Differential Induction of UDP-Glucuronosyltransferase Activity and Gene Expression in Rat Liver

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INTRODUCTION

The UDP-glucuronosyltransferases (UGT) are a superfamily of isozymes catalyzing the glucuronidation of endogenous and exogenous substrates (1,2). In rats, UGT exists as at least ten isozymes that can be classified into two broad families based on evolutionary divergence (3). Although some UGT exhibit substrate overlap, other isozymes appear to possess greater substrate selectivity (4). Small planar phenols and bilirubin are generally considered substrates for UGT Family 1, while steroids, bile acids, and NSAIDS apparently are substrates for Family 2 isoforms. As the determinants of UGT isozyme substrate selectivity become better characterized, it may be possible through *in vitro* studies to qualitatively predict the influence of induction on the *in vivo* clearance. The present studies utilized the rat as a model system to examine the effect of treatment with different enzyme inducers on the UGT activity and mRNA levels. A greater understanding of the determinants of UGT regulation would be relevant, particularly to the toxicological evaluation of drugs eliminated by the UGT. The agents examined were: phenobarbital, 2[3]-t-butyl-4-hydroxyanisole, a phenolic antioxidant (5), 3-methylcholanthrene, a polycyclic aromatic hydrocarbon (6), clofibric acid, a peroxisome proliferator (7), and indole 3-carbinol, a chemopreventative component of cruciferous vegetables that exhibits some properties of antioxidant and aromatic hydrocarbon receptor compounds (8).

MATERIALS AND METHODS

Materials. Phenobarbital, clofibric acid, 2[3]-t-butyl-4-hydroxyanisole (BHA), 3-methylcholanthrene (3MC), indole-3-carbinol (I3C), UDP-glucuronic acid (UDPGA), 1-naphthol, 17α-ethinylestradiol (EES), and 4-hydroxyestrone were purchased from Sigma (St. Louis, MO). Propafol was obtained from Aldrich. 4(methylnitrosamine)-1-(3-pyridyl)-butan-1-ol (NNAL) was kindly provided by Dr. Stephen S. Hatch (American Health Foundation, Valhalla, NY).

Induction Treatments. Male Sprague-Dawley rats, purchased from Taconic Farms (Germantown, NY), were housed under standard conditions and allowed free access to food and water. Phenobarbital 80 mg/kg, indole-3-carbinol 30 mg/kg, and BHA 600 mg/kg were administered daily for 4 days. Clofibric acid 250 mg/kg was given daily for 3 days. 3-Methylcholanthrene was given as a single 80 mg/kg dose. The dosing vehicle was corn oil, except for phenobarbital and clofibric acid which were dissolved in water. Control animals received corn oil. All treatments were administered intraperitoneally to groups of three animals.

Microsome Preparation. Liver microsomes for each individual animal were obtained from one-half of the tissue by differential centrifugation as previously described (9). Following ultracentrifugation, the microscomes were resuspended in 10 mM potassium phosphate, 0.25 M sucrose (pH 7.4) to final protein concentration of 20 mg/ml, frozen in liquid nitrogen, and stored at -70°C.

Enzymatic microsomal activity was assessed using various glucuronidation substrates: 1-naphthol (1000 μM), propafol (1000 μM), 4-hydroxyestrone (1000 μM), EES (1000 μM), and NNAL (333 μM). Reaction mixtures consisted of 1 mM UDPGA, 0.1 to 0.2 μCi UDP-[^{14}C]glucuronic acid, and 0.1% Triton-X 100, microsomes 0.5 to 1 mg protein/ml in a total volume of 150 μl 100 mM tris-HCl, 5 mM $MgCl_2$ (pH 7.4) buffer. Microsomes were activated by gentle agitation for 15 minutes at 5°C and preincubated for 5 min at 37°C. Reactions were initiated by the addition of the substrates. Incubations were run for 15 - 30 minutes, quenched with 500 μl ethanol, and stored at -70°C until analysis.

Glucuronide conjugates were quantified using the general method of Coughtrie *et al.* (10) with minor modifications as previously described (11). Samples were centrifuged 10 min at 13000 x g and 400 μl of the supernatant was injected onto the column. The radioactivity flow detector was a Radiomatic Flo-one 515A (Meriden, CT). Counting efficiency as a function of mobile phase composition was determined using a [^{14}C]labeled standard (Packard). Glucuronides of naphthol and EES served as retention time standards.

Isolation and Analysis of RNA. On the day of sacrifice, one-half of the liver was immediately frozen in liquid nitrogen and then stored at -70°C for one week. Total RNA was extracted from a pooled sample of *ca.* 1 g tissue using a kit based on the guanidine-thiocyanate method of Chomczynski and Sacchi (Promega, Madison, WI; 12). The RNA was subjected to electrophoresis in denaturing formaldehyde/1% agarose gel and transferred onto nylon membranes (GeneScreen, DuPont-NEN, Boston, MA). Following prehybridization, the membranes were hybridized with [^{32}P]-labeled cDNA probes: *mUGTcom* (13), *mUGTml* (14), and human β-actin (purchased from ATCC). A separate blot was obtained for each UGT probe. After hybridization, washed membranes were visualized by autoradiography at -70°C. Other details of the Northern blotting procedure were as given in Maniatis *et al.* (15) with minor modifications (11). Relative mRNA levels (β-actin-normalized) were deter-

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mined by densitometry (UltroScan XL, Pharmacia, Piscataway, NJ).

The cDNA probes have been described previously (13,14). Briefly, *mUGTcom* consisted of the 1.2 kilobase (kb) fragment that contains the common domain of mouse UGTbr1 and UGTp4 and which is 95% identical to the equivalent portion of the Family 1 rat gene (13). The other probe (*mUGTml*) was a full length clone of 1860 base pairs that is 85% identical to UGTr3 and UGTr4 of the rat UGT Family 2 genes (14) and was kindly provided by Dr. Ida S. Owens (NIH).

RESULTS AND DISCUSSION

Control animals contained measurable UGT activity towards 1-naphthol, EES, 4-hydroxyestrone, NNAL, and propafol (Table I). Phenobarbital treatment increased enzymatic microsomal activity towards 1-naphthol, EES, and NNAL. Administration of BHA doubled activity towards naphthol, the steroids, 4-hydroxyestrone and EES, while leaving NNAL activity unchanged. Treatment with 3-MC strongly induced activity towards 1-naphthol consistent with previous studies (5), while leaving other activities unchanged. Clofibric acid induced activity towards EES, but not 1-naphthol. There was a trend towards increased activity towards 4-hydroxyestrone following treatment with phenobarbital and clofibric acid, but due to the small numbers and variability, the differences were not statistically reliable ($p>0.05$, unpaired t-test). No effect of indole 3-carbinol was observed on UGT activity toward these substrates, but this could be due to the lower dose that was used here compared to those which induce cytochrome P450 (16). None of the treatments affected activity towards propafol.

Hybridization of RNA to a *mUGTcom* cDNA probe that will detect UGT family 1 (phenol/bilirubin) mRNA, showed a prominent transcript of about 2.5 kb (Fig. 1A). Following treatment with I3C, 3MC, BHA, phenobarbital, and clofibric acid, this transcript was present in levels 0.69, 3.30, 0.57, 1.75, and 1.66 times that of control, respectively (β-actin normalized; Figure 1B). 3-Methylcholanthrene and phenobarbital treatment increased family 1 mRNA and 1-naphthol activity to a similar extent, which is consistent with the view that isoforms which conjugate planar phenols reside in this family. Following administration of clofibric acid, increased family 1 UGT mRNA was accompanied by a similar increase in EES activity. Although steroids are generally substrates for family 2 UGT isoforms, this may reflect the ability of the fibrate class of compounds to specifically induce bilirubin isoforms of UGT family 1 which share EES as a substrate (8, 17).

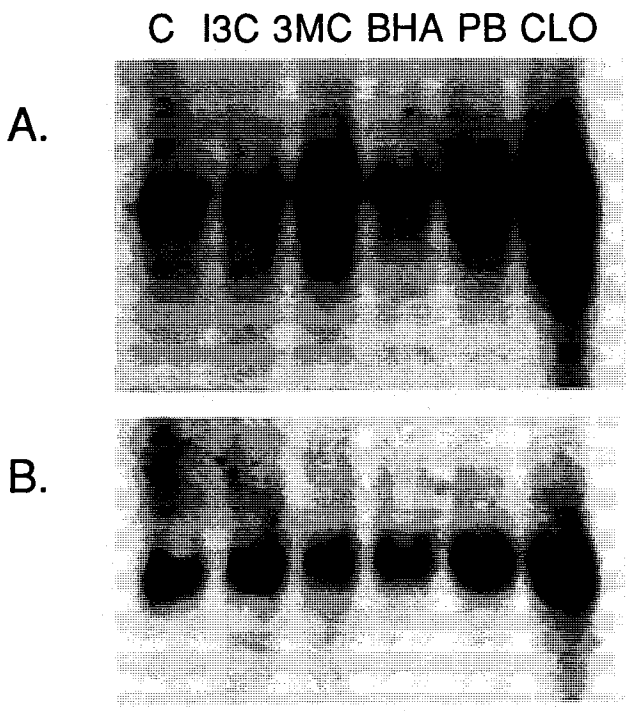

Fig. 1. Effect of inducer treatments on (A) *mUGTcom* RNA levels in rat liver. Normalization for gel loading was with a β-actin probe (B). Abbreviations for lane markers: C - control, I3C - indole 3-carbinol, 3MC - 3-methylcholanthrene, BHA - butylated hydroxyanisole, PB - phenobarbital, and CLO - clofibric acid.

Table I. Effect of Prototype Inducers on UGT Activity in Rat Liver Microsomes

Treatment	1-Naphthol	EES	4-hydroxy estrone nmol/min/mg protein	NNAL	Propafol
Control	11 ± 2	0.43 ± 0.04	0.12 ± 0.06	0.10 ± 0.04	1.0 ± 0.3
Phenobarbital	16* ± 0	1.4* ± 0.3	0.26 ± 0.07	0.21* ± 0.02	0.81 ± 0.10
Clofibric acid	12 ± 2	0.67* ± 0.07	0.34 ± 0.17	0.14 ± 0.05	1.4 ± 0.3
BHA	25* ± 3	0.83* ± 0.14	0.30* ± 0.04	0.096 ± 0.039	1.3 ± 0.6
3-MC	26* ± 2	0.51 ± 0.08	0.14 ± 0.07	0.14 ± 0.02	1.4 ± 0.4
Indole 3-carbinol	14 ± 5	0.50 ± 0.22	0.19 ± 0.21	0.095 ± 0.047	0.92 ± 0.22

* $p < 0.05$, different from control by unpaired t-test. Shown as mean ± S.D. of $n = 3$ individual animals per treatment group.

Hybridization of the RNA to a *mUGTml* (UGT2B5) cDNA probe that detects UGT family 2 mRNA showed a prominent transcript of 2.5 kb (Figure 2A). Normalized mRNA levels for this transcript after treatment with I3C, 3MC, BHA, phenobarbital, and clofibric acid were 1.1, 1.2, 1.0, 3.2, and 0.89 times that of control, respectively. The extent of increases in activity toward EES and family 2 mRNA levels were comparable in phenobarbital treated rat livers, consistent with the view that the increased enzymatic activity arose from induction of one or more family 2 isoforms. It has been recently reported by Radominska *et al* (4) that estradiol is a substrate for UGTr3 (UGT2B3) and that phenobarbital induces UGT activity toward estrone and estradiol in Fisher 344 male rats, which is consistent with the present observations in Sprague-Dawley rats.

Simultaneous measurement of UGT activity and mRNA levels provides additional insight regarding the mode of induction for this gene superfamily. The treatment associated increased steady-state mRNA levels were consistent with enzyme induction through increased gene transcription. Administration of the antioxidant BHA for four days increased microsomal glucuronidation activity toward three substrates while leaving mRNA levels decreased or unchanged, which raised the possibility of nontranscription regulatory actions, perhaps such as has been reported for glutathione S-transferase P1-I (18). In contrast to these results, it was recently reported that dietary administration of BHA for two weeks increases family 1 UGT mRNA levels in male Wistar rats using an oligonucleotide probe coding for the common 3′-domain of the rat bilirubin phenol UGT gene (19). The difference could be due to the strains of rats, dose and route of administration, as well as the probes that were used. Future studies involving nuclear run-on techniques and examination of dose and route-dependence would further clarify the actions of BHA in UGT induction in the rat.

The UGT isoforms acting on small phenols were apparently differentially induced. UGT-Naphtol activity was increased by treatment with phenobarbital, BHA, and 3-methylcholanthrene, while enzymatic activity toward propafol remained unchanged. These two planar phenols are preferred substrates for different isoforms in humans (20). Although UGT are generally considered inducible pathways, the isoform which mediates propafol conjugation appears less broadly susceptible to induction. The inducibility of this isoform would be further defined by mRNA and protein analyses using specific modes of detection.

The regulation of UGT gene expression is a complex phenomenon given the multigene family, species differences, and tissue specificity. In this report, differential induction of the two UGT families by a structurally diverse group of chemicals was demonstrated in rat liver. Examination of UGT induction in animal models may have use in the study of carboxylic acid drugs and environmental chemicals which form glucuronides, some of which may be reactive and potentially toxic (21). However, further studies are needed to characterize the extent of species differences between rodent and human isoforms and the regulation of their expression by xenobiotics.

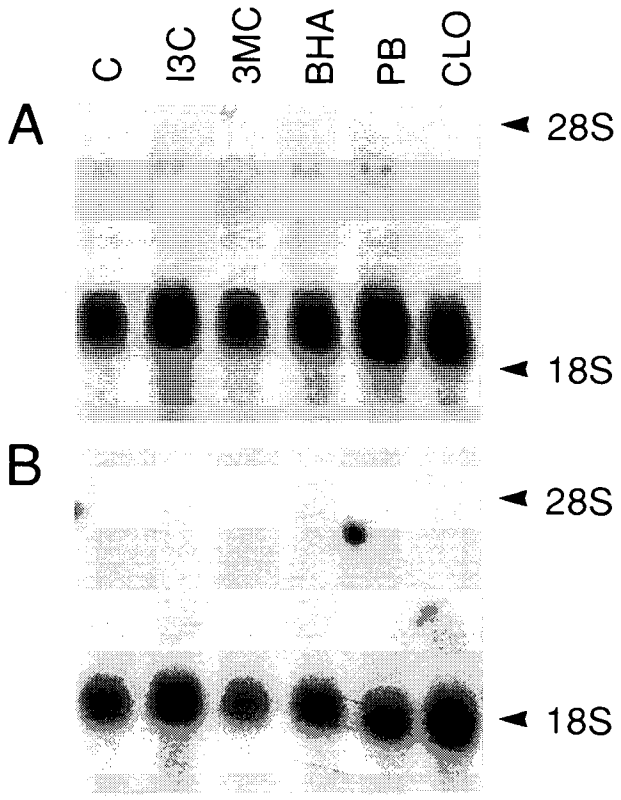

Fig. 2. Effect of inducer treatments *mUGTml* RNA levels in rat liver. Normalization was with β-actin (B). Lane markers are described in Figure 1.

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