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SHORT COMMUNICATION

Mono- and Diglucuronide Formation from Benzo[*a*]pyrene and Chrysene Diphenols by AHH-1 Cell-Expressed UDP-Glucuronosyltransferase UGT1A7

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ABSTRACT. Polycyclic aromatic hydrocarbon (PAH)-type compounds induce at least two rat UDP-glucuronosyltransferase isoforms, UGT1A6 and UGT1A7. Among the glucuronidation reactions of PAH metabolites studied, mono- and diglucuronide formation of benzo[*a*]pyrene and chrysene-3,6-diphenol showed the highest induction factors in rat liver microsomes. Availability of AHH-1 cells stably expressing UGT1A7 allowed us to study whether this PAH-inducible isoform could catalyze benzo[*a*]pyrene and chrysene-3,6-diphenol glucuronidation. It was found that UGT1A7 indeed catalyzed mono- and diglucuronide formation of both benzo[*a*]pyrene and chrysene 3,6-diphenols. V79 cell-expressed rat UGT1A6 also catalyzed these reactions, except for chrysene diphenol diglucronide formation (Bock *et al.*, *Mol Pharmacol* **42:** 613–618, 1992). Enzyme kinetic studies of the glucuronidation of 6-hydroxychrysene (used as a stable PAH phenol) indicated that UGT1A7 conjugated this compound with a lower apparent K_m value (0.1 μM) than UGT1A6 (10 μM). The results suggest that the two PAH-inducible UGTs may cooperate in conjugating PAH metabolites, but that UGT1A7 is more efficient. BIOCHEM PHARMACOL **57**;6:653–656, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. benzo[*a*]pyrene-3,6-diphenol; chrysene-3,6-diphenol; 6-hydroxychrysene; glucuronidation; UGT1A7

The metabolism of BP‖, a prototypic carcinogenic PAH and widespread environmental contaminant, has been extensively studied, in particular the formation of the major ultimate carcinogenic metabolite, BP-7,8-dihydrodiol-9,10-epoxide [1, 2]. BP-7,8-dihydrodiol-9,10-epoxide formation is known to occur via BP-7,8-dihydrodiol by sequential oxidation and hydrolysis reactions catalyzed by cytochrome P450 and microsomal epoxide hydrolase. It has been shown previously that the UDP-glucuronosyltransferase isoform UGT1A7 is able to conjugate BP-7,8-dihydrodiol, thereby reducing its further oxidation to BP-7,8-dihydrodiol-9,10-epoxide [3]. Much less is known about the fate of quantitatively major BP metabolites including phenols and quinones. Phenols can be further oxidized to quinones. In turn, quinones are reduced to diphenols, which are efficiently eliminated in bile following conjugation with glucuronic acid [4]. It is noteworthy that BP diphenols are not found in the usual analysis of BP metabolites, since they are rapidly autoxidized to quinones.

The role of glucuronidation and glutathione conjugation in BP metabolism has been studied in various systems such as hepatocyte cultures [5] and perfused liver [6]. Liver and intestinal mucosa play a major role in BP metabolism and disposition after dietary uptake of BP [7, 8]. Furthermore, Ah receptor-controlled induction of drug-metabolizing enzymes (cytochromes P450, quinone oxidoreductase, UDP-glucuronosyltransferases, and glutathione S-transferases) represents key adaptive mechanisms of the organism to PAH exposure [9]. The role of enzyme induction was demonstrated in studies with inbred mouse strains (C57BL/6 and DBA/2) carrying high-affinity or low-affinity Ah receptors, respectively. When BP was given orally, the inducible strain was more resistant to BP bone marrow toxicity, due to more efficient first-pass metabolism [10].

At least two rat UGT isoforms of family 1 (UGT1A6 and UGT1A7) are known to be PAH-inducible [11]. These UGT isoforms and two isoforms of family 2 (UGT2B1 and UGT2B2) have been shown to catalyze glucuronidation of BP phenols, diphenols, and dihydrodiols [3, 12, 13]. (For nomenclature of UGTs see [14]). DNA adducts from BP were found to be enhanced in Gunn rats, a mutant rat strain in which all UGT isoforms of family 1 are absent or markedly reduced, suggesting that glucuronide formation by

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‖ *Abbreviations:* BP, benzo[*a*]pyrene; PAH, polycyclic aromatic hydrocarbon; UGT, UDP-glucuronosyltransferase; MG, monoglucuronide; and DG, diglucuronide.

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UGT1A6 and UGT1A7 is able to significantly reduce the level of BP DNA adducts *in vivo* [15]. Formation of BP- and the structurally related chrysene-3,6-diphenol DGs showed the highest induction factors in rat liver microsomes after treatment with PAH-type inducers: 40- and over 100-fold for BP- and chrysene-3,6-diphenol DG, respectively [16]. This reaction may be important *in vivo*, since intratracheally administered BP is rapidly eliminated in bile as glucuronide and glutathione conjugates; in particular, it has been found that BP-3,6-diphenol MGs and DGs represent the major biliary glucuronides [4]. Biliary elimination of their conjugates sequesters BP phenols and diphenols and may prevent their subsequent oxidation to DNA-binding metabolites. It also prevents toxic redox-cycling between quinones and quinols associated with the generation of semiquinone radicals and reactive oxygen species [16–18]. The importance of BP-3,6-diphenol glucuronidation has also been demonstrated in previous mutagenicity studies [9].

Availability of AHH-1 cells stably expressing UGT1A7 [3] now makes it possible to study whether this isoform is involved in MG and DG formation of BP-3,6-diphenol. The reaction was also studied using the more stable chrysene-3,6-diphenol and 6-hydroxychrysene as substrates. The results demonstrate that UGT1A7 indeed appears to be very efficient in conjugating PAH mono- and diphenols.

MATERIALS AND METHODS

Chemicals

6-Hydroxychrysene and chrysene-3,6-diphenol were synthesized as described [12] and identified by high-field proton NMR spectroscopy and mass spectrometry. Their purity was found to be >98%, based on HPLC analysis. BP-3,6-quinone was obtained from the Carcinogen Reference Standard Repository, National Institutes of Health (Bethesda, MD, USA).

Cells

UGT1A7-transfected AHH-1 cells were grown in culture flasks containing 50 mL RPMI 1640 medium including 1% (w/v) penicillin/streptomycin, 10% (v/v) horse serum and hygromycin B (5 mg) for selection of the transfected colonies. The selection medium was changed every 2–3 days. To harvest the cells, they were centrifuged at 800 *g* and washed in ice-cold PBS. Washed cells were resuspended in PBS and stored at −80°. After thawing, lysates were prepared either by homogenization in 0.25 M sucrose containing 10 mM Tris–HCl, pH 7.4, using a Dounce homogenizer and brief sonication to disperse aggregates or by suspension in lysis buffer containing 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, and 10 mM KCl. Cells were allowed to stand at 4° for 10 min and homogenized.

UGT Assays

Described methods were used for the assay of 4-methylumbelliferone (0.5 mM [19]), 6-hydroxychrysene (0.2 mM [12, 20]), BP-3,6-diphenol, which was obtained by reduction from the corresponding quinone (0.05 mM [16]), and chrysene-3,6-diphenol (0.02 mM [12]). In brief, lysates from AHH-1 cells were incubated in the presence of 0.1 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$ in a total volume of 500 μL. As UGT activity could not be activated by Brij 58 in cell lysates, the detergent was omitted. The reaction was started by addition of UDP-glucuronic acid (final concentration 3 mM). After incubation at 37° for 20 min, the reaction was stopped by rapid mixing with 2 mL chloroform [in the case of BP-3,6-diphenol, chloroform extraction could be omitted], followed by centrifugation. Glucuronides were measured fluorimetrically in the aqueous phase, except for UGT activity towards chrysene-3,6-diphenol. In the latter case, the glucuronides were measured after HPLC separation, since the two MGs cannot be distinguished fluorimetrically. In this case, aqueous extracts were filtered through a Millex HV_{13} (0.45 μm) filter (Millipore) before injection in the HPLC system. Because endogenous UGT activities of untransfected AHH-1 cells were low (ca. 2%) compared to UGT1A7-transfected cells, these UGT activities were neglected. Care was taken to measure initial rates. In cell lysates, UGT activities were linear up to 40 min (20 min for the unstable BP-3,6-diphenol). Under these conditions, the limit of detection was ca. 0.1 pmol/min/mg protein. Protein was determined according to Lowry *et al.* [21], using BSA as protein standard.

HPLC Determination of Mono- and Diglucuronides of Chrysene- and Benzo[a]-pyrene-3,6-diphenols

The system established previously was used [12]. It consisted of a Waters HPLC system (Millipore) equipped with two 501/510 pumps and a variable wavelength fluorescence detector (model S3400, Sykam). Glucuronides were separated by a Grom reverse phase column (C_{18}, 250 × 4.6 mm, 5 μm), and acquired data were evaluated using an IBM XT computer (model 486) together with Maxima 820 and Baseline Chromatography Software (version 3.30). The mobile phase was composed of two solvents. Solvent A consisted of 0.05 M ammonium acetate, pH 6.4, and solvent B of 0.05 M ammonium acetate, pH 6.4/acetonitrile (70/30, v/v). A linear gradient of 100% solvent A to 100% solvent B (15 min for benzo[*a*]pyrene glucuronides and 10 min for chrysene glucuronides) was used, followed by an isocratic elution with 100% solvent B. Fluorescent peaks were identified by comparison with the corresponding glucuronides generated with liver microsomes of 3-methylcholanthrene-treated rats, as described [12].

RESULTS AND DISCUSSION

MG and DG formation of both BP- and chrysene-3,6-diphenol could be clearly demonstrated in incubation mixtures containing lysates of UGT1A7-expressing cells (Table 1). Formation of these glucuronides has also been observed in UGT1A6-expressing V79 cells, with the nota-

TABLE 1. Benzo[*a*]pyrene- and chrysene-3,6-diphenol mono- and diglucuronide formation by UGT1A7

Substrate	UGT activities (nmol/min/mg protein)
BP-3,6-diphenol	
MG formation	0.75 ± 0.09*
DG formation	0.09 ± 0.01
Chrysene-3,6-diphenol	
3-MG formation	0.020 ± 0.005
6-MG formation	0.037 ± 0.004
DG formation	0.033 ± 0.003

*Data represent means ± SD of 4 experiments.

ble exception that chrysene-3,6-diphenol DG formation was not detectable [12]. Kinetic characteristics were determined with 6-hydroxychrysene as a stable PAH phenol and compared with those found with a widely used model substrate, 4-methyl-umbelliferone. Apparent kinetic constants were determined at a concentration of 3 mM UDP-glucuronic acid. As shown in Fig. 1 and Table 2, K_m values were much lower with UGT1A7 than with UGT1A6, underlining the importance of UGT1A7 for conjugating phenolic metabolites of PAHs. UGT activity in transfected cells obviously depends upon the number of integrated gene copies. Therefore, V_{max} determined with UGT1A7-expressing AHH-1 and UGT1A6-expressing V79 cells cannot be compared without determination of the levels of expressed proteins with monospecific antibodies which are not yet available. However, K_m values may be characteristic of the integrated UGT enzymes.

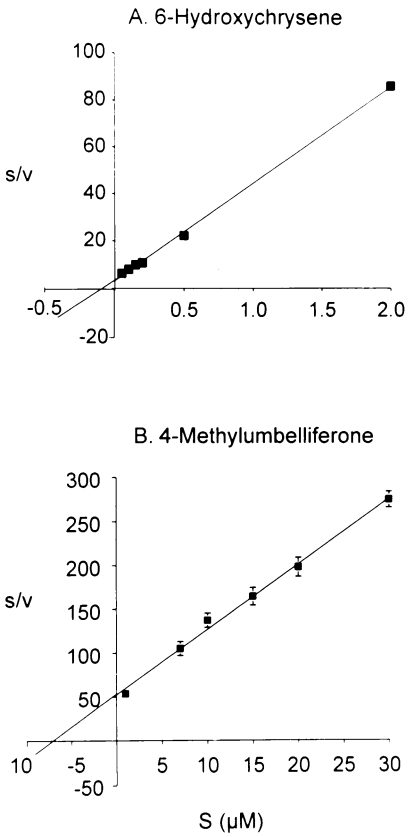

FIG. 1. Kinetic characteristics of UGT activities towards 6-hydroxychrysene and 4-methyl-umbelliferone in AHH-1 cells expressing UGT1A7. Apparent K_m values and V_{max} were determined in cell lysates.

TABLE 2. Comparison of kinetic characteristics of UGT1A6 and UGT1A7*

UGT isoform Substrate	K_m (μM)	V_{max} (nmol/min/mg protein)
UGT1A7		
6-Hydroxychrysene	0.1	0.03
4-Methylumbelliferone	7	0.11
UGT1A6		
6-Hydroxychrysene	10	0.18
4-Methylumbelliferone	47	6.00

*Apparent K_m and V_{max} values were determined using Hanes plots, as listed in Fig. 1. Kinetic characteristics of UGT1A6 were determined in UGT1A6-expressing V79 cells, as described [20]. Hence, V_{max} values are not comparable to those obtained with UGT1A7-expressing AHH-1 cells as discussed in the text. Means of 3 experiments are listed.

The present study demonstrates that UGT1A7 is able to catalyze glucuronidation of PAH phenols and diphenols, the latter to DGs. DG formation of chrysene-3,6-diphenol appears to be a selective property of UGT1A7. There is general agreement that UGT1A6 preferentially uses simple phenols and planar phenols of PAHs as substrates, whereas UGT1A7 also accepts bulky substrates [22]. PAH diphenol MGs certainly represent bulky substrates. This study also demonstrates that the K_m value for glucuronidation of 6-hydroxychrysene is much lower with UGT1A7 than with UGT1A6, suggesting a major role of UGT1A7 in the conjugation of PAH phenols and diphenols. The two PAH-inducible UGTs may cooperate in sequestering and inactivating BP metabolites.

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References

1. Miller EC and Miller JA, Searches for ultimate chemical carcinogens and their reactions with cellular macromolecules. *Cancer* **47:** 2327–2345, 1982.
2. Conney AH, Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G.H.A. Clowes memorial lecture. *Cancer Res* **42:** 4875–4917, 1982.
3. Grove AD, Kessler FK, Metz RP and Ritter JK, Identification of a rat oltipraz-inducible UDP-glucuronosyltransferase (UGT1A7) with activity towards benzo[*a*]pyrene-7,8-dihydrodiol. *J Biol Chem* **272:** 1621–1627, 1997.
4. Bevan DR and Sadler VM, Quinol diglucuronides are predominant conjugated metabolites found in bile of rats following intratracheal instillation of benzo[*a*]pyrene. *Carcinogenesis* **13:** 403–407, 1992.
5. Burke MD, Vadi H, Jernström B and Orrenius S, Metabolism of benzo[*a*]pyrene with isolated hepatocytes and the forma-

tion and degradation of DNA-binding derivates. *J Biol Chem* **252:** 6424–6431, 1977.
6. Bock KW, Bock-Hennig BS, Lilienblum W and Volp RF, Release of mutagenic metabolites of benzo[*a*]pyrene from the perfused rat liver after inhibition of glucuronidation and sulfation by salicylamide. *Chem-Biol Interact* **36:** 167–177, 1981.
7. Bock KW, v.Clausbruch UC and Winne D, Absorption and metabolism of naphthalene and benzo[*a*]pyrene in the rat jejunum *in situ*. *Med Biol* **57:** 262–264, 1979.
8. Wall KL, Gao W, te Koppele JM, Kwei GY, Kauffman FC and Thurman RG, The liver plays a central role in the mechanism of chemical carcinogenesis due to polycyclic aromatic hydrocarbons. *Carcinogenesis* **12:** 783–786, 1991.
9. Bock KW, Lipp H-P and Bock-Hennig BS, Induction of drug-metabolizing enzymes by xenobiotics. *Xenobiotica* **20:** 1101–1111, 1990.
10. Legraverend C, Harrison DE, Ruscetti FW and Nebert DW, Bone marrow toxicity induced by oral benzo[*a*]pyrene: Protection resides at the level of the intestine and liver. *Toxicol Appl Pharmacol* **70:** 390–401, 1983.
11. Ikushiro S-I, Emi Y and Iyanagi T, Identification and analysis of drug-responsive expression of UDP-glucuronosyltransferase family 1 (UGT1) isozyme in rat hepatic microsomes using anti-peptide antibodies. *Arch Biochem Biophys* **324:** 267–272, 1995.
12. Bock KW, Gschaidmeier H, Seidel A, Baird S and Burchell B, Mono- and diglucuronide formation from chrysene and benzo[*a*]pyrene phenols by 3-methylcholanthrene-inducible phenol UDP-glucuronosyltransferase (UGT1A1). *Mol Pharmacol* **42:** 613–618, 1992.
13. Mackenzie PI, Rodbourn L and Iyanagi T, Glucuronidation of carcinogen metabolites by complementary DNA-expressed uridine 5′-diphosphate glucuronosyltransferases. *Cancer Res* **53:** 1529–1533, 1993.
14. Mackenzie PI, Owens IS, Burchell B, Bock KW, Bairoch A, Belanger A, Fournel-Gigleux S, Green M, Iyanagi T, Lancet D, Louisot P, Magdalou J, Roy Chowdhury J, Ritter JK, Schachter H, Tephly TR, Tipton KF and Nebert DW, The UDP glycosyltransferase gene superfamily: Recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics* **7:** 255–269, 1997.
15. Hu Z and Wells PG, *In vitro* and *in vivo* biotransformation and covalent binding of benzo[*a*]pyrene in Gunn and RHA rats with a genetic deficiency in bilirubin uridine diphosphate-glucuronosyltransferase. *J Pharmacol Exp Ther* **263:** 334–342, 1992.
16. Lilienblum W, Bock-Hennig BS and Bock KW, Protection against toxic redox cycles between benzo[*a*]pyrene-3,6-quinone and its quinol by 3-methylcholanthrene-inducible formation of the quinol mono- and diglucuronide. *Mol Pharmacol* **27:** 451–458, 1985.
17. Lorentzen RJ, Lesko SA, McDonald K and Ts'o POP, Toxicity of metabolic benzo[*a*]pyrenediones to cultured cells and the dependence upon molecular oxygen. *Cancer Res* **39:** 3194–3198, 1979.
18. Chesis PL, Levin DE, Smith MT, Ernster L and Ames BN, Mutagenicity of quinones: Pathways of metabolic activation and detoxification. *Proc Natl Acad Sci* USA **81:** 1696–1700, 1984.
19. Bock KW and White INH, UDP-glucuronyltransferase in perfused rat liver and in microsomes: Influence of phenobarbital and 3-methylcholanthrene. *Eur J Biochem* **46:** 451–459, 1974.
20. Bock KW, Forster A, Gschaidmeier H, Brück M, Münzel P, Schareck W, Fournel-Gigleux S and Burchell B, Paracetamol glucuronidation by recombinant rat and human phenol UDP-glucuronosyltransferases. *Biochem Pharmacol* **45:** 1809–1814, 1993.
21. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
22. Ebner T and Burchell B, Substrate specificities of two stably expressed human liver UDP-glucuronosyltransferases of the UGT1 gene family. *Drug Metab Disp* **21:** 50–55, 1993.