapolis, MN), University of Florida (Gainesville, FL), and the CHU–Québec Research Centre (Quebec, Canada). Written informed consent was obtained from all participants. All authors had access to the data for studies NCT00083369 and NCT00575042.

**Analytical method.** Concentrations of 11 BA-G (CDCA-3G, CDCA-24G, CA-24G, LCA-3G, LCA-24G, DCA-3G, DCA-24G, HDCA-6G, HDCA-24G, HCA-6G, and HCA-24G) were determined using high-performance LC–MS/MS as extensively described in **Supplementary Information S1** online. The chromatographic system used an Alliance 2690 HPLC instrument (Waters, Milford, MA), and the MS/MS system was an API4000 mass spectrometer (Applied Biosystems, Concord, Canada). As detailed in **Supplementary Information S1** online, HCA-6G was quantified with a standard curve constructed using CDCA-3G. All other species were quantified with the appropriate standard curve. Limits of quantifications were CDCA-3/24G, DCA-3/24G, and HDCA-6/24G: 1.76 nmol/l; LCA-3/24G: 1.81 nmol/l; and CA-24G and HCA-24G: 1.71 nmol/l. GOLDN samples were also analyzed for a series of 17 unconjugated, taurine-, glycine-, or sulfate-conjugated BA species using LC–MS/MS quantification (see **Supplementary Information S4** online). In the context of the present study, these concentrations were used to calculate the relative abundance (percentage) of BA-G and the MR for each glucuronide.

**UGT genotyping.** Genomic DNA was isolated from peripheral blood leukocytes from the GOLDN study participants using Puregene DNA reagents according to the manufacturer’s instructions (Qiagen, Germantown, MD). Targeted UGT polymorphisms including UGT2B4 1374T>A (rs13119049, n = 197), UGT2B7 802C>T (rs7439366, n = 142), and UGT1A3 31T>C (rs3821242, n = 227) were determined at the University of Minnesota BioGenomic Medicine Center (http://www.bmgc.umn.edu/Genotyping/index.htm) using the iPLEX Gold method and the previously reported genotyping strategies (see **Supplementary Information S1** online for additional experimental information).

**Cell culture and mRNA level determination.** Cryopreserved human primary hepatocytes from three donors (see **Supplementary Information S1** online) were obtained from Celsis-InVitro Technologies (Baltimore, MD) and cultured as reported previously. For RNA analyses, 350,000 hepatocytes per well were seeded in 12-well plates and cultured in the Invitro Gro CP medium for 48 h. Cells were then treated with vehicle (dimethyl sulfoxide, 0.1% vol/vol) or activators (250 µmol/l fenofibrate or 75 µmol/l Wy14,643) for 48 h. For glucuronide formation, 700,000 hepatocytes per well were seeded in six-well plates, treated with vehicle (dimethyl sulfoxide, 0.1% vol/vol) or Wy14,643 (75 µmol/l) for 48 h, and then cultured with CDCA (100 µmol/l) for 2 h. Culture media were analyzed for CDCA-3 and CDCA-24G through LC–MS/MS (see above). A total of 10⁶ HepG2 cells were treated as indicated with the PPAR-α activator, in the absence or presence of cycloheximide (20 µg/ml) or actinomycin D (1 µg/ml). Total RNA was isolated according to the TRI Reagent acid phenol protocol as specified by the supplier (Molecular Research Center, Cincinnati, OH). The reverse transcription and quantitative PCR reactions were performed as previously described and detailed in **Supplementary Information S1** online.

**MTS reduction assays and fluorescence-activated cell sorting.** HepG2 cells (20,000 cells per well) were seeded in 96-well plates and treated for 24–72 h with 100 µmol/l BAs. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reduction was evaluated using the CellTiter 96 AQueous One Solution Cell Proliferation Assay according to the manufacturer’s instructions (Promega, Madison, WI).

Living cell quantification was achieved through fluorescence-activated cell sorting analyses using the annexin V/propidium iodide colabeling method as reported. Labeled cells were then analyzed using a BD fluorescence-activated cell sorting Canto II instrument (BD biosciences, San Jose, CA).

**Production of HEK293 cells overexpressing the UGT1A4 alleles, microsome isolation, and western blot analyses.** HEK293 cells expressing the UGT1A4 T31V, V89, and W11 variant alleles were generated by site-directed mutagenesis of the WT UGT1A4 (R181P34,48)–pcDNA6v5-His vector. UGT1A4 and HEK293 cell lines were obtained by transfecting 2 µg of the UGT1A4 plasmids with the ExGen reagent according to the manufacturer’s instructions (Invitrogen, Burlington, Canada). Stable transfectants were selected in a medium containing 10 µg/ml blasticidin–HCl as previously described.

**Glucuronidation assays.** All assays were performed for 1 h as previously reported, using 100 µmol/l CDCA and 10 µmol of commercial baculosomes, liver microsomes, or microsomes purified from UGT1A4- and UGT2B7-HK293 cells. The formation of CDCA-3 and CDCA-24G was quantified by LC–MS/MS as previously described. Velocity values obtained with the UGT1A4 and UGT2B7 allozymes were normalized according to the UGT protein levels as determined by western blot (WT proteins arbitrarily set at 1).

**Data analyses.** BA-G levels were calculated as mean ± SEM for noncholestatic volunteers or mean ± SD for PBC patients. BA-G concentrations did not satisfy the normal distribution according to the Shapiro–Wilk test; therefore, the matched-pairs Wilcoxon signed-rank test was used for statistical analyses of the response to treatment. Comparisons of baseline profiles and the response to treatment between men and women were made using the Wilcoxon Mann–Whitney rank-sum test (JMP V7.0.1, SAS Institute Cary, NC). For genotyping analyses, Hardy–Weinberg equilibrium was tested with the Allele Procedure in SAS, version 9.2 (SAS Institute). BA-G values were transformed before their distribution was normalized, and a repeated analysis of variance model was used to evaluate the effect of each genotype and the genotype by response interaction effect (SAS Institute). These analyses were adjusted for the effects of age and sex.

The statistical significance of differences in mRNA levels, CDCA-24G formation, and cell viability between control and treated cells, as well as in glucuronidation activity between WT and variants, was determined using the nonparametric Student t-test using the JMP V7.0.1 program (SAS Institute).

**SUPPLEMENTAL MATERIAL** is linked to the online version of the paper at http://www.nature.com/cpt

**ACKNOWLEDGMENTS**

The authors thank Virginie Bocher for critical reading of the manuscript. B.S. is a member of the Institut Universitaire de France. This study was supported by grants from the Canadian Institute of Health Research (CIHR; MOP-84338 and MOP-229488), the Canadian Liver Foundation and the Canadian Foundation for Innovation (10469). The Genetics of Lipid Lowering Drugs and Diet Network (GOLDN) study was supported by National Heart, Lung, and Blood Institute (National Institutes of Health) grant U01HL72524, Genetic and Environmental Determinants of Triglycerides. J.T. is holder of a scholarship from CIHR. M.P. is holder of a scholarship from the Fonds pour la Recherche en Santé du Québec and a scholarship from the Canadian Institutes of Health Research.
4. Wagner, M., Zollner, G. & Trauner, M. Nuclear receptor regulation of the bile acid pool in cholestatic liver disease. *J. Exp. Med.* **2009**, *206*, 1511–1524.

5. Nakai, S., Masaki, T., Kurokohchi, K., Deguchi, A. & Nishioka, M. Combination therapy of bezafibrate and ursodeoxycholic acid in primary biliary cirrhosis: a preliminary study. *Am. J. Gastroenterol.* **95**, 326–327 (2000).

6. Ohira, H., Sato, Y., Ueno, T. & Sata, M. Fenofibrate treatment in patients with primary biliary cirrhosis. *Am. J. Gastroenterol.* **97**, 2147–2149 (2002).

7. Levy, C. et al. Pilot study: fenofibrate for patients with primary biliary cirrhosis and an incomplete response to ursodeoxycholic acid. *Aliment. Pharmacol. Ther.* **33**, 235–242 (2011).

Study Highlights

**WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?**

- Toxic BAs accumulate in the cholestatic liver.
- Glucuronidation, catalyzed by UGT enzymes, detoxifies BAs.
- Fenofibrate ameliorates liver biochemistry in cholestatic patients.

**WHAT QUESTION DID THIS STUDY ADDRESS?**

Does fenofibrate stimulate BA glucuronidation in humans?

**WHAT THIS STUDY ADDS TO OUR KNOWLEDGE**

This study provides:

- Clinical evidence that circulating BA-Gs are affected by gender and genetic mutations in UGT genes.
- A clear picture of the contribution of human UGT enzymes in BA glucuronidation.
- A first clinical assessment of the fenofibrate-dependent stimulation of BA glucuronidation in clinics.
- A pharmacological explanation of these effects.

**HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY AND THERAPEUTICS**

The present study further supports the use of fibrate drugs for chronic cholestasis and supports the search for more potent inducers of BA glucuronidation for cholestasis treatment.
rations and effects of ursodeoxycholate conjugates. *Dig. Dis. Sci.* **38**, 1543–1548 (1993).

29. Gall, W.E. *et al.* Differential glucuronidation of bile acids, androgens and estrogens by human UGT1A3 and 2B7. *J. Steroid Biochem. Mol. Biol.* **70**, 101–108 (1999).

30. Caron, P., Trottier, J., Verreault, M., Bélanger, J., Kaeding, J. & Barbier, O. Enzymatic production of bile Acid glucuronides used as analytical standards for liquid chromatography-mass spectrometry analyses. *Mol. Pharm.* **3**, 293–302 (2006).

31. Hofmann, A.F. Why bile acid glucuronidation is a minor pathway for conjugation of endogenous bile acids in man. *Hepatology* **45**, 1083–1084; author reply 1084 (2007).

32. Regan, S.L., Maggs, J.L., Hammond, T.G., Lambert, C., Williams, D.P. & Park, B.K. Acyl glucuronides: the good, the bad and the ugly. *Ther. Drug Monit.* **25**, 1–16 (2003).

33. Zhang, D. *et al.* Plasma stability-dependent circulation of acyl glucuronide metabolites in humans: how circulating metabolite profiles of muraglitazar and pelaglitazar can lead to misleading risk assessment. *Drug Metab. Dispos.* **39**, 123–131 (2011).

34. Buckley, D.B. & Klaassen, C.D. Mechanism of gender-divergent UDP-glucuronosyltransferase mRNA expression in mouse liver and kidney. *Drug Metab. Dispos.* **37**, 834–840 (2009).

35. Trottier, J. *et al.* The human UGT1A3 enzyme conjugates norursodeoxycholic acid into a C23-ester glucuronide in the liver. *J. Biol. Chem.* **285**, 1113–1121 (2010).

36. Verreault, M. *et al.* Regulation of endobiotics glucuronidation by ligand-activated transcription factors: physiological function and therapeutic potential. *Drug Metab. Rev.* **42**, 110–122 (2010).

37. Erichsen, T.J., Aehlen, A., Ehmer, U., Kalthoff, S., Manns, M.P. & Strassburg, C.P. Regulation of the human bile acid UDP-glucuronosyltransferase A3 by the farnesoid X receptor and bile acids. *J. Hepatol.* **52**, 570–578 (2010).

38. Lévesque, E., Beaulieu, M., Hum, D.W. & Bélanger, A. Characterization and substrate specificity of UGT2B4 (E458): a UDP-glucuronosyltransferase encoded by a polymorphic gene. *Pharmacogenetics* **9**, 207–216 (1999).

39. Lévesque, E. *et al.* Extensive splicing of transcripts encoding the bile acid-conjugating enzyme UGT2B4 modulates glucuronidation. *Pharmacogenet. Genomics* **20**, 195–210 (2010).

40. Moffit, J.S., Aleksunes, L.M., Maher, J.M., Scheffer, G.L., Kläassen, C.D. & Manautou, J.E. Induction of hepatic transporters multidrug resistance-associated proteins (Mrp) 3 and 4 by clofibrate is regulated by peroxisome proliferator-activated receptor alpha. *J. Pharmacol. Exp. Ther.* **317**, 537–545 (2006).

41. Regan, S.L., Maggs, J.L., Hammond, T.G., Lambert, C., Williams, D.P. & Park, B.K. Acyl glucuronides: the good, the bad and the ugly. *Ther. Drug Monit.* **25**, 1–16 (2003).

42. Pauli-Magnus, C., Stieger, B., Meier, Y., Kullak-Ublick, G.A. & Meier, P.J. Enterohepatic transport of bile salts and genetics of cholestasis. *J. Hepatol.* **43**, 342–357 (2005).

43. Iwasaki, S., Akisawa, N., Saibara, T. & Onishi, S. Fibrate for treatment of primary biliary cirrhosis. *Hepatol. Res.* **37** (suppl. 3), S515–S517 (2007).

44. Rieger, A.M., Nelson, K.L., Konowalchuk, J.D. & Barreda, D.R. Modified annexin V/propidium iodide apoptosis assay for accurate assessment of cell death. *J. Vis. Exp.* (2011); e-pub 24 April 2011.

45. Benoit-Biancamano, M.O. *et al.* A pharmacogenetics study of the human glucuronosyltransferase UGT1A4. *Pharmacogenet. Genomics* **19**, 945–954 (2009).