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Contribution of the Ah receptor to the phenolic antioxidant-mediated expression of human and rat UDP-glucuronosyltransferase UGT1A6 in Caco-2 and rat hepatoma 5L cells

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Abstract

UDP-glucuronosyltransferases (UGTs) represent major phase II enzymes of drug metabolism which are regulated in a tissue-specific manner by endogenous and environmental factors. Among the latter, aryl hydrocarbon receptor (AhR) agonists such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and phenolic antioxidants such as *tert*-butylhydroquinone (tBHQ) are known to induce the expression of human UGT1A6 in Caco-2 cells. While binding of the TCDD-activated AhR to one xenobiotic response element (XRE) in the 5′-flanking regulatory region of UGT1A6 was characterised previously, the mechanism responsible for tBHQ induction is unknown. Therefore, it was investigated whether antioxidant response elements (AREs) are involved in tBHQ induction of UGT1A6. Transfectants of 3 kb of its regulatory region and its deletion mutants were treated with tBHQ. These studies suggested a region with approximately 2-fold induction, including an ARE-like motif, 15 bp downstream of the previously characterised XRE. Transfectants of the point-mutated ARE-like motif showed marginally reduced response to tBHQ, but surprisingly, loss of response to TCDD, suggesting interference of flanking proteins with the AhR/Arnt complex. Coordinate responses of UGT activity after treatment with TCDD or tBHQ were also observed in rat hepatoma 5L cells, mutants without the AhR and with recomplemented AhR. The results suggest a contribution of the AhR pathway and of proteins binding to the XRE flanking region to the induction of human UGT1A6 by both AhR agonists and phenolic antioxidants.

Keywords: UDP-glucuronosyltransferase; Human and rat UGT1A6; *tert*-Butylhydroquinone; TCDD; Caco-2 cells; Rat hepatoma 5L cells; Ah receptor

1. Introduction

Mammalian microsomal UGTs are major phase II enzymes of drug metabolism. They convert hundreds of lipophilic endobiotics and xenobiotics (drugs, dietary plant constituents, carcinogens, etc.) into hydrophilic and excretable conjugates [1]. Based on evolutionary divergence human UGTs have been grouped into two families consisting of nine family 1 members encoded on chromosome 2q37 and greater than six family 2 members encoded on chromosome 4q13 [2]. UGTs are regulated in a tissue-specific manner by endogenous and environmental factors [3,4]. Among the latter, AhR agonists such as TCDD and phenolic antioxidants such as tBHQ have been found to induce rat and human UGT1A6 [5–8]. Induction by TCDD has been shown to be mediated by binding of the activated AhR/Arnt complex to one consensus xenobiotic response element (XRE; GCGTG) in the 5′-regulatory region of rat [9] and human UGT1A6 [10].

tBHQ has been found to be a prototype inducer of a novel, non-receptor signalling pathway triggered by oxidative/electrophile stress. This signalling pathway selectively induces phase II enzymes and therefore has been termed

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Abbreviations: UGT, UDP-glucuronosyltransferase; AhR, aryl hydrocarbon receptor; Arnt, Ah receptor nuclear translocator; ARE, antioxidant response element; XRE, xenobiotic response element; tBHQ, *tert*-butylhydroquinone; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; GST, glutathione *S*-transferase; NQO1, NADPH quinone oxidoreductase-1; Nrf2, NR-E2-related factor.

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monofunctional induction, in contrast to AhR-mediated bifunctional induction in which both phase I and II enzymes are transcriptionally activated [11]. In the case of rodent GSTs and rat and human NAD(P)H quinone oxidoreductase-1 (NQO1), AREs have been characterised in their 5′-regulatory regions [12–15]. Studies with NR-E2-related factor (Nrf2) knockout mice suggested that this basic leucine zipper protein is a key factor of the protein complex binding to AREs [16–18]. Evidence has been obtained that Nrf2 is normally present in the cytosol in a latent complex with the chaperone keap-1 [19]. Oxidative/electrophile stress has been proposed to activate protein kinases, i.e. MAP kinases, disrupting the Nrf2/keap-1 complex after which Nrf2 is translocated to the nucleus where it binds to AREs [20]. Moreover, studies with Nrf2 knockout mice suggested that Nrf2 may be involved in the regulation of mouse UGT1A6 induction [21–23].

UGT1A6 has been shown to efficiently conjugate benzo[*a*]pyrene diphenols, thereby preventing toxic quinone/quinol redox cycles [24,25]. Hence, UGTs such as UGT1A6 may play important roles in preventing electrophilic stress. Dietary monofunctional inducers of phase II enzymes, including phenolic antioxidants, currently receive a lot of interest in the efforts of cancer chemoprotection [23]. However, mechanisms responsible for UGT1A6 induction by phenolic antioxidants are still unclear. To investigate whether a putative ARE-like motif is functional, it was mutated by site-directed mutagenesis. The results suggest a contribution of the AhR pathway and of proteins binding to the XRE flanking region to the induction of UGT1A6 by both TCDD and phenolic antioxidants.

2. Material and methods

2.1. Cell culture, treatment and preparation of cell homogenates

Human Caco-2 cell, clone TC7, was maintained as described [6]. Briefly, cells were cultured on 100 mm Falcon dishes in DMEM supplemented with 20% foetal calf serum and non-essential amino acids. Cells were treated with 80 μM tBHQ, 50 μM β-naphthoflavone (BNF) or 10 nM TCDD when they reached confluency and were harvested after 42 hr, the optimal time for measuring UGT1A6 mRNA. Solvent controls contained 0.1% DMSO. Before harvest, cells were washed with PBS and stored at −80°. UGT activity was measured in cells cultured for 72 hr, with daily changes of medium and daily addition of inducers.

Rat hepatoma 5L cells, AhR-deficient 5L-derived BP8 cells and AhR-complemented BP8 cells [26] were cultured as described [27]. They were treated with 1 nM TCDD or 20 μM tBHQ for 48 hr. Untreated controls contained 0.1% DMSO.

Preparation of cell homogenates: Cell monolayers were washed with cold 0.9% sodium chloride and harvested by scraping with 0.25 M sucrose containing 0.01 M Tris–HCl, pH 7.4 (buffered sucrose). Thereafter, the cells were centrifuged for 5 min at 1000 *g*, resuspended with buffered sucrose and hand-homogenised with a Dounce homogeniser. The homogenate was centrifuged for 10 min at 10,000 *g*.

2.2. Duplex RT–PCR

Total RNA was isolated using Trizol (Invitrogen) and duplex RT–PCR was performed as described [6]. In short, the primers used for amplification were the following: UGT1A6 (EMBL J04093): forward primer (nt 1) 5′-ATGGCCTGCCTCCTTCGCTCATT-3′ (nt 23), reverse primer (nt 906) 5′-CCATTGATCCCAAAGAGAAAACC-3′ (nt 928). β-Actin was used for semiquantification of UGT1A6 expression. Human β-actin (EMBL X00351): forward primer (nt 940) 5′-CTGGCGGCACCACCATGTACCCT-3′ (nt 962); reverse primer (nt 1223) 5′-GGAGGGGCCGACTCGTCATACT-3′ (nt 1145). PCR was carried out in a Perkin-Elmer 2400 thermal cycler (PCR comprised 32 cycles for UGT1A6). The hot start modification was used to avoid non-specific priming by heating the master mix containing cDNA samples to 94° for 1 min; Taq DNA polymerase was heated to 90° separately and added to the samples. Annealing was performed at 65° for 40 s and extension at 72° for 2 min. Thereafter, a 10-min elongation step at 72° was included.

2.3. Generation of UGT1A6 reporter constructs and of deletions mutants

Reporter gene constructs were generated using PCR amplification of a −38 to 2996 bp fragment of the 5′-flanking region of the UGT1A6 gene [10] and mismatched primers introducing *Hin*dIII and *Bgl*II sites followed by directed ligation into the base luciferase vector (pGL3-Basic, Promega). Shorter deletion constructs −2330; −1806; −1550; −1449; −765; −434 and −225 bp were obtained using the endonucleases *Stu*I, *Sac*I, *Apa*I, *Kpn*I, *Mlu*NI, *Pvu*II and *Bpu*10I, respectively, together with *Sma*I, followed by T4 DNA polymerase-directed blunt end generation and subsequent religation.

2.4. Generation of point mutants of ARE3′ and of XRE by site-directed mutagenesis

ARE3′: A double point mutation in the ARE3′ motif of the UGT1A6 reporter plasmid (Fig. 2B) was generated according to the instructions of the manufacturer using Chameleon™ Double stranded, Site-Directed Mutagenesis Kit (Stratagene). For PCR the following oligonucleotides were used [28] (GenBank AF297093): sense (nt 108,108) 5′-CGTGCCAGCCAGGTGTGCA**A**GACTA**C**CTCTGGGCAAGTCTG-3′ (nt 108,148); antisense 5′-CAGACTTGCCCAGAG**G**TAGTC**T**TGCACACCTGGCTGGCACG-3′. The construct was sequenced in both directions to ensure

the mutation had been introduced at the correct site during PCR.

XRE: A single point mutation in the XRE motif of the UGT1A6 reporter plasmid (Fig. 2B) was generated as described for ARE3′. For PCR the following oligonucleotides were used: sense (nt 108,089) 5′-GGGTGGGA-ACAGGAACTCGC**T**TGCCAGCCAGGTGTGCATG-3′ (nt 108,128); antisense 5′-CATGCACACCTGGCTGG-CA**A**GCGAGTTCCTGTTCCCACCC-3′. The construct was sequenced in both directions to ensure that the mutations (bold) had been introduced at the correct sites during PCR.

2.5. Transfection of UGT1A6 reporter plasmids and expression assay

Two transgenes and respective mutants were studied: Transgene I (p1A6/2996 ARE3′ Luc) consisted of the longest reporter gene construct (Fig. 2A). Transgene II (p1A6/396 ARE3′), a short, thymidine kinase-driven p1A6 ARE3′ wt tk-Luc plasmid containing ARE3′ (regions −1810 to −1441 bp), was generated as follows: HSV-tk promoter derived from pRLTK (Promega) was inserted between *Bgl*II and *Hin*dIII sites of pGL3-Basic and was subsequently cut by *Eco*RI and *Bgl*II resulting in tk-Luc. Short 369 bp fragments containing wild-type and mutated ARE3′ from deletion construct −1806 were obtained by cleavage with Asp718 and inserted in tk-Luc upstream of the tk promoter.

Electroporation was used for transfection. Briefly, Caco-2 cells were trypsinized and diluted by culture medium. Caco-2 cells (10^7 cells/mL) were suspended in cytomix (60 mM KCl, 75 mM $CaCl_2$, 5 mM PO_4^{3-}, 1 mM EGTA, 2.5 mM $MgCl_2$, 2 mM ATP, 5 mM glutathione and 12 mM HEPES buffer, pH 7.6). UGT1A6 transgenes (16 pmol/mL) were added and electroporation was carried out at 4° with 659 V/cm and exponential pulse half-time of 35 ms. For controlling the efficiency of transfection (ca. 10%) and effects of inducers on cell growth, Renilla luciferase gene expression was monitored using pRLTK (94 fmol/mL; Promega). Cells were suspended immediately in prewarmed culture medium and plated at approximately 70% confluency. The transfected cells (4 dishes/group) were then treated with the inducers and further cultivated for 42 hr. Luciferase gene expression, normalised by Renilla luciferase activity was analysed using the Dual Luciferase Kit (Promega).

2.6. Electrophoretic mobility shift analysis

Preparation of nuclear extracts was performed as described [10]. Nuclear protein extracts from cells treated for 42 hr with tBHQ or from solvent controls were incubated for 30 min at 20° in 20 mM HEPES buffer (pH 7.6) containing 120 mM KCl, 5 mM $MgCl_2$, 10%(v/v) glycerol, 1 mM dithiothreitol and ^{32}P-endlabelled, double-stranded ARE-like oligonucleotides (ARE1′ to ARE3′; listed in Fig. 2B) of human UGT1A6 (100,000 cpm = 20 fmol) or human NQO1-ARE [15].

2.7. UGT assay (1-naphthol or 4-methylumbelliferone as substrate)

UGT activity was measured with 0.5 mM 1-naphthol or 4-methylumbelliferone as substrates using cell homogenates of rat hepatoma 5L or human Caco-2 cells, activated by the detergent Brij 58, as previously described [6].

2.8. Statistics

For statistical analysis the Student's *t*-test was used.

3. Results and discussion

3.1. Induction of human UGT1A6 expression by treatment with tBHQ, TCDD and BNF

Treatment of Caco-2 cells with tBHQ or BNF clearly increased UGT1A6 expression (Fig. 1A and B). The effect of BNF was stronger possibly due to the fact that BNF is both an AhR agonist and a monofunctional inducer, after efficient metabolism by BNF-induced CYP1A1 to electrophilic metabolites. The results were supported by UGT activity data using 4-methylumbelliferone or 1-naphthol (not shown) as substrates which are, however, overlapping substrates of several UGT isoforms (Fig. 1C). BNF was not studied further since it is a mixed-type inducer and it interfered with the luciferase assay. Previously, it was shown that induction of UGT1A6 by the selective inducer TCDD (10 nM) was similar to that of tBHQ [6].

3.2. Functional analysis of the UGT1A6 5′-flanking region with tBHQ as inducer

To investigate underlying mechanisms, the 3 kb regulatory region of UGT1A6 and deletion mutants were cloned into the pGL3-luciferase reporter plasmid and transfectants were treated with tBHQ (Fig. 2A). Moderate induction (ca. 2-fold) was observed with transfectants between −2996 and −1550 bp. Searching for putative consensus ARE motifs (TGACnnnGC) revealed several ARE-like motifs (Fig. 2A). ARE1′ and ARE2′ were not investigated since induction was low. Induction by tBHQ was nevertheless significant ($P < 0.05$). Functionality of these AREs cannot be excluded. In fact, the remaining tBHQ response observed after site-directed mutagenesis of ARE3′ and the XRE may be explained by ARE1′ and ARE2′. No other consensus AREs were detected in the 10 kb 5′-flank of UGT1A6 [28]. Although ARE3′ had only two base pairs as spacer between the TGAC and GC parts of the ARE motif, it appeared to be interesting since it was close

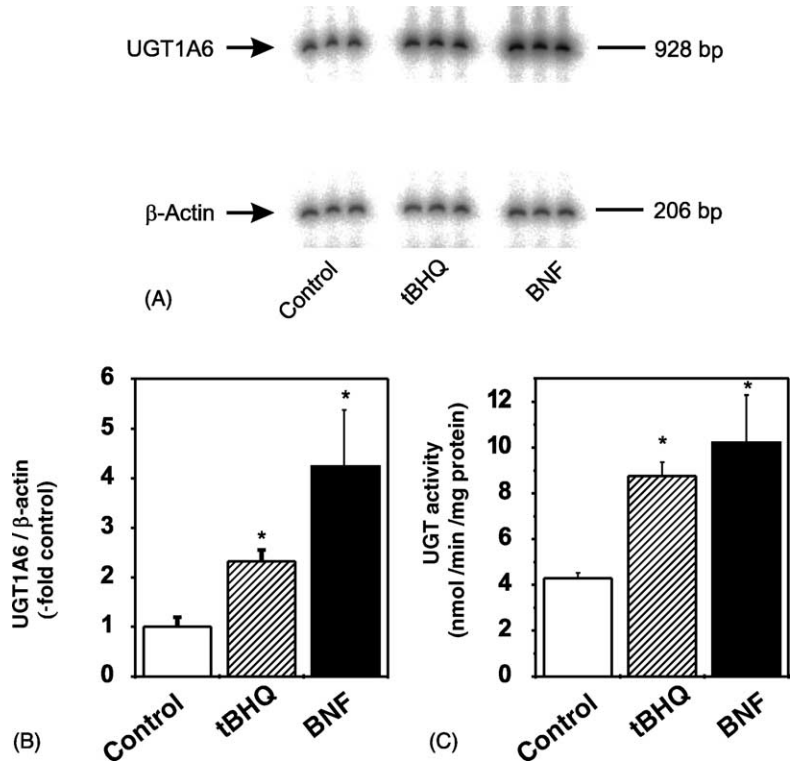
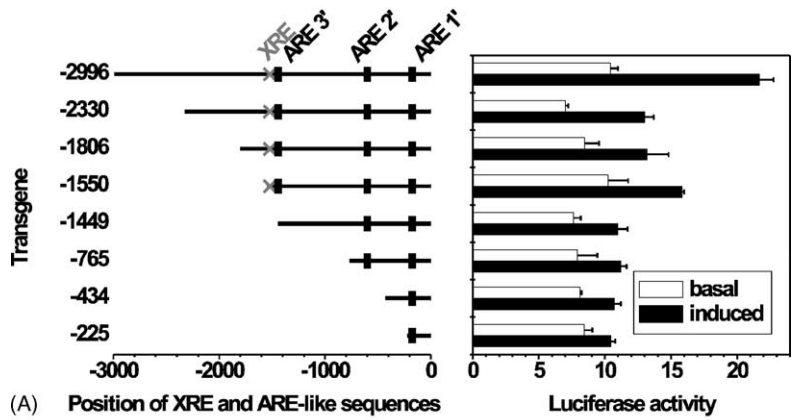


Fig. 1. Induction of UGT1A6 expression (A) and (B) and of UGT activity (C) by treatment with tBHQ or BNF. In studies of UGT expression Caco-2 cells were treated for 48 hr and in UGT activity studies for 72 hr with 80 μM tBHQ or 50 μM BNF. Duplex RT–PCR was carried out using β-actin as internal control. Means ± SD of three experiments are shown. $^{*}P < 0.05$.



(B)

hUGT1A6	
ARE 1'	cagaagctcaggtgaagc**TGAC**acg**GC**catagt
ARE 2'	ttacccacaacttctgtc**TGAC**ttg**GC**aaaaat
XRE-ARE 3'	aactc**GCGTG**ccagccaggtgtgca**TGAC**t-a**GC**tctggg
XRE-ARE 3' mut	aactc**GCGTG**ccagccaggtgtgca**AGAC**t-a**CC**tctggg
XRE mut-ARE 3'	aactc**GCTTG**ccagccaggtgtgca**TGAC**t-a**GC**tctggg
rGSTA2 [13]	aaatggcattgctaatgg**TGAC**aaa**GC**aacttt
hNQO1 [15]	aaatcgcagtcacag**TGAC**tca**GC**agaatc
ARE consensus [13]	**TGAC**nnn**GC**

Fig. 2. Functional analysis of 3 kb of the UGT1A6 5′-flanking sequence. (A) Basal and tBHQ-induced expression of UGT1A6-Luc transgenes. Transfected Caco-2 cells were treated with tBHQ for 42 hr. Means ± SD of three transfection experiments (4 dishes/group) are listed. (B) Putative ARE-like sequences in the 5′-flanking region of human UGT1A6 are shown as well as the studied point-mutated ARE3′ and XRE.

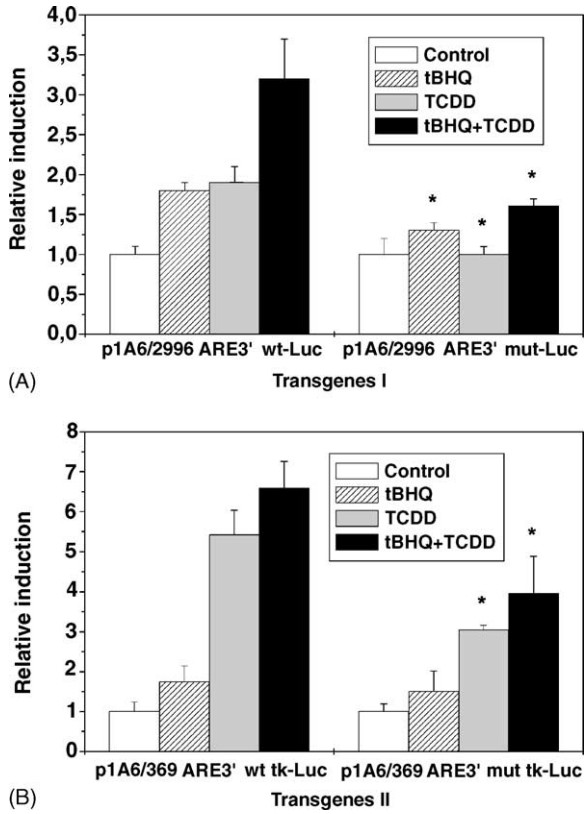


Fig. 3. (A) Inducer response of wild-type and ARE3′-mutated p1A6/2996-Luc transfectants (transgenes I). Transfected Caco-2 cells were treated with 80 μM tBHQ, 10 nM TCDD or both for 42 hr. Means ± SD of four transfection experiments (4 dishes/group) are listed. (B) Inducer response of wild-type and ARE3′-mutated p1A6/369-tk-Luc transfectants (transgenes II). Treatment was carried out as in panel A. Means ± SD of two independent experiments (4 dishes/group) are listed. $^{*}P < 0.05$, inducer responses of ARE3′-mutated vs. wild-type transfectants.

(15 bp) to the previously characterised XRE. Moreover, addition of one C to this spacer would lead to an AP1 motif, similar to that found in NQO1 (Fig. 2B). The mutant of ARE3′, listed in Fig. 2B, was generated by site-directed mutagenesis and transfectants were treated with tBHQ, TCDD or both. While the effect of the mutant on tBHQ induction was marginal, effects of TCDD and TCDD plus tBHQ were clearly reduced using transgene I (Fig. 3A). These findings were confirmed in transfection experiments with transgene II, a thymidine kinase-driven 369 bp fragment of wild-type and mutated ARE3′ (plus XRE) but lacking possibly interfering regions, including ARE1′ and ARE2′ (Fig. 3B).

As expected, site-directed mutagenesis of XRE clearly reduced the inducibility of the UGT1A6 reporter by TCDD and by TCDD plus tBHQ (Fig. 4). Reduction of the TCDD response in ARE3′-mutated plasmids was unexpected since the wild-type XRE was present. These findings suggest that proteins bound to ARE3′ interfered with the function of the AhR pathway.

Gel mobility shift analysis demonstrated that the band observed with ARE3′ moved faster than bands observed with the consensus NQO1-ARE or with ARE1′ and ARE2′

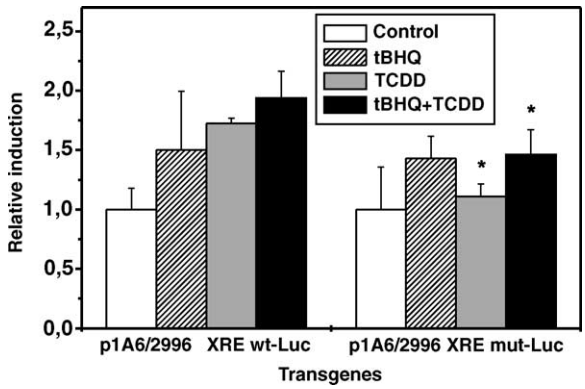


Fig. 4. Inducer response of wild-type and XRE-mutated UGT1A6-Luc transfectants. Transfected Caco-2 cells were treated with 80 μM tBHQ, 10 nM TCDD or both for 42 hr. Means ± SD of three experiments (4 dishes/group) are listed. $^{*}P < 0.05$, inducer response of XRE-mutated vs. wild-type transfectants.

(Fig. 5, positions a and b, respectively). Binding of labelled oligonucleotides was abolished by adding unlabelled oligonucleotides in 40-fold excess (not shown). Protein binding appeared to be constitutive; only the band obtained with the ARE motif of NQO1 was slightly enhanced by pretreatment of the cells with tBHQ. Interestingly, using an oligonucleotide with the spacer TCA instead of TA of ARE3′ led to a band with mobility similar to that of the NQO1-ARE (not shown).

The findings suggest a role of the AhR pathway in the antioxidant response of human UGT1A6. Proteins binding 3′-downstream of the AhR/Arnt complex appear to influence its functionality in the sense of a composite element. Since protein binding to ARE3′ is not affected by tBHQ treatment, it may be activated by protein phosphokinases which have been shown to be important in transducing the antioxidant signal *via* oxidative/electrophile stress to cellular effectors [20,29]. Further work is needed to establish

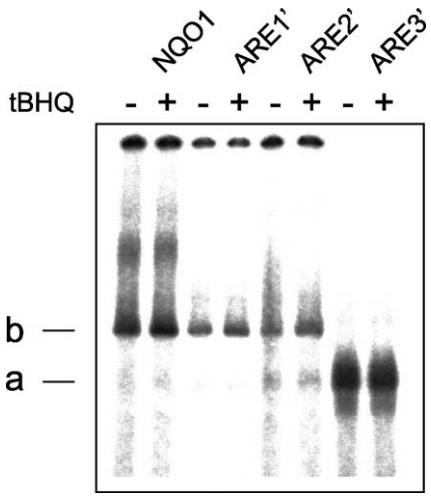


Fig. 5. Electrophoretic mobility shift analysis of putative ARE-like motifs of human UGT1A6 compared to ARE of NQO1. Experiments with nuclear protein extracts from untreated (−) and 42 hr tBHQ-treated Caco-2 cells (+) are compared. A representative blot of three experiments is shown.

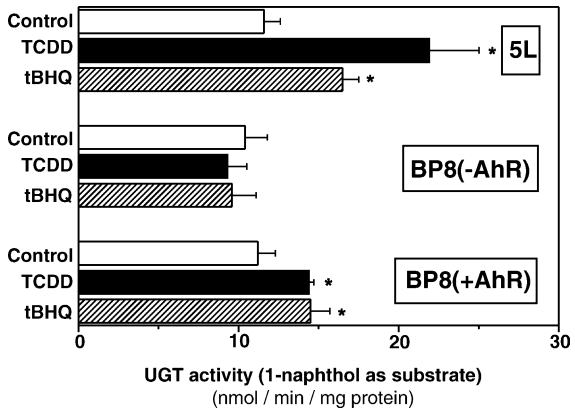


Fig. 6. Coordinated tBHQ and TCDD responses of UGT activity in wild-type rat hepatoma 5L cells, AhR-deficient (BP8 − AhR) and AhR-complemented rat hepatoma cells (BP8 + AhR). Cells were treated with 20 μM tBHQ or 1 nM TCDD for 48 hr. UGT activity (1-naphthol as substrate) was determined in cell homogenates. Data represent means ± SD of four different experiments. $^{*}P < 0.05$.

the role of kinases in the tBHQ-mediated activation of the AhR pathway. In this context, it is important to note that tBHQ effects are not abolished by site-directed mutagenesis of ARE3′ and of the XRE. Hence, we cannot exclude that ARE1′ and ARE2′ contribute to the induction of human UGT1A6 by tBHQ. No other AREs were found in the 5′-enhancer region of human UGT1A6.

3.3. Contribution of the AhR to tBHQ-induced UGT activity in rat hepatoma 5L cells

Cooperativity between the responses of AhR agonists and antioxidants was also observed in rat hepatoma 5L cells (preliminary results are described in ref. [27]). While in wild-type 5L cells TCDD and tBHQ moderately induced UGT activity (1-naphthol as substrate), both responses were lost in AhR-deficient 5L-derived BP8 cells (Fig. 6). Moreover, both effects were partly regained in AhR-complemented BP8 cells. Similar results were obtained using 4-methylcholanthrene as substrate (not shown) or with 6-hydroxychrysene [27]. UGT activity using these substrates is known to be mediated by both rat UGT1A6 and UGT1A7. To differentiate between these isoforms, mRNA was measured by selective duplex RT–PCR. It was found that TCDD and tBHQ induced expression of UGT1A6 and UGT1A7 isoforms to similar extents, 1.5- and 3-fold, respectively.[1] A contribution of the AhR pathway to rat UGT1A6 induction was also demonstrated by the mixed-type inducer oltipraz in primary hepatocyte cultures and in HepG2 cells [30].

In conclusion, our results in the human Caco-2 cell model suggest a contribution of the AhR pathway and of proteins binding to the XRE flanking region to the induction of human UGT1A6 by both TCDD and tBHQ. Supportive data of a contribution of the AhR to tBHQ induction of UGT1A6 were observed in rat hepatoma 5L cells.

[1] F.T. Raschko, P.A. Münzel and K.W. Bock, unpublished.

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