Studies on the Unusual 1\(\beta\)-Hydroxylated Bile Acid Biosynthesis in Infants

Yukihiro Nomura,*a Hiroyuki Murata,a Hiroaki Sasai,a Akihiko Kimura,b Takao Kurosawa,c Takahiro Sasaki,c and Tsuyoshi Murai*a

*aDrug Metabolism and Pharmacokinetics Research Laboratories, Central Pharmaceutical Research Institute, Japan Tobacco Inc.; Osaka 569–1125, Japan. bDepartment of Pediatrics and Child Health, Kurume University School of Medicine; 67 Asahi-machi, Kurume 830–0011, Japan. and cSchool of Pharmaceutical Sciences, Health Sciences University of Hokkaido; Kanazawa, Ishikari-Tobetsu, Hokkaido 061–0293, Japan.

Received December 6, 2017; accepted January 10, 2018

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Unusual bile acids (1\(\beta\)-hydroxylated bile acids), particularly 1\(\beta\)-hydroxyl-cholic acid (CA-1\(\beta\)-ol) and 1\(\beta\)-hydroxyl-chenodeoxycholic acid (CDCA-1\(\beta\)-ol), have been detected in the urine of infants. These acids are conjugated with amino acids, such as taurine, and are then excreted mainly via the urine. CA-1\(\beta\)-ol and CDCA-1\(\beta\)-ol are the predominant bile acids during infancy and are present in relatively large amounts in the urine. However, the biosynthetic pathway of 1\(\beta\)-hydroxylated bile acids in infants remains unclear. To investigate the biosynthetic pathway of 1\(\beta\)-hydroxylated bile acids during infancy, we performed a metabolic reaction using infant hepatocytes at 3 months after delivery. Glyco- and tauro-CA-1\(\beta\)-ol were identified by LC/tandem mass spectrometry (MS/MS) analysis of the extracted culture medium incubated with cholic acids (CAs). Further, we identified that ketoconazole suppressed CA 1\(\beta\)-hydroxylation and that the CYP3A subfamily was the primary group of enzymes responsible for CA-1\(\beta\)-ol formation. The present study provides new information about the biosynthetic pathway of 1\(\beta\)-hydroxylated bile acid conjugates in human infants.

Key words 1\(\beta\)-hydroxylated bile acid; LC/tandem mass spectrometry (MS/MS); CYP3A

Numerous polyhydroxylated bile acids have been detected in the urine of pregnant women and newborns.1) 1\(\beta\)-Hydroxyl-cholic acids (CA-1\(\beta\)-ol) are present at relatively high levels and are major polyhydroxylated bile acids in the urine of infants.2) In adults, CA and chenodeoxycholic acid (CDCA) are conjugated with glycine and taurine, but in infants, 1\(\beta\)-hydroxylated bile acids are mainly conjugated with taurine.3) It has been reported that bile acids hydroxylated at the 1-position are predominantly found in the taurine fraction.4) It has been reported that the quantification of plasma or urine bile acids has been accomplished mostly with GC-MS methods.5) However, these procedures have some disadvantages, such as tedious sample clean-up and insufficient information concerning the conjugation mode of bile acids. In recent years, it has been reported that simple, sensitive, and specific HPLC-electrospray ionization coupled with tandem mass spectrometry (LC/ESI-MS/MS) method for the analysis of the bile acid.6) In particular, LC/ESI-MS/MS, which makes the prior deconjugation unnecessary, appears to be suitable for determining 1\(\beta\)-hydroxylated bile acid conjugates in human biological fluids.

Bile acids play an important role in the absorption of fat and have a pronounced hepatotoxic effect.7) Newborn infants have high serum levels of bile acids and low excretion of bile acids to the intestine.8) Therefore, bile acids need to be metabolized into more polar compounds to enhance their urinary excretion.

1\(\beta\)-Hydroxylated bile acids, particularly CA-1\(\beta\)-ol, may be excreted into the urine as the main detoxification pathway of serum CA.9) The major hydroxylated metabolite of deoxycholic acid (DCA) in human liver microsomal incubations was identified by ultra-high-performance LC-high-resolution MS as 1\(\beta\)-hydroxyl-deoxycholic acid. During this previous study, this metabolic reaction was also shown to be catalyzed in vitro by recombinant CYP3A4, CYP3A5, and CYP3A7, although no data were presented on primary bile acids, such as CA and CDCA.10 The mechanism underlying CA and CDCA 1\(\beta\)-hydroxylation thus remains unclear. It has been reported that CYP3A7 is the main fetal form of CYP3A, which is downregulated after birth.11) Against this background, the present study deals with a highly sensitive method for determining the levels of conjugated and unconjugated 1\(\beta\)-hydroxylated bile acids, including common bile acids, using LC/ESI-MS/MS. The specific aim of the present study was to investigate the biosynthetic pathway of 1\(\beta\)-hydroxylated bile acids during infancy and CA 1\(\beta\)-hydroxylation by human hepatocytes and to assess the contribution of individual cytochromes using human recombinant P450 enzymes.

MATERIALS AND METHODS

Chemicals and Reagents Cholic acid (CA), CDCA, taurocholic acid (T-CA), taurochenodeoxycholic acid (T-CDCA), glycocholic acid (G-CA), and glycochenodeoxycholic acid (G-CDCA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and used after chromatographic purification. Tauro-1\(\beta\),3\(\alpha\),7\(\alpha\),12\(\alpha\)-tetrahydroxy-5\(\alpha\)-cholan-24-oic acid (T-CA-1\(\beta\)-ol), glyco-1\(\beta\),3\(\alpha\),7\(\alpha\),12\(\alpha\)-tetrahydroxy-5\(\alpha\)-cholan-24-oic acid (G-CA-1\(\beta\)-ol), and 1\(\beta\),3\(\alpha\),7\(\alpha\),12\(\alpha\)-tetrahydroxy-5\(\alpha\)-cholan-24-oic acid (G-CDCA-1\(\beta\)-ol) were synthesized as described previously (Tôhma et al.). Chemical structures of all bile acids and their abbreviations are shown in Fig. 1.

* To whom correspondence should be addressed. e-mail: yukihiro.nomura@jt.com
Recombinant human cytochromes CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A13, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, CYP3A7, CYP4A11, CYP4F2, and CYP4F3 were obtained from CyRx Ltd. (Dundee, Scotland). Cryopreserved primary human hepatocytes (PHHs) from different donors (YJX and Hu8105) were purchased from Bioreclamation IVT (New York, NY, U.S.A.) and Life Technologies Japan (Tokyo, Japan). Acetonitrile (HPLC grade) and ammonium acetate (JIS special grade) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Incubation of CA with a Panel of 19 Recombinant Human Cytochromes** An assay mixture containing CA and each recombinant human cytochrome in potassium phosphate buffer (100 mmol/L, pH 7.4) and reduced nicotinamide adenine dinucleotide phosphate (NADPH)-regenerating system solution including nicotinamide adenine dinucleotide phosphate (NADP)+ (13 mmol/L), glucose-6-phosphate (33 mmol/L), magnesium chloride (33 mmol/L), and glucose-6-phosphate dehydrogenase (40 U/mL) in potassium phosphate buffer was prepared. After 5 min of pre-incubation at 37°C, the NADPH-regenerating system solution was added to the assay mixture to make incubation mixtures containing CA (25 µmol/L), each recombinant human cytochrome at a protein concentration of 0.5 mg/mL, NADP+ (1.3 mmol/L), glucose-6-phosphate (3.3 mmol/L), magnesium chloride (3.3 mmol/L), and glucose-6-phosphate dehydrogenase (4 U/mL). The reaction was conducted for 60 min and was stopped by adding equivalent volume of ice-cold 90% acetonitrile. The mixture was centrifuged at 4000 × g for 5 min at 4°C. The obtained supernatant was then analyzed by LC/MS/MS.

**Incubation of CA with Human Hepatocytes** Cryopreserved primary human hepatocytes from two donors, YJX and Hu8105, were seeded at a density of 0.5 × 10^6 cells/500 µL/well and 0.4 × 10^6 cells/500 µL/well, respectively, in 24-well plates. In the study with taurine pretreatment, cells were incubated with 100-µmol/L taurine or a vehicle in 5% CO₂ at 37°C for 1 h. After pre-incubation, CA was added to each well, and the plates were incubated in 5% CO₂ at 37°C for 24 h. Each incubation mixture contained 25-µmol/L CA. The reactions were stopped by adding a volume of ice-cold 90% acetonitrile equivalent to the reaction volume. The mixture was centrifuged at 8900 × g for 5 min at 4°C. The supernatant was then analyzed by LC/MS/MS.

**RESULTS**

**LC/MS Analysis** Figure 1 shows the chemical structures of the 10 variants of unconjugated and conjugated C24 bile acids examined in this study. In general, CA and CDCA are produced from hepatic cells and are conjugated with amino acids, such as glycine and taurine. It has been reported that CA-1β-ol conjugates with taurine in the urine during early infancy. A method for determining conjugated and unconjugated CA-1β-ol and related bile acids in human hepatocytes has been developed using LC/ESI-MS/MS. The most sensitive transitions that could be optimized by directly injecting the standard solution are listed in Table 1. Typical selected reaction monitoring (SRM) chromatograms for authentic samples of compounds are shown in Fig. 2, indicating the simultaneous separation and determination of all bile acids within 10 min.

**Identification of Glyco- and Tauro-1β-hydroxylated Bile Acids** G-CA-1β-ol and T-CA-1β-ol standard solutions, peak A, peak B, and unknown peaks of SRM chromatograms are shown in Fig. 3. G-CA-1β-ol and T-CA-1β-ol were detected at retention times of 5.4 and 5.6 min on the MS chromatogram of m/z 480 and 530 in the negative ion mode, respectively. When product ions were measured using m/z 480 as the precursor ion, fragment ions were detected at m/z 416 and 74, and when they were measured using m/z 530 as the precursor ion, fragment ions were detected at m/z 124, 107, and 80 (Figs. 4, 5). Retention times on the MS chromatogram and...
MS and MS/MS spectra of peaks A and B in the sample were consistent with those of the standard substance (G-CA-1β-ol and T-CA-1β-ol). Therefore, peaks A and B were identified as G-CA-1β-ol and T-CA-1β-ol, respectively.

Formation Rate of Glyco- and Tauro-1β-hydroxylated Bile Acids Formation rates of G-CA-1β-ol and T-CA-1β-ol after incubation for 24 h were 2.0 (taurine+) and 2.6 (taurine−) pmol/min/10^6 cells and those of T-CA-1β-ol were 2.9 (taurine+) pmol/min/10^6 cells and not detected (taurine−), respectively. In infant (3 months old) hepatocytes, formation rates of G-CA-1β-ol after incubation for 24 h were 11.7 (taurine+) and 14.9 (taurine−) pmol/min/10^6 cells and those of T-CA-1β-ol were 13.6 (taurine+) and 0.2 (taurine−) pmol/min/10^6 cells, respectively. Formation rates of G-CA-1β-ol and T-CA-1β-ol in infants were higher than those in adults. The formation rate of T-CA-1β-ol was markedly improved by adding taurine (Table 2).

Identification of P450 Enzymes Responsible for CA-1β-Hydroxylation The contribution of individual P450 enzymes to 1β-CA biotransformation was evaluated using a panel of 19 human recombinant P450 enzymes. Studies on the inhibition of human hepatocytes showed that co-incubation with ketoconazole reduced the production of 1β-hydroxylated CA (Fig. 6). These experiments illustrated that the CYP3A7 and CYP3A subfamily was the principal enzyme responsible for the formation of 1β-hydroxylated CA. CA-1β-ol was not detected upon incubation with other investigated CYPs (Fig. 6).

Infant and Adult Hepatocyte mRNA Expression Levels Figure 7 shows a composite of CYP3A expression in infant and adult hepatocyte samples. As expected, compared with infant hepatocytes, adult human hepatocytes exhibited low levels of CYP3A7. Hepatocytes from human infants showed extremely high expression of the CYP3A7 gene and low expression of the CYP3A4 gene.
Fig. 3. SRM Chromatograms of G-CA-1β-ol (A: m/z 480.2→74.1) and T-CA-1β-ol (B: m/z 530.2→124.0)
Upper panels mean standard sample, and under panels mean cryopreserved infancy human hepatocytes.

Fig. 4. MS/MS Spectrum of G-CA-1β-ol (A) Obtained by Chemical Synthesis and (B) Produced from Incubations with Cryopreserved Infancy Human Hepatocytes

Fig. 5. MS/MS Spectrum of T-CA-1β-ol (A) Obtained by Chemical Synthesis and (B) Produced from Incubations with Cryopreserved Infancy Human Hepatocytes

Table 2. The Formation Rate of T-CA-1β-ol and G-CA-1β-ol in Infant and Adult Human Hepatocytes

<table>
<thead>
<tr>
<th>Donor age</th>
<th>Taurine&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Taurine&lt;sup&gt;−&lt;/sup&gt;</th>
<th>Taurine&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Taurine&lt;sup&gt;−&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>33 years</td>
<td>2.9</td>
<td>ND</td>
<td>13.6</td>
<td>0.2</td>
</tr>
<tr>
<td>3 months</td>
<td>2.0</td>
<td>2.6</td>
<td>11.7</td>
<td>14.9</td>
</tr>
<tr>
<td>T-CA-1β-ol</td>
<td>2.6</td>
<td>ND</td>
<td>0.75</td>
<td>0.78</td>
</tr>
<tr>
<td>G-CA-1β-ol</td>
<td>2.6</td>
<td>ND</td>
<td>11.7</td>
<td>14.9</td>
</tr>
</tbody>
</table>
DISCUSSION

Few previous studies have reported on the biosynthetic pathway of 1β-hydroxylated bile acids during infancy because of difficulty in synthesis and lack of sensitive methods for analyzing glycine- and taurine-conjugated polyhydroxy bile acids.12) We previously reported on the synthesis of 1β-hydroxylated bile acids.13) First, we developed simple and sensitive methods for analyzing conjugated and unconjugated CA-1β-ol and CDCA-1β-ol by LC/MS/MS. One-step sample preparation using liquid-phase extraction was used to extract bile acids (BA). This method is sensitive, with a limit of quantification of approximately 1 nmol and has a short run time (11 min).

It has been reported that the major hydroxylated metabolite of DCA in human liver microsomal incubations was 1β-hydroxy-DCA.10) However, few in vitro studies have been reported on hydroxylated metabolite primary bile acids, such as CA and CDCA. In this study, we successfully identified 1β-hydroxy-G-CA and T-CA using infant hepatocytes by simple and sensitive LC/MS/MS methods. Unconjugated 1β-hydroxylated CA was not detected after 24 h of incubation, and 1β-hydroxylated CA was the predominant metabolite formed in conjugation with amino acids, such as glycine and taurine. Glyco, Tauro and unconjugated-CDCA-1β-ol were not detected by LC/MS/MS analysis of the extracted culture medium (data not shown). Results obtained in this study suggest that unknown peaks A and B are produced from CA by hydroxylation at C-2, C-6, or C-4.15)

Kimura et al. have reported that the mean polyhydroxylated bile acid (1β-CA)/Cr ratio rapidly increases during the first week of life.15) However, at 11–12 months after birth, the mean polyhydroxylated bile acid/Cr ratio declines to values approaching those observed in healthy adults.9) The percentage of 1β-hydroxylated bile acids in the urine is significantly higher at 7 d of age than at 3 months, 6–12 months, 1–2 years, or 2–4 years of age.16)

In this study, formation rates of 1β-hydroxylated bile acids in infant hepatocytes were higher than those in adult hepatocytes (Table 2). The cause for the only small amounts of 1β-hydroxylated bile acids in adult human hepatocytes seems to be high conjugation activity.

In infant, we have concluded that the activity of hydroxylation at C-1 is higher than the amino conjugation activity. This is further supported by the observation that 1β-hydroxylated bile acids are predominant during early infancy and decline over time. This indicates that the metabolism of bile acids significantly changes from infancy to adulthood.

In the study by Bodin et al., the importance of CYP3A4 in this process was confirmed by incubation with recombinant
CYP3A4 or human liver microsomes, both of which efficiently converted DCA into 1β-hydroxydeoxycholic acid. In this study, we established that CA 1β-hydroxylation is specifically catalyzed by CYP3A7 and CYP3A4 using a panel of 19 human recombinant P450 enzymes. It has been reported that the CYP3A7 accounts for 87 to 100% total CYP3A in fetal liver. In contrast to the sharp decline in CYP3A7 levels from early gestation to infancy, CYP3A4 levels increase slowly with age, and the contribution of CYP3A4 to total CYP3A content is highly variable. Dehydroepiandrosterone-3-sulfate (DHEA-3S) is a compound showing high catalytic activity at CYP3A7 compared with CYP3A4 and CYP3A5. Fetal growth is suppressed when DHEA-3S is present at high concentration in the fetus. It has been reported that the metabolism of DHEA-3S by CYP3A7 is one of fetal defense mechanisms.

We showed that the adult human hepatocytes used in this experiment exhibited low expression of CYP3A7 genes compared with that in infant hepatocytes (Fig. 7). Recent evidence experiment exhibited low expression of CYP3A7 genes combination in the fetus. It has been reported that the metabolism of β-taurine conjugates in fetuses, in contrast to the glycine conjugates mainly observed in adults. Our results showed that the adult human hepatocytes used in this study, taurine concentration upon the incubation of human hepatocytes was 59-fold higher than those without adding taurine. In this in vitro study, taurine concentration upon the incubation of human hepatocytes was extremely low (data not shown). The finding that the presence of taurine markedly improved T-CA-1β-ol production is interesting. In the liver and intestine, transporters play a critical role in maintaining the enterohepatic circulation and bile acid homeostasis. Sodium taurocholate co-transporting polypeptide and bile salt export pump (BSEP) are two key transporters for hepatic bile acid uptake and excretion. In general, BSEP is also expressed in the canalicular membrane and TCA is a good substrate of BSEP. Yamaguchi et al. have reported that T-CA-1β-ol was not a substrate of BSEP. It is assumed that T-CA-1β-ol is not maintained within the enterohepatic circulation by the combined actions of transporter systems in the liver and intestine. Reportedly, 1β-hydroxylation of bile acids in a predominant pathway enhances urinary excretion in infants with inefficient biliary excretion of bile acids.

We suggested that T-CA-1β-ol facilitates enhanced urinary bile excretion during the period when the mechanisms for hepatic excretion of bile acids are poorly developed. The cause of the enhanced urinary excretion associated with T-CA-1β-ol requires further investigation.

In conclusion, we identified that the hydroxylated CA upon incubation of human hepatocytes was tauro-CA-1β-ol, as revealed by LC/ESI-MS/MS analysis. Our results clearly showed that CA-1β-ol formation from CA is predominantly catalyzed by CYP3A7 and CYP3A4 enzymes. The results provide useful information on a major biosynthetic pathway of bile acids during infancy.

**Conflict of Interest** The authors declare no conflict of interest.


21) Verner A, Craig S, McGuire W. Effect of taurine supplementation on growth and development in preterm or low birth weight infants.


