Involvement of the Xenobiotic Response Element (XRE) in Ah Receptor-mediated Induction of Human UDP-glucuronosyltransferase 1A1*

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UDP-glucuronosyltransferase 1A1 (UGT1A1) plays an important physiological role by contributing to the metabolism of endogenous substances such as bilirubin in addition to xenobiotics and drugs. The UGT1A1 gene has been shown to be inducible by nuclear receptors steroid xenobiotic receptor (SRX) and the constitutive active receptor, CAR. In this report, we show that in human hepatoma HepG2 cells the UGT1A1 gene is also inducible with aryl hydrocarbon receptor (Ah receptor) ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), β-naphthoflavone, and benzo[a]pyrene metabolites. Induction was monitored by increases in protein and catalytic activity as well as UGT1A1 mRNA. To examine the molecular interactions that control UGT1A1 expression, the gene was characterized and induction by Ah receptor ligands was regionalized to bases −3338 to −3287. Nucleotide sequence analysis of this UGT1A1 enhancer region revealed a xenobiotic response element (XRE) at −3381/−3299. The dependence of the XRE on UGT1A1-luciferase activity was demonstrated by a loss of Ah receptor ligand inducibility when the XRE core region (CAGGA) was deleted or mutated. Gel mobility shift analysis confirmed that TCDD induction of nuclear proteins specifically bound to the UGT1A1-XRE, and competition experiments with Ah receptor and Arnt antibodies demonstrated that the nuclear protein was the Ah receptor. These observations reveal that the Ah receptor is involved in human UGT1A1 induction.

Glucuronidation has evolved in vertebrates to catalyze the transfer of glucuronic acid from uridine 5′-diphosphoglucuronic acid to soluble non-lipid dependent substances generated as byproducts of dietary and cellular metabolism (1). Some of the endogenous agents that are targets for glucuronidation are bilirubin and many of the steroids as well as several phenolic neurotransmitters. In addition, hundreds of drugs and xenobiotics are subject to glucuronidation (2, 3). The vast numbers of endogenous and exogenous substances that are susceptible to glucuronidation in humans are catalyzed by the family of UDP-glucuronosyltransferases (UGTs). A comparison of the deduced amino acid sequence of the UGTs in mammalian species has helped in classifying these proteins as members of the UGT1 or UGT2 gene family (4). In humans, 16 cDNAs have been identified and shown through expression experiments in tissue culture to encode proteins that display functional glucuronidation activity (3). It is generally felt that evolutionary constraints associated with the UGT1 family of proteins leads to more efficient glucuronidation of drugs and xenobiotics, whereas the UGT2 family of proteins displays far greater catalytic diversity toward endogenous agents such as steroids.

Regulation of the UGTs in human tissues is tightly controlled. Analysis of RNA expression patterns has demonstrated that no two tissues display the same pattern of UGT gene expression, indicating that regulatory control occurs in a tissue-specific manner (5). In addition, environmental influences on gene control clearly indicate that the UGTs are capable of undergoing differential regulation resulting in enhanced glucuronidation capacity. The treatment of Caco-2 cells with the antioxidant tert-butylhydroquinone leads to induction of UGT1A6, UGT1A9, and UGT2B7 (6, 7). Transcriptional regulation of UGT1A6 and UGT1A9 occurs after exposure to Ah receptor ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (6, 8). Human UGT1A1 has recently been shown to be under control by agents that induce gene expression in concordance with the constitutive active receptor (CAR) (9) and the steroid xenobiotic receptor (SRX) (33). The treatment of HepG2 and Caco-2 cells with the flavonoid chrysin leads to the induction of UGT1A1 (10–12). Interestingly, flavonoids have also been shown to induce CYP1A1 (13) in a CYP1A1-luciferase reporter HepG2 cell line (14), implicating a potential role for the induction of UGT1A1 through a similar mechanism. One potential mechanism that may link the expression of UGT1A1 and CYP1A1 by flavonoids is the ability of these agents to activate the Ah receptor. Although the mechanisms surrounding expression of CYP1A1 after activation of the Ah receptor are well documented (15–17), there is little information linking expression of the human UGT1A1 gene through an Ah receptor-dependent mechanism. Experiments were undertaken in this study to examine the actions of several Ah receptor ligands to modulate expression of the UGT1A1 gene.

EXPERIMENTAL PROCEDURES

Materials—1-Naphthol, 17α-ethynylestradiol, o-nitrophenyl-β-D-galactopyranoside and β-naphthoflavone (BNF) were purchased from Sigma. TCDD, 1-hydroxybenzo[a]pyrene (1B[a]P), 2B[a]P, 3B[a]P, 4B[a]P, 6B[a]P, 7B[a]P, 8B[a]P, 9–9B[a]P, 10B[a]P, benzo[a]pyrene-cis/zol[a]pyrene: Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; DTT, dithiothreitol, XRE, xenobiotic response element, DRE, drug response element.

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1 The abbreviations used are: UGT, glucuronosyltransferase; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; CAR, constitutive active receptor; SXR, steroid xenobiotic receptor; BNF, β-naphthoflavone; B[a]P, benzo[a]pyrene; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; DTT, dithiothreitol, XRE, xenobiotic response element, DRE, drug response element.
characterized previously (38). The restriction enzyme sites SacI and XhoI were incorporated at the 5′ end of the sense and antisense primers, respectively. The PCR product for the ~3712/~7 UGT1A1 promoter was generated with oligonucleotides 5′-tttgggctTCAGACAAAGGAA3′ and 5′-ttcttggctgATGCCCCCTCTC3′, digested with SacI/XhoI (the sites are in lowercase and underlined) and subcloned into SacI/XhoI-digested PGL3-basic vector. This plasmid is identified as plUGT1A1. Using the plUGT1A1 plasmid, promoter activity was determined in the liver (14), the neomycin gene was removed and cloned into the SacI site of pLUGT1A1, generating the pLUGT1A1 plasmid.

The sequences of the primers used for the enhancers are as follows: E1, 5′-atattgggctAAAGAGAACAACT-3′ and 5′-atatttcgGGAATGATCCTTT-3′; E2, 5′-atattgggctTTGTCTGGGC-3′ and 5′-aattttctgGGAACATAGCTGTT-3′; E3, 5′-ttttgggctTCAGACAAAAG3′ and 5′-tatattggctGACTTCCTCCTC3′; E5, 5′-aaggggctgTAACGGTTCTAAA3′ and 5′-aatattttctgGTTACTTCAAA3′ and 5′-aatttgctgGTTATGTAATCAGA-3′. Each of these amplified inserts was digested with SacI and XhoI site and subcloned into the SacI/XhoI digested PGL3-promoter vector.

For construction of the mutant UGT1A1-XRE enhancer plasmid, E4 was used as template. The primers used for amplification of the insert were 5′-ttttgggctTTTTTGACTGCGA-3′ and 5′-aattttctgCTCATC-TCTTCTCTC-3′. The two internal primers that carried the mutations were 5′-CTTTGCTAGAGCCATAGGAC-3′ and 5′-GTTCTGATTCC-GGAGACTAAG-3′. The underlined region represents the area of the Ah receptor core binding region and the bold and italicized boxes are those that were changed form the normal XRE sequence to disrupt the Ah receptor binding region (see Fig. 4A). After digestion of the amplified sequence with SacI and XhoI, the insert was cloned into these same sites in the PGL3-promoter vector.

Transfection Assays—HepG2 cells were plated in 12-well tissue culture plates at 30–40% confluence and transfectionated after 24 h using LipofectAMINE Plus reagent as described by the manufacturer's protocol (Invitrogen). In general, transfection mixtures contained 500 ng of UGT1A1-reporter plasmid and 300 ng of β-galactosidase expression vector (PCMVβ) as an internal control to monitor for transfection efficiency. The day after transfection, the cells were treated with 20 μM BNF, 20 μM TCDD, or MeSO for 48 h. The cells were harvested, lysed, and analyzed for luciferase and β-galactosidase activity. Luciferase activities were assessed by the methods described previously (22) using a Monolight 2001 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Briefly, HepG2 cells were harvested in lysis buffer (1% Triton, 25 mM Tricine, 15 mM MgSO4, 4 mM EDTA, and 1 mM DTT). Cell lysates were centrifuged, and 10 μl of the supernatant were used as 20 μM TCV, 20 μM TCDD, or 20 μM BNF, as substrates (18).

Northerm Blot Analysis—Nuclear proteins from HepG2 cells were harvested in lysis buffer (1% Triton, 25 mM Tricine, 15 mM MgSO4, 4 mM EDTA, and 1 mM DTT). Nuclear extracts were prepared as described previously (22) using a Monolight 2001 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Briefly, HepG2 cells were harvested in lysis buffer (1% Triton, 25 mM Tricine, 15 mM MgSO4, 4 mM EDTA, and 1 mM DTT). Cell lysates were centrifuged, and 10 μl of the supernatant were used as 20 μM TCV, 20 μM TCDD, or 20 μM BNF, as substrates (18).

Preparation of Nuclear Proteins—Nuclear extracts from HepG2 cells were isolated as described previously (22), with all of the procedures performed at 4 °C. After 48 h of treatment with 10 nM TCDD, 20 μM BNF, or MeSO, HepG2 cells were washed twice with 10 mM HEPES buffer, pH 7.5, collected by scraping into MDH buffer (3 mM MgCl2, 1 mM DTT, 25 mM HEPES, pH 7.5), and homogenized with a Potter-Elvehjem tissue grinder driven by an electric motor. The homogenate was centrifuged at 1000 × g for 5 min, and the pellet was washed with MDH buffer (3 mM MgCl2, 1 mM DTT, 25 mM HEPES, pH 7.5, 0.1 mM KCI) 3 times. The pellet was then lysed in HDK buffer (25 mM HEPES, 10 mM Tris, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1 mM DTT) and sonicated on a 10-s pulse cycle for 5 min. The supernatant, which was 5 × 107 cells/ml, was then centrifuged at 10 000 × g for 10 min at 4 °C.

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**RESULTS**

Induction of UGT1A1 by TCDD and BNF—The small phenolic compound 1-naphthol was used as a substrate to examine UGT activity in HepG2 cells (Fig. 1A). Treatment of HepG2 cells with 10 nM TCDD for 72 h led to a time-dependent increase in 1-naphthol UGT activity that consistently was determined to be 3-fold over untreated cells. Similar treatment of cells with 20 μM BNF resulted in a 4–5-fold increase in 1-naphthol UGT activity. Simple phenols have been shown to be glucuronidated by most of the UGT1A proteins (3), with a preference for UGT1A1, UGT1A6, UGT1A8, and UGT1A9. Glucuronidation of 17α-ethynylestradiol, a substrate that is preferentially glucuronidated by UGT1A1, was increased 2.5–5-fold in TCDD- or BNF-treated cells (Fig. 1A). Quantitation of UGT1A1 RNA transcripts by Northern blot analysis demonstrated that both TCDD and BNF induced UGT1A1 (Fig. 1B) in a time-dependent fashion. Slightly greater increases in RNA were observed with BNF-treated cells, a pattern that was also reflected in catalytic activity. It was also observed that induction of UGT1A1 RNA and 17α-ethynylestradiol glucuronidation by TCDD and BNF correlated with increased levels of UGT1A1 protein (Fig. 1C), with BNF generating slightly greater levels of induced UGT1A1 in microsomes. In HepG2 cells, TCDD and BNF are capable of inducing CYP1A1, as shown by induction of CYP1A1 (Fig. 1C) and activation of the human CYP1A1-luciferase gene in TV101 cells (Fig. 2). Induction of CYP1A1-luciferase in TV101 cells has been linked to activation of the Ah receptor (22, 23). Although maximal CYP1A1-luciferase activity is achieved between 8–24 h in TV101 cells with TCDD and BNF, maximal levels of UGT1A1...
RNA and protein are evident at around 48 h (Fig. 1C), indicating that slightly different regulatory events may control the CYP1A1 and UGT1A1 genes. Combined, these results indicate that induction of UGT1A1 may occur through an Ah receptor-dependent mechanism.

**Characterization of the UGT1A1 Promoter and Ah Receptor Binding Site**—To examine the mechanism of UGT1A1 induction, an 11-kilobase region of the UGT1A1 promoter was cloned from a human BAC containing the entire UGT1A1 locus. UGT1A1 promoter and enhancer regions, cloned by PCR, were subcloned into the pGL3 basic or pGL3 promoter vectors, respectively. Portions of the regulatory region including the promoter constituted a fragment from 8134 (Enhancer 1, E1), 8533 to 4738 (Enhancer 2, E2), and 3712 to 2081 (Enhancer 3, E3). Each plasmid was transiently transfected into HepG2 cells, and expression of luciferase activity was determined after treatment of cells for 48 h with TCDD or BNF (Fig. 3). Our selection of 48 h for the treatment time was based on our observation that adequate accumulation of both RNA and protein in TCDD/BNF-treated HepG2 cells. The UGT1A1 −3712/+7 luciferase promoter fragment was induced after treatment with TCDD and BNF. An enhancer sequence from −3712 to −2081 (E3) relative to the transcriptional start site was also responsive. Enhancer sequences E2 and E1, which covered a region from −10998 to −4738, were refractory to both TCDD and BNF.

Induction of the −3712/+7 promoter-luciferase construct with TCDD indicates that the transcriptional activation may occur through an Ah receptor-dependent mechanism. Compounds that have been shown to be ligands for the Ah receptor are classically polycyclic aromatic hydrocarbons. To examine this possibility further, we developed MH1A1L cells carrying the UGT1A1-luciferase plasmid and demonstrated that classical polycyclic aromatic hydrocarbons composed of hydroxylated benzo[a]pyrene were capable of inducing UGT1A1−driven luciferase. We examined 1, 2, 3, 4, 6, 7, 8, 9, and 10-hydroxylated isomers of benzo[a]pyrene in addition to cis- and trans-4,5-dihydrodiol benzo[a]pyrene (Fig. 4). Along with TCDD induction, we observed a 2–5-fold induction of luciferase activity with the 3- and 9-hydroxybenzo[a]pyrene and the trans-4,5-dihydrodiol serving as the most efficient inducers. The use of cell lines deficient in Ah receptor function show that polycyclic aromatic hydrocarbons induce gene expression in an Ah receptor-dependent fashion (24). It has also been demonstrated through the use of reporter gene assays that are controlled by the Ah receptor enhancer sequence that polycyclic aromatic hydrocarbons induce transcription through activation of the Ah receptor (14, 25, 26). Combined, the results of TCDD, BNF, and B[a]P induction of the UGT1A1 promoter constructs strongly indicates that these agents elicit transcriptional activation through and Ah receptor-dependent pathway.

To localize the region on the UGT1A1 gene that controls induction, a reporter analysis on the E4 clone demonstrated that a sharp drop in induction was observed between 3525 and 3309 (Fig. 5A). Sequence analysis in this region revealed the presence of a single copy of the Ah receptor XRE motif (CACGCA) starting at position −3309 (Fig. 5B). Using DNA fragments spanning −3525 to −3144, site-directed mutagenesis was carried out on the conserved UGT1A1 XRE sequence, altering CACGCA to ACCGCA. Transient transfection of this plasmid demonstrated that the mutated UGT1A1-XRE resulted in a loss of inducibility (Fig. 5C) by TCDD and BNF.

Regulation of UGT1A1 by the XRE core sequence indicates that the CACGCA motif may be a binding site for the Ah receptor. Binding of Ah receptor complex to the XRE response element in the UGT1A1 promoter region was examined by gel
The human \textit{UGT1A1} gene plays an important role in normal physiology by serving as the only source for the glucuronidation of bilirubin (28), the byproduct of heme degradation. The gene is expressed differentially in a tissue-specific fashion in humans (29–31), indicating that multiple regulatory factors are involved in \textit{UGT1A1} expression. Several recent findings demonstrate that the induction of \textit{UGT1A1} by the Ah receptor is controlled in part by binding of the activated Ah receptor-Arnt complex to the \textit{UGT1A1}-XRE sequence.

**DISCUSSION**

The human \textit{UGT1A1} gene plays an important role in normal physiology by serving as the only source for the glucuronidation of bilirubin (28), the byproduct of heme degradation. The gene is expressed differentially in a tissue-specific fashion in humans (29–31), indicating that multiple regulatory factors are involved in \textit{UGT1A1} expression. Several recent findings demonstrate that the induction of \textit{UGT1A1} by the Ah receptor is controlled in part by binding of the activated Ah receptor-Arnt complex to the \textit{UGT1A1}-XRE sequence.

**Fig. 5. Functional characterization of the UGT1A1-XRE sequence.** A, an additional series of expression plasmids were generated from E1 (Fig. 3) to identify the TCDD-responsive region. A region of −200 bases (E5) was identified that supports enhancer activity after treatment with TCDD (blue bars) and BNF (brown bars). B, nucleotide sequence of a 130 base pair region spanning from −3425 to −3295. Shown in bold are binding regions for SXR, CAR, NR1 (9), and the Ah receptor (XRE). C, activity of an enhancer region that contains a mutation in the XRE sequence. The reporter plasmid containing either wild type or mutated UGT1A1-XRE (see “Experimental Procedures”) was inserted into the PGL3-promoter vector and then used in transient transfections. The core binding sequence of CACCGA was changed to ACCCGA. This mutation resulted in a lack of TCDD-dependent induction of transcriptional activity.

**Fig. 6. Ah receptor binding to UGT1A1-XRE.** HepG2 cells were treated with MeSO or 10 nM TCDD for 48 h. As outlined under “Experimental Procedures,” nuclear extract was isolated from MeSO-treated (DMSO-E) or TCDD-treated (TCDD-E) HepG2 cells, and 10 μg of protein from each extract was incubated with labeled UGT1A1-XRE or CYP1A1-DRE probe (indicated at the bottom of the autoradiographs) and subjected to 6% non-denaturing acrylamide gel electrophoresis. Competition was performed in the presence of a 50-fold excess of unlabeled UGT1A1-XRE (XRE×50) or CYP1A1-DRE (DRE×50). To determine whether the induced nuclear protein represented the Ah receptor/Arnt complex, binding reactions were also carried out in the presence of antibody generated toward the mouse Ah receptor (Anti-AhR) or mouse Arnt (Anti-Arnt). Control experiments were also conducted with an antibody generated toward the UDP-glucuronosyltransferases (Anti-UGT). The arrow indicates the TCDD inducible protein-DNA complex.

**Fig. 7. Ah receptor binding to UGT1A1-XRE.** A, an additional series of expression plasmids were generated from E1 (Fig. 3) to identify the TCDD-responsive region. A region of −200 bases (E5) was identified that supports enhancer activity after treatment with TCDD (blue bars) and BNF (brown bars). B, nucleotide sequence of a 130 base pair region spanning from −3425 to −3295. Shown in bold are binding regions for SXR, CAR, NR1 (9), and the Ah receptor (XRE). C, activity of an enhancer region that contains a mutation in the XRE sequence. The reporter plasmid containing either wild type or mutated UGT1A1-XRE (see “Experimental Procedures”) was inserted into the PGL3-promoter vector and then used in transient transfections. The core binding sequence of CACCGA was changed to ACCCGA. This mutation resulted in a lack of TCDD-dependent induction of transcriptional activity.

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such as metabolites of B[a]P are capable of inducing UGT1A1. In addition, there is building evidence that some of the flavonoids modulate gene regulation in part through the Ah receptor. Chrysirin is a potent inducer of UGT1A1 (10) and is able to induce the expression of CYP1A1, as demonstrated through induction of CYP1A1-luciferase in TV101 cells.8 Studies in rats show that Ah receptor ligands such as 3-methylcholanthrene are capable of inducing intestinal Ugt1a1 (34), and it is well known that 3-methylcholanthrene is a potent Ah receptor ligand. Omeprazole, a benzimidazole used in the treatment of peptic ulcer disease, activates the Ah receptor and induces CYP1A1 (23). Although not directly demonstrating induction of UGT1A1, omeprazole therapy has been shown to increase duodenal 3-hydroxybenzo[a]pyrene UGT activity greater than 5-fold (35). UGT1A1 is abundantly expressed in the small intestine (31). However, it is important to appreciate that dual regulation of UGT1A1 and CYP1A1 may not always occur. Apigenin, a flavonoid that is a potent inducer of human UGT1A1 (32), has very limited capacity to induce CYP1A1, as measured by induction of CYP1A1-luciferase in TV101 cells (13). Apigenin may regulate UGT1A1 in a manner that is independent of the Ah receptor.

As described by Sugatani et al. (9) and expanded by these studies and others (33), the UGT1A1 gene can be regulated by ligands that activate nuclear receptors CAR, SRX, and the Ah receptor. These cis-acting regulatory elements are positioned within a 125-base pair region on the UGT1A1 gene between bases −3424 and −3299. The location of these xenobiotic receptors in close proximity to each other may serve an important biological role in maintaining adequate expression levels UGT1A1. SRX and CAR are part of the orphan nuclear receptors that are structurally related to nuclear hormone receptors. It has been proposed that the xenobiotic nuclear receptors compose a family of regulatory proteins that are involved in steroid and xenobiotic sensing, leading to altered gene expression patterns essential for normal homeostasis (36, 37). Originally postulated to regulate CYP3A genes, these nuclear receptors are now known to regulate a number of phase I and phase II xenobiotic enzymes. Although not part of the nuclear receptor family, the Ah receptor also serves to modulate phase I and phase II enzymes in response to environmental stimuli. Thus, regulation of UGT1A1 can be controlled by numerous endogenous agents that are ligands for SRX and CAR as well as xenobiotics that are ligands for SRX, CAR, and the Ah receptor.

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