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Activity of rat UGT1A1 towards benzo[*a*]pyrene phenols and dihydrodiols

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Abstract

Four UDP-glucuronosyltransferases from the rat UGT1A family were tested for activity towards benzo[*a*]pyrene phenols and dihydrodiols. UGT1A1 and UGT1A7 were found to be broadly active towards BaP metabolites. Antisera recognizing rat UGT1A1 and UGT1A7 were used to assess UGT levels in relation to UGT activity towards benzo[*a*]pyrene-7,8-dihydrodiol (BPD). The rank BPD UGT activities were liver = intestine ≫ kidney, whereas UGT1A1 was highest in liver and UGT1A7 was highest in intestine. Phenobarbital, an inducer of hepatic UGT1A1, only slightly increased BPD UGT activity, whereas UGT1A7 inducers more potently increased the activity. Inhibition studies using the differential UGT1A1 inhibitor, bilirubin, suggest that UGT1A1 is not a major contributor to the constitutive BPD glucuronidating activity of control rat liver microsomes. These data suggest that multiple UGT1A enzymes contribute to glucuronidation of BPD and other BaP metabolites, and that their relative contributions depend on tissue- and environmental-specific factors.

Keywords: UDP-glucuronosyltransferase; Benzo[*a*]pyrene; Glucuronidation; Environmental carcinogenesis

1. Introduction

BaP is a known animal carcinogen, teratogen, and immunotoxicant and is a suspected human carcinogen. Exposure occurs through cigarette smoking, burning of fossil fuels, and charbroiled foods (Gelboin, 1980; Dipple et al., 1990). BaP is metabolized to its toxic and non-toxic metabolites by a number of organs, including liver, kidney, lung, skin, and intestine. Cytochrome P450s (CYPs) are phase I enzymes that add functional groups onto BaP, converting it to many different metabolites including phenols, diphenols, quinones, and dihydrodiols. The metabolite BaP-7,8-oxide is the first step in the formation of the mammalian tumorigenic metabolite. Epoxide hydrolase converts the BaP-7,8-oxide to BaP-7,8-dihydrodiol, which can subsequently be metabolized to BPD-9,10-epoxide, a highly potent genotoxin considered to be the ultimate carcinogenic form of BaP. BPD-9,10-epoxide binds to guanosine residues in DNA forming adducts that lead to mutations and tumor formation.

Glucuronide conjugates are major phase 2 metabolites of BaP as demonstrated in pharmacokinetic studies using rats and in studies using isolated rat hepatocytes and intestinal epithelial cells (Burke et al., 1977; Stohs et al., 1977). Enzymes that mediate conjugation of BaP metabolites such as the UDP-glucuronosyltransferases (UGTs) may be critical determinants of BaP-induced cytotoxicity, covalent binding, and mutagenicity (Hu and Wells, 2004). UGTs catalyze the transfer of glucuronic acid provided by the donor substrate, UDP-glucuronic acid, to eligible functional groups on the acceptor substrate. Phenolic BaP metabolites are mutagenic in bacterial genotoxicity assays but lack significant carcinogenicity in mammalian systems, potentially due to the high efficiency of glucuronidation of phenolic metabolites. UGTs that mediate the glucuronidation of BaP dihydrodiols may be particularly important for BaP cytotoxicity and/or genotoxicity (Hu and Wells, 2004). In the rat, the polycyclic aromatic hydrocarbon-inducible UGT1A7 form was shown to be active towards BaP metabolites including BPD (Grove et al., 1997), but other UGT1A family enzymes have not been characterized.

The purpose of this study was to characterize the activities of the three major UGT1A family enzymes expressed

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in rat liver towards BaP metabolites. UGT1A1 was identified as a second major active UGT1A family member with broad activity towards BaP metabolites in general. The finding that UGT1A1 was active towards BPD led to further studies to assess the contribution of UGT1A1 to BPD glucuronidation.

2. Materials and methods

2.1. Materials

Authentic standards for BaP metabolites including (−) *trans* BPD were obtained from the NCI Carcinogen Standard Repository (Midwest Research Laboratory, Kansas City, MO). Nickel NTA-resin was obtained from Qiagen. The purity of BPD estimated by high performance liquid chromatography was >95%.

2.2. Animal handling and microsome preparation

All experiments using animals in this report were performed using a protocol approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. Rats were obtained from Harlan Sprague–Dawley and allowed one week to acclimate during which they were given free access to standard rat chow and water. Microsomes were prepared from liver and kidney as described (Kessler and Ritter, 1997). For intestine, the method of Harmsworth and Franklin using soy trypsin inhibitor was used (Harmsworth and Franklin, 1990). Microsomal protein concentrations were determined using the BCA (bicinchoninic acid) method and a commercially available kit (Pierce Chemical Co.).

2.3. Preparation of recombinant UGT-expressing membrane preparations

For the analysis of UGT activity towards BaP metabolites, preparations from clonal human embryonic kidney 293B cell lines stably expressing UGT1A1, UGT1A5, UGT1A6, and UGT1A7 were used (Kessler et al., 2002). For the analysis of UGT1A activities towards BPD, preparations were from human hepatoma HepG2 cells expressing rat UGTs 1A1, 1A2, 1A3, 1A5, 1A6, 1A7, and 1A8 after infection with recombinant, replication-defective adenoviruses which will be described elsewhere. Microsomes were prepared as follows: after washing monolayers with ice-cold 1× phosphate-buffered saline, cells were harvested by scraping in 5 mL 0.1 M potassium phosphate buffer, pH 7.5, containing 10 mM EDTA and 20% glycerol and were subjected to three consecutive freeze-thaw cycles followed by 10 passes using a Potter-Elvejhem glass homogenizer fitted with a Teflon pestle. Microsomes were prepared by centrifuging the cell lysates at 8000 × *g* for 10 min at 4 °C, collecting the supernatant, and recentrifuging for 45 min at 105,000 × *g* at 4 °C. Microsomal pellets were resuspended in 0.1 M potassium phosphate buffer, pH 7.5 containing 10 mM EDTA and 10% glycerol. Protein concentrations were estimated using the BCA method.

2.4. Assay for activity of recombinant UGT1A isoforms towards BaP metabolites

The unified [^{14}C]-UDP-glucuronic acid method (Bansal and Gessner, 1980) was used. Reactions contained the following: 50 mM Tris–Cl, pH 7.5, 5 mM $MgCl_2$, HEK cell microsomes (0.5 mg/mL), alamethicin (50 μg/mL), and BaP metabolite (100 μM). Reactions were initiated by addition of [^{14}C]-UDP-glucuronic acid (0.1 μCi, final concentration 1.5 mM) and were stopped after either 15 min (UGT1A7) or 60 min (UGT1A1, UGT1A5, and UGT1A6) by addition of ice-cold methanol (1:1, v/v). Reaction contents were placed on dry ice for 10 min and centrifuged (14,000 × *g*, 2 min). The supernatants were collected and applied to silica thin layer chromatography (TLC) plates which were subsequently developed using the TLC mobile phase of Nemoto and Gelboin (Nemoto and Gelboin, 1976). After air drying, the plates were exposed to a Molecular Dynamics phosphorimaging cassette for 12 h. The resulting image was densitometrically analyzed using the Image Quant program. UGT activities were calculated based on the ratio of -[^{14}C]-glucuronic acid incorporated into the glucuronide band. UGT activity data were normalized for the level of UGT expression of individual over-expressed UGT1A enzymes as described (Webb et al., 2004). Differences in enzyme expression level were determined by immunoblot analysis using an antibody recognizing UGT1 common exon sequence.

2.5. Microsomal UGT activities towards BPD

Microsomal BPD UGT activity was determined using a high performance liquid chromatography (HPLC)-based methodology. Reactions contained 50 mM Tris–Cl, pH 7.5, 5 mM $MgCl_2$, microsomal protein (1 mg/mL), alamethicin (50 μg/mL), and 100 μM (−) *trans* BPD. After equilibrating the reactions at 37 °C, the reactions were initiated by addition of UDP-glucuronic acid (final concentraiton 3 mM). After 120 min, the reactions were stopped by addition of concentrated perchloric acid to 5.7% (v/v). Samples were placed on ice for 10 min and centrifuged at 14,000 × *g* for 5 min to remove the precipitated protein. A 20 μL aliquot of reaction supernatant was injected onto a Waters Model 510 HPLC system equipped with a U6K injector, a Whatman Partisil 10 ODS-2 column (4.6 mm × 250 mm) and a Lambda Max Model 481 LC spectrophotometer. The mobile phase was 35% acetonitrile: 65% 10 mM potassium phosphate, pH 2.3 at a flow rate of 1.5 mL/min. BPD glucuronide was detected by optical density at 254 nm. The identity of the glucuronide peak was confirmed by performing control reactions with and without BPD or UDP-glucuronic acid. The BPD glucuronide peak was found to be 100% hydrolyzed by *E. coli* β-glucuronidase hydrolysis. Peak quantification was carried out as previously described (Grove et al., 2000) using BPD glucuronide standard generated in our laboratory.

2.6. Development of specific anti-rat UGT1A1 antiserum

An anti-rat UGT1A1 antisera, anti-r1A1$_{29–162}$, was raised in mice using the same fusion protein strategy described previously for preparing anti-human UGT1A1 (Ritter et al., 1999). Briefly, an amino terminal 6×-His tagged fusion protein containing residues 29–162 of rat UGT1A1 was expressed in *E. coli* strain XL1-Blue bacteria using pQE30 vector and purified by affinity chromatography using Ni-NTA Sepharose (Qiagen). The source of UGT1A1 coding sequence for expression was UGT1A1 cDNA clone p1–16 (Kessler et al., 2002). Female B6C3F1 mice were subjected to weekly injections with fusion protein. After three months, the mice were anesthetized and blood was collected by cardiac puncture. After clotting, serum was obtained by centrifuging at 14,000 × *g* for 2 min and collecting the supernatant. Aliquots of anti-r1A1$_{29–162}$ (0.1 mL) were stored at −80 °C.

2.7. Analysis of rat liver microsomal samples and recombinant UGTs by Western immunoblotting

Liver microsomal samples were subjected to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis-western immunoblotting as described previously (Ritter et al., 1999). Primary antisera raised in mice against either UGT1A1 residues 29–162 or UGT1A7 residues 23–169 (Grove et al., 2000) were used at 1:1000 and 1:2000 dilution, respectively. The secondary antibody was horseradish peroxidase-conjugated sheep anti-mouse IgG purchased from Amersham (Arlington Heights, IL). Antibody complexes were detected using enhanced chemiluminescence with a commercially available kit (ECL, Amersham).

2.8. Statistical analysis

Statistical analyses were performed using one way analysis of variance testing (SigmaStat software). When statistically significant differences between groups were found, Tukey post hoc analysis were performed to identify specific groups with differences. The significance level was $p < 0.05$.