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ACCELERATED COMMUNICATION

Nuclear Uptake of the Ah (Dioxin) Receptor in Response to Omeprazole: Transcriptional Activation of the Human *CYP1A1* Gene

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SUMMARY

In the presence of halogenated and polycyclic aromatic hydrocarbons, the *CYP1A1* gene is regulated through induction after ligand binding to the cytosolic Ah receptor (AhR). Ligand-dependent AhR activation leads to nuclear translocation and binding of the receptor to dioxin-responsive element (DRE) sequences, an event that initiates transcriptional activation of the *CYP1A1* gene. We recently established a human hepatoma cell line stably integrated with the human *CYP1A1* promoter and 5′-flanking enhancer sequences fused to the firefly luciferase gene. This cell line, 101L, was used to determine whether the induction of CYP1A1 by omeprazole, a gastric proton pump inhibitor, is AhR mediated. Treatment of 101L cells with either 50 μM omeprazole or 5 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin for 12–72 hr resulted in maximal activity at 24 hr for both inducers. A dose-response curve for omeprazole induction at 24 hr was determined and the EC_{50} for omeprazole induction of the human *CYP1A1* gene was estimated to be 100 μM. The induction of the *CYP1A1* gene by omeprazole corresponds to increases in CYP1A1 mRNA. To examine whether omeprazole-initiated transcriptional activation of the *CYP1A1* gene correlates with nuclear accumulation of the AhR, binding of nuclear proteins to the DRE was examined. When gel mobility shift assays were performed using nuclear extracts isolated from 101L cells treated with omeprazole or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, specific binding of the AhR to the DRE was observed. These studies demonstrate that omeprazole initiates AhR activation and that induction of the human *CYP1A1* gene by omeprazole is AhR dependent.

Polycyclic aromatic hydrocarbons and halogenated aromatic hydrocarbons exert their effects through binding to an intracellular protein, the AhR. The prototypic ligand for the AhR is TCDD, a compound that displays profound toxicity in humans, including teratogenicity, immunotoxicity, porphyria, chloracne, and body weight loss (1). TCDD is also a potent inducer of the CYP1[2] family, which consists of CYP1A1 and CYP1A2 (2). Binding of ligand to the AhR results in the release of the heat shock protein 90 and activation of the receptor to a DNA-binding form (3, 4), followed by translocation of the ligand-receptor complex to the nucleus (5–7). Once in the nucleus, the AhR associates with specific *cis*-acting enhancer sequences that promote the activation of gene transcription. With regard to the *CYP1A1* gene, multiple AhR-specific enhancer sequences, referred to as DREs, have been identified upstream of the *CYP1A1* promoter and have been shown to be required for ligand-dependent gene activation (8–13). Together, these studies have shown clearly that the AhR is a ligand-dependent transcription factor. Therefore, transcriptional activation of the *CYP1A1* gene as a result of exposure to potential inducers is linked to the actions of the AhR.

Omeprazole, a substituted benzimidazole, suppresses gastric acid secretion by inhibiting the H^+/K^+-ATPase in gastric parietal cells. The reduction in gastric acidity is a useful therapy for individuals suffering from gastroesophageal reflux disease (14). The clinical effectiveness of omeprazole in preventing acid-related disorders has led to many investigations directed at examining the physiological and biochemical properties of this compound. With regard to drug metabolism, the secondary effects of omeprazole have been reported to both inhibit and induce phase I metabolism (15). For example, one of the actions in humans results in prolongation of the half-lives of drugs such as aminopyrene, diazepam, and phenytoin (16, 17). Along with the observation that omeprazole interferes *in vitro* with

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[2] The nomenclature used for the designation of the cytochromes P450 and their genes is as outlined (42).

ABBREVIATIONS: AhR, dioxin (Ah) receptor; DRE, dioxin-responsive element; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

mixed-function monooxygenase reactions, it is speculated that one of the *in vivo* actions of omeprazole leads to the inhibition of oxidative drug metabolism (18). However, in contrast to its inhibitory actions, when human primary hepatocytes in culture were exposed to omeprazole, activities catalyzed by CYP1A1 and CYP1A2, as well as CYP1A1 and CYP1A2 mRNA, were significantly induced (19). In addition, it has recently been demonstrated that the administration of omeprazole to humans leads to induction of CYP1A mRNAs in the alimentary tract (20).

The induction of CYP1A1 by traditional planar molecules such as 3-methylcholanthrene and TCDD occurs exclusively through interaction of the compounds with the AhR (21). Omeprazole is a compound that does not conform to the structural features associated with AhR ligands. Interestingly, the induction of human CYP1A1 and CYP1A2 by omeprazole was reported not to occur through linkage with the AhR (22), which would indicate that the inducing actions are most likely the result of a nontranscriptional mechanism such as mRNA stabilization. To examine in greater detail the cellular and molecular events associated with induction of the human *CYP1A1* gene by omeprazole, a stable cell line was used that responds to CYP1A1 inducers by an increase in luciferase activity mediated through the DREs (23). Using sensitive techniques to detect the nuclear accumulation of the AhR as well as transcriptional activation of the DRE-mediated luciferase activity, results are presented to demonstrate that the actions of omeprazole on the induction of the *CYP1A1* gene are dependent upon the AhR.

Experimental Procedures

Chemicals and biochemicals. TCDD was obtained from Chemsyn Science Laboratories (Lenexa, KS). Omeprazole was obtained from Dr. Michael Franklin, University of Utah. Luciferin was obtained from Analytical Luminescence Laboratory (San Diego, CA) and G418 from GIBCO BRL (Gaithersburg, MD). The remaining chemicals were of the highest quality available. Precautions for safe handling of TCDD in the laboratory were followed, based on guidelines from Dow Chemical (CRI B-600-134-79).

Cell culture and Northern blot analysis. The 101L cells are stable cells derived from the human hepatoma cell line HepG2, into which the human *CYP1A1* promoter and 5′ flanking sequences, fused to the firefly luciferase gene, were stably integrated. A detailed description of this cell line is found in Ref. 23. Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (Irvine Scientific, Irvine, CA) and 0.4 mg/ml G418. All inducers were dissolved in dimethylsulfoxide, and this solvent was added to control cells at 0.1%. Total RNA was isolated (24) and used for Northern blot analysis (25). The DNA probes used to screen the Northern blots were a 5′ *Pst*I fragment from the human CYP1A1 cDNA (26) and a *Bam*HI-*Sal*I fragment from the human actin cDNA (27).

Luciferase assay. Luciferase assays were as described previously (23), with the exception that 101L cells were lysed with a lysis buffer consisting of 1% Triton, 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, and 1 mM dithiothreitol (28). Cell lysates were centrifuged at 14,000 rpm and the supernatant was used in luciferase and protein assays. Luciferase assays were performed by mixing 10 μl of the lysates with 300 μl of 0.1 M potassium phosphate buffer, pH 7.8, containing 5 mM ATP and 10 mM $MgCl_2$. Reactions were started by injection of 100 μl of luciferin, dissolved in 0.1 M potassium phosphate buffer, pH 7.8, into the reaction mixture. Light output was measured for 10 sec at 25° with a Monolight 2001 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Luciferase activity is expressed as relative light units/μg of protein. Protein was determined by the method described by Bradford (40).

Isolation of cytosolic and nuclear proteins and gel mobility shift assay. Nuclear extracts were prepared from control, omeprazole-treated, and TCDD-treated 101L cells after 12 hr of treatment, as outlined (11). For gel mobility shift assays, 10 μg of nuclear extracts were used. Gel mobility shift assays were performed exactly as described by Denison *et al.* (11), with the exception that gel electrophoresis was conducted using 1× TBE (10 mM Tris-borate, 10 mM boric acid, 1 mM EDTA) buffer. The double-stranded oligonucleotide probe corresponding to the mouse DRE3 sequence (11) was labeled with T4 polynucleotide kinase and [γ^{32}P]ATP. The sequence of the probe is as follows: 5′-GAGCTCGGAGTTGCGTGAGAAGAGCC-3′. For competition experiments, a double-stranded oligonucleotide corresponding to a sequence in the human α-antitrypsin gene (29) was used as a nonspecific competitor DNA. The sequence of the oligonucleotide is 5′-GATCACCTTGGTTAATATTCACCAG-3′.

For AhR activation experiments, cytosols from 101L cells were prepared in HEDG (25 mM HEPES, pH 7.5, 1 mM EDTA, 10%, v/v, glycerol, 1 mM dithiothreitol) buffer and used for activation of the AhR, as outlined (30). TCDD at 10 nM and omeprazole at 100 μM (final concentration) were added in dimethylsulfoxide. In these experiments, 40 μg of cytosolic protein were used. Analysis of the DNA-binding complex by gel retardation analysis was performed as outlined above.

Results

Omeprazole induces the human *CYP1A1* gene through transcriptional activation. The induction of *CYP1A1* gene expression by halogenated aromatic hydrocarbons, such as TCDD, occurs by transcriptional activation mediated through the activated AhR (8–13). Increases in CYP1A1 mRNA are a reflection of the resulting transcriptional activity. The treatment of human primary hepatocytes with omeprazole has been shown to induce CYP1A1 mRNA (19). To confirm that omeprazole also induces CYP1A1 mRNA in human HepG2 101L cells, the cells were treated with either TCDD or omeprazole for 12 hr and total RNA was isolated. Northern blot analysis shows that omeprazole does induce the expression of human CYP1A1 mRNA in 101L cells (Fig. 1). Because constitutive expression of CYP1A1 was not detected in this experiment, we were unable to estimate the magnitude of the induction response. However, results of densitometric scanning of autoradiograms of the Northern blot probed with cDNAs to human CYP1A1 and to human actin indicated that omeprazole induced the expression of CYP1A1 by approximately 30% of the response observed with TCDD.

To determine whether the induction of CYP1A1 mRNA by omeprazole occurs through gene activation, we examined the transcriptional activity of the *CYP1A1* gene in 101L cells. These cells are unique, in that the human CYP1A1 promoter and flanking DNA containing the AhR-specific DREs were stably integrated with the luciferase reporter gene such that an increase in nuclear liganded AhR results in an increase in detectable luciferase activity. Analysis of luciferase activity driven by the *CYP1A1* DREs is a direct reflection of transcriptional activity, because the half-life of firefly luciferase is only 3 hr in mammalian cells (41). When 101L cells were treated with 50 μM omeprazole and assayed for luciferase activity at 12, 24, 48, and 72 hr, omeprazole induced a steady increase in luciferase activity that reached maximal levels of approximately 20-fold by 24 hr (data not shown). The dose response of the *CYP1A1*-luciferase activity in 101L cells was determined by exposing cells to different concentrations of omeprazole for 24

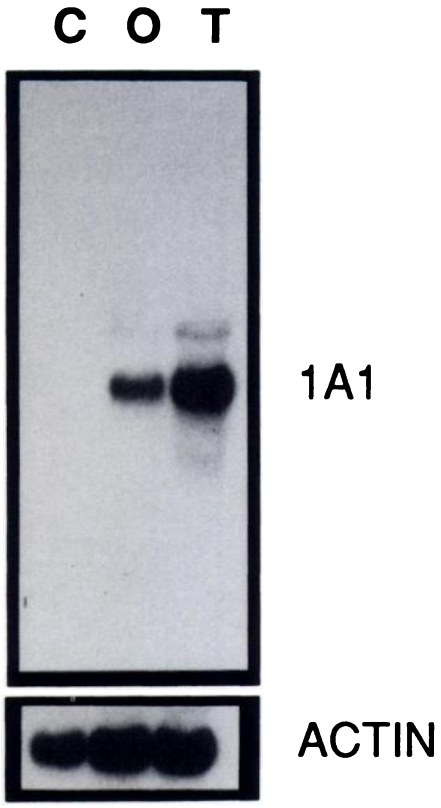

Fig. 1. Human CYP1A1 mRNA expression induced by omeprazole and TCDD. Human HepG2 101L cells were treated with 100 μM omeprazole or 10 nM TCDD for 12 hr. *Lane C*, untreated cells; *lane O*, omeprazole-treated cells; *lane T*, TCDD-treated cells. The cDNA probes were either human CYP1A1 (*upper*) or human β-actin (*lower*).

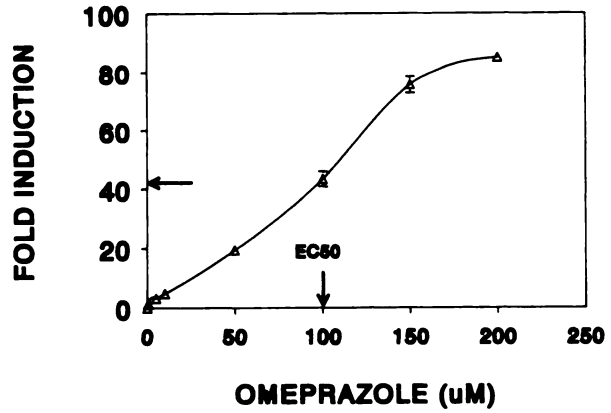

Fig. 2. Dose-dependent induction of luciferase activity by omeprazole. Human HepG2 101L cells were treated for 24 hr with concentrations of omeprazole ranging from 0.5 to 200 μM. Fold induction refers to relative light units/μg of protein for induced cells divided by relative light units/μg of protein for noninduced cells. Luciferase activity in untreated cells ranges from 25 to 50 luciferase units/μg of protein. In the experiment presented, each data point is the mean ± standard error of two independent experiments, performed in triplicate.

hr (Fig. 2). An increase in *CYP1A1*-luciferase activity was shown to be dose dependent over the range of 0.5–200 μM omeprazole, with an EC_{50} estimated to be approximately 100 μM. A dose of 100 μM elicited a 50-fold increase in luciferase activity, a value that is approximately 25% of the maximal response seen with 5 nM TCDD and 90% of the maximal response seen with 10 μM 3-methylcholanthrene (23). The induction of CYP1A1 mRNA and the transcriptional activation of the *CYP1A1* gene by omeprazole are strong indicators that this drug elicits its response through interaction with the AhR.

Omeprazole is a ligand for the AhR. Formation of a nuclear receptor complex is dependent on the interaction of ligand with cytosolic receptor and subsequent activation to a DNA-binding form. To determine whether the increase in luciferase activity produced by the activation of the *CYP1A1* gene is mediated directly through the AhR, we determined whether nuclear extracts from cells treated with omeprazole contained the activated AhR complex. In this experiment, nuclear extracts were isolated from 101L cells treated with omeprazole or TCDD for 12 hr. As shown in Fig. 3, nuclear extract from omeprazole-treated cells contained an inducible protein that binds to the mouse DRE3 sequence. This interaction is specific, because binding to the labeled DRE can be completely inhibited if the incubations are performed in the presence of excess unlabeled DRE sequence but is not inhibited in incubations with DNA that does not contain DRE sequences. These results demonstrate that omeprazole induces the nuclear translocation of the cytosolic AhR.

To examine whether omeprazole serves directly as a substrate for the AhR, omeprazole was used to examine whether the cytosolic AhR can be transformed *in vitro* to a form that binds to the DREs. Transformation *in vitro* is dependent upon ligand binding. Cytosolic extracts from 101L cells were incubated with the labeled DRE in the presence of TCDD and omeprazole at 22° for 2 hr, as outlined (30). The concentrations used in these experiments were similar to those used by Daujat *et al.* (22) to measure binding of omeprazole to the AhR. When the products were assayed by gel shift analysis, results similar to those shown in Fig. 3 were observed (data not shown). Because it has previously been shown that the magnitude of *CYP1A1* induc-

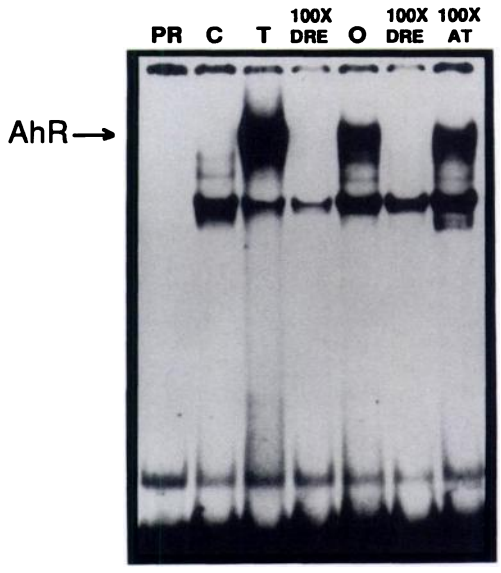

Fig. 3. Gel mobility shift assay. The 5′ end-labeled double-stranded oligonucleotide corresponding to the mouse DRE3 was incubated with nuclear extracts from untreated (*C*), omeprazole-treated (*O*), and TCDD-treated (*T*) HepG2 101L cells. Cells were treated for 12 hr before isolation of nuclear extracts. For competition, extracts were incubated with a 100-fold molar excess of unlabeled DNA, as indicated, before addition of the probe. Competitor DNA was either unlabeled DRE3 or nonspecific DNA [α-antitrypsin (*AT*)]. *Arrow*, position of the TCDD-induced protein-DNA complex. PR, probe only.

tion is correlated with the levels of liganded nuclear receptor (2), the results presented in Fig. 3 demonstrate that the observed increase in *CYP1A1*-mediated luciferase activity in omeprazole-treated cells can be attributed to the interaction of the omeprazole-AhR with *CYP1A1*-DRE sequences. This is further evidence that the actions of omeprazole *in vivo* stimulate accumulation of CYP1A1 mRNA and transcription of the gene through an interaction of the drug with the AhR.

Discussion

Omeprazole, a substituted benzimidazole, does not bind to cytosolic proteins or compete with TCDD for binding to the AhR (22). These *in vitro* assays indicate that omeprazole is not a suitable ligand for the AhR. However, omeprazole does induce human CYP1A1 and CYP1A2 mRNA in human hepatocytes (19) and, as we have demonstrated in this report, efficiently induces the accumulation of human CYP1A1 mRNA in HepG2 cells. With concentrations of omeprazole comparable to those used to treat human primary hepatocytes (19), the actions of omeprazole in human HepG2 101L cells lead to the nuclear accumulation of a DNA-binding form of the AhR and transcriptional activation of the *CYP1A1* gene. The cellular and molecular events that lead to activation of the *CYP1A1* gene mimic those that are observed when cells or animals are exposed to TCDD and certainly demonstrate that omeprazole induces the *CYP1A1* gene through an interaction with the AhR.

The EC_{50} of omeprazole for induction of the *CYP1A1* gene was 100 μM. For the most part, the AhR affinity of a ligand is correlated with the ability of that agent to induce the *CYP1A1* gene (31). The EC_{50} of omeprazole for induction of *CYP1A1* is approximately 15–20-fold higher than those of 3-methylcholanthrene and benzo[*a*]pyrene and 10,000-fold higher than that of TCDD (23). AhR activation *in vitro* to a DNA-binding form can be accomplished with similar concentrations of 3-methylcholanthrene, benzo[*a*]pyrene, TCDD, and omeprazole. Other agents such as food-derived heterocylic amines, which are weak inducers of *CYP1A1* in rats (32), are able to activate the AhR to a DNA-binding form (33) at concentrations comparable to the concentrations of omeprazole needed to induce the *CYP1A1* gene in 101L cells or to activate the human AhR to a DNA-binding form. Although omeprazole could not be shown to bind directly to a cytosolic protein or displace TCDD from the AhR (22), the results presented in this report would suggest that omeprazole is a ligand for the AhR.

Omeprazole has been shown to be extensively metabolized in mice, rats, dogs, and humans (14, 15, 34). The major metabolites are formed from reactions involving aliphatic and aromatic hydroxylations, *O*-demethylation, sulfoxide oxidation, and conjugation with glutathione. Little unchanged drug is observed in the urine (14). Because omeprazole was shown not to bind to the AhR (22), it was speculated that a metabolite of omeprazole induced the induction of the *CYP1* genes in hepatocytes in a fashion that was not dependent upon a functional AhR. However, the results presented in this report demonstrate that an *in vivo* detection system such as tissue culture can be used to examine the nuclear accumulation of AhR after omeprazole treatment. Although we cannot rule out conclusively the possibility that a metabolite of omeprazole serves as the ligand for the AhR, this seems unlikely because the drug is able to activate the AhR to a DNA-binding form in cytosolic extracts from HepG2 cells. We can only speculate that the inability to detect omeprazole binding to the AhR using cytosolic preparations from primary hepatocytes (22) indicates that the methods used to detect classical ligand binding to the AhR, such as sucrose density gradient centrifugation and competition with labeled TCDD, are not as sensitive as those techniques used to examine omeprazole-induced nuclear accumulation of the AhR in tissue culture or activation of the AhR to a DNA-binding form.

Experiments conducted in mice after treatment with TCDD demonstrated that the nuclear accumulation of the AhR corresponded to transcriptional activation of both the *Cyp1a-1* and *Cyp1A-2* genes (2). When TCDD was administered at different doses over 12 hr, there was a concordant increase in the nuclear accumulation of the AhR and transcriptional activation of the *Cyp1* genes. Although the *CYP1A2* gene is not expressed in terminally differentiated hepatoma cells such as the human HepG2 cell line (35) or mouse wild-type hepatoma cells, transcriptional activation of the *CYP1A2* gene has been demonstrated in rat primary hepatoma cells (36). These results suggest that, upon nuclear accumulation of the AhR in response to treatment with an appropriate ligand, concordant increases result in transcriptional activation of the *CYP1A1* and *CYP1A2* genes. In studies that require chronic treatment with agents such as omeprazole, it is anticipated that induction of *CYP1A1* and *CYP1A2* would occur in tissues where both genes are expressed. This has recently been demonstrated in humans, where the administration of omeprazole leads to induction of CYP1 mRNAs in the alimentary tract (20). In addition, analysis of caffeine metabolism, which occurs predominately through the actions of CYP1A2 (37, 38), has shown CYP1A2 to be induced in humans after omeprazole treatment (39). Thus, it is proposed that the induction of the *CYP1* gene family in humans by imidazole and imidazole-containing compounds results from the actions of the AhR and transcriptional activation of these genes.

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