GLUCURONIDATION OF RETINOIDS BY RAT RECOMBINANT UDP: GLUCURONOSYLTRANSFERASE 1.1 (BILIRUBIN UGT)

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ABSTRACT:

Rat liver recombinant BR$^{+}$UGT1.1 was found to have significant activity toward retinoid substrates. UGT1.1 glucuronidation activity was 91 ± 18 pmol/mg × min for atRA and 113 ± 19 pmol/mg × min for 5,6-epoxy-atRA. The apparent $K_{m}$ and $V_{max}$ of atRA acid glucuronidation by UGT1.1 were 59.1 ± 5.4 μM and 158 ± 43 pmol/mg × min, respectively. SDS-PAGE and Western blot analysis of UGT1.1-transfected HK293 membrane proteins photolabeled with [11,12-$^{3}$H]atRA revealed a protein of ~56 kDa that was labeled by [3]$^{3}$H]-atRA, detected by anti-pNP UGT antibody and not present in membranes from nontransfected HK293 cells. Liver microsomes from Gunn rats, which lack UGT1.1, had significant activity toward atRA (111 ± 28 pmol/mg × min).

The retinoids are physiologically and pharmacologically important substrates for glucuronidation. Physiologically, retinoids are active in maintenance of such functions as vision, reproduction, and differentiation (1), whereas pharmacologically they are used for treatment of acne (2, 3) and are under study for use in cancer chemotherapy and chemoprevention (4–6). Retinoid glucuronides have been identified as products of in vivo retinoid metabolism in several species (7–11), but the precise physiological significance of this mechanism has not been established. Its major role may be to reduce toxicity and teratogenicity (12), to produce a bioactive metabolite (13), to facilitate retinoid transport and presentation to target tissues, and/or to modulate retinoid concentrations. Whatever its role, data available on the enzymology of the retinoid conjugation process or identification of the UGT(s) catalyzing the reaction are limited. The carboxylic function of atRA has been shown to be effectively glucuronidated in vitro by rat liver microsomes (14–17), and the in vitro biosynthesis of the $\beta$-glucuronide of retinol has also been demonstrated (14), but the specific UGT isoenzymes have yet to be identified. The availability of rat and human recombinant UGTs has allowed the screening of different UGT isoforms for their ability to glucuronidate retinoids. In the present study, we identify recombinant UGT1.1 from rats and humans as one isoform involved in the glucuronidation of atRA and 5,6-epoxy-atRA. Enzymatic assays, photoaffinity labeling, and immunoprecipitation studies reveal that, although the contribution of rat UGT1.1 is significant, other, as yet unidentified, isoforms also catalyze the glucuronidation of these substrates.

This work was supported in part by Grants DK45123 and DK49715 (to A.R.), GM26221 (to T.R.T.), and ES06765 (V.S.) from the National Institutes of Health. 1 Abbreviations used are: BR, bilirubin; UGT, UDP-glucuronosyltransferase; atRA, all-trans-retinoic acid; 5,6-epoxy-atRA, 5,6-epoxy-all-trans-retinoic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; pNP, 4-nitrophenol; UDP-GlcUA, UDP-glucuronic acid; SD, Sprague-Dawley; BSA, bovine serum albumin; RA, retinoic acid; RLM, rat liver microsome.

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Materials and Methods

[11,12-$^{3}$H]atRA was purchased from Dupont-New England Nuclear (Boston, MA). [$^{14}$C]Lithocholic acid and [$^{14}$C]UDP-GlcUA were from American Radiolabeled Chemicals (St. Louis, MO). [$^{3}$H]Hyodeoxycholic acid, [$^{3}$H]Hepatic acid, and [$^{3}$H]iso-etanic acid were synthesized as previously described (18–20). Unlabeled atRA, Brij 58, UDP-GlcUA, saccharolactone, BR, and alamethicin were from Sigma Chemical Co. (St. Louis, MO). 5,6-Epoxy-atRA was synthesized from atRA by oxidation with monoperophthalic acid as described by Wertz et al. (21), and its identity and purity were established by NMR and MS analysis. Protein G-Sepharose 4 FastFlow was from Pharmacia Biotech (Piscataway, NJ).

RLMs and Recombinant UGT. Male SD, Wistar, and Gunn rats were fasted overnight before kiilling, livers were excised, and microsomes were prepared as described by Vanstapel and Blanckaert (22), except that they were not further subfractionated into rough and smooth endoplasmic reticulum fractions. Conditions for stable expression of rat and human UGTs in HK293 cells were as previously described (23). Enriched membrane fractions were prepared as described by Battaglia et al. (24). The resulting membrane fractions were stored in small aliquots at ~80°C until needed.

Enzymatic Assays. Retinoid glucuronidation activity was measured as described in ref. 17. Preliminary experiments established that neither alamethicin or detergent had a significant activating effect on recombinant UGT activity toward the substrates tested; therefore, these compounds were omitted from recombinant UGT incubations. All manipulations were conducted under yellow light. Experiments to assess kinetic parameters were done using the same basic method over a range of concentrations of [3]$^{3}$H]-atRA (1–100 μM) at a constant concentration of UDP-GlcUA (4 mM). Data were analyzed and apparent $K_{m}$ and $V_{max}$ determined using the program “EnzymeKinetics” (Trinity Software Compton, NH).

Bile acid glucuronidation activity was determined as previously described (18, 25). For determination of BR glucuronidation activity, BR dissolved in 0.2 N NaOH and bound to BSA (final concentration: 0.34 mM BR, 8 mg/ml BSA) was incubated in a total volume of 100 μl containing 20 mM Tris-Cl (pH 7.6), 5 mM MgCl₂, 5 mM saccharolactone, 0.05% Brij-58, and 50 μg of microsomal protein. Reactions were started by the addition of [14$^{3}$C]UDP-GlcUA (final concentration: 2 mM and 0.07 μM). After 30 min at 37°C, the reaction was stopped by adding 0.4 M HCl-glycine (pH 2.7; 400 μl), and the samples were diacitized and extracted using the method of Heirwegh et al. (26), with all volumes reduced by a factor of 5. Extracts were evaporated under nitrogen, redissolved in extraction solvent, and aliquots were chromatographed...
as described by Jansen (27). Labeled metabolites were localized by color and autoradiography as previously described.

Photoaffinity Labeling. Photolabeling with [11,12-3H]atRA was done using the method of Bernstein et al. (28), modified as follows. [3H]atRA (30–50 μM in ethanol) was added (final concentration of 1.5–2 μM; 2.0 μCi) to rat liver microsomal or UGT1.1 membrane protein (100 μg) in 100 mM HEPES (pH 7.0) containing 5 mM MgCl₂ and 60 μg amelamin/mg protein in a total volume of 25 μL. The reaction was incubated on ice for 10 min, followed by irradiation with a hand-held long-wave UV lamp (366 nm, UVP-21; Ultraviolet Products, Inc., San Gabriel, CA) for 15 min on ice. All experiments involving RA were conducted under yellow light. Reactions were stopped with 10% trichloroacetic acid (150 μL) and processed for SDS-PAGE on 10% gels as previously described (29). Gels were treated with Autofluor autoradiography enhancer (National Diagnostics, Atlanta, GA) according to the manufacturer’s directions before drying. Labeled protein bands were detected by autoradiography at −80°C for 3–7 days. Autoradiographs were analyzed by densitometry with a BioRad Imaging Densitometer. For Western blot analysis (30), proteins were electroblotted from gels to nitrocellulose that was then probed with a rat anti-pNP-UGT antibody.

**Results**

**Enzymatic Glucuronidation.** Three rat and three human recombinant UGTs (rat UGT1.1, 2B1, and 2B12; human UGT1.1, 1.4, and 2B15) were assayed for atRA glucuronide activity. Of the six UGTs, only rat and human UGT1.1 had measurable activity toward atRA. Because activity of the human enzyme was very low (<5 pmol/mg × min), rat UGT1.1 was used to characterize retinoid glucuronidation by this isoenzyme.

Figure 1 is an autoradiogram of a TLC plate from a representative assay of atRA glucuronidation by SD microsomes and membrane fractions from UGT1.1 transfected and untransfected (control) HK293 cells. This figure shows that [3H]atRA glucuronidation was completely separated from unreacted [3H]atRA, the reaction was dependent on the presence of UDP-GlcUA, and control HK293 cells did not glucuronidate atRA. Alamethicin, although a very effective activator of microsomal atRA glucuronidation activity, had no effect on the activity in UGT1.1 membrane fractions.

The substrate specificity of rat UGT1.1 glucuronidation is summarized in table 1. UGT1.1 glucuronidated BR at rates comparable with published values (23). Both atRA and 5,6-epoxy-atRA were glucuronidated at similar rates by UGT1.1. Expressed UGT1.1 had no detectable enzymatic glucuronidation activity toward any of the substrates in nontransfected HK293 cells. Kinetic analysis gave an apparent Km of 59 ± 5.4 μM and an apparent Vmax of 158 ± 43 pmol/mg × min for atRA glucuronidation by rat UGT1.1.

Rates of glucuronidation of BR, atRA, and 5,6-epoxy-atRA by liver microsomes from the Gunn rat, a mutant of the Wistar strain of rat deficient in UGT1.1, are compared in table 2 with rates from control Wistar and SD RLM. In contrast to both Wistar and SD RLMs, there was no evidence of BR glucuronidation by either intact or detergent-treated Gunn RLMs. On the other hand, untreated Gunn RLMs had measurable (35 pmol/mg × min) atRA glucuronidation activity that was significantly increased after activation with alamethicin (111 pmol/mg × min; table 2). Glucuronidation of 5,6-epoxy-atRA by Gunn RLMs was undetectable in the absence of alamethicin, and glucuronidation rates were still quite low and variable after activation by alamethicin (table 2). The activated activity in Gunn RLMs toward atRA was less than one-fourth and toward 5,6-epoxy-atRA was less than one-tenth of the rates seen in control Wistar RLMs.

**Photoaffinity Labeling.** SD RLMs and enriched membrane fractions from control and UGT1.1 transfected HK293 cells were directly photolabeled with [3H]atRA. Figure 2A is an autoradiograph from a representative experiment of labeled proteins separated by SDS-PAGE. In membranes from cells expressing rat UGT1.1 (fig. 2A, lanes 5 and 6), a protein band at ~56 kDa photoincorporated [3H]atRA. A corresponding photolabeled band was present in RLMs (fig. 2A, lanes 2 and 3), but absent in membrane proteins from untransfected cells (fig. 2A, lanes 8 and 9). Photoincorporation of [3H]atRA

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**TABLE 1**

| Substrate Enzymatic Activity (pmol/mg × min) |
|---------------------------------|-----------------|
| BR                              | 210 ± 6         |
| atRA                            | 91 ± 18         |
| 5,6-Epoxy-atRA                  | 113 ± 19        |
| Retinol                         | ND              |
| Lithocholic acid                | ND              |
| (3α,6α-dihydroxy-5β-cholanoic acid) | ND          |
| Hyodeoxycholic acid             | ND              |
| (3α,6β-dihydroxy-5β-cholanoic acid) | ND          |
| Etianic acid                    | ND              |
| (3α-hydroxy-5β-androstan-17β-carboxylic acid) | ND         |
| Iso-etianic acid                | ND              |

*ND, not detectable.*
into microsomal or membrane proteins was significantly UV-dependent (compare lanes 1 and 2, and lanes 4 and 5) and unaffected by pretreatment of the proteins with alamethicin (compare lanes 2, 5, and 6).

**Immunoblot Analysis.** Figure 2B is a Western blot of SDS-PAGE-separated protein from samples identical to those in fig. 2A. Probing with an anti-pNP UGT antibody revealed an immunoreactive protein of $\approx 56$ kDa in membrane proteins from cells expressing UGT1.1 (fig. 2B, lanes 4–6) and in RLMs (fig. 2B, lanes 1–3), but not in control HK293 cells (fig. 2B, lanes 7–9). The immunoreactive UGT proteins corresponded with the $56 \text{ kDa} \ [{}^3 \text{H}]\text{atRA-labeled proteins in UGT1.1 membranes (fig. 2A, lanes 5 and 6) and in RLMs (fig. 2A, lanes 2 and 3).}

**Discussion**

This study is a systematic effort to identify UGT isoforms active in retinoid glucuronidation and reports that the recombinant rat liver BR-specific UGT, UGT1.1, catalyzes the biosynthesis of acyl glucuronides of atRA and 5,6-epoxy-atRA. Progress in recombinant DNA technology has led to expression of cloned UGT isoenzymes in various tissue culture cell lines, providing the opportunity to study the substrate specificity of isoenzymes in the absence of other interfering UGT activities. Human and rat BR-specific UGT1.1 have been cloned and expressed, and their activity toward BR has been defined (23, 31, 32). The identification of UGT1.1 as one isoform involved in the glucuronidation of retinoids came from screening a series of available recombinant UGTs. Previous work has shown that several rat liver UGTs from the 2B (steroid) family (UGT2B1, 2B2, 2B3, and 2B6), expressed in COS 7 cells, did not accept RA as a substrate (33). In this study, we found that rat liver 2B1 and 2B12 and human liver 2B15, expressed in HK293 cells, did not demonstrate retinoid glucuronidating activity (data not shown). However, screening of isoforms from the phenol/BR family 1 revealed that both rat and human UGT1.1 are involved in the glucuronidation of retinoids. Recombinant UGT1A4, previously designated as the minor BR isoform, was not active. We selected the rat recombinant UGT1.1 for detailed characterization of retinoid glucuronidation. The glucuronidation of BR, compared with the two RA substrates, indicated that the retinoids are glucuronidated at approximately one-half of the activity found toward BR. Bile acids, endogenous UGT substrates with a carboxylic acid group, were not glucuronidated by UGT1.1, nor was retinol. Kinetic analysis of retinoid glucuronidation catalyzed by UGT1.1 showed that the $K_m$ value observed for atRA ($\approx 50 \mu\text{M}$) is in the same range as that of BR. TLC analysis of the products of enzymatic glucuronidation (fig. 1) indicated that one major polar metabolite of atRA was produced that cochromatographed with the product of microsomal glucuronidation.

**TABLE 2**

Glucuronidation of BR and retinoids by RLMs

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzymatic Activity (pmol/mg × min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wistar RLMs</td>
</tr>
<tr>
<td>BR</td>
<td>2600</td>
</tr>
<tr>
<td>atRA</td>
<td>524</td>
</tr>
<tr>
<td>5,6-epoxy-atRA</td>
<td>958</td>
</tr>
</tbody>
</table>

$^a$ Protein activated with 0.05% Brij 58.

$^b$ Protein activated with 60 $\mu$g alamethicin/mg protein.

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For example, the table compares the glucuronidation of different substrates by RLMs from different strains. The results are expressed as means ± SD for at least two separate duplicate determinations.

**Fig. 2.** $[{}^3 \text{H}]\text{atRA}$ photoaffinity labeling and Western blot of SD RLMs (lanes 1–3), rat recombinant UGT1.1 (lanes 4–6), and control HK293 cells (lanes 7–9).

Autoradiogram from SDS-PAGE separation of proteins photolabeled with $[{}^3 \text{H}]\text{atRA.}$ (B) Western blot analysis of the same proteins using anti-pNP-UGT IgG. In both (A) and (B), lanes 1, 4, and 7 are controls without UV irradiation; lanes 2, 5, and 8 are samples with UV irradiation, but without alamethicin; and lanes 3, 6, and 9 are samples with UV irradiation and 60 $\mu$g alamethicin/mg protein. Arrows indicate position of $56 \text{ kDa}$ protein.
of atrA previously identified by HPLC as a carboxyl-linked glucuronic acid (17).

Additional characterization of recombinant UGT1.1 was obtained from photoaffinity labeling with RA. The fact that recombinant UGT1.1 can be effectively photolabeled with [3H]atrA in a UV-dependent manner and that protection against photoincorporation is provided by the presence of unlabeled substrate is additonal confirmation that this isoform has a strong affinity for RA. The light dependence and protection indicate that the photolabeling is directed to the RA binding site of the enzyme.

To investigate the contribution of this isoform to total microsomal RA-specific glucuronidation, hepatic microsomal activities from SD, Wistar, and Gunn rats—a Wistar-derived mutant that does not have UGT1.1—were characterized. Data presented herein showing that Gunn rat microsomes have significant RA glucuronidating activity, indicate that other isoforms contribute to total RA glucuronidation. This activity might be catalyzed by other family 1 isoforms that are induced by phenobarbital or 3-methylcholanthreine treatment. Biochem. Pharmacol. 47, 485–492 (1994).


