REGULATION OF CYP1A1 GENE EXPRESSION BY THE ANTIOXIDANT TERT-BUTYLHYDROQUINONE

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ABSTRACT:

CYP1A1, a major phase I enzyme, plays an important role in the metabolism of polycyclic aromatic hydrocarbons and in the chemical activation of xenobiotics to carcinogenic derivatives. The phe- nolic antioxidant tert-butylhydroquinone (tBHQ), often used as a food preservative, is generally considered to act only as a monofucntional inducer of phase II enzymes, thereby exerting chemoprotection. However, we recently observed that tBHQ elevated the activity of an aryl hydrocarbon receptor (AhR) response element (DRE)-driven luciferase reporter in human colon carcinoma cells (Caco-2). Therefore, we studied the effects of tBHQ on the activity of a DRE-driven reporter, CYP1A1 mRNA expression, and CYP1A enzyme activity in Caco-2 cells and human HepG2 hepatoma cells.

We found tBHQ caused induction of reporter activity and CYP1A1 expression and activity in Caco-2 and HepG2 cells. Moreover, tBHQ combined with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) increased reporter activity and mRNA expression in Caco-2 cells in an additive manner. By contrast, tBHQ decreased TCDD-mediated induction of reporter activity and CYP1A1 mRNA expression in HepG2 cells. Resveratrol, an AhR antagonist, repressed the induction of CYP1A1 by tBHQ. Cotransfection of HepG2 cells with a dominant negative AhR nuclear translocator mutant abolished the tBHQ-induced CYP1A1 reporter activity. These findings indicate that CYP1A1 may be induced by the antioxidant tBHQ via an AhR-dependent mechanism.

The CYP1A subfamily catalyzes the metabolic activation of polycyclic aromatic hydrocarbons (PAHs), which generates genotoxic metabolites that bind to DNA, thus mediating PAH-induced carcinogenesis. Polyhalogenated PAHs, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), are also responsible for a variety of toxic responses, such as immune suppression, birth defects (cleft palate), and endocrine disruption (Poland and Knutson, 1982). Classically, transcription of several members of the CYP1A subfamily, such as CYP1A1/2 (Denison et al., 1989; Nebert and Jones, 1989; Quattrochi and Tukey, 1989) and CYP1B1 (Sutter et al., 1992), is induced by PAHs such as TCDD via activation of the aryl hydrocarbon receptor (AhR) (Poland et al., 1974), also called the dioxin receptor. TCDD binds to the cytosolic AhR ligand binding subunit, which undergoes an activation process involving several steps: translocation into the nucleus, release of 90 kDa heat-shock proteins, and heterodimerization with a partner protein, the AhR nuclear translocator (Arnt) (Denison and Nagy, 2003). This heterodimer interacts with a 5'-GGCGT-3' DNA sequence, the core binding motif of the xenobiotic response element or dioxin response element (DRE) (Fujisawa-Sehara et al., 1987; Kubota et al., 1991), located and present in multiple copies in the upstream region of the CYP1A1 gene promoter. The human CYP1A1, the mouse Cyp1a2, and the mouse Cyp1b1 genes harbor 10, 12, and 11 DRE motifs (Zhang et al., 2003) in their respective upstream regions; however, not all the DREs are functional. Recently, we have identified four functional DREs within a 1.4-kilobase (kb) 5'-regulatory region of the human CYP1A1 gene enhancer (Kress et al., 1998).

AhR-mediated signaling pathways provide a first line of defense against potentially toxic environmental contaminants. However, induction of metabolic processes by the AhR can also produce highly carcinogenic metabolites, creating a link between AhR activation and chemical carcinogenesis. AhR-mediated induction can transcriptionally activate genes encoding enzymes of phase I and phase II and is therefore termed bifunctional, whereas antioxidants selectively induce phase II enzymes and are therefore termed monofunctional (Prochaska and Talalay, 1988). The antioxidant tert-butylhydroquinone (tBHQ), the major metabolite of 3-tert-butyl-hydroxyanisol in dogs, rats, and humans (Nakamura et al., 2003), has been found to be a prototype inducer of a novel, nonreceptor signaling pathway triggered by oxidative/electrophile stress. In the rodent glutathione S-transferases (GSTs) and rat and human NADPH quinone oxidoreductase-1 (NQO1), antioxidant response elements (AREs) have been characterized in their 5'-regulatory regions mediating their transcriptional activation (Rushmore et al., 1990; Jaiswal, 1994). Oxidative/electrophile stress has also been proposed to activate via

ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbon; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon receptor nuclear translocator; DRE, dioxin response element; tBHQ, tert-butylhydroquinone; GST, glutathione S-transferase; NQO1, NAPDH quinone oxidoreductase-1; ARE, antioxidant response element; Nr2f, nuclear factor erythroid 2-related factor 2; dNArnt, dominant negative aryl hydrocarbon receptor nuclear translocator; NAC, N-acetyl-L-cysteine; PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide; EROD, ethoxyresorufin O-deethylase.
protein kinase (i.e., mitogen-activated protein kinases) transcription factors such as nuclear factor erythroid 2-related factor 2 (Nrf2), which binds to the ARE (Tony-Kong et al., 2001), thereby inducing the respective phase II enzymes.

Recent observations in Caco-2 cells (clone TC7) have shown induction of CYP1A activity (Münzel et al., 1999) and CYP1A1 reporter gene activity either by tBHQ alone or combined with TCDD. CYP1A1 transcriptional induction by oltipraz in Caco-2 cells was also recently described by Le Ferrec et al. (2002) as AhR- and calcium-dependent.

To study the mechanism of CYP1A1 induction by tBHQ, DRE-containing reporter vectors were transiently transfected into cells of two selected lines representing hepatocytes in liver (HepG2) and enterocytes in colon (Caco-2). These cell types should mimic the in vivo situation where environmental and food contaminants accumulate in the gastrointestinal tract, which expresses CYP1A metabolic capacity (de Waziers et al., 1990), and are further passed to the liver.

In addition to DRE-containing reporter constructs, induction of CYP1A1 by tBHQ was investigated in mRNA and enzyme activity levels. The possible AhR/Arnt-DRE mechanism was examined using the AhR antagonist resveratrol, a dominant negative Arnt (dnArnt) mutant, and mutated DRE reporter constructs. Our findings provide strong evidence for an involvement of the AhR/Arnt-DRE mechanism in induction of CYP1A1 by the antioxidant tBHQ.

Materials and Methods

Materials. tBHQ was purchased from Fluka (Buchs, Switzerland). TCDD was provided by Okomet (Bayreuth, Germany). Resorufin, resveratrol, N-acetyl-l-cysteine (NAC), dicumarol, and Tris-(hydroxymethyl) aminomethane hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). TRZol reagent, nonessential amino acids, and trypsin/EDTA solution were purchased from Invitrogen (Carlsbad, CA). Oligonucleotide primers were purchased from MWG (Ebersberg, Germany). Dulbecco’s modified Eagle’s medium and penicillin/streptomycin were purchased from Biochrom (Berlin, Germany). Agarose psqGold Universal, DNA 1-kb ladder, reverse transcriptase psqGold AMV, and GenePorter were purchased frompeqLab (Erlangen, Germany). The polymerase chain reaction (PCR) kit LightCycler Fast Start DNA Master SYBR Green I was purchased from Roche Diagnostics (Mannheim, Germany). All the other chemicals were purchased from Merck (Darmstadt, Germany).

Cell Culture and Treatments. Human Caco-2 cells, clone TC7, were maintained as described previously (Münzel et al., 1999). Briefly, cells were cultured on 100-mm dishes in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal calf serum and nonessential amino acids. The human hepatoblastoma cell line HepG2 (American Type Culture Collection, Manassas, VA) was cultured in α-minimum essential medium (Life Technologies, Eggenfelden, Germany) supplemented with 10% fetal calf serum, and cells were maintained at 37°C in a humidified atmosphere of 5% CO₂/air. All the reagents for cell treatment were dissolved in dimethyl sulfoxide (DMSO). Cells were treated with the indicated substances and harvested after 24 or 48 h for RNA isolation or for measuring CYP1A activity as ethoxyresorufin-O-deethylase (EROD) activity, respectively. Transfected cells were treated after the pulse in prewarmed culture medium and plated at approximately 70% confluence. The transfected cells (4 dishes/group) were then treated with the inducers and further cultivated for 42 h. Alternatively, chemical transfection with GenePorter (peqLab) was used for HepG2 cells according to the manufacturer’s instructions. One microgram of luciferase reporter plasmid, 23.8 ng of pTKRL reference vector, and 5 μl of transfection reagent were used for 1 well of a 24-well plate. Firefly luciferase gene expression, normalized for Renilla luciferase activity, was analyzed using the Dual Luciferase Kit (Promega).

Real-Time PCR. Cells were washed twice with ice-cold 0.9% NaCl solution and scraped off the plate. RNA was isolated using TRizol (Invitrogen) and quantified spectrophotometrically at 260 nm and 280 nm. Five hundred nanograms of total RNA was heated at 95°C for 5 min and cooled on ice. RNA-derived synthesis of CYP1A1 cDNA was carried out with the following reaction conditions: 0.5 μg of RNA, 5 μM MgCl₂, 1 mM dNTP, 0.5 U Rnase (Promega), 12.5 U psqGold avian myeloblastosis virus reverse transcriptase (peqLab), and 0.25 μg of oligo(dT)₁₈ and 0.25 μg of random hexamers as primers in a final volume of 20 μl of GeneAmp PCR buffer II (Applied Biosystems, Foster City, CA). The samples were incubated at 42°C for 60 min, and reverse transcriptase was inactivated by heating at 95°C for 5 min. PCR was carried out in the LightCycler instrument using the FastStart DNA Master SYBR Green I kit (Roche). PCR reactions contained 2.4 mM MgCl₂ and 0.5 μM concentration of both forward and reverse primers: human CYP1A1 (GenBank: NM_000499) forward primer, 5'-GTCTTCTTCTCCTCCGGTGATC-3' and human CYP1A1 reverse primer, 5'-TACCTGGTGCTCTCCTGGGATTTTC-3' and reverse primer 5'-CCGAGGAGGAGGCTTGAG-3'. The resulting 1.2-kb PCR fragment was cloned into vector pCR 2.1-TOPO (Invitrogen), and orientation and identity were confirmed by sequencing. The resulting vector and the reporter vector pT81Luc were digested with Xhol and HindIII, and after purification and ligation of the fragments they resulted in plasmid pT81Luc/ChYPCYP1A1-5'-wt. pT81 Luc was a gift of Dr. F. Gaunitz (Leipzig, Germany). The pT81/3DRE reporter construct was generated using the motif of DRE3 from the CYP enhancer region (Kress et al., 1998) fused in triplicate tandem array into pT81Luc.

Determination of Enzyme Activities. Cells were washed twice and harvested by scraping off in cold NaCl solution (0.9%). The cells were then centrifuged for 10 min at 500g and at 4°C. The supernatant was removed; 0.25 M sucrose-0.01 M Tris-HCl, pH 7.4 (buffered sucrose), was added; and the cells were homogenized by sonication for 2 × 5 s. CYP1A1 activity was measured as EROD activity according to Lubet et al. (1985). Protein concentration was measured...
Results

**tBHQ-Mediated Induction of CYP1A1 Reporter Constructs in HepG2 Cells.** HepG2 cells were transiently transfected with one of the following reporter constructs: 1) pT81/hCYP1A1-5’-wt, containing nine DREs, including the four functional DREs, 3, 4, 5, and 6 (also termed C, D, E, and F); 2) a mutant construct derived thereof by site-directed mutagenesis harboring knockouts of the four functional DREs, 3, 4, 5, and 6 (termed pT81/CDEF); and 3) an artificial construct containing three DRE motifs (pT81/3xDRE). Cells were incubated in the presence of varying concentrations of tBHQ (20–100 μM) for 48 h. The activities of the pT81/hCYP1A1-5’-wt and pT81/3xDRE reporters were inducible by tBHQ: in a concentration-dependent manner in the case of pT81/3xDRE (up to 3-fold at 80 μM tBHQ), whereas in the case of pT81/hCYP1A1-5’ no concentration dependence was observed (Fig. 1). For all the further experiments, a concentration of 80 μM tBHQ was chosen, at which significant induction of luciferase reporters and CYP1A1 mRNA was seen (Münzel et al., 1999), and no cytotoxic effects were observed in HepG2 and Caco-2 cells. Reporter activity was almost completely abolished in the CDEF mutant (Fig. 1), indicating that DRE motifs in the enhancer region of CYP1A1 contribute to transcriptional activation of the gene by the antioxidant tBHQ. Comparable effects on reporter activity were obtained in Caco-2 cells when using the same reporter constructs and tBHQ concentrations (data not shown).

**Effects of tBHQ, TCDD, and Resveratrol on CYP1A1 in HepG2 Cells.** Treatment of HepG2 cells with tBHQ (80 μM) or TCDD (10 nM) increased the activity of a transiently transfected pT81Luc/3xDRE reporter 7-fold and 25-fold, respectively, compared with DMSO-treated controls (Fig. 2A). However, combined treatment with TCDD and tBHQ reduced the TCDD-mediated induction by 30% (Fig. 2A). After 24 h of treatment with tBHQ, TCDD, or both, total RNA was isolated and CYP1A1 mRNA expression was determined using real-time PCR. tBHQ induced CYP1A1 mRNA expression 22-fold compared with the level seen in DMSO-treated cells (Fig. 2B). TCDD elevated CYP1A1 mRNA about 800-fold. Only a small decline in the TCDD-elicted response could be detected by combined treatment with tBHQ. Finally, CYP1A1 activity was measured as EROD activity after treatment of HepG2 cells with tBHQ, TCDD, or a combination of both compounds for 48 h (Fig. 2C).

**Effect of dnArnt on Induction of pT81/3xDRE by tBHQ and TCDD in HepG2 Cells.** HepG2 cells were transiently cotransfected with pT81/hCYP1A1-5’-wt and pcDNA3.1 or pCDEF. Figure 3 illustrates the effect of dnArnt on luciferase activity of pT81Luc/3xDRE after treatment of cells with tBHQ, TCDD, or both compounds with mock (pcDNA3.1) cotransfected controls. In the presence of dnArnt, the activity of the pT81/3xDRE reporter was almost totally blocked, regardless of whether cells were treated with tBHQ, TCDD, or a combination of both.

**Effects of tBHQ, TCDD, and Resveratrol on pT81/3xDRE Reporter Activity, CYP1A1 mRNA Expression, and EROD Activity in Caco-2 Cells.** To compare the results obtained in the liver-derived...
HepG2 cell line with a second cell system in which xenobiotic metabolizing enzymes play an important role in chemoprotection and activation of carcinogens, the gastrointestinal colon-derived cell line Caco-2 was chosen. Caco-2 cells, transiently transfected with pT81/3xDRE and treated with tBHQ, exhibited a 6-fold elevated reporter activity when compared with the DMSO control (Fig. 4A). Treatment of cells with TCDD resulted in a lower increase in reporter activity (7-fold) (Fig. 4A) than the one that was obtained in HepG2 cells (22-fold) (Fig. 2A). In contrast to what was seen in HepG2 cells, combined treatment with tBHQ and TCDD resulted in a 22-fold induction of reporter activity, suggesting a more than additive effect (Fig. 4A).

Treatment of Caco-2 cells with tBHQ significantly increased CYP1A1 mRNA levels, an effect that could be completely abolished by simultaneous treatment with resveratrol (Fig. 4B). The same inhibitory effect of resveratrol was observed when it was administered to Caco-2 cells along with TCDD. CYP1A1 activity, measured as EROD activity, was also reduced to control values when tBHQ or TCDD was administered together with resveratrol (Fig. 4C). Simultaneous tBHQ and TCDD treatment of Caco-2 cells led to a reduction in EROD activity by almost 50% when compared with values obtained by incubation of cells with TCDD alone. Resveratrol was again strongly inhibitory in these treatment regimens (Fig. 4C).

Comparability between HepG2 and Caco-2 Cells. Caco-2 cells express a nonfunctional form of NQO1 (NQO1*2 allele), which plays a role in detoxification of quinones (Bonnemsen et al., 2001). Therefore, we incubated HepG2 cells with the NQO1 inhibitor dicumarol (Ermster, 1967) to mimic the situation in Caco-2 cells. No difference excluding a crucial role of NQO1 possibly elicited by tBHQ was observed between untreated and dicumarol-treated HepG2 cells (data not shown). The glutathione precursor NAC is known as an antioxidant scavenging reactive oxygen species. NAC was used in a set of experiments to test whether tBHQ-produced reactive oxygen species are important in the HepG2 system. No effect of NAC on CYP1A1 inducibility by tBHQ was observed (data not shown).

Discussion

In our present study, we have investigated the effect of the antioxidant tBHQ on expression and activity of CYP1A1 in two human cell systems, HepG2 and Caco-2 cells, representing models for liver and gastrointestinal cells. This study was initiated because we had previously observed that phenolic antioxidants can induce human UDP-glucuronosyltransferase 1A6 by a mechanism in which DRE binding of the AhR plays some role (Münzel et al., 2003). Moreover, two recent studies report on an inducing effect of tBHQ on CYP1A1 in HepG2 cells (Sugatani et al., 2004) and Hepa1c1c7 cells (Gharavi and El-Kadi, 2005).

Phenolic antioxidants act through activation of Nrf2, and interaction between Nrf2 and AhR signal transduction has been reported to occur during induction of NQO1 (Ma et al., 2004). Therefore, we were interested to examine whether and, if so, how antioxidants such as tBHQ could regulate CYP1A1 expression. The results of our study now clearly show that higher concentrations of tBHQ can induce CYP1A1 both in HepG2 and Caco-2 cells. CYP1A promoters/enhancers do not contain any sequences that would match the ARE consensus sequence, suggesting that their transcriptional activation by antioxidants involves an ARE-independent pathway. To examine the possible involvement of DREs in CYP1A1 induction by tBHQ, transfection experiments with three different DRE-containing reporter constructs were performed: pT81/hCYP1A1-5′ containing 1.2 kb of the upstream region with four functional DRE core motifs (Kress et al., 1998), pT81/3xDRE with three functional DREs in tandem-like array, and a pT81/CDEF reporter in which the four functional DREs were mutated. Treatment of HepG2 cells with tBHQ (10–100 μM) caused induction of the DRE-driven reporters, whereas no such effect was seen when using the construct pT81/CDEF containing the four mutated DREs (Fig. 1). These results strongly suggested that induction of the reporters containing the correct DRE sequences by tBHQ is DRE-mediated. The missing concentration dependence with the pT81/hCYP1A1-5′ construct may indicate that additional factors are involved in regulation of the wild-type promoter.

To further elucidate the possible involvement of the AhR in tBHQ-mediated induction of CYP1A1, experiments were conducted in which HepG2 and Caco-2 cells were simultaneously exposed to tBHQ and TCDD. Combined TCDD/tBHQ treatment of HepG2 cells led to a decrease in all the parameters measured (DRE reporter activity, CYP1A1 mRNA expression, and CYP1A1 activity) when compared with values obtained with TCDD alone. tBHQ has been shown to bind and activate the AhR (Gharavi and El-Kadi, 2005). However, it may behave as a partial agonist that, when present at a very high concen-


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