Glucuronidation of oxidized fatty acids and prostaglandins B_1 and E_2 by human hepatic and recombinant UDP-glucuronosyltransferases

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Abstract Arachidonic acid (AA) can be metabolized to various metabolites, which can act as mediators of cellular processes. The objective of this work was to identify whether AA, prostaglandin (PG) B_1 and E_2, and 15- and 20-hydroxyeicosatetraenoic acids (15- and 20-HETE) are metabolized via glucuronidation. Assays with human recombinant UDP-glucuronosyltransferase 1A (UGT1A) isoforms revealed that AA and 15-HETE were glucuronidated by UGT1A1, 1A3, 1A4, 1A9, and 1A10, whereas 20-HETE was glucuronidated by UGT1A1 and 1A4 and PGB_1 was glucuronidated by UGT1A1, 1A9, and 1A10. All substrates were glucuronidated by recombinant UGT2B7, with AA and 20-HETE being the best substrates. Kinetic analysis of UGT1A1 and 1A9 with AA resulted in K_m values of 37.9 and 45.8 μM, respectively. PGB_1 was glucuronidated by UGT1A1 with a K_m of 26.3 μM. The K_m values for all substrates with UGT2B7 were significantly higher than with the UGT1A isoforms. Liquid chromatography-mass spectrometry of glucuronides biosynthesized from PGB_1 and 15-HETE showed that hydroxyl groups were the major target of glucuronidation. **This work demonstrates a novel metabolic pathway for HETEs and PGs and the role of UGT1A isoforms in this process. These results indicate that glucuronidation may play a significant role in modulation of the availability of these fatty acid derivatives for cellular processes.**—Little, J. M., M. Kurkela, J. Sonka, S. Jäntti, R. Ketola, S. Bratton, M. Finel, and A. Radominska-Pandya. **Glucuronidation of oxidized fatty acids and prostaglandins B_1 and E_2 by human hepatic and recombinant UDP-glucuronosyltransferases. *J. Lipid Res.* 2004.** 45: **1694–1703.**

Supplementary key words uridine diphosphate-glucuronosyltransferase • prostaglandins • hydroxyeicosatetraenoic acid • human liver • human intestine • liquid chromatography-mass spectrometry

Glucuronide conjugation is one of the major pathways of biotransformation of steroid and thyroid hormones, bilirubin, bile acids, retinoids, xenobiotics, and several classes of drugs (1–5). The majority of endogenous compounds that are substrates for UDP-glucuronosyltransferases (UGTs) contain a carboxylic function. Bilirubin, bile acids, retinoids, and the recently identified new substrates for glucuronidation, Fatty acids (FAs) (6–10), all form acyl glucuronides. Acyl glucuronides may bind to albumin and other proteins to form adducts that can act as antigens to stimulate an immunoreaction (11). This covalent binding has been demonstrated for a number of carboxylic acid-containing drugs (11). However, carboxyl-linked glucuronides of endogenous compounds have not been shown to form similar adducts.

Glucuronidation is considered a detoxification pathway; however, glucuronidation of some endogenous/exogenous compounds (e.g., morphine, lithocholic acid, and estrogens) (12–14), can result in an increase in biological activity and/or toxicity. Although glucuronidation in general may terminate the biological activity of some compounds, it, like hydroxylation and sulfation, may also be involved in the bioactivation of compounds to toxins, mutagens, and carcinogens.

To date, 52 individual UGT gene products have been identified from different species. They are classified into two families, UGT1A and UGT2B (15, 16), based on the similarity of their DNA sequences (15, 17). The isoforms in family 1A are derived from a single gene locus that extends over 100 kb on human chromosome 2 (18–20). In contrast, UGT2B isoforms are encoded by separate genes clustered on human chromosome 4 and exhibit differ-

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Abbreviations: AA, arachidonic acid; GlcUA, glucuronic acid; HETE, hydroxyeicosatetraenoic acid; HI, human intestine; HL, human liver; 13-HODE, 13-hydroxyoctadecadienoic acid; LA, linoleic acid; LC-MS, liquid chromatography-mass spectrometry; OFA, oxidized fatty acid; 13-OXO, 13-oxooctadecadienoic acid; PG, prostaglandin; UDP-GlcUA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.

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ences in amino acid sequence throughout their length, including the carboxy-terminal domain (15, 17, 21).

FAs are a relatively complex class of compounds that includes saturated and monounsaturated or polyunsaturated compounds and are energy-rich molecules that are also an integral part of cellular membrane components. FAs can directly and indirectly modulate specific cell-signaling pathways (22). FAs serve as dietary precursors of prostanoids and other eicosanoids and, thus, are of great significance in health and the modulation of disease conditions.

The biotransformation of FAs via glucuronidation in humans is a relatively new concept. The first glucuronides of oxidized fatty acids (OFAs) were isolated from and identified in human urine (23–25) and primary human hepatocyte cultures (26). Glucuronides of dihydroxylated linoleic acid (LA) were first isolated from urine of patients with generalized peroxisomal disorders and were considered detoxification products of these cytotoxic diols (24). The major compounds isolated were LA-9,10- and LA-12,13-diol glucuronides. A monohydroxylated arachidonic acid (AA) derivative, 20-hydroxyeicosatetraenoic acid (20-HETE), was also isolated as a glucuronide from urine from normal subjects and, at significantly higher levels, from patients with hepatic cirrhosis (23, 25). The biosynthesis of the glucuronides of several eicosanoids by isolated human and rat hepatocytes has been described (26, 27).

Recently, our laboratory has become interested in the characterization of the glucuronidation of free FAs (FFAs) and OFAs by reporting the first studies demonstrating that OFA glucuronides can be biosynthesized by human hepatic and intestinal microsomes (7, 8, 28). These studies have demonstrated that LA, LA diols, 13-hydroxyoctadecadienoic acid (13-HODE), and 13-oxooctadecadienoic acid (13-OXO), as well as AA, are conjugated with glucuronic acid (GlcUA) in vitro (7, 8, 28, 29). The human UGT2B7 isoform was identified as the only available UGT isoform with the ability to glucuronidate both the hydroxyl and carboxyl functions of FA. In addition to our experiments with OFAs, our in vitro studies with human microsomes and recombinant UGT2B7 established that LA and AA, the parent compounds in FA oxidative processes, are also glucuronidated with relatively high activity at the carboxyl function by human UGTs (7 and the present study).

In recent studies by Turgeon et al. (10), the glucuronidation of leukotriene B_4, as well as 5-, 12-, and 15-HETE, by human liver (HL) microsomes and several recombinant UGTs was also demonstrated. In this project, we have extended our studies on FA glucuronidation to the in vitro characterization of the glucuronidation of the prostaglandins PGB_1, PGE_2, and 15- and 20-HETE (structures are shown in **Fig. 1** and the biosynthetic pathways from AA are shown in **Fig. 2**), compounds with recognized physiological significance, for comparison with the glucuronidation of the corresponding parent compound AA. HL and intestinal microsomes and UGT2B7 were found to glucuronidate all of the substrates with varying activities. These data suggest that human UGT2B7, constitutively expressed in several tissues, biotransforms OFA at high concentrations. Therefore, UGT2B7 can be characterized as a low-affinity, high-capacity isoform. Human recombinant UGTs from the 1A family, including 1A1, 1A3, 1A4, 1A9, and 1A10, have been shown for the first time to accept these FA derivatives as substrates and can be classified as high-affinity, low-capacity components of the glucuronidation system.

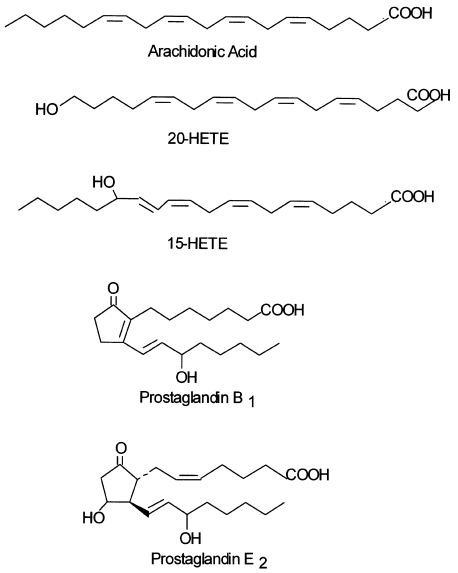

Fig. 1. Structures of the substrates used in these studies. HETE, hydroxyeicosatetraenoic acid.

EXPERIMENTAL PROCEDURES

Materials

[^{14}C]UDP-glucuronic acid ([^{14}C]UDP-GlcUA; specific activity, 325 mCi/mmol), [^{3}H]UDP-GlcUA (specific activity, 10.2 Ci/mmol), and [^{3}H]AA (specific activity, 98.6 Ci/mmol) were purchased from Perkin-Elmer Life Sciences (Boston, MA). Unlabeled UDP-GlcUA and AA were obtained from Sigma (St. Louis, MO). 20-HETE, 15-HETE, PGE_2, and PGB_1 were from Cayman Chemicals (Ann Arbor, MI). All other chemicals and solvents were of the highest quality commercially available.

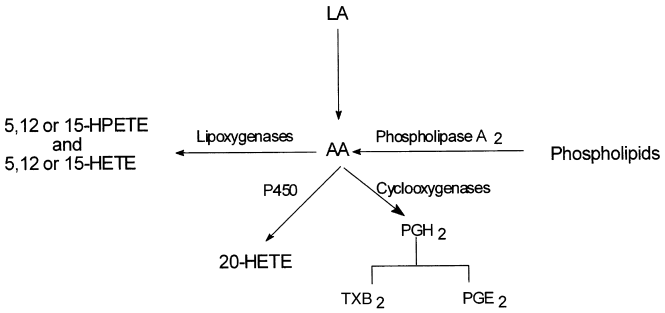

Fig. 2. Metabolism of arachidonic acid (AA). Schematic representation of the major pathways in the metabolism of AA. HPETE, hydroperoxyeicosatetraenoic acid; LA, linoleic acid; PGE_2, prostaglandin E_2; PGH_2, prostaglandin H_2; TxB, thromboxane B_2.

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Stable expression of UGT2B7

Human UGT2B7, a gift from Dr. T. Tephly (Department of Pharmacology, University of Iowa), was expressed in HK293 cells as previously described (30). HK293 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose, 10 mM HEPES, 110 μg/ml sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (all from Invitrogen, Carlsbad, CA) in a humidified atmosphere containing 5% CO_2 at 37°C.

Cloning and expression of human UGTs from the UGT1A family in baculovirus-infected insect cells

The cloning and expression of human hepatic UGTs as fusion proteins with a carboxy-terminal extension containing an enterokinase cleavage site and a His tag have recently been described (31). The cloning of exon 1 of the extrahepatic human UGTs 1A7, 1A8, and 1A10 and the construction of complete genes using exons 2–5 of UGT1A9 are detailed by Kuuranne et al. (32).

Human hepatic and intestinal microsomes and human recombinant UGTs

Liver and intestinal tissues were obtained from organ donors by transplant surgeons at the University of Arkansas for Medical Sciences under a protocol approved by the Human Research Advisory Committee. HL3 and human intestine (HI) microsomes (HI55) were prepared from the same donor, a 49 year old male who died of a stroke, using protocols described previously (33, 34). Small intestine (segment S-2) and colon microsomes from additional donors were used as detailed in the legend to **Fig. 3**.

For recombinant UGT2B7 expressed in HK293 cells, a membrane fraction was prepared as described by Battaglia et al. (35) and aliquots were stored at −70°C until use. Total membrane fractions from insect cells expressing each UGT isoform used were prepared as previously described (31). Briefly, frozen washed cells were disrupted by osmotic shock and homogenization, followed by centrifugation at 40,000 *g* for 120 min. The pellets were suspended and homogenized in a small volume of 25 mM Tris-Cl and 0.5 mM EDTA and stored at −70°C until use.

Enzyme assays with human microsomal fractions and recombinant UGT isoforms

UGT activities toward PGB_1 and PGE_2, AA, and 15- and 20-HETE in human hepatic and intestinal microsomes were assayed using [^{14}C]UDP-GlcUA or [^{3}H]UDP-GlcUA as the sugar donor as described in detail previously (9). Briefly, 100 μM [^{3}H]AA and unlabeled UDP-GlcUA (4 mM) or 100 μM unlabeled eicosanoid and [^{14}C]UDP-GlcUA or [^{3}H]UDP-GlcUA (4 mM) were incubated in 100 mM HEPES, 5 mM $MgCl_2$, and 5 mM saccharolactone with 50 μg of microsomal protein or 10 μg of recombinant UGT at 37°C for 10–30 min. For the assays with human hepatic and intestinal microsomes, the HETEs were prepared as micelles with Brij 58, which served to both solubilize the substrates and activate UGTs. When detergent was omitted from the incubation mixture, no detectable glucuronidation was observed. The glucuronidation activities for all recombinant proteins with AA and PGs were assayed in the absence of detergent. These lipid substrates were introduced to the reaction mixture in DMSO (3% final concentration). Products and unreacted substrates were separated by TLC on silica gel plates (Baker Si250-PA, 19C) in chloroform-methanol-acetic acid-water (65:25:2:4) as previously described (9).

Enzyme kinetic measurements

Apparent enzyme kinetic parameters (K_m and V_{max}) were determined using recombinant human UGT2B7, 1A1, and 1A9 at a fixed concentration of UDP-GlcUA (4 mM) and varied substrate concentrations (10–750 μM) with an incubation time of 10 min. Kinetic parameters were determined using Prism4 software (GraphPad Software, San Diego, CA).

Identification of the position of the glucuronide

Initial identification of the position of glucuronidation (hydroxyl or carboxyl group) was determined by TLC. Two identical reaction mixtures were prepared; after incubation, the reaction was stopped as usual in one sample while the other was incubated with 0.1 N NaOH at room temperature overnight. Both samples were chromatographed as described above. After development, the plates were subjected to autoradiography. Because carboxyl-linked glucuronides are alkali-labile, comparison of the recovery of product in each sample (hydroxyl- and carboxyl-linked glucuronides from the control sample; only hydroxyl-linked glucuronides from the hydrolyzed sample) allows for a tentative assignment of the position of glucuronidation.

Preparation of individual glucuronide conjugates for mass spectrometric analysis

For the preparation of the glucuronide samples for mass spectrometry, several standard analytical incubations were performed with HL3 microsomes and 15-HETE, 20-HETE, PGB_1, and PGE_2. After incubation of substrates with UDP-GlcUA for 1 h at 37°C, reactions were stopped by the addition of 0.1 M glycine-TCA, pH 2.8. (The low pH prevents acyl glucuronides from being hydrolyzed.) Samples were applied to a C18 solid-phase extraction cartridge (BondElut; Varian, Palo Alto, CA) and treated as previously described (36). The biosynthesized glucuronide(s) and any unreacted aglycon were eluted with methanol, which was then evaporated under nitrogen. For alkaline hydrolysis studies, samples incubated as above were brought to 0.1 M NaOH, incubated at room temperature for 1 h, and purified on BondElut cartridges as described above.

Liquid chromatography coupled with mass spectrometry

Samples containing glucuronides biosynthesized as described above were analyzed by HPLC coupled with mass spectrometry. The HPLC system consisted of an XTerra MS C18 column (2.5 μm, 30 × 4.6 mm; Waters, Milford, MA), a model 1100 binary pump, and an auto sampler (Agilent Technologies, Waldbronn, Germany). The mobile phase consisted of solvents A [methanol/water/50 mM ammonium acetate, pH 4 (1:8:1)] and B [methanol/50 mM ammonium acetate, pH 4 (9:1)], and a gradient of 40–100% solvent B was developed over 14 min at 0.7 ml/min. The HPLC system was coupled with a 1:100 splitter to an API 3000 triple quadrupole instrument (Applied Biosystems/MDS Sciex Instruments, Foster City, CA) with an electrospray ionization source set to the negative ion mode with ion spray voltage at −4,200 V and orifice voltage at −25 V. Ion chromatograms of the aglycons and glucuronides were obtained using selected ion monitoring or as an extracted ion chromatogram when the total ion chromatogram *m/z* 50–900 was acquired. The product ion spectra were obtained using an orifice voltage of −25 V and a collision energy of −35 V, and the data were analyzed with Analyst 1.3 software (Applied Biosystems/MDS Sciex Instruments).

RESULTS

Glucuronidation of lipid substrates by human recombinant UGTs

Human hepatic and intestinal microsomes from a single donor, recombinant UGT isoforms from the 1A family

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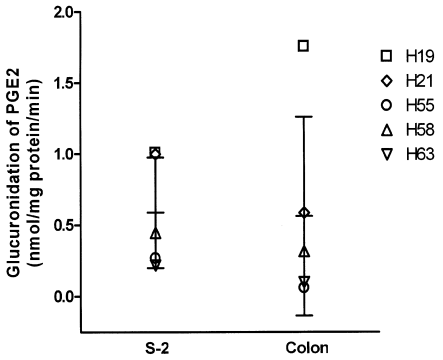

Fig. 3. Comparison of the glucuronidation of PGE_2 by small intestine (segment S-2) and colon microsomes from five donors. Assays were carried out by incubating microsomal proteins (50 μg) with PGE_2 (100 μM) and [^{3}H]UDP-glucuronic acid ([^{3}H]UDP-GlcUA; 4 mM) for 15 min at 37°C. Reactions were stopped by the addition of ethanol, and products were separated by TLC. Silica gel containing glucuronides, as identified by autoradiography of the TLC plate for 3–5 days at −70°C, was transferred to vials, and radioactivity was quantitated by liquid scintillation counting. The donors were as follows: H19, an 18 year old male who died of a subarachnoid hemorrhage; H21, a 61 year old female who died of a subarachnoid hemorrhage; H55, a 49 year old male who died of a stroke; H58, a 59 year old male who died of gunshot wound to the head; H63, a 39 year old male who died in a motor vehicle accident. Each point represents the mean of at least two sets of duplicate determinations of specific activity of S-2 or colon toward PGE_2 from an individual donor. The error bars represent the mean and SD of the glucuronidation activity of the donor sample set.

expressed in Sf9 cells as His tag proteins, and UGT2B7 expressed in HK293 cells were screened for their ability to glucuronidate AA, 15-HETE, 20-HETE, PGE_2, and PGB_1; the results are summarized in **Table 1**. Glucuronidation assays using UGTs from the 1A family identified UGT1A1, 1A3, 1A4, 1A9, and 1A10 as being active in the glucuronidation of both AA and 15-HETE. AA, which is glucuronidated on the carboxyl function, was the best substrate for all recombinant UGTs. Interestingly, 20-HETE was not accepted as a substrate by UGT1A9 or 1A10 under the experimental conditions used.

Both PGB_1 and PGE_2 were glucuronidated by all recombinant UGTs under investigation, with the exception of UGT1A4. PGB_1, which contains only one hydroxyl group, as opposed to PGE_2, which contains two hydroxyl groups, one on the cyclic ring and one on the side chain, was a much better substrate for all recombinant UGTs. UGT1A3 was able to glucuronidate both HETE derivatives; however, 20-HETE was the better substrate by a factor of almost 2. UGT1A4 glucuronidated AA and both HETEs equally, although the activities were among the lowest measured. UGT1A9 glucuronidated AA and 15-HETE with the highest activities; PGB_1 was also glucuronidated by this isoform, but the activity was low. AA was an excellent substrate for UGT1A10, but the activity of this isoform toward 15-HETE and PGB_1 was relatively low. As also shown in Table 1, all five substrates were glucuronidated by recombinant UGT2B7 expressed in HK293 cells, and the activities varied significantly for the different substrates.

Glucuronidation of lipid substrates by human hepatic and intestinal microsomes

We also included human hepatic and intestinal microsomes from the same donor in these studies (Table 1). With HL3, PGB_1 was the best substrate, followed by 20-HETE > PGE_2 > 15-HETE > AA; with HI55 (segment S-2), the order was PGB_1 > PGE_2 > 20-HETE > 15-HETE > AA. TLC analysis before and after alkaline hydrolysis of the products of incubation of the substrates indicated that hydroxyl glucuronides of all substrates were formed, and only 20-HETE showed evidence of a relatively minor acyl glucuronide product.

The intestinal studies were extended to include small

TABLE 1. Glucuronidation of AA and its derivatives by recombinant UGTs expressed in either baculovirus-infected Sf9 cells (UGT1A isoforms) or HK293 cells (UGT2B7) and human hepatic and intestinal microsomes

	Substrate				
UGT Source	AA	15-HETE	20-HETE	PGB_1	PGE_2
	nmol/mg protein/min				
UGT1A1	1.82 ± 0.12	0.76 ± 0.26	1.11 ± 0.19	1.47 ± 0.30	0.20 ± 0.02
UGT1A3	3.04 ± 1.02	0.54 ± 0.40	0.97 ± 0.16	0.76 ± 0.29	0.14 ± 0.02
UGT1A4	0.30 ± 0.03	0.44 ± 0.46	0.33 ± 0.09	nd	nd
UGT1A9	5.45 ± 1.25	1.17 ± 0.42	nd	0.87 ± 0.17	0.04 ± 0.01
UGT1A10	1.84 ± 0.02	0.30 ± 0.03	nd	0.23 ± 0.02	0.05 ± 0.02
UGT2B7	5.08 ± 0.81	0.34 ± 0.10	1.00 ± 0.15	0.74 ± 0.07	0.49 ± 0.14
HL3	0.50 ± 0.14	0.54 ± 0.40	1.54 ± 0.22	1.70 ± 0.36	0.60 ± 0.24
HI55 S-2	0.13 ± 0.08	0.25 ± 0.07	0.33 ± 0.02	0.53 ± 0.14	0.40 ± 0.14

Assays were carried out by incubating 50 μg of microsomal protein or 10 μg of recombinant protein with 100 μM substrate and [^{3}H]UDP-glucuronic acid ([^{3}H]UDP-GlcUA; 4 mM) for 20 min at 37°C. Reactions were stopped by the addition of ethanol, and products were separated by TLC. Silica gel containing glucuronides, as identified by autoradiography of the TLC plate for 3–5 days at −70°C, was transferred to vials, and radioactivity was quantitated by liquid scintillation counting. Results are expressed as means ± SD of at least two sets of duplicate determinations. AA, arachidonic acid; 15-HETE and 20-HETE, 15- and 20-hydroxyeicosatetraenoic acid; HI, human intestine; HL, human liver; nd, not detected; PGB_1, prostaglandin B_1; PGE_2, prostaglandin E_2; UGT, UDP-glucuronosyltransferase.

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TABLE 2. Apparent kinetic constants for AA and its derivatives

Substrate	K_m	V_{max}	V_{max}/K_m
	μM	*nmol/mg protein/min*	*μl/mg protein/min*
AA (1A9)	45.8 ± 10	1.1 ± 0.1	24.0
AA (1A1)	37.9 ± 29	0.5 ± 0.2	13.2
AA (2B7)	161.4 ± 69	2.5 ± 0.4	15.5
20-HETE (2B7)	48.4 ± 17	0.9 ± 0.1	18.6
15-HETE (2B7)	64.2 ± 30	0.5 ± 0.1	7.8
15-HETE (1A9)	70.1 ± 27	1.0 ± 0.1	14.3
PGB_1 (2B7)	161.9 ± 100	0.8 ± 0.2	4.9
PGB_1 (1A1)	42.7 ± 20	1.3 ± 0.1	31.2
PGE_2 (2B7)	216.2 ± 64	1.2 ± 0.2	5.6

Kinetic analyses were carried out at increasing concentrations of substrate at a constant concentration of UDP-GlcUA (4 mM), and constants were determined using GraphPad Prism4 software.

intestinal (segment S-2) and colon microsomes from five different donors. These were assayed for activity toward PGE_2, which has been implicated in the development of colon cancer (37–39). The results shown in Fig. 3 reveal that PGE_2 was glucuronidated by microsomes from small intestine and colon of all donors studied; however, there were large differences between donors. Activities were generally lower in colon than in small intestine except for donor H19, a generally healthy 18 year old male who had the highest values for both segments.

Kinetic analysis

The kinetic analyses were carried out with UGT2B7 for all substrates and were also measured for those UGT1A isoforms having the highest activities toward certain substrates. Specifically, kinetic parameters were also determined for UGT1A9 with AA and 15-HETE and for UGT1A1 with AA and PGB_1. The apparent kinetic parameters (K_m, V_{max}, and V_{max}/K_m) presented in **Table 2** were estimated by plotting the measured initial reaction velocity values as a function of substrate concentration (**Fig. 4**) and fitting these to the Michaelis-Menten equation using Prism4 software.

Our previous experiments have demonstrated that LA is a good substrate for UGT2B7, with a K_m of 146 ± 15 μM and a V_{max} of 5.29 ± 0.3 nmol/mg/min (28). The K_m for AA demonstrated in the present studies with the same isoform (162 ± 69 μM) was very close to that of LA; however, the V_{max} value was only half that of LA. Kinetic analysis of the glucuronidation of 15- and 20-HETE with UGT2B7 gave K_m values that were similar, 64.2 ± 29 and 48.3 ± 17 μM, respectively. However, the V_{max} for 20-HETE, 0.94 ± 0.1 nmol/mg/min, was twice as high as that for 15-HETE, 0.5 ± 0.1 nmol/mg/min. Thus, the efficiency of glucuronidation of these substrates was 7.8 ± 0.5 and 18.6 ± 1.7 μl/mg/min for 15- and 20-HETE, respectively.

The glucuronidation of AA by UGT1A9 and 1A1 has been demonstrated here for the first time. The K_m value with UGT1A9 was 45.8 ± 10 μM, one of the lowest values obtained in the present studies, indicating that this isoform might be highly specific for the glucuronidation of AA. AA was also glucuronidated by UGT1A1 with very high affinity; however, the V_{max} value was half of that obtained for 1A9 (0.5 ± 0.2 vs. 1.1 ± 0.1 nmol/mg/min, respectively). Additionally, it was shown that UGT1A9 glucuronidated 15-HETE with the same affinity as UGT2B7; however, the V_{max} was double that of the value with UGT2B7.

Glucuronidation of PGB_1 by UGT2B7 and 1A1 has also been demonstrated for the first time in this work. Kinetic analysis showed a K_m of 161.9 ± 100 μM for PGB_1 with UGT2B7, approximately two to four times higher than for the two HETEs. The V_{max} of 1.2 ± 0.2 nmol/mg/min for PGB_1 was almost 2-fold higher than for the HETEs. We also showed that UGT1A1 glucuronidated PGB_1 with high efficiency: the K_m value for this isoform was 42.7 ± 20 μM. It can be concluded that UGT1A1 is the isoform that has the greatest affinity for this substrate.

The kinetics obtained for the glucuronidation of PGE_2 by UGT2B7 resulted in the highest K_m value measured (216.2 ± 64 μM). It is probable that other unidentified UGTs may be involved in the glucuronidation of this PG.

Detection of glucuronides by liquid chromatography-tandem mass spectrometry

At present, the development of precise and sensitive HPLC-mass spectrometry methods makes it possible to isolate and identify lipid glucuronides biosynthesized in vitro (10). Here, we have presented a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the in vitro determination of lipid substrate glucuronides. The spectral analysis by LC-MS/MS was carried out for 15- and 20-HETE, PGB_1, and PGE_2. The different ions that were detected by LC-MS/MS analyses of the tested glucuronides are listed in **Table 3**. The product ion spectra of the glucuronides showed the respective aglycons (attributable to the neutral loss of *m/z* 176) as well as ions at *m/z* 175 and 113, which are specific glucuronide ions (Table 3). Representative data obtained by LC-MS/MS analysis of the 15-HETE and PGB_1 glucuronides are shown in Figs. 5 and 6. Single monoglucuronides of 15-HETE (**Fig. 5**), as reported recently (10), and PGB_1 (**Fig. 6**) were detected by LC-MS as the respective deprotonated molecular ions (Table 3). Similar results from the LC-MS analyses of the products of 15-HETE glucuronidation were obtained previously by Turgeon et al. (10). In the case of 20-HETE, however, two distinct monoglucuronides, a minor and a major product (Table 3), were detected. TLC analysis (as discussed above) indicated that the major glucuronide product was a hydroxyl glucuronide and the minor product was the acyl (or carboxyl) glucuronide. No diglucuronides were detected for any of the aglycons that were examined in this study.

Analytical glucuronidation assays showed that only a single compound was biosynthesized from PGE_2. However, this could not be confirmed by LC-MS analysis. Instead of PGE_2 glucuronide, LC-MS/MS analysis using multiple reaction monitoring showed ion pairs of 509/333 amu and 509/175 amu with same retention time. The 509 amu mass is consistent with a glucuronide of PGA_2, not PGE_2, which gives rise to two daughter ions at 175 and 333 amu, having the masses of dehydrated glucuronic acid and aglycon, respectively. This can be explained, as has

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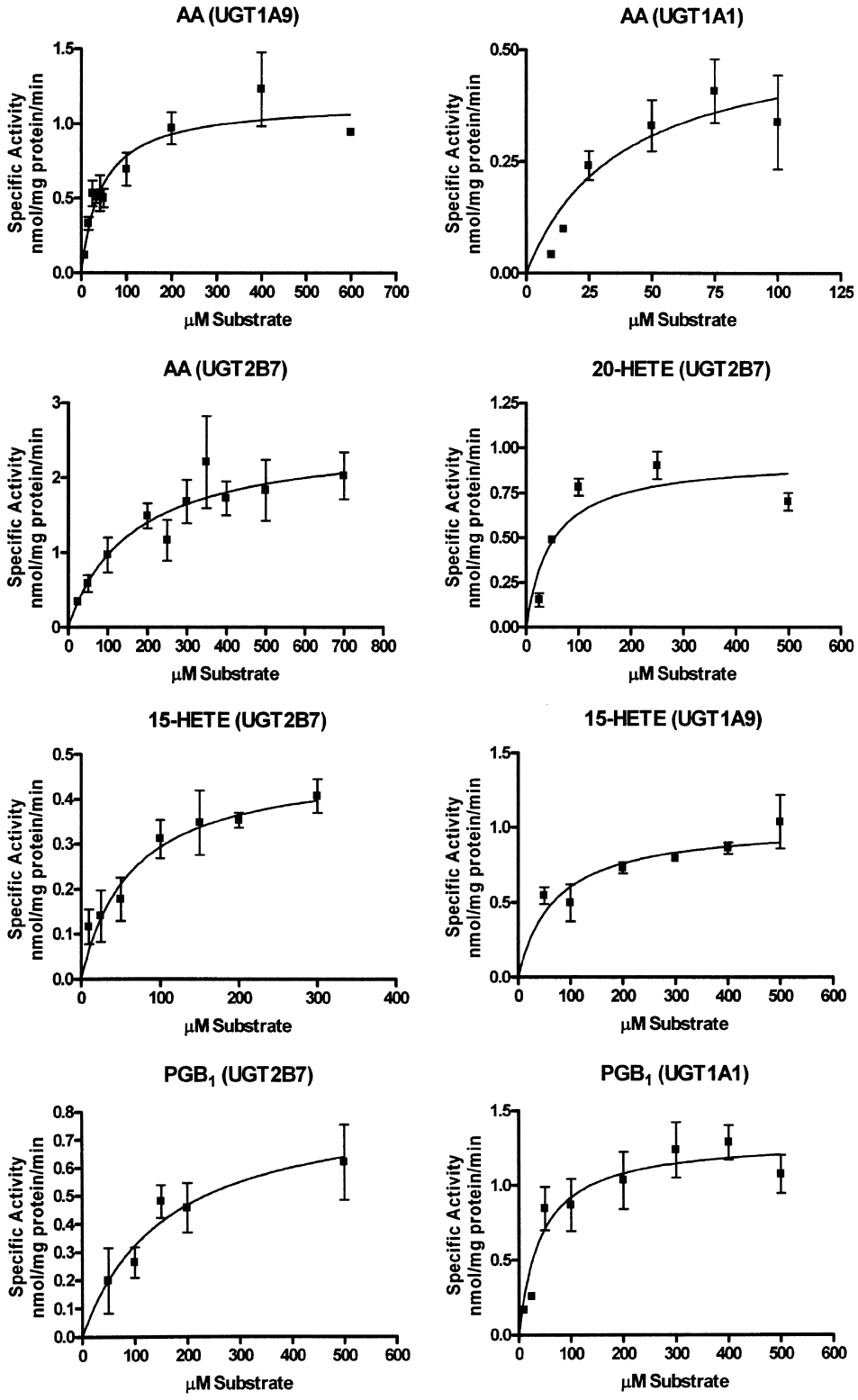

Fig. 4. Kinetic analysis of the glucuronidation of AA, 15-HETE, 20-HETE, PGB_1, and PGE_2 by UDP-glucuronosyltransferase 1A (UGT1A) isoforms expressed as His tag proteins in insect cells and UGT2B7 expressed in HK293 cells. The graphic fits of the data from each of the analyses with each substrate (means ± SD of four to six determinations) are shown. Assays were carried out by incubating membrane fractions containing recombinant UGTs (10 µg) with increasing concentrations (as indicated) of each substrate and [^{3}H]UDP-GlcUA (4 mM) for 10 min at 37°C. Reactions were stopped by the addition of ethanol, and products were separated by TLC and identified by autoradiography. Curve fits were determined using GraphPad Prism 4 software.

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TABLE 3. Identification of FA glucuronides by tandem mass spectrometry

Ions	15-HETE	20-HETE (Major Peak)	20-HETE (Minor Peak)	PGB_1
		m/z		
Precursor ion				
$[M-H]^-$	495	495	495	511
Product ions				
$[M-H-H_2O]^-$	477	nd	477	493
[M-H-176 *m/z*]$^-$	319	319	319	335
[M-H-glucuronic acid]$^-$	301	301	301	317
[Glucuronic acid-H]$^-$	193	193	193	193
[Gluc fragment]$^-$	175	175	175	175
[Gluc fragment]$^-$	157	nd	157	157
[Gluc fragment]	113	113	113	113

Gluc, glucuronic acid. The samples were prepared by incubation of the aglycons with HL microsomes (HL3). The products were partially purified by solid-phase extraction and subsequently subjected to HPLC and identified by negative ion electrospray ionization tandem mass spectrometry. Deprotonated aglycon substrate ions [M-H-176 *m/z*]$^-$ and the loss of 194 *m/z*, indicating the loss of glucuronic acid [M-H-glucuronic acid]$^-$ from the precursor ions $[M-H]^-$, were found in all spectra. Other abundant ions seen in all of the spectra were [glucuronic acid-H]$^-$ and the diagnostic glucuronide moiety fragments at *m/z* 175 and 113. nd, not detected.

been described previously by Hankin, Wheelan, and Murphy (27), by PGA_2 being produced by dehydration of PGE_2 at the low pH (2.8) used for the preparation of the sample for MS. At present, we cannot confirm this because chemically synthesized standards of these glucuronides are not available for LC-MS identification. Therefore, the fragmentation patterns and spectra of these glucuronides are not included in this paper.

DISCUSSION

The present work is a continuation of our former studies that clearly indicated that FFAs and their hydroxylation products, all of biological and pharmacological importance, are excellent substrates for glucuronidation. Now, we have extended our investigation to the study of the biotransformation of HETEs and PGs biosynthesized from AA, as shown in Fig. 2.

The availability of human UGT isoforms expressed as His tag conjugates in Sf9 cells allowed us to directly compare the glucuronidation of AA and its oxidized derivatives by these isoforms. As shown in Table 1, enzymes expressed as His tag proteins have glucuronidating efficiencies similar to, or in some cases higher than, those of UGT2B7 expressed in HK293 cells. Therefore, this set of proteins can be successfully used for the evaluation of the glucuronidation of other endogenous and exogenous UGT substrates.

The evaluation of glucuronidation activity with microsomes from a single HL and HI is not representative of

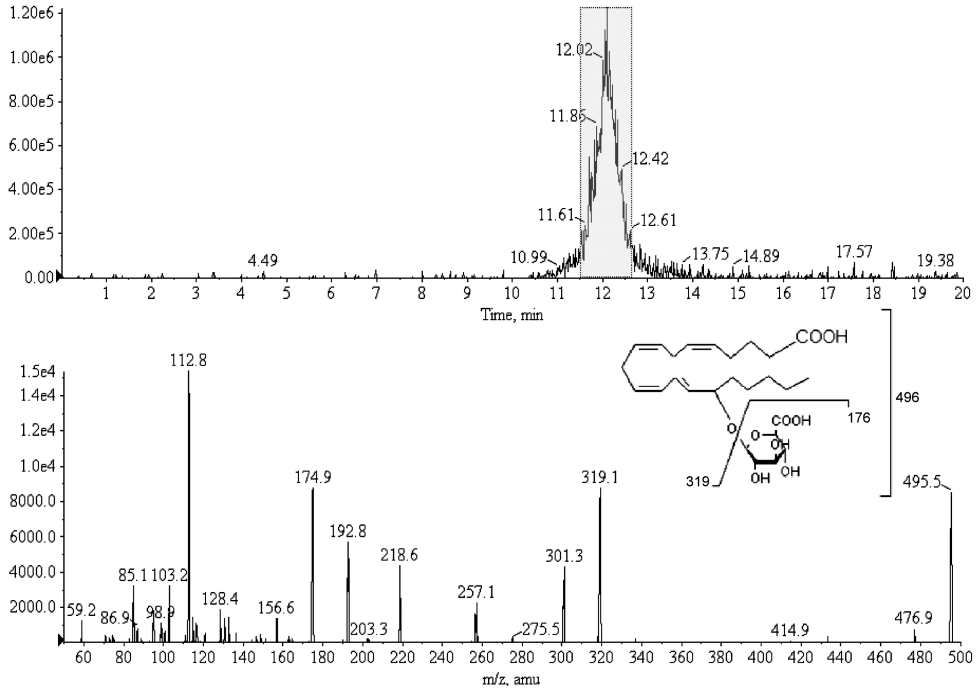

Fig. 5. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of 15-HETE glucuronide biosynthesized by human liver (HL) microsomes. Glucuronides were prepared by incubation of HL microsomes (HL3) (50 μg) with 15-HETE (200 μM) and UDP-GlcUA (4 mM) for 2 h at 37°C. Reactions were stopped with 0.1 M glycine-TCA, pH 2.8, samples were partially purified by solid-phase extraction on C18 cartridges, and the products were separated and analyzed by HPLC coupled with MS. The glucuronidation product was detected by daughter ion scanning of the deprotonated glucuronide at *m/z* 495 (upper panel). The ion in the MS/MS spectrum (lower panel), seen at *m/z* 319.1, corresponds to deprotonated 15-HETE that was formed after the loss of the glucuronide moiety (176 amu). The product ions at *m/z* 174.9 and 112.8 are specific marker ions (fragments) of the glucuronide moiety.

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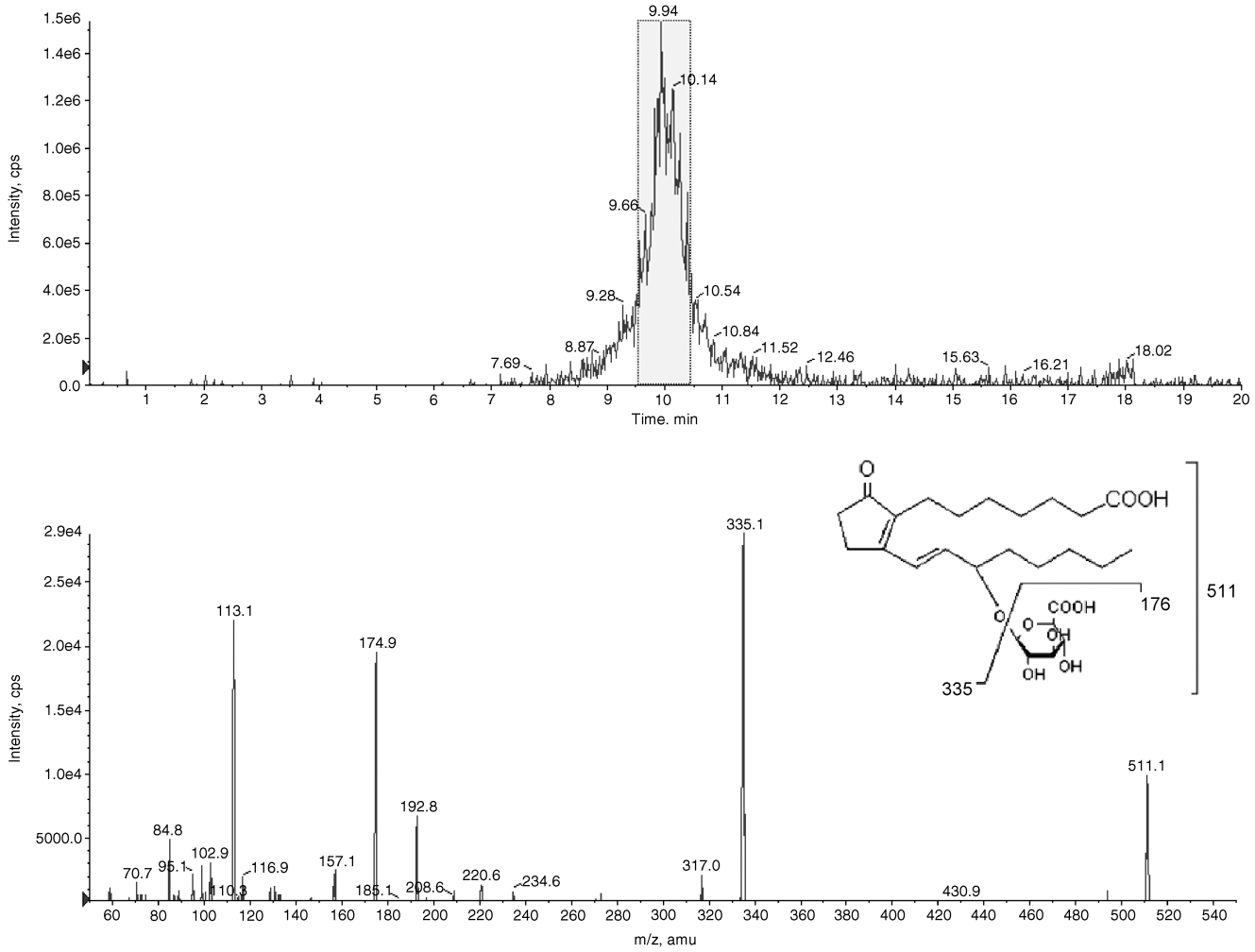

Fig. 6. LC-MS/MS analysis of the PGB_1 glucuronide biosynthesized by HL microsomes. Microsomal proteins (50 μg) from HL3 (see text for details) were incubated with 200 μM PGB_1 and 4 mM UDP-GlcUA at 37°C for 30 min. Samples were partially purified on C18 cartridges, and the products were separated and analyzed by HPLC coupled with MS. The glucuronidation product was detected by daughter ion scanning of deprotonated glucuronic acid conjugate at *m/z* 511 (upper panel). The ion in the MS/MS spectrum (lower panel), seen at *m/z* 335, corresponds to deprotonated PGB_1 that was formed after the loss of the glucuronide moiety (176 amu). The product ions at *m/z* 174.9 and 113.1 are specific marker ions (fragments) of the glucuronide moiety. cps, counts per second.

the activity in tissues from other donors, because we have shown that there is a great deal of interindividual variation in the activity of human hepatic and intestinal UGTs (28, 34, 40). These initial studies were carried out to assess the general ability of human UGTs to glucuronidate these substrates, and the hepatic microsomes were later used for preparative synthesis of the glucuronide conjugates for LC-MS/MS. We also checked the ability of small intestinal and colon microsomes from five different donors to glucuronidate PGE_2. The rationale for these experiments was that several studies have shown that PGE_2 is involved in the development of malignancy in human colon (37–39) and we were interested in checking the ability of UGTs in the colon to detoxify this compound.

Our present studies have identified new, physiologically important substrates, AA, PGB_1, PGE_2, and 20-HETE, for human microsomal and recombinant UGTs. As shown in Table 1, AA, which is glucuronidated at the carboxyl group, the only function available for glucuronidation, is the best substrate for both recombinant UGT1A isoforms and UGT2B7. This is the first demonstration that UGT1A1, 1A3, 1A4, 1A9, and 1A10 glucuronidate AA in the nanomolar range.

Analysis of 20- and 15-HETE, as well as the PG glucuronidation kinetic data (Table 2, Fig. 4), clearly demonstrated that, although UGT2B7 is involved in the glucuronidation of all substrates used in this work, the K_m values are relatively high (except with the HETEs) and probably do not reflect physiological concentrations of substrates. Our studies have also confirmed the results obtained by Turgeon et al. (10) that 15-HETE is glucuronidated by UGT1A1, 1A3, 1A9, and UGT2B7. The K_m values obtained in both laboratories with UGT2B7 were nearly identical.

Although it has previously been reported that 20-HETE glucuronide is present in urine from normal human subjects at concentrations much higher than PGE_2 (23), this

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is the first report that 20-HETE glucuronide is formed in vitro by human UGT isoforms. 20-HETE is formed as a result of the cytochrome P450-mediated metabolism of AA. 20-HETE is a potent vasoconstrictor (41–43) and may also play an important role in renal function and the pathogenesis of hypertension (44). Hydroxylated derivatives of AA such as 15- and 20-HETE are usually produced in the body after the consumption of foods rich in fats or under certain pathological conditions (25). The glucuronidation of HETEs can be considered a biotransformation process that results in the excretion of these lipids from the body after conversion to more soluble compounds. However, because HETEs are also important signaling molecules, their biotransformation via glucuronidation could be, as in the case of PGs, considered a termination of biological activity.

Our studies have identified human recombinant UGT2B7 as being capable of forming a PGE_2 glucuronide. It has been demonstrated previously that metabolism of PGE_2 in isolated hepatocytes leads to a series of products including glucuronide conjugates (27). However, the in vitro formation of this compound is a new discovery. PGE_2 is a major product of AA metabolism via cyclooxygenase-2 and is also the major prostanoid product of AA metabolism in colorectal tissue (45). Because PGE_2 is actively glucuronidated by human intestinal mucosa, one can speculate about the significance of this reaction. Because UGT2B7 is the only isoform that has been identified to date that is able to glucuronidate PGE_2, and because it is a major isoform expressed in human colon, the increased expression of UGT2B7 in the colon may protect against the accumulation of this PG. On the other hand, lower expression may promote the accumulation of this PG and, thereby, the development of cancer. As presented in Fig. 4, the glucuronidation of PGE_2 in small intestinal segment S-2 and colon is relatively high in some donors but demonstrates very significant individual variation. High expression of UGT2B7 in small intestine and colon, and the ability of this isoform to glucuronidate PGE_2, could be a very important colon cancer protective pathway.

Additionally, we examined the glucuronidation of another PG, PGB_1, for which the physiological role is not yet clearly defined. The fact that PGB_1 is glucuronidated with relatively high affinity by UGT1A1, and with slightly lower affinity by UGT2B7, is also a new discovery. To our knowledge, this is the first demonstration of glucuronidation of PGB_1 by human UGTs and demonstrates a new biotransformation pathway for this product of AA metabolism. This may represent a detoxification step or, if PGB_1 is a ligand for nuclear receptors, as are other OFAs, may provide additional evidence that UGTs, like other drug-metabolizing enzymes, are involved in controlling steady-state concentrations of signaling molecules and/or ligands for nuclear receptors, as has been discussed by Nebert (46, 47).

In summary, our present studies have identified novel substrates for in vitro glucuronidation: AA, PGB_1, PGE_2 and 20-HETE. We have demonstrated previously that LA and its derivatives, LA-9,10- and LA-12,13-diols, as well as 13-HODE and 13-OXO, are excellent substrates for UGT2B7 (7, 8, 28). This work establishes the role of human recombinant UGT2B7 and several isoforms from the UGT1A family in the glucuronidation of physiologically and pharmacologically important lipid compounds, such as PGs, HETEs, and AA. Because glucuronidation is the most effective detoxification process, it can be postulated that UGT2B7 is involved in the glucuronidation of these lipids when they accumulate above normal physiological concentrations. On the other hand, the UGTs from the 1A family, especially 1A1 and 1A9, may be responsible for the glucuronidation of oxidized lipid substrates at physiological concentrations.

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