Differential Expression of the Phenol Family of UDP-Glucuronosyltransferases in Hepatoma Cell Lines

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INTRODUCTION

UDP-glucuronosyltransferases (UGTs)* are phase II drug metabolizing enzymes that conjugate a variety of endogenous and exogenous compounds such as steroid hormones (i.e., androgens), bile acids (e.g., hyodeoxycholic acid), drugs (e.g., acetaminophen and propranolol), bilirubin, and carcinogens (e.g., *N*-hydroxy-2-acetylaminofluorene and hydroxylated benz[a]pyrene) (1,2,3). The UGTs are a superfamily of enzymes that reside in the endoplasmic reticulum of many cells including the liver, kidney, intestinal mucosa and lung. These enzymes covalently attach glucuronic acid to a vast array of lipophilic compounds and conjugation with glucuronic acid confers greater polarity and water solubility on the parent agents, thereby facilitating biliary and/or urinary excretion and detoxification. UGT can be divided into two families based on similarity of their amino acid sequences and divergent evolution of the genes (3). Family 1 contains isoforms that glucuronidate bilirubin [e.g., UGT1*1 (HUG-Br1)] and planar phenols [e.g., UGT1*6 (hlugP1)] and have identical carboxy-terminal domains. The transcripts encoding these isoforms appear to be derived from a single gene as a result of differential alternative splicing (4). Family 2 consists of isoforms that glucuronidate steroids and bile acids, and are encoded by separate genes (3). The differential regulation of gene expression of the different UGTs by xenobiotics are not well characterized and regulation of drug metabolizing enzymes such as UGT plays an important role in the biotransformation of many pharmaceutical agents as well as in toxicology of many environmental pollutants and carcinogens (3).

Compared to *in vivo* studies using animals, mammalian cell culture systems are convenient models with which to investigate the regulation of gene expression of drug-metabolizing enzymes. The primary culture of rat hepatocytes is probably the most widely used model. However, the hepatocytes can rapidly lose their most differentiated functions within 24–72 hours of culture including expression of several drug metabolizing enzymes (5). Alternatively, established mammalian hepatoma cell lines are attractive as potential tools to investigate various aspects of cellular and molecular biology of drug metabolizing enzymes. Hepatoma cell lines are easily available, immortal, phenotypically stable, and relatively easy to handle. Most importantly they express certain inducible cytochrome P450 genes and phase II enzymes such as the glutathione *S*-transferases (6).

The aim of the present study was to investigate the gene expression of UGT in hepatoma cell lines. Three hepatoma cell lines, Fao (rat), Hepa 1c1c7 (mouse) and Hep G2 (human), were utilized as models to study the regulation of UGT gene expression by xenobiotics. The effects of treatment with anti-oxidants (hydroquinone [HQ] and 3-(2)-*tert*-butyl-4-hydroxyanisole [BHA]), and indole-3-carbinol (I3C) on the gene expression of two UGT were studied. Northern blot analysis of total RNA and functional assays using phenolic substrates; 1-naphthol (1NA) and 4-*tert*-butylphenol (4BP) quantifying isoenzyme activity of UGT-p1 and UGT-p4, respectively (8), were performed.

EXPERIMENTAL

Materials

Hepa 1c1c7, and Hep G2 cell lines were obtained from ATCC (Rockville, MD, USA) and Fao was obtained from Dr. Barry Goldstein (Thomas Jefferson University). Cells were grown in humidified air with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, penicillin 100 IU/ml and streptomycin 0.1 mg/ml (Gibco-BRL, Bethesda, MD, USA) until 80–90% confluent and at which time they were split 1:2 into 10-cm dishes. The medium was renewed routinely every 3 days after passage.

Induction

When the cells were about 70% confluent, inducing agents were added in 2 μl of absolute ethanol (<0.05% v/v). Control cells were treated with the vehicle alone. The inducing agents and final concentrations were: hydroquinone (HQ) [50 μM], 3-(2)-*tert*-butyl-4-hydroxyanisole (BHA) [60 μM], and indole-3-carbinol (I3C) [10 μM] (purchased from Aldrich, Milwaukee, WI, USA). The next day, fresh medium containing the inducing agents were replaced. After 48 hr of treatment with inducers, 5×10^6 cells/10-cm dish were harvested by scraping with rubber policeman in ice-cold phosphate buffered saline (PBS). Following centrifugation and

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* UGT(s) is used to denote UDP-glucuronosyltransferase protein(s) and italic *UGT* with subscript, e.g., UGT_{Br1}, indicates the gene that encodes the protein.

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aspiration of PBS, the cells were resuspended in 50 mM Tris-HCL buffer, according to published procedures for the glucuronidation assay (7,8). Cell membranes were disrupted mechanically with 20 strokes of a 7-ml tight-fitting (B) Dounce glass homogenizer and followed by twice freezing the cells in dry ice/ethanol for 5 min and then thawing for 5 min at 37°C. Protein concentrations were determined by the BCA method (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as the standard. Homogenates were stored at −70°C until analysis.

Glucuronidation Assay

UGT enzyme activities were measured in cell homogenates prepared in 50 mM Tris-HCL buffer as described in Jin et al. (7). Glucuronidation was assessed using 500 μM 1-naphthol (1NA) and 500 μM 4-*tert*-butylphenol (4BP) quantifying UGT-p1 and UGT-p4 activities, respectively (8). Enzyme incubations were done in 1.5 ml plastic tubes containing 500 μM [^{14}C] UDP-glucuronic acid (UDPGA, 0.2 μCi, NEN-Dupont), 5 mM $MgCl_2$, 0.1% v/v Triton X-100, 0.5–1 mg/ml of cell homogenate protein in a total volume of 150 μl of 50 mM Tris-HCL buffer (pH 7.4), and were incubated at 25°C for 16 hr as described by previous investigators (7,8). The reactions were terminated by adding 400 μl of absolute ethanol and stored at −70°C until analyses.

Glucuronides of 1NA and 4BP were assayed using the method of Coughtrie et al. (9). Chromatographic equipment consisted of a Waters model 991 diode array detector, 600E pump, and 175 autosampler. The column was a Partisil 5 PAC 4.6 × 250 mm column (Whatman, Clifton, NJ) preceded by a guard column of the same packing meterial. Elution was performed with a linear gradient mobile phase of 10 mM tetrabutylammonium hydrogen sulfate (TBAHS; pH 2.7) and acetonitrile (linear increase from 0 to 100% 10 mM TBAHS from 0 to 20 min, hold at 100% 10 mM TBAHS from 20 to 45 min) at a flow rate of 1.5 ml/min. Radioactivity was continuously monitored with a Packard model 515A flow detector using Ultima-Flo M as scintillant at a flow rate of 4.5 ml/min. Counting efficiency was determined using a ^{14}C-labelled standard (Spec-Chec). The glucuronide conjugate of 1-naphthol (Sigma Chemical Co., St. Louis, MO), served as chromatographic retention time standard.

Isolation of Total RNA and "Northern Blot" Analysis

Total RNA from hepatoma cell lines was isolated by the guanidine-thiocyanate method as described by Chomczynski and Sacchi (10) using the RNAgents Total RNA Isolation Kit (Promega, Madison, WI). The RNA was subjected to electrophoresis in denaturing formaldehyde/1% agarose gel and transferred onto GeneScreen nylon membranes (NEN-Dupont, Boston, MA). These membranes were prehybridized, then hybridized with a [^{32}P]-labelled $mUGT_{com}$ cDNA probe which contains the 1.2 kilobase (kb) fragment of the 3′ common region of the mouse bilirubin/phenol family of UGT $mUGT_{br/p}$ (11), to detect Family 1 *UGT* mRNA transcripts. Filters were washed and autoradiographed at −70°C. The Northern blotting hybridization and washing conditions are detailed in Maniatis et al. (12) with minor modifications as previously described (13).

RESULTS AND DISCUSSION

Table 1 shows the results of the UGT enzyme activities towards phenolic substrates in the three hepatoma cell lines treated for 48 hr with the different inducing agents. Control Fao cells contained measurable level of UGT activities towards 1-naphthol (1NA) and 4-*tert*-butylphenol (4BP), which were significantly induced by BHA, HQ and I3C. [Addition of <0.05% v/v ethanol alone to all three cell lines had no effect on either UGT activity or mRNA levels; data not shown]. In the Hepa 1c1c7 cells, detectable level of UGT activities towards 1NA and 4BP were also observed in the control group. Hepa 1c1c7 cells expressed about the same level of UGT-p1 activity (1NA) as Fao, but higher UGT-p4 activity (4BP) than Fao at the basal state. These activities remained either unchanged or slightly decreased upon treatment with the anti-oxidants, BHA and HQ; however, the activities were increased slightly after treatment with I3C. In the Hep G2 cell line, no UGT activity were detected against either substrate with the untreated cells, but a slight UGT-p1 activity (1NA) was detected after treatment with HQ and I3C.

Figure 1 shows the results of the Northern blot analysis of total RNA obtained following the different treatments. Hybridization of the nitrocellulose membrane to a $mUGT_{com}$ cDNA probe (which will detect *UGT* family 1 mRNA) (Fig. 1A), revealed a prominent mRNA transcript of about 2.5 kb in the control Fao cells; this transcript was 2.52, 1.08, and 6.37 times that of control (quantitated by UltroScan XL, Pharmacia, Piscataway, NJ) following treatments with BHA, I3C and HQ, respectively. Hybridization of the same blot to

Table I. UDP-glucuronosyltransferase Activity Towards 1-Naphthol and 4-*tert*-Butylphenol in Fao, Hepa1 and Hep G2 Cells after Treatments with BHA, HQ, I3C for 48 Hours. Results are from Duplicate Experiments: Activity are expressed as pmol/min/mg protein

Activity	Control	BHA	HQ	I3C
1-Naphthol				
Fao	30 ± 23	280 ± 24 (850%)[2]	160 ± 110 (420%)	240 ± 68 (720%)
Hepa1	27 ± 14	15 ± 1 (−45%)	26 ± 10 (−5.8%)	61 ± 12 (120%)
Hep G2	N.D.[1]	N.D.	0.44 ± 0.62	0.24 ± 0.33
4-*tert*-butylphenol				
Fao	3.6 ± 0.8	29 ± 5 (720%)	10 ± 0 (180%)	27 ± 4 (650%)
Hepa1	22 ± 2	9.4 ± 6.5 (−60%)	8.9 ± 5.6 (−60%)	28 ± 7 (28%)
Hep G2	N.D.	N.D.	N.D.	N.D.

[1] Not Detectable.
[2] Percent change versus control.

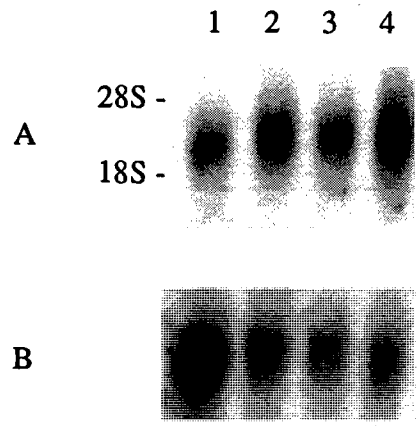

Fig. 1. Northern blot analysis of total RNA (about 20 μg) prepared from Fao cells treated for 48 hr with control (lane 1), 60 μM BHA (lane 2), 10 μM I3C (lane 3), and 50 μM HQ (lane 4). The blot was hybridized to (A) a 1.2 kb fragment of the 3′ common region of the mouse bilirubin/phenol family of UGT, $mUGT_{br/p}$ (11), and (B) human β-actin (ATCC).

a human β-actin cDNA probe (ATCC) showed even loading (Fig. 1B). [Due to nonspecificity, the control lane seems to have higher signal than the other lanes. Ethidium bromide staining of the 18S and 28S ribosomal RNA, indicated that all lanes were loaded evenly (data not shown). This experiment has been repeated twice and the same magnitude of induction was observed]. No message was detected in Hepa 1c1c7 and Hep G2 cells (data not shown).

Comparing the results of functional assay of UGT activity towards 1NA and 4NP (Table I) and the Northern blot analysis of mRNA (Figure 1), *UGT* family 1 mRNA was highly expressed in control Fao cells and induced by BHA, I3C and HQ between 1.08 to 6.37 times that of control. However, UGT-p1 (1NA) activity was induced 4.2–8.5 fold, and UGT-p4 (4BP) activity were induced 1.8–7.2 fold (Table I). The difference between the observed UGT activities and steady state mRNA level could be due to: (1) the induction of *UGT* family 2 gene(s) that possess activity towards these substrates; or (2) post-translational stabilization of protein with slight induction of family 1 *UGT* mRNA. Future studies would include Northern blot analysis using specific *UGT* family 1, and family 2 cDNA probes.

Untreated Fao cells expressed high level of UGT phenolic activities towards 1NA and 4BP which correspond to UGT-p1 and UGT-p4 activities, respectively (8). Here we reported for the first time that phenolic UGT activities are induced after the anti-oxidant treatments with BHA and HQ, as well as following I3C treatment in hepatoma cell lines. [I3C is a naturally occuring component of the human diet found in high quantity in plants of the family Cruciferae, e.g., cabbage, brocoli etc. (14), which has been shown to exhibit chemopreventive property against cancer (15).] Other genes such as glutathione *S*-transferase (16), NAD(P):quinone oxidoreductase (17), γ-glutamylcysteine synthetase (18), epoxide hydrolase (19), and aldo-keto reductase (20) are regulated by this group of anti-oxidative compounds. The specific mechanism for induction is not known, although it has been noted that all such inducing chemicals have in common the ability to act as electrophilic Michael acceptors (21). Presence of a consensus sequence element in the 5′-flanking region of many of these genes, identified as "anti-oxidant responsive element" or ARE (14), provides a fundamental basis for a common mechanism of gene regulation among these various enzymes. The induction of UGT activities towards phenolic substrates such as 1NA and 4BP, suggest that *UGT* may be similarly regulated, at least in the Fao cells. [Similar findings were observed in male Sprague-Dawley rat livers after anti-oxidants treatment—unpublished observations]. Induction of UGT activities by I3C could be mediated by both the ARE and the aromatic hydrocarbon (Ah) receptor (22). Cloning of the 5′ flanking region of the genomic DNA of the *UGT* genes will delineate this finding.

In summary, treatment of the highly differentiated Fao hepatoma cell line with antioxidants (BHA, and HQ) and I3C increases UGT-p1 and UGT-p4 activities. This increase in UGT phenolic activities likely arises from transcriptional activation (induction) of the *UGT* gene since the mRNA transcripts of UGT family 1 were elevated after anti-oxidants treatment. It remains to be seen whether induction is mediated through the anti-oxidant responsive element at the 5′-flanking region of the *UGT* genes.

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