Interaction between Oxidative Stress Sensor Nrf2 and Xenobiotic-activated Aryl Hydrocarbon Receptor in the Regulation of the Human Phase II Detoxifying UDP-glucuronosyltransferase 1A10*

Sandra Kalthoff, Ursula Ehmer, Nicole Freiberg, Michael P. Manns, and Christian P. Strassburg

From the Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, 30625 Hannover, Germany

The defense against oxidative stress is a critical feature that prevents cellular and DNA damage. UDP-glucuronosyltransferases (UGTs) catalyze the glucuronidation of xenobiotics, mutagens, and reactive metabolites and thus act as indirect antioxidants. Aim of this study was to elucidate the regulation of UGTs expressed in the mucosa of the gastrointestinal tract by xenobiotics and the main mediator of antioxidant defense, Nrf2 (nuclear factor erythroid 2-related factor 2). Xenobiotic (XRE) and antioxidant (ARE) response elements were detected in the promoters of UGT1A8, UGT1A9, and UGT1A10. Reporter gene experiments demonstrated XRE-mediated induction by dioxin in addition to tert-butylhydroquinone (ARE)-mediated induction of UGT1A8 and UGT1A10, which are expressed in extrahepatic tissue in humans in vivo. The responsible XRE and ARE motifs were identified by mutagenesis. Small interfering RNA knockdown, electrophoretic mobility shifts, and supershifts identified a functional interaction of Nrf2 and the aryl hydrocarbon receptor (AhR). Induction of UGT1A8 and UGT1A10 requires Nrf2 and AhR. It proceeds by utilizing XRE as well as ARE-binding motifs. In summary, we demonstrate the coordinated AhR- and Nrf2-dependent transcriptional regulation of human UGT1As. Cellular protection by glucuronidation is thus inducible by xenobiotics via AhR and by oxidative metabolites via Nrf2 linking glucuronidation to cellular protection and defense against oxidative stress.

Molecular oxygen is essential for the survival of almost all eukaryotes. Under normal physiological conditions, reactive oxygen species (ROS), including hydrogen peroxide, superoxide, peroxyxnitrite, and hydroxyl radicals, are generated as metabolic by-products. However, increased levels of ROS can lead to oxidative stress and cell injury. During evolution, mammalian cells have developed a variety of inducible genetic programs to adapt to the presence of ROS. As a first cellular reaction in response to oxidative/electrophilic stress, an array of defense genes is activated (1, 2), which, in most instances, leads to the neutralization of oxidative stress, its effects, and finally to survival. In the absence of appropriate defense mechanisms, the accumulation of ROS and electrophiles can lead to membrane and DNA damage, mutagenicity, degeneration of tissues, premature aging, apoptotic cell death, cellular transformation, and cancer (3–5).

Cellular antioxidant defense employs a number of proteins (e.g. enzymes) and small molecules (e.g. vitamins C and E), to restrict ROS at levels, which are not critical for the organism. Enzymes with antioxidant capabilities capable of inactivating ROS and preventing ROS-initiated reactions include superoxide dismutases, catalase, and glutathione peroxidase and belong to the group of “direct” antioxidants (6–8). In contrast, phase 2 detoxifying (conjugating) enzymes are classified as “indirect” antioxidants based upon their role in maintaining redox balance and thiol homeostasis. They contribute to biosynthesis and the recycling of thiols or facilitate the excretion of oxidized, reactive secondary metabolites (quinines, epoxides, aldehydes, and peroxides) through reduction/conjugation reactions during the process of xenobiotic detoxification (9). Phase 2 enzymes with antioxidant capability include glutathione S-transferase isozymes and NADP(H):quinone oxidoreductase (NQO1), ɣ-glutamyl cysteine synthetase, and UDP-glucuronosyltransferases (UGTs).

UGTs facilitate the elimination of a broad array of endogenous and exogenous substances by glucuronidation. Cytochromes P450 generate ROS as a result of oxidative metabolism. The resulting reactive metabolites are among the major substrates for conjugation with glucuronic acid, which is catalyzed by UGTs. Glucuronidation leads to biologically inactive glucuronides in most instances. The human UGT1A enzyme family encoded on chromosome 2 comprises nine members (UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10) (10). The proximal 1 kb of the UGT1A8–10 promoters are highly similar, displaying >75% of sequence identity (11). The UGT1A10 gene product detoxifies many xenobiotic substrates and drugs as well as 7-hydroxy-benz[a]pyrene, known as a precursor of ROS. UGT1A10 is expressed exclusively in the extrahepatic gastrointestinal tract (12–15). UGT1A9 is the only isoform within the...
Coordinate Regulation of UGT1A10 by Nrf2 and AhR

UGT1A7–10 gene cluster, which is expressed in the liver. It is also highly expressed in kidneys and catalyzes the glucuronidation of estragole, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and compounds such as phenylbutazone (16–18).

The signal transduction pathways responsible for sensing oxidative stress and activating the appropriate defense genes are still not completely understood in eukaryotes. The transcription factor Nrf2 appears to represent a key regulator in oxidative stress that is activated by ROS (1, 2). Nrf2 is a member of the Cap’n’Collar family of BZIP proteins and recognizes the antioxidant response element (ARE) in the promoter of its target genes (19). Under normal basal conditions, Nrf2 is bound to its inhibitor, the cytoskeleton-associated protein Keap1, which represses Nrf2 by facilitating its proteasomal degradation (20). Upon stimulation by antioxidants such as tert-butylhydroquinone (tBHQ), Nrf2 is released from Keap1 and translocates into the nucleus, followed by heterodimerization with other transcription factors, such as Jun and small Maf (21–23).

Recent data provide evidence for a cross-talk between the Nrf2 pathway and the pathway leading to the induction of XRE-driven genes and the aryl hydrocarbon receptor (AhR). AhR is a basic helix-loop-helix transcription factor that, prior to ligand binding, is stabilized in the cytoplasm by direct interaction with HSP90 (heat shock protein 90), XAP2 (X-associated protein 2), and HSP90 co-chaperone p23 (24). Upon ligand binding (e.g., 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), phytochemicals, and sterols) the AhR ligand complex translocates into the nucleus and dimerizes with the Arnt (aryl hydrocarbon receptor nuclear translocator) (25). The AhR/Arnt dimer binds to XRE DNA-binding motifs located in the promoter of many drug-metabolizing enzymes. A number of studies have investigated the cross-talk between Nrf2 and AhR. Mutagenesis of ARE-binding elements in the promoter of human UGT1A6 showed that prior to treatment. Yeager et al. (27) showed in mice that TCDD induction of Ugt1a5, Ugt1a6, and Ugt1a9 was dependent on Nrf2, whereas TCDD induction of Ugt1a1 was not. There are several possibilities to explain this dependence. Miao et al. (28) demonstrated that nrf2 gene transcription is directly modulated by AhR through XRE-elements in the nrf2 gene promoter. A second possible mechanism is a direct interaction between AhR and Nrf2 proteins, but evidence that the two transcription factors can physically interact has not been presented to date. A third possibility is an interaction between Nrf2- and AhR-associated proteins or an interaction between AhR- and Nrf2-associated proteins.

As the major site of first entry for xenobiotics, the gastrointestinal tract, and subsequently the liver are continuously exposed to a broad array of compounds with ROS capability. Mucosal metabolism can lead to metabolites with increased toxicity and increases the susceptibility of the gastrointestinal tract and the liver for oxidative metabolites, chemical toxicity, and potentially carcinogenesis. Both organ systems harbor molecular defense mechanisms to detoxify reactive intermediates and minimize oxidative stress. Because UGTs are expressed directly in the intestinal mucosa as well as in the liver, we hypothesized that they are regulated by antioxidant signaling pathways. In this study, the human UGT1A8/UGT1A10 (extrahepatic expression in humans in vivo) and UGT1A9 (hepatic expression in humans in vivo) were studied, and their regulation by Nrf2 and/or AhR was identified and characterized.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—In all experiments, human esophageal squamous cell carcinoma (KYSE70) cells were used. They were previously established from the poorly differentiated invasive esophageal squamous cell carcinoma resected from middle intrathoracic esophagus of a 77-year-old Japanese man prior to treatment. KYSE70 cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum. Esophageal epithelium derived KYSE70 cells were used because the esophageal mucosa exclusively expresses UGT1A7, UGT1A8, UGT1A9, and UGT1A10 (10), the regulation of which was the aim of the experimental characterization of this study. The cells were maintained at 37 °C under an atmosphere of 5% CO2 and 95% air.

RNA Isolation and Reverse Transcription-PCR—KYSE70 cells were treated with test compounds (5 nM TCDD, 100 μM tBHQ, or vehicle dimethyl sulfoxide) for 24 h, and total RNA was prepared with TRizol (Invitrogen), 5 μg of RNA were used for the generation of cDNA in an oligo(dT)-primed Superscript III reverse transcriptase reaction according to the manufacturer’s instructions (Invitrogen).

PCR of UGT1A10, Nrf2, and AhR—The influence of TCDD and tBHQ on UGT1A10-, AhR-, and Nrf2-mRNA levels was shown by co-amplification of the gene of interest and β-actin. Amplification of UGT1A10 from cDNA was performed with an initial denaturation for 5 min at 94 °C followed by 35 cycles of denaturation for 30 s at 94 °C, primer annealing for 30 s at 59 °C, and an extension reaction for 1 min at 72 °C, followed by a final extension reaction of 7 min at 72 °C. Amplification of Nrf2 and AhR was carried out using the same protocol except for the annealing temperature (58 °C) and the number of cycles (AhR, 31 cycles; Nrf2, 25 cycles). β-Actin primers were added after 10 (UGT1A10 and AhR) or 5 (Nrf2) cycles. All primers are listed in supplemental Table 3.

Generation of UGT1A8, UGT1A9, and UGT1A10 Luciferase Reporter Gene Constructs—A 500-bp (UGT1A8 and UGT1A10) and a 530-bp (UGT1A9) DNA fragment of each UGT1A 5′-upstream sequence were amplified by PCR from a healthy blood donor (all primers are shown in supplemental Table 4). The PCR fragments were cut by XhoI and NheI and ligated into pGL3 vector (Promega, Mannheim, Germany). Mutagenesis of putative AhR- and Nrf2-binding sites was performed by primer extension using primers specified in supplemental Table 5. All inserts were sequenced in full using the Dye Terminator Cycle Sequencing kit 1.1 (Applied Biosystems, Darmstadt, Germany) and the ABI 310 automated sequencer (Applied Biosystems, Darmstadt, Germany).

Luciferase Assays—KYSE70 cells were seeded in 12-well plates and transfected with UGT1A8-, UGT1A9-, and UGT1A10 constructs (800 ng/well) in addition to the pRL-TK.
plasmid using Lipofectin Transfection reagent (Invitrogen) to perform a Dual-Luciferase assay (Dual-Reporter Assay; Promega, Mannheim, Germany). For transfection of siRNA (100 nM) Lipofectamine 2000 (Invitrogen) was used. Transfection of the reporter gene constructs using Lipofectin followed 6 h later. On the next day, cells were treated with 5 nM TCDD or 100 μM tBHQ for 48 h if not stated otherwise. All experiments were performed in triplicate in at least 3–10 independent experiments. Results were analyzed using Microsoft Excel software and are shown as luciferase activity relative to empty pGL3 vector or as fold induction where indicated. Error bars represent S.D. Statistical analysis was performed using Student’s t test for comparisons between groups. Significance was determined across all performed experiments. Differences were considered significant when p values were <0.05.

Transfection of siRNA—200 pmol of siRNA (MWG Biotech, Ebersberg, Germany) against Nrf2 (AAGAGUAUGAGCGAAAAACTT), AhR (AAGCGGCAUAGAGACCGACUU), or nonsilencing control (UAAUGUAUUGGCAAATTT) were transfected within 2 ml of OPTI-MEM (Invitrogen) into KYSE70 cells seeded into 6-well plates using Lipofectamine 2000 according to the manufacturer’s instructions. Consequently, final concentration of siRNA was 100 nM. Knockdown efficiency was determined by semiquantitative Western blot analysis (see below).

TABLE 1
Sequence of used oligonucleotides in EMSAs

| Primer Position | Sequence |
|-----------------|----------|
| ARE consensus   | AGA ATG CTG AGT CAC GGT G (forward), CAC CTT GAC TCA GCA TTC T (reverse) |
| XRE consensus   | CAAAAGCTTCTGA (forward), TGACCCTTGTG (reverse) |
| 1A10 XRE-101    | bp−114 to −85 |
| 1A10 ARE-149    | bp−159 to −130 |
| 1A10 XRE-136    | bp−144 to −120 |

*PPARγ, peroxisome proliferator-activated receptor γ.*

FIGURE 1. A, time-dependent regulation of UGT1A10 mRNA by TCDD (5 nM) and tBHQ (100 μM) in comparison to solvent (dimethyl sulfoxide, DMSO) in KYSE70 cells. The highest induction of UGT1A10 mRNA by TCDD was detectable after 48 h. Maximal tBHQ inducibility was observed after 24 h. B, time-dependent regulation of the UGT1A10 500-bp 5′-upstream region by TCDD and tBHQ in luciferase assay in KYSE70 cells. A maximal up-regulation of luciferase activity was observed after 48 h both by TCDD and tBHQ. WT, wild type.
Western Blot—20 μg of total cell lysates from KYSE70 cells treated with either 5 nM TCDD or 100 μM tBHQ were boiled for 10 min in Laemmli sample buffer (2% sodium dodecyl sulfate, 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, and 0.001% bromphenol blue) and separated by 8% SDS-PAGE prior to electrotransfer onto a nitrocellulose membrane. Blocking was performed in
RESULTS

Time-dependent Inducibility of Human UGT1A10 mRNA by TCDD (AhR) and tBHQ (Nrf2)—A semiquantitative PCR was performed for UGT1A10 mRNA in relation to actin mRNA. KYSE70 cells treated with TCDD or tBHQ for different lengths of time showed an UGT1A10 mRNA induction by TCDD after 3 h and a maximum after 48 h (Fig. 1A). tBHQ induced UGT1A10 mRNA after 24 h of incubation, which is the point of strongest induction. Luciferase reporter gene assays similarly showed a time-dependent induction of a UGT1A10:500-bp construct by TCDD and tBHQ (Fig. 1B). The strongest TCDD and tBHQ inducibility was detectable after 48 h. Induction was 6.4-fold with TCDD and 2.5-fold with tBHQ. These data indicate a previously undescribed time-dependent induction of human UGT1A10 mRNA and of the UGT1A10 5′-upstream region, most likely by the transcription factors AhR and Nrf2, which was further clarified.

Identification of XRE- and ARE-binding Elements in UGT1A10 5′-upstream Region—To identify the responsible DNA binding motifs 5′-upstream DNA sequence was analyzed. Four potential XRE-binding sites and one ARE-binding site were identified in the human UGT1A10 5′-upstream region within the first 500 bp of the UGT1A10 promoter that were highly similar to XRE and ARE consensus sequences (Table 2). All potential binding sites were mutagenized individually to examine their specific effects on TCDD and tBHQ inducibility. If the transcription factors AhR or Nrf2 bind specifically to the examined binding elements, mutagenesis would be expected to lead to the prevention of transcription factor binding and therefore result in abolished TCDD or tBHQ induction. Mutagenesis of the XRE-176 and XRE-256 site did not affect UGT1A10 induction by AhR or Nrf2 (Fig. 2A). By mutagenesis of XRE-binding elements, a reduction of TCDD induction was expected. Interestingly, the mutagenesis of the XRE-101 or XRE-136 motif led to a simultaneous decrease of both TCDD- and tBHQ-mediated induction. Similarly, the mutagenesis of the ARE-149 motif resulted in a simultaneous decrease of both TCDD- and tBHQ-mediated induction of UGT1A10.

TCDD and tBHQ Inducibility of Highly Homologous UGT1A8 and UGT1A9 and Identification of Respective XRE- and ARE-binding Elements—The experiments were expanded to include the highly homologous UGT1A8 and UGT1A9 constructs are shown in Table 2.) In luciferase assays, UGT1A10 promoter inducibility by TCDD and tBHQ was significantly reduced with an ARE-containing Thr instead of Gly, in comparison to wild type construct. B, chimeric mutagenesis of the UGT1A9 ARE to correspond to the sequence of the UGT1A10 ARE led to tBHQ inducibility previously not observed with the wild type UGT1A9 sequence. (The sequences of the constructs are shown in Table 2.) Significance is determined in comparison to wild type construct. KYSE70 cells were treated with TCDD (5 nM) and tBHQ (100 μM) for 48 h (A and B). DMSO, dimethyl sulfoxide; WT, wild type.
genes. Despite a promoter sequence homology of >75%, UGT1A9 is mainly expressed in liver and kidneys, whereas UGT1A8 and UGT1A10 are exclusively expressed in the extra-hepatic gastrointestinal tract in humans in vivo (14, 15). Luciferase activity assays showed a similar TCDD (6.4-fold) and tBHQ (2.7-fold) inducibility for UGT1A8 in comparison to UGT1A10 (Fig. 2B). In contrast, liver expressed UGT1A9 was only induced 4.3-fold by TCDD, and no tBHQ-mediated induction was detectable. The corresponding XRE-101 and ARE-149 binding elements were mutagenized in both the UGT1A8 and UGT1A9 5′-upstream regions. Mutagenesis of the XRE-101 and ARE-149 sites of UGT1A10 led to a significant reduction of TCDD and tBHQ inducibility similar to that seen for UGT1A9 (Fig. 2C). When the corresponding XRE-101 element in the UGT1A9 promoter was mutated, TCDD inducibility decreased significantly (Fig. 2D). However, mutagenesis of the corresponding ARE-143 site did not affect TCDD inducibility, as shown for UGT1A8 and UGT1A10. We conclude that there is coordination between TCDD (AhR)- and tBHQ (Nrf2)-mediated induction of the UGT1A8 and UGT1A10 genes, which is absent for the TCDD inducibility of UGT1A9.

Conversion of the UGT1A10 ARE-149 Binding Element to the Respective UGT1A9 ARE-143 Binding Element—The XRE-101 and ARE-149 sites of UGT1A10 are identical to those of UGT1A8 and UGT1A10 differ from those found in UGT1A8 and UGT1A10 with respect to a single base pair, which is located directly in the binding element, and within three base pairs, which are located within the adjoining sequence. When Gly at position 143 of UGT1A10 was mutated into Thr corresponding to the UGT1A9 ARE motif (Table 2), both TCDD and tBHQ inducibility were abolished comparable with complete mutation of ARE-149 in luciferase assays (Fig. 3A). The UGT1A10 construct “ARE-149 mut-like 1A9 all” contains all four base pair
Coordinate Regulation of UGT1A10 by Nrf2 and AhR

Changes corresponding to the UGT1A9 sequence including those of the surrounding sequence. In luciferase assay, this construct caused no further reduction in inducibility in comparison to the single mutagenesis of Gly → Thr at position -143. Interestingly, conversion of Thr at position -137 in ARE-143 of UGT1A9 led to a 1.8-fold inducibility and mutation of the additional 3 bp to a 2.1-fold tBHQ (Nrf2) inducibility, which was not observed for the wild type UGT1A9 promoter (Fig. 3B). In summary, these data suggest that the difference of one base pair in the ARE DNA binding motifs of UGT1A10 and UGT1A9 are associated with the ability of tBHQ (ARE/Nrf2) inducibility or its absence. This analysis identifies the molecular determinant of differential regulation of these two genes.

siRNA-mediated Knockdown of Nrf2 and AhR Impacts TCDD and tBHQ Inducibility of UGT1A10, UGT1A8, and UGT1A9—The analysis was expanded to provide evidence for a direct involvement of AhR and Nrf2 by siRNA knockdown. The efficiency of specific AhR and Nrf2 siRNA was tested by Western blot at different time points. Using 100 nM AhR/Nrf2 siRNA, AhR and Nrf2 protein amounts were strongly reduced after 24 and 48 h of incubation with either TCDD or tBHQ compared with control siRNA (Fig. 4A). In luciferase assays, the AhR knockdown resulted in a complete loss of both TCDD and tBHQ inducibility of UGT1A10 (Fig. 4B). Similarly, Nrf2 knockdown caused a loss of tBHQ (Nrf2) and TCDD (AhR)-mediated induction. Similar to UGT1A10, the single use of either AhR or Nrf2 siRNA led to a simultaneous reduction of TCDD and tBHQ inducibility of UGT1A8 (Fig. 4C). However, Nrf2 siRNA was not able to affect TCDD inducibility of UGT1A9, although AhR siRNA reduced TCDD induction, which is in agreement with the previous induction studies (Fig. 4D). The UGT1A9 construct “ARE-143 like 1A10 "all" containing all four base pair changes corresponding to the ARE of UGT1A10 (and a restoration of tBHQ inducibility, Fig. 3B) showed, as expected, a simultaneous decrease in AhR- as well as Nrf2-mediated induction using only AhR siRNA. In contrast, Nrf2 knockdown resulted in an abolishment of tBHQ inducibility but did not affect the induction by TCDD. Therefore, we conclude that both transcription factors, AhR and Nrf2, are required for either TCDD- and tBHQ-mediated induction. These data suggest that AhR and Nrf2 interact to perform a coordinate regulation of UGT1A8 and UGT1A10. In contrast, TCDD inducibility of UGT1A9 appears not to be dependent on the presence of Nrf2. Even if the ARE of UGT1A9 is mutated to correspond to the ARE of UGT1A10, Nrf2 inducibility depends on AhR, and Nrf2 does not appear to be essential for AhR-mediated induction.

Specificity of TCDD- and tBHQ-mediated Induction of AhR and Nrf2 and Specificity of Used AhR and Nrf2 siRNA—TCDD is a ligand of AhR and is responsible for the separation of AhR from its inhibitor in the cytoplasm. tBHQ is a creator of oxidative stress and treatment results in separation of Nrf2 from its inhibitor Keap1 and migration in the nucleus. It has been reported that AhR-mediated induction of Nrf2 was associated with an XRE-binding element in the nrf2 promoter (28). In this case, TCDD treatment would not only lead to a specific activation of AhR but also to mRNA induction of Nrf2. To test the specificity of TCDD- and tBHQ-mediated AhR and Nrf2 induction, a Western blot was performed for both transcription factors (Fig. 5A). Our data indicate that under the experimental conditions used in this study, TCDD only activated AhR and not Nrf2, and tBHQ mediated only a specific induction of Nrf2. To confirm these data, mRNA levels of AhR and Nrf2 were examined (Fig. 5B). Neither TCDD nor tBHQ induced AhR or Nrf2 on the mRNA level, therefore, there is no evidence for the up-regulation of gene expression of Nrf2 by TCDD in the employed KYSE70 cells. Furthermore, the specificity of siRNA used in this study was examined. Nrf2 siRNA did not affect the amount of AhR protein and use of AhR siRNA led not to a decrease of Nrf2 protein amount (Fig. 5A). These data show conclusively that the coordinated regulation of the UGT1A8 and UGT1A10 genes by Nrf2 and AhR are not the result of cross-reactivity.

Binding of AhR and Nrf2 to UGT1A10 XRE-101 and ARE-149 Binding Sites Confirmed by Gel Shift Assay—To examine a direct interaction of the transcription factors AhR and Nrf2 with the identified UGT1A10 XRE-101 and ARE-149 binding element, EMSA was performed (Fig. 6A). Gel shift bands were observed with both UGT1A10 XRE/ARE motifs and consensus...
XRE/ARE elements. The UGT1A10 XRE-101 band was competed by consensus XRE and UGT1A10 ARE-unlabeled oligonucleotides but not by unlabeled peroxisome proliferator-activated receptor-γ consensus sequence used as a control. Similarly, the band of UGT1A10 ARE-149 was competitively reduced by unlabeled consensus ARE and the UGT1A10 XRE-101 but not by the unlabeled peroxisome proliferator-activated receptor-γ oligonucleotide. The specificity of transcription factor binding to the XRE and ARE elements of UGT1A10 was established by using AhR- and Nrf2-specific antibodies (Fig. 6B). Interestingly, a supershift for the XRE-101 site was formed with both AhR and Nrf2 antibody. In a similar way, both antibodies were able to produce a supershift with the ARE-binding element. An unspecific IgG was not able to produce a supershifted band. A similar experiment was also performed for the XRE-136 binding motif, which also demonstrated a simultaneous binding of both Nrf2 and AhR (Fig. 6C).

In summary, these data show that AhR and Nrf2 simultaneously bind to UGT1A10 XRE-101 element as well as to the ARE-149 element. This suggests an interaction between these two transcription factors in the regulation of UGT1A10 and agrees with the coordinate regulation observed in this study.

**DISCUSSION**

Oxidative stress represents an imbalance between oxidant production and antioxidant mechanisms within a tissue. The effects of oxidative stress depend on the extent and duration of changes in redox balance and the ability of the cell to regain a physiological balance. Severe oxidative stress contributes to aging and age-related diseases such as cardiovascular disease, chronic inflammation, neurodegenerative diseases, and cancer. Levels of oxidized proteins, phospholipids, and DNA increase in these processes. In addition, DNA-damaging
electrophiles are often carcinogens (4, 29–31). Induction of a family of oxidative stress-related genes that protect against damage by electrophiles and ROS is a key element in the maintenance of cellular redox homeostasis and in reducing oxidative damage (32, 33). These genes encode various antioxidant and detoxifying enzymes and are regulated through the cis-acting ARE in their 5′-flanking promoter regions. Nrf2 is the central transcription factor that regulates both constitutive and inducible ARE-related gene expression (32). Nrf2 knock-out mice have a deficiency in this protective genetic program and have a higher susceptibility to oxidative damage (34, 35). Recent studies have shown a connection between the Nrf2 and the AhR pathways but the nature of this potential cross-talk has not been described specifically to date. In this study, we showed and characterized the interaction between Nrf2 and AhR in the regulation of human glucuronidation, which is a key mechanism of indirect antioxidant action and plays an important role for the maintenance of the redox balance in eukaryotic cells. These data show that glucuronidation is not only regulated by its xenobiotic substrates but also by subsequent oxidative metabolism and ensuing oxidative stress, which indicates a closely regulated mechanism of cellular defense.

In humans, the UGT1A10 gene is expressed throughout the extrahepatic gastrointestinal tract that establishes first contact to a host of compounds. In the presented in vitro studies, it is conclusively shown to be inducible by TCDD (AhR) and tBHQ (Nrf2). It was surprising that both TCDD and tBHQ induction were abolished when either XRE-101 or XRE-136 DNA-binding elements in the promoter of UGT1A10 were eliminated, which was also observed when the ARE-149 binding motif was absent (Fig. 2A). Although a dependence of TCDD induction on an intact ARE element has been reported for the human UGT1A6 gene (26), the reduction of tBHQ inducibility as a result of XRE mutagenesis described here was unexpected and adds new insight into the tightly coordinated regulation involving both pathways.

The expression pattern of UGT1A8 is very similar to that of UGT1A10 (36). The first 500 bp of the promoter of UGT1A8 are 89.6% similar to those of UGT1A10, and the ARE-149 and XRE-101 DNA-binding sites are identical in both promoters. Therefore, it is not surprising that UGT1A8 is also up-regulated by TCDD and tBHQ and that mutagenesis of either XRE-101 or ARE-149 in the UGT1A8 5′-upstream region leads to the simultaneous loss of TCDD and tBHQ inducibility as in the case of UGT1A10 (Fig. 2C).

However, in contrast to UGT1A8, the first 500 bp of the UGT1A9 and UGT1A10 promoters share only 82.6% similarity, and the ARE-143 of UGT1A9 differs from the ARE of UGT1A10 in one base pair (Table 2). UGT1A9 is the only UGT within the very homologous UGT1A7–10 cluster, which is expressed in the liver. It differs in its absence of significant tBHQ inducibility, although a motif in its promoter is similar to the ARE consensus sequence (GCNNGTCA). Our promoter analyses indicate that the difference of one base in the ARE of UGT1A9 and UGT1A10 accounts for tBHQ inducibility, which was confirmed by chimeric mutagenesis experiments (Fig. 3).

These data also provide evidence for a molecular mechanism determining differential inducibility of different UGT1A genes. This may also contribute to the observation of tissue-specific UGT1A expression despite high degrees of sequence homology.

The siRNA experiments in our study indicate that the presence of AhR is essential for Nrf2-mediated induction of UGT1A10 and UGT1A8 and vice versa (Fig. 4, B and C). If one transcription factor is eliminated, induction by both TCDD and tBHQ are abolished simultaneously. In contrast, TCDD inducibility of UGT1A9 was not affected by the knockdown of Nrf2 (Fig. 4D). However, Nrf2 inducibility of the UGT1A9 construct, with ARE mutated to correspond to the UGT1A10 ARE, appeared to be dependent on the presence of AhR (Fig. 4D), whereas AhR inducibility does not depend on the presence of Nrf2. To exclude that the interdependency of Nrf2 and AhR in the regulation of UGT1A10 and UGT1A8 occurred as a result of transcription factor up-regulation, their levels were examined (Fig. 5, A and B) and failed to show an unspecific effect. However, Miao et al. (28) showed an up-regulation of Nrf2 mRNA by TCDD in mice. This discrepant result may be a consequence of the fact that Miao et al. (28) used a mouse nrf2 promoter and different mouse cell lines. Species specific and/or a tissue specific regulation of Nrf2 may be an explanation. The results of this study suggest that a direct or indirect interaction between AhR and Nrf2 is the likely mechanism. This was further substantiated by EMSA and supershift experiments with Nrf2- and AhR-specific antibodies. In the promoter of UGT1A10, both Nrf2 and AhR are capable of binding to XRE as well as ARE DNA-binding motifs.

A previously reported study analyzing the murine glutathione S-transferase A1 (Gsta1) promoter hypothesized that a coordinate regulation of AhR and Nrf2 by physical interaction or an adapter protein may exist (37). Our data demonstrate the presence of both proteins in supershift assays with ARE as well as XRE DNA-binding motifs. It can be hypothesized that they bind directly; however, the sequence specific binding of both transcription factors is extremely well conserved throughout mammalian organisms, and it is questionable whether this specificity is lost regarding human UGT1A regulation. In this study, the Nrf2/AhR interaction is conclusively demonstrated in the presence of the UGT1A10 XRE-101, XRE-136, and ARE-149 DNA motifs by electrophoretic mobility shifts and specific antibodies, and this is further suggested by induction and

FIGURE 6. Nuclear extracts for all EMSA experiments were prepared from KYSE70 cells treated with either 5 nm TCDD (for all experiments using XRE-probes) or 100 μM tBHQ (for all experiments using ARE-probes) for 48 h. A, electrophoretic mobility shift assay for AhR (XRE-101) and Nrf2 (ARE-149) binding elements. Shifts are shown in comparison to published consensus sequences and can be competed by excess unlabeled consensus sequence but not by peroxisome proliferator-activated receptor-γ (PPARγ; control) sequence. The ARE-binding site can be competed with excess of unlabeled XRE sequence and the other way around. B, supershift of labeled UGT1A10 oligonucleotides with the addition of specific antibody for AhR and Nrf2. A supershift of UGT1A10 XRE- and ARE-binding site with AhR as well as Nrf2 antibody (Ab) suggests binding of both transcription factors to both sites. C, electrophoretic mobility shift assay for UGT1A10 XRE-136 binding element. A supershifted band is observed with both AhR and Nrf2 antibody, and XRE-136 site can be competed with an excess of unlabeled ARE-149 sequence.
knockdown studies. Whether additional proteins are involved in this binding will require additional studies.

The findings of this study are of importance for the understanding of the metabolic defense against xenobiotics and oxidative stress. Nrf2 is critical for cytoprotection by initiating the activation of detoxification genes, which is relevant for the pathogenesis of toxicity reactions and inflammatory diseases. This activation is likely to determine the susceptibility to oxidative and chemical-induced injury. In addition, xenobiotics activate the AhR pathway, which regulates metabolism by cytochrome P450 and, as shown previously as well as in this study, also of detoxification enzymes such as the UGT (38–40). For cellular homeostasis, a critical balance between oxidative metabolism and detoxification, i.e., by glucuronidation, is critical. According to the data provided here, in cell culture experiments, the regulation of UGTs expressed in the mucosa of the digestive tract, which is continuously exposed to xenobiotics, can proceed directly by xenobiotics using XRE-binding motifs, and indirectly, when ROS have been generated by oxidative metabolism using ARE-binding motifs. This way, both AhR- and Nrf2-mediated signal transduction influences detoxification by glucuronidation. Their coordinated influence on this critical mechanism of cellular defense is therefore biologically plausible and conclusively demonstrated in this study. Coordinated regulation was shown for UGT1A8 and UGT1A10, which in humans are expressed in esophagus, small intestine, and colon.

In summary, this study identifies and characterizes the coordinated regulation of UGT1A8 and UGT1A10 by the oxidative stress sensor Nrf2 and the xenobiotics-induced AhR, including the identification of the responsible DNA-binding elements. In contrast to the regulation of human UGT1A9, UGT1A8 and UGT1A10 require the simultaneous presence of Nrf2 and AhR. These data provide an important link between xenobiotic and drug metabolism, oxidative stress, cellular defense and, potentially, between toxicity and disease disposition. The elucidation of the biological mechanisms of cellular defense is of value for the future development of specific therapeutic strategies of modulating oxidative stress and cellular injury for the prevention of inflammatory and neoplastic diseases.

REFERENCES

1. Dhaeshinamoorthy, S., Long, D. J., 2nd, and Jaiswal, A. K. (2000) Curr. Top Cell Regul. 36, 201–216
2. Jaiswal, A. K. (2000) Free Radic Biol. Med. 29, 254–262
3. Ward, J. F. (1994) Int. J. Radiat Biol. 66, 427–432
4. Goetz, M. E., and Luch, A. (2008) Cancer Lett 266, 73–83
5. Strassburg, C. P., Manns, M. P., and Tukey, R. H. (1997) Cancer Res. 57, 2979–2985
6. Auten, R. L., O’Reilly, M. A., Oury, T. D., Nozik-Grayck, E., and Whorton, M. H. (2006) Am. J. Physiol. Lung Cell Mol. Physiol. 290, L32–40
7. Ho, Y. S., Xiong, Y., Ma, W., Spector, A., and Ho, D. S. (2004) J. Biol. Chem. 279, 32804–32812
8. Koo, H. C., Davis, J. M., Li, Y., Hatzis, D., Osimhos, H., Pollack, S., Strayer, M. S., Ballard, P. L., and Kazzaz, J. A. (2005) Am. J. Physiol. Lung Cell Mol. Physiol. 288, L718–726
9. Holtzclaw, W. D., Dinkova-Kostova, A. T., and Talalay, P. (2004) Adv. Enzyme Regul. 44, 335–367
10. Strassburg, C. P., Kalthoff, S., and Ehmer, U. (2008) Crit. Rev. Clin. Lab. Sci. 45, 485–530
11. Gregory, P. A., Lewinsky, R. H., Gardner-Stephen, D. A., and Mackenzie, P. I. (2004) Mol. Pharmacol. 65, 953–963
12. Breimer, I. H. (1990) Mol. Carcinog. 3, 188–197
13. Venkatraman, M., Konga, D., Peramaiyan, R., Ganapathy, E., and Dhana-pal, S. (2008) Biol. Pharm. Bull. 31, 1639–1645
14. Strassburg, C. P., Manns, M. P., and Tukey, R. H. (1998) J. Biol. Chem. 273, 8719–8726
15. Moijariabi, B., and Mackenzie, P. I. (1998) Biochem. Biophys. Res. Com-mun. 247, 704–709
16. Iyer, L. V., Ho, M. N., Shinn, W. M., Bradford, W. W., Tanga, M. J., Nath, S. S., and Green, C. E. (2003) Cancer Sci. 73, 36–43
17. Dellinger, R. W., Chen, G., Blevins-Primeau, A. S., Krzeminski, J., Amin, S., and Lazarus, P. (2007) Careineogenesis 28, 2412–2418
18. Nishiyama, T., Fujishima, M., Masuda, Y., Iizawa, T., Ohnuma, T., Ogura, K., and Hiratsuka, A. (2008) Arch. Biochem. Biophys. 478, 75–80
19. Yu, X., and Kessler, T. (2005) Mutat Res. 591, 93–102
20. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., and Yamamoto, M. (1999) Genes Dev. 13, 76–86
21. Venugopala, R., and Jaiswal, A. K. (1998) Oncogene 17, 3145–3156
22. Marini, M. G., Chan, K., Casula, L., Kan, Y. W., Cao, A., and Moi, P. (1997) J. Biol. Chem. 272, 16490–16497
23. Nguyen, T., Sherratt, P. J., and Pickett, C. B. (2003) Annu. Rev. Pharmacol. Toxicol. 43, 233–269
24. Petrisl, J. R., and Perdew, G. H. (2002) Chem. Biol. Interact 141, 25–40
25. Whitlock, J. P., Jr. (1990) Annu. Rev. Pharmacol. Toxicol. 30, 251–277
26. Münzel, P. A., Schmohl, S., Buckler, F., Jaehrling, F. T., Köhle, C., and Bock, K. W. (2003) Biochem Pharmacol 66, 841–847
27. Yeager, R. L., Reisman, S. A., Aleksunes, L. M., and Klaassen, C. D. (2009) Toxicol. Sci. 111, 238–246
28. Miao, W., Hu, L., Scrivens, P. J., and Batist, G. (2005) J. Biol. Chem. 280, 20340–20348
29. Videan, E. N., Heward, C. B., Chowdhury, K., Pummerer, J., Su, Y., and Cutler, R. G. (2009) Comp. Med. 59, 287–296
30. Segal, B. H., Davidson, B. A., Hutson, A. D., Russo, T. A., Holm, B. A., Mullan, B., Habitruether, M., Holland, S. M., and Knight, P. R., 3rd. (2007) Am. J. Physiol. Lung Cell Mol. Physiol. 292, L760–768
31. Reddy, V. P., Zhu, X., Perry, G., and Smith, M. A. (2009) J. Alzheimers Dis. 16, 763–774
32. Nguyen, T., Sherratt, P. J., Huang, H. C., Yang, C. S., and Pickett, C. B. (2003) J. Biol. Chem. 278, 4536–4541
33. Itoh, K., Kondo, K. I., and Yamamoto, M. (2004) Free Radic Biol. Med. 36, 1208–1213
34. Ramos-Gomez, M., Kwak, M. K., Dolan, P. M., Itoh, K., Yamamoto, M., Talalay, P., and Kessler, T. W. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 3410–3415
35. Chen, K., and Kan, Y. W. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 12731–12736
36. Cheng, Z., Radominska-Pandya, A., and Tephly, T. R. (1998) Arch. Bioch. Biophys. 356, 301–305
37. Vasiliiou, V., Puga, A., Chang, C. Y., Tabor, M. W., and Nebert, D. W. (1995) Biochem Pharmacol 50, 2057–2068
38. Quattrocchi, L. C., and Tukey, R. H. (1993) Mol. Pharmacol. 43, 504–508
39. Lankisch, T. O., Pillman, T. C., Erichsen, T. J., Ehmer, U., Kalthoff, S., Freiber, N., Munzel, P. A., Manns, M. P., and Strassburg, C. P. (2008) Arch. Toxicol. 82, 573–582
40. Erichsen, T. J., Ehmer, U., Kalthoff, S., Lankisch, T. O., Müller, T. M., Munzel, P. A., Manns, M. P., and Strassburg, C. P. (2008) Toxicol Appl. Pharmacol. 230, 252–260