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Omeprazole Stimulates the Induction of Human Insulin-Like Growth Factor Binding Protein-1 through Aryl Hydrocarbon Receptor Activation

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Abstract

5-Methoxy-2-[(4-methoxy-3,5-dimethyl-pyridin-2-yl)methylsulfinyl]-3H-benzimidazole (omeprazole), a benzimidazole-derived gastric H⁺/K⁺-ATPase proton pump inhibitor (PPI) extensively prescribed for the treatment of gastroesophageal acid reflux disease, can stimulate the expression of CYP1A1 via activation of the human aryl hydrocarbon receptor (hAhR) in an apparent nonligand-binding manner. Here, we have examined the effect of nonclassical, i.e., nonligand binding, AhR activation by omeprazole upon human insulin-like growth factor binding protein (hIGFBP)-1, a secreted phosphoprotein involved in regulation of insulin-like growth factor-I/II bioavailability and mitogenic activity. Analysis of the proximal promoter of the hIGFBP-1 gene reveals the presence of an aryl hydrocarbon binding/dioxin response element (DRE). Quantitative mRNA analysis revealed hIGFBP-1 expression to be responsive to both ligand (TCDD) and nonligand (omeprazole) modes of hAhR activation in the human hepatocarcinoma HepG2 cell line. Furthermore, mutagenesis of the DRE renders the hIGFBP-1 promoter unresponsive to both compounds in HepG2 cells. Likewise, small interfering RNA-mediated hAhR ablation inhibits TCDD and omeprazole-dependent hIGFBP-1 induction, as determined by quantitative mRNA analysis. Cotreatment with cycloheximide further suggests a direct transcriptional role for hAhR at the hIGFBP-1 promoter. Omeprazole exposure prompted a significant increase in both hIGFBP-1 mRNA and secreted protein from HepG2 cells. In addition, we present in vitro evidence indicating that omeprazole at a concentration comparable with that found circulating in subjects undergoing PPI therapy can stimulate the expression of hIGFBP-1. These data demonstrate that activation of hAhR by pharmaceuticals such as omeprazole can alter IGFBP-1 expression and thus may influence IGFBP-1-dependent physiological processes.

The hAhR, a ligand-activated transcription factor, is primarily classified as a xenobiotic sensor, initiating the metabolic clearance of toxins, via enhanced hepatic expression of phase I/II/III enzymes (Ramadoss et al., 2005). The hAhR adheres to the classical ligand-activated transcription factor paradigm. In the absence of ligand, hAhR is predominantly cytosolic, held in a core complex comprising two molecules of 90-kDa heat shock protein and a single molecule of the cochaperone hepatitis X-associated protein-2 (Chen and Perdew, 1994). Ligand binding promotes a conformational change in the AhR complex exposing a nuclear localization signal and facilitating redistribution to the nucleus. In the nucleus, AhR associates with its dimerization partner, the AhR nuclear translocator, forming a competent transcription factor capable of binding dioxin response elements (DRE) and initiating transcription of AhR-dependent genes.

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AhR activation can be initiated by diverse chemicals, including halogenated hydrocarbons, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) being the prototypical example; polycyclic aromatic hydrocarbons, e.g., benzo[a]pyrene; indoles; and tryptophan derivatives (Denison and Nagy, 2003). Such compounds are characterized as direct ligands for AhR and initiate AhR-dependent transcription. There also exist a class of compounds capable of activating but not binding AhR, e.g., 5-methoxy-2-[(4-methoxy-3,5-dimethyl-pyridin-2-yl)methylsulfinyl]-3H-benzimidazole (omeprazole) (Daujat et al., 1992). Omeprazole, a selective gastric H⁺/K⁺-ATPase inhibitor is prescribed to ameliorate hyperchlorhydria associated with gastroesophageal acid reflux disease (Robinson et al., 1991; Richter et al., 2000). Reports demonstrate that omeprazole can induce human AhR-dependent genes involved in xenobiotic metabolism, e.g., cyp1a1 (Daujat et al., 1992). However, in the absence of AhR binding, the mechanism and nature of hAhR activation has remained elusive.

Establishment of *ahr*^(-/-) mouse models has revealed involvement of AhR in physiological processes not directly linked to metabolism (Schmidt et al., 1996). *ahr*^(-/-) mice, although viable, exhibit multiple organ dysfunction (Schmidt et al., 1996), abnormal vasculature (Harstad et al., 2006), compromised immune system (Rodríguez-Sosa et al., 2005), and reduced life span and limited fertility (Abbott et al., 1999). In line with these phenotypic abnormalities, the repertoire of AhR-regulated genes, the “AhR battery” now incorporates targets not directly involved in detoxification. There is increasing evidence supporting the integration of AhR signaling either directly in a DRE-dependent manner or via “cross-talk” with other signal transduction pathways, e.g., estrogen receptor signaling (Matthews and Gustafsson, 2006), to modulate physiological processes.

TCDD and 1,2,3,4,7,8-hexachlorodibenzo-p-dioxin, both AhR ligands, have been demonstrated to diminish insulin-like growth factor (IGF) signaling (Croutch et al., 2005). Furthermore, human IGF binding protein (hIGFBP)-1 has been reported to be a direct transcriptional target of hAhR after activation with TCDD, thus incorporating hIGFBP-1 into the AhR battery (Adachi et al., 2004; Marchand et al., 2005).

hIGFBP-1 is a member of a family made up of six homologous proteins (IGFBP1-6) that exhibit multiple functions but whose defining feature is high-affinity binding of IGFs-I/II (Baxter, 2000). IGFs-I/II are evolutionary conserved signaling factors with wide-ranging physiological functions. IGFs-I/II represent potent mitogens, stimulating cell proliferation via cell surface receptors. In addition, IGFs have been shown to affect adipocyte and myoblast differentiation (Foulstone et al., 2004), glucose uptake (Clemmons, 2006), and apoptosis (Kooijman, 2006).

IGF activity/bioavailability is modulated (positively and negatively) in a context-specific manner by interaction with IGFBP-1 (Baxter, 2000). Insights from IGFBP-1^(-/-) and transgenic overexpression models illustrate the importance of IGFBP-1 in physiology (Silha and Murphy, 2002). *igfbp-1*^(-/-) animals fail to exhibit any overt phenotype, due to functional rescue and redundancy by other members of the IGFBP family. However, in common with the other IGFBPs, IGFBP-1 overexpression leads to intrauterine and generalized growth retardation through enhanced sequestration and inhibition of circulating IGFs, diminishing their mitogenic potential (Crossey et al., 2002). In addition, global IGFBP-1 overexpression affects female reproductive capacity; animals are fertile but they produce smaller litters with reduced body weight of the offspring (Dai et al., 1994). Tissue-specific over-expression has revealed hepatic-derived IGFBP-1 to influence brain weight and morphology, glucose homeostasis, bone demineralization, and kidney function (Dai et al., 1994). It is of interest that some of these effects converge with toxic endpoints associated with TCDD exposure, i.e., reduced litter size, increased catabolism, and wasting syndrome. Furthermore, long-term exposure of rodents to omeprazole results in suppression of weight gain and skeletal demineralization (Cui et al., 2001); a causal link with AhR or IGFBP-1 has thus far not been investigated.

The potential for AhR to modulate the expression of hIGFBP-1 may directly affect IGF-1 signaling and have consequences for IGF-I/II-mediated physiological processes. Furthermore, inappropriate activation of AhR and subsequent hIGFBP-1 regulation may contribute to the development of disease. Given that omeprazole is widely prescribed and an activator of hAhR, its effect upon hIGFBP-1 warrants investigation.

Materials and Methods

Materials

TCDD was a generous gift provided by Dr. Stephen Safe (Texas A&M, College Station, TX). Omeprazole was obtained commercially (Sigma-Aldrich, St. Louis, MO).

Cell Culture

The human hepatoma HepG2 cell line was routinely maintained in α -minimal essential medium (Sigma-Aldrich) supplemented with 8% fetal bovine serum (HyClone Laboratories, Logan, UT), 100 IU/ml penicillin, and 100 μ g/ml streptomycin (Sigma-Aldrich). Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

RNA Isolation and Reverse Transcription

Total RNA was isolated from HepG2 cells cultured in six-well plates using TRIzol (Invitrogen, Carlsbad, CA). RNA concentration was determined via spectrophotometry at 260 and 280 nm. Two micrograms of total RNA was reverse transcribed to cDNA using High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA).

Quantitative PCR

PCR was performed on a DNA Engine Opticon system using DyNAmo SYBR Green reagent (New England Biolabs, Ipswich, MA). PCR primers (Integrated DNA Technologies, Coralville, IA) used in this study are listed in Table 1. In all cases, melting point analysis revealed amplification of a single product. Data acquisition and analysis were achieved using MyIQ software (Bio-Rad, Hercules, CA).

Plasmids

The proximal (–500/+165 bp) promoter region of hIGFBP-1 was amplified from MCF-7 human genomic DNA using the primer pair hIGFBP-1^{Pro} (forward and reverse) (Integrated DNA Technologies) listed in Table 1. The 665-base pair fragment was subcloned into the XhoI/HindIII site of pGL3basic-Luc (Promega, Madison, WI) to generate the pGL3basic/hIGFBP-1^{Pro} WT-Luc construct. pGL3basic/hIGFBP-1^{Pro} DRE-Luc, lacking a functional DRE was generated from the wild-type parent construct by site-directed mutagenesis using the following mutagenic primer pair: hIGFBP-1^{Pro} DRE (forward and reverse), listed in Table 1, and the QuikChange (Stratagene, La Jolla, CA) mutagenesis kit. Wild-type and mutant constructs were verified by DNA sequencing.

Quantitative Western Immunoblot Analysis

Total cellular and secreted proteins were isolated using TRIzol (Invitrogen) according to the manufacturer's protocol. Protein pellets were resuspended in 1% SDS, and concentrations were determined using the DCq Protein Assay reagent (Bio-Rad). Protein was resolved on 3% stacking/8% resolving Tricine/SDS polyacrylamide gels. Proteins were transferred to Immobilon PVDF membrane (Millipore Corporation, Billerica, MA), visualized with Ponceau S stain, and blocked with 3% bovine serum albumin. Where indicated, membranes were probed at recommended dilutions for 1 h with the following primary antibodies: α -hIGFBP-1 mouse IgG (MAB675; R&D Systems, Minneapolis, MN), α -hAhR mouse IgG (MA1-514; Affinity

BioReagents, Golden, CO), --rS6 rabbit IgG (sc-20085; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and --albumin rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA). Secondary antibody detection was achieved using species-appropriate biotin-conjugated IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Tertiary detection was achieved through incubation with 0.03 μ Ci/ml 125 I-streptavidin (GE Healthcare, Piscataway, NJ). Blots were exposed to BioMAX (Eastman Kodak, Rochester, NY) film and developed. Protein expression was quantified using the Cyclone storage phosphor system (PerkinElmer Life and Analytical Sciences, Shelton, CT). Data acquisition and analysis were achieved with the Opti-Quant software package (PerkinElmer Life and Analytical Sciences).

Transient Transfection and Reporter Assays

Cells were seeded in six-well plates and cultured to ~80% confluence. Twenty-four hours before transfection, cells were washed with phosphate-buffered saline and transferred to --minimal essential medium supplemented with 0.5% fetal bovine serum and 5 mg/ml bovine serum albumin. Transient transfection assays were performed in triplicate using Lipofectamine/Lipofectamine Plus (Invitrogen) following the manufacturer's recommendations, and DNA quantity was maintained across transfections through addition of pcDNA3 (Invitrogen). Transfection efficiency was monitored through cotransfection with the constitutively active pSV/--Gal reporter (Promega). Twenty-four hours after transfection, cells were washed with phosphate-buffered saline, and fresh reduced serum media were added containing the indicated treatments. Cells were treated for 24 h, and then they were lysed in 200 μ l of lysis buffer [25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% (v/v) glycerol, and 1% (v/v) Triton X-100]. Lysate (20 μ l) were combined with 80 μ l of Luciferase Reporter Substrate (Promega), and luciferase activity was measured with a TD-20e luminometer (Turner Designs, Sunnyvale, CA). --Galactosidase activity was determined by addition of 100 μ l of lysate to an equal volume of --galactosidase assay buffer and by measuring absorbance at ~410 nm. Luciferase activity was normalized with respect to --galactosidase activity.

siRNA Studies

Specific RNA-mediated knockdown studies were performed using siGENOME ON-TARGETplus hAhR (sense and antisense) siRNAs (Dharmacon RNA Technologies, Lafayette, CO), listed in Table 1. HepG2 cells seeded in six-well plates were cultured to ~80% confluence, and then they were transferred to antibiotic-free media for 24 h. Cells were transfected with 100 nM siRNA/well using 6 μ l of DharmaFect-1 reagent (Dharmacon RNA Technologies) for 30 h. Control transfections were performed using siRNAs for green fluorescent protein (GFP) (New England Biolabs) listed Table 1.

Statistical Analyses

In all cases, studies were performed in triplicate. Statistical analyses of data were performed using Prism graphing and statistical analysis software (GraphPad Software Inc., San Diego, CA). Data presented in Figs. 2, 3, and 4 were analyzed using Student's t test. Data presented in Figs. 5, 6, and 7 were analyzed using one-way ANOVA and Tukey's multiple comparison test. In all cases, $P < 0.05$ values were deemed statistically significant and are indicated by an asterisk.

Results

hIGFBP-1 mRNA Is Induced by TCDD and Omeprazole

Analysis of the hIGFBP-1 promoter has revealed the presence of numerous regulatory elements (Fig. 1) (Suwanichkul et al., 1990). It has been reported that TCDD, a potent ligand for AhR

can induce hIGFBP-1 transcription and that such induction is facilitated by a single core consensus DRE element (5'-TAGCGTG-3') situated at position -89/-82bp within the hIGFBP-1 proximal promoter (Marchand et al., 2005).

Before investigating the effect of omeprazole on hIGFBP-1 expression, we wanted to confirm this observation. HepG2 human hepatoma cells were incubated in 0.5% reduced serum media to reduce the effective concentration of insulin, a major negative regulator of hepatic hIGFBP-1 expression; cells were exposed to increasing doses of TCDD, ranging from 0 to 100 nM for 24 h. Analysis of hIGFBP-1 mRNA levels by RTq-PCR revealed a clear dose-responsive induction by TCDD (Fig. 2), achieving 20-fold induction at the highest dose (100 nM) tested, with significant ($P < 0.05$) induction occurring at 0.1 nM.

Confirmation of TCDD stimulated induction led us to explore the effect of nonclassical AhR activation by omeprazole on hIGFBP-1. Twenty-four-hour exposure to increasing doses of omeprazole (0-300 μ M) resulted in a dose-responsive elevation in hIGFBP-1 transcription by HepG2 cells (Fig. 3). Significant ($P < 0.05$) induction was observed at 3 μ M, whereas at the maximum dose tested (300 μ M) omeprazole evoked a similar, i.e., 20-fold, increase to that previously observed with 100 nM TCDD. It is interesting to note that plasma concentrations of omeprazole in patients undergoing standard PPI therapy for acid reflux disease are in the low micromolar range (Yin et al., 2004), suggesting that the observed 2-fold induction of hIGFBP-1 mRNA by 3 μ M omeprazole may be physiologically relevant.

Omeprazole-Mediated hIGFBP-1 Induction Is Independent of Ongoing Translation

We wanted to determine whether the induction of hIGFBP-1 by omeprazole is a direct transcriptional event rather than a consequence of enhanced translation of a secondary factor that could subsequently modulate hIGFBP-1 promoter activity. To address this issue, we again analyzed hIGFBP-1 mRNA levels in HepG2 cells after 24-h treatment with omeprazole but in the context of cotreatment with the ribosomal inhibitor cycloheximide to inhibit peptide translation. In the absence of cycloheximide, 300 μ M omeprazole reproduced the ~20-fold induction in hIGFBP-1 transcription. Preincubation (1 h) with 1 μ g/ml cycloheximide followed by 24-h cotreatment with omeprazole revealed a similar level of induction, which was not significantly different ($P < 0.05$) from that observed with omeprazole alone (Fig. 4). The failure of cycloheximide to affect omeprazole-mediated induction of hIGFBP-1 mRNA is indicative of a direct transcriptional event not dependent upon ongoing translation.

Omeprazole-Mediated hIGFBP-1 Induction Is hAhR-Dependent

The observation that hIGFBP-1 mRNA induction by omeprazole is dose responsive, in conjunction with the previous finding that TCDD, acting directly via AhR, can also stimulate induction led us to examine the possible involvement of hAhR in mediating the effect of omeprazole. To investigate this issue, we generated a luciferase reporter construct (pGL3basic/hIGFBP-1^{PRO}-WT-Luc) harboring the wild-type proximal (-500/-169 bp) hIGFBP-1 promoter, previously shown to contain the DRE responsible for mediating TCDD-AhR induction. A mutant version (pGL3basic/hIGFBP-1^{PRO}- Δ DRE-Luc) was also constructed, in which the AhR recognition/binding half-site of the DRE was mutated from the wild-type consensus sequence (TAGCGTG) to (CGACGTG), thus eliminating the AhR binding site and rendering it potentially AhR-insensitive. These constructs were transfected into HepG2 cells, which were then treated with vehicle (DMSO), 10 nM TCDD, or 300 μ M omeprazole for 24 h. HepG2 cells, transfected with wild-type reporter, responded to 24 h TCDD or omeprazole with a similar significant ($P < 0.05$) 2.5-fold induction of luciferase activity (Fig. 5). By contrast, cells transfected with the Δ DRE promoter construct similarly exposed to vehicle (DMSO), 10 nM TCDD, or 300 μ M omeprazole displayed a marked inhibition of reporter activity (Fig. 5). TCDD failed to significantly ($P < 0.05$) increase luciferase expression, whereas omeprazole-

exposed cells exhibited a slight but insignificant ($P > 0.05$) repression below that observed with vehicle alone. These data are consistent with previous findings demonstrating the role of an AhR-DRE interaction in mediating hIGFBP-1 induction by TCDD, and they also indicate that the same DRE is required to facilitate omeprazole-mediated induction.

The requirement of a functional DRE is suggestive but not conclusive evidence for the involvement of hAhR in mediating the effect of omeprazole. To confirm hAhR involvement, we decided to examine hIGFBP-1 expression in the context of diminished hAhR levels. HepG2 cells were transfected with siRNA oligonucleotides targeted against hAhR (siRNA-hAhR) or green fluorescent protein as a control (siRNA-GFP Ctrl) followed by exposure to vehicle (DMSO), 10 nM TCDD, or 300 μ M omeprazole for 24 h. Quantitative Western immunoblot analysis revealed a significant ($P < 0.05$) 50% reduction in hAhR protein levels in siRNA-hAhR samples compared with control (Fig. 6, A and B), such levels of siRNA-mediated AhR knockdown are consistent with previous reports (Abdelrahim et al., 2003). The absence of complete hAhR knockdown at the protein level required us to examine the affect of a 50% reduction upon hAhR signaling. RTq-PCR analysis of hCYP1A1 mRNA levels revealed greater than 90% reduction in TCDD and omeprazole sensitivity with regard to CYP1A1 mRNA induction in siRNA-hAhR cells compared with similarly treated siRNA-GFP Ctrl counter-parts (Fig. 6C). The almost complete loss of hCYP1A1 induction suggests that a 50% reduction in hAhR protein expression is sufficient to inhibit hAhR-dependent transcription.

Confirmation of functional knockdown of hAhR by siRNA thus allowed us to examine the role of hAhR in mediating the induction of hIGFBP-1 by omeprazole. HepG2 cells transfected with siRNA-GFP as a control and subsequently treated with TCDD or omeprazole exhibited 20- and 30-fold hIGFBP-1 mRNA induction, respectively (Fig. 6D). By contrast, hAhR-specific siRNA-treated cells similarly exposed to TCDD or omeprazole for 24 h could only elicit 2- and 5-fold increase in hIGFBP-1 transcription (Fig. 6D). These data indicate that loss of hAhR expression, albeit by 50%, significantly diminishes the capacity of TCDD or omeprazole to modulate hIGFBP-1 transcription. The dual requirements of a functional level of hAhR expression and an intact cognate DRE to mediate hIGFBP-1 mRNA induction by omeprazole clearly suggest direct involvement of hAhR.

Enhanced hIGFBP-1 Secretion in Response to Omeprazole

We have demonstrated that activation of hAhR either via classical ligand binding with TCDD or nonclassical (i.e., nonligand binding) with omeprazole can induce hIGFBP-1 mRNA in the human hepatoma HepG2 cell line. hIGFBP-1 exerts its biological activity predominantly through the local and systemic modulation of IGF-I/II bioavailability. For hIGFBP-1 to interact with IGFs, it must first be secreted. We wanted to demonstrate that omeprazole-mediated hAhR activation and subsequent transcription of hIGFBP-1 are reflected by enhanced secretion of hIGFBP-1 peptide. To this end, HepG2 cells were exposed to vehicle (DMSO), 3 μ M omeprazole, or 30 μ M omeprazole for 24 h, and hIGFBP-1 protein in the conditioned media was determined by quantitative Western immunoblot analysis (Fig. 7). Exposure to vehicle alone (Fig. 7A, lane 1) resulted in an observable level of immunoreactivity at 30 kDa, corresponding to hIGFBP-1 and thus demonstrates that the HepG2 cell line retains the capacity to express and secrete hIGFBP-1. Twenty-four-hour exposure of HepG2 cells to omeprazole prompted an increase in hIGFBP-1 peptide accumulation into the media at both doses (3 and 30 μ M) examined (Fig. 7). Omeprazole at 3 μ M resulted in a significant ($P < 0.05$) 230% increase in hIGFBP-1 peptide secretion (Fig. 7A, lane 2; and B), whereas 30 μ M omeprazole was able to elicit a 300% accumulation of hIGFBP-1 peptide (Fig. 7A, lane 3; and B). These data indicate that the positive action of omeprazole (and hAhR activation) on hIGFBP-1 transcription is reflected at the level of peptide synthesis and secretion.

Discussion

Its role as an environmental sensor has required the AhR to evolve the capacity to respond to a diverse array of chemicals to initiate adaptive responses to chemically induced stress. As a result, many chemicals, pharmaceuticals, and persistent environmental pollutants have the capacity to activate the AhR. Sustained and/or aberrant activation of the AhR is known to be detrimental in various respects to many species, including humans, often resulting in the phenomenon of endocrine disruption (Safe et al., 1998). In this report, we have examined the potential for the widely prescribed pharmaceutical omeprazole, acting via the hAhR to modulate endocrine gene expression.

Omeprazole is an enigmatic activator of hAhR; its mode of activation has thus far remained elusive. Direct binding assays using ^{14}C -labeled omeprazole fail to show a physical interaction with hAhR (Daujat et al., 1992); similarly, competitive ligand binding studies demonstrate that even at high micromolar concentrations omeprazole is incapable of displacing high-affinity ^3H -labeled AhR ligands (Gerbal-Chaloin et al., 2006). Indeed, omeprazole does not adhere to the classical structural requirements, i.e., planar, polycyclic composition, common to most high-affinity AhR ligands. Thus, by inference omeprazole is not considered to be a direct ligand for hAhR. Nonetheless, omeprazole does promote hAhR nuclear translocation and association with AhR nuclear translocator together with subsequent DRE-dependent DNA binding, thus driving hAhR-dependent gene expression (Quattrochi and Tukey, 1993). Therefore, the likelihood exists that omeprazole may share the endocrine-disrupting properties of prototypical AhR ligands, such as TCDD.

Several facets of endocrine signaling have been demonstrated to be affected by TCDD exposure, including steroid synthesis and metabolism; steroid receptor function and peptide hormone expression. In recent studies, AhR ligands have been shown to negatively affect insulin and IGF signaling (Croutch et al., 2005). The observation that hIGFBP-1, a major regulator of IGF action, is regulated via AhR (Adachi et al., 2004; Marchand et al., 2005) led us to examine whether omeprazole could activate hAhR to elicit a similar induction of hIGFBP-1 expression to that observed previously with classical AhR binding agonists. Our data clearly demonstrate that omeprazole, in the context of HepG2 cells, has the capacity to stimulate the expression of hIGFBP-1 mRNA and that such induction is mediated by hAhR acting directly at the hIGFBP-1 promoter. Furthermore, our observation that 24-h exposure to omeprazole can induce transcription of hIGFBP-1 mRNA is enhanced by the subsequent finding that such induction can be extrapolated with regard to hIGFBP-1 peptide secreted by HepG2 cells. In addition, we reveal that at concentrations comparable with those found circulating in patients undergoing PPI therapy (Shimizu et al., 2006), omeprazole can significantly elevate hIGFBP-1 secretion. The mechanism behind omeprazole-mediated hAhR activation is beyond the scope of this study; however, it has been proposed that omeprazole, acting through an unidentified tyrosine kinase(s), may alter rat AhR phosphorylation, specifically Tyr³²⁰, to provoke ligand-independent AhR activation (Backlund and Ingelman-Sundberg, 2005). The assimilation of hIGFBP-1 into the AhR battery (Adachi et al., 2004; Marchand et al., 2005), together with the colocalized hepatic expression patterns of hIGFBP-1 and hAhR (Lindros et al., 1997; Hazel et al., 1998), suggests a certain level of integration between these two signal transduction pathways. The physiological significance of such a connection and the consequences of aberrant omeprazole-mediated, AhR-dependent hIGFBP-1 induction warrant further investigation. Furthermore, the role that the AhR may have in regulation of IGFBP-1 expression in the absence of exogenous activators remains to be addressed.

Despite extensive investigation, bone fide endogenous activators that mediate normal physiological regulation of AhR, whether direct high-affinity ligands or otherwise, have not

been unambiguously identified for the AhR. However, the phenotypic differences observed with *ahr*^(-/-) mice compared with wild-type counterparts indicate that AhR has intrinsic transcriptional activity under exogenous ligand-free conditions; thus, it is not inconceivable for AhR to contribute to normal physiological IGFBP-1 expression. It is of interest to note that an increase in circulating IGFBP-1 is often associated with stress such as tissue damage and inflammation (Leu et al., 2001). Increasing IGFBP-1 is thought to serve to limit peripheral glucose use by diminishing the effect of insulin. We could speculate that AhR-dependent increases in hepatic IGFBP-1 expression serves a similar function, thus allowing the primary AhR stress response, i.e., induction of phase I/II/III metabolism, to protect from toxic insults.

The identification of IGFBP-1 as a gene target of AhR (Adachi et al., 2004; Marchand et al., 2005) may in part, represent a causative link with the documented inhibition of IGF signaling and reduction in body weight after exposure to AhR ligands, such as TCDD (Croutch et al., 2005). Likewise, AhR-mediated modulation of IGFBP-1 levels by exogenous activators, including omeprazole, may affect IGF-independent actions of IGFBP-1 via its association with integrin signaling to a greater degree. Studies have revealed the major fraction of circulating IGF-I/II to be in a bound state with IGFBPs. Therefore, an increase in IGFBP-1 prompted by activation of AhR may not greatly influence systemic IGF bioavailability but could impinge upon local or paracrine IGF activity. Modulation of local IGF and insulin activity may become particularly evident in extrahepatic tissues that exhibit AhR expression. The current study has focused upon classical and nonclassical hAhR-mediated hIGFBP-1 expression by the hepatic-derived human hepatocarcinoma HepG2 cell line. Although the liver represents the predominant site of expression for AhR and IGFBP-1, both display wide-spread tissue expression. Intriguingly, PPI therapy is associated with an increased predisposition to the development of fundic polyps within the gastric epithelium (el-Zimaity et al., 1997). The AhR is known to be present in gastric tissue, the intended target for omeprazole intervention; moreover, increased AhR expression within the stomach has been reported to promote the development of gastric tumors (Ma et al., 2006). A mechanistic relationship among omeprazole, AhR, and IGFBP-1 with regard to the development of such gastric cell proliferation remains to be addressed.

Transgenic and other studies with rodents have provided evidence implicating important roles for both AhR and IGFBP-1 in female reproductive physiology, including placental development, blastocyst implantation, and embryonic growth (Safe, 1993; Silha and Murphy, 2002). A large body of evidence supports a correlation between reproductive toxicity and exposure to persistent environmental pollutants known to be AhR ligands, such as TCDD and polychlorinated biphenyls (Safe, 1993). Although clinical studies have been performed to assess the safety of omeprazole therapy during pregnancy (Diav-Citrin et al., 2005), none have identified any overt detrimental pre- or postnatal effects such as spontaneous abortion or growth retardation that could be attributed to aberrant IGFBP-1 levels. However, most of the subjects in these cohorts were prescribed omeprazole after conception after onset of pregnancy-associated acid reflux. Data concerning omeprazole use at time of conception is limited (Brunner et al., 1998), but they show no apparent effects of omeprazole on the probability of conception or pregnancy outcome.

It is interesting to note that elevated levels of hIGFBP-1 are known to contribute toward the progression of various pathophysiological conditions, including diabetes, certain cancers, and renal disease. It remains to be addressed whether hAhR activation, either classically or nonclassically with omeprazole (or other xenobiotics) could have pathophysiological consequences via a mechanism involving the induction of hIGFBP-1. Although beyond the scope of this present study, the inductive action of omeprazole with regard to hIGFBP-1 may be exacerbated by additional factors. Omeprazole metabolism may affect the potential for increased hIGFBP-1 synthesis. Metabolism of omeprazole is achieved primarily through the

action of hepatic CYP2C19, and in humans, it is highly variable. Such variability arises from the existence of multiple polymorphic cyp2c19 alleles. Depending upon cyp2c19 genotype, subjects can be efficient, intermediate, or poor metabolizers, which may affect the potential for hIGFBP-1 induction. Furthermore, the recent observation that activation of AhR within gastrointestinal tissues leads to a whole body elevation in CYP1A1 and by inference AhR activity (Ito et al., 2007), raises the potential for omeprazole to activate gastric hAhR before being metabolized by the liver and hence stimulate hIGFBP-1 expression even in the context of a high metabolizing cyp2c19 genotype.

In conclusion, the present study reveals hIGFBP-1 to be an AhR-dependent target after exposure to classical and nonclassical modes of hAhR activation. Furthermore, exposure to the widely prescribed pharmaceutical omeprazole, at doses comparable with those observed in plasma of patients undergoing acid suppression therapy can elicit a significant increase in the level of secreted hIGFBP-1 and may have physiological consequences. As such, consideration should perhaps, under certain circumstances, be given to the clinical use of omeprazole. Effective PPI therapy requires a regular dosing regime, perhaps over a period of weeks or months. Hence, repeated exposure to omeprazole and sustained hAhR activation may in the absence of any counter-balancing mechanism permit hIGFBP-1 to accumulate, a situation that could be further confounded if associated with the cyp2c19 genotype linked to poor omeprazole metabolism. This study further serves to emphasize the increasing role(s) of AhR in mediating biological processes not associated directly with xenobiotic metabolism. The expansion of the gene repertoire associated with hAhR together with its promiscuity with regard to ligand binding, present the potential for aberrant gene regulation arising from environmental, nutritional and pharmaceutical activation of hAhR.

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ABBREVIATIONS

hAhR, human aryl hydrocarbon receptor
 AhR, aryl hydrocarbon
 DRE, dioxin response element(s)
 TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin
 IGF, insulin-like growth factor
 hIGFBP, human insulin-like growth factor binding protein
 Luc, luciferase
 PCR, polymerase chain reaction
 Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine
 bp, base pair(s)
 PVDF, polyvinylidene difluoride
 siRNA, small interfering RNA
 GFP, green fluorescent protein
 ANOVA, analysis of variance
 RTq, quantitative reverse transcription
 PPI, proton pump inhibitor
 DMSO, dimethyl sulfoxide
 Ctrl, control
 hrL13a, human ribosomal protein L13a

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hIGFBP-1 (-500/+165bp) proximal promoter

-500 GGGCTTTTCCACAGATAACCTGCTAGGGCTCGCAGAGACCTGGATTCTAACCCCATCTCGCCTTTCCTCACC
 -428 TGGGGCATTGTTTTCTGCGTTTGAGAACTGCTGGGTGGGCTGGTGTACCCAGGATTTTAAATTTTGCAG
 -356 GAGACGCTTTCAGGAGATCTCTAGGGCTTTTTCTGTGTCAATTAAAGAGCCTGGCCAGCTGGACCTGGGC
 -284 TGTCTTTTGGACAAAAAACAAC**CGTCAT**CCCCCTCCAGCTGAGCACTTGTAGAACTGGACTTTAACTGAGG
 -211 GCCTGAACCCCTAACAA**CGGGACAAACAGTAT**GAAATCAAAACCGTTTACCCTCCTCCCACCAGCGGTTTG
 -141 CGTAGGGCCTTGGGT**GCACTAGCAAAACA**AACTTATTT**TGAACACTCAGCTCC****TAGCGT**GCGGCGCTGCCAA
 -69 **TCATTAACT**CTCCTGGTGCAAGTGGCGCGCCTGTGCC**TTTATAA**GGTGCAGCTGTGTCCAGCGAGCATCG
 +5 GCCACCGCCATCCCATCCAGCGAGCATCTGCCCGCGCGCCGCCACCCTCCAGAGAGCACTGGCCACCG
 +77 CTCCACCATCACTTGCCAGAGTTTGGGCCACCGCCCGCCACCAGCCAGAGAGCATCGGCCCTGTCT
 +149 GCTGCTCGCGCCTGGAG-**ATG**

Fig. 1.

Schematic of the hIGFBP-1 proximal promoter. Nucleotide sequence spanning -500/+169 base pairs of the hIGFBP-1 proximal promoter. Key regulatory elements are indicated: cAMP response element (CRE); glucocorticoid response elements I/II (GRE-I/II); insulin response sequences A/B (IRS-A/B); TATA element (TATA); hepatocyte nuclear factor-1 element (HNF-1), and DRE. Transcription start site (-1) is indicated with an arrow. Start ATG codon is illustrated in bold.

Fig. 2.

Regulation of hIGFBP-1 transcription by TCDD is dose-responsive. HepG2 cells were maintained in routine culture media to 80% confluence and then transferred to 0.5% reduced serum media. After overnight incubation, cells were exposed, as indicated to vehicle (DMSO), 0.1, 1, 10, or 100 nM TCDD for 24 h. Total RNA was isolated and reverse transcribed. cDNAs were amplified by quantitative PCR using a hIGFBP-1-specific primer set. hIGFBP-1 mRNA levels were normalized against hrL13a mRNA. Data represent mean -fold hIGFBP-1 mRNA induction $\pm$ S.E.M. over vehicle-treated control. Data were subjected to the Student's *t* test, and $P < 0.05$ values were deemed statistically significant and are indicated by an asterisk.

Fig. 3.

Regulation of hIGFBP-1 transcription by omeprazole is dose-responsive. HepG2 cells were cultured in routine culture media to 80% confluence and then transferred to 0.5% reduced serum media. After overnight incubation, cells were exposed, as indicated to vehicle (DMSO), 0.3, 3, 30, or 300 μ M omeprazole for 24 h. Total RNA was isolated and reverse transcribed. cDNAs were amplified by quantitative PCR using a hIGFBP-1-specific primer set. hIGFBP-1 mRNA levels were normalized against hL13a mRNA. Data represent mean $\pm$ fold hIGFBP-1 mRNA induction $\pm$ S.E.M. over vehicle-treated control. Data were subjected to the Student's *t* test, and *P* $\leq$ 0.05 values were deemed statistically significant and are indicated by an asterisk; n.s., not significant.

Fig. 4.

Induction of hIGFBP-1 mRNA by omeprazole does not require translation. HepG2 cells were maintained in routine culture media to 80% confluence and then transferred to 0.5% reduced serum media. After overnight incubation, cells were exposed, as indicated to vehicle (DMSO), 300 μM omeprazole, 1 μg/ml cycloheximide (CHX), or omeprazole plus cycloheximide for 24 h. Cells receiving cotreatment were pre-exposed to cycloheximide for 1 h before addition of omeprazole. Total RNA was isolated and reverse transcribed. cDNAs were amplified by quantitative PCR using a hIGFBP-1-specific primer set. hIGFBP-1 mRNA levels were normalized against hrL13a mRNA. Data represent mean -fold hIGFBP-1 mRNA induction ± S.E.M. over vehicle (DMSO)-treated control. Data were subjected to the Student's t test, and $P < 0.05$ values were deemed statistically significant and are indicated by an asterisk.

Fig. 5.

Disruption of the hIGFBP-1 DRE inhibits TCDD and omeprazole-mediated hIGFBP-1 promoter activity. HepG2 cells were transiently transfected with luciferase reporter constructs harboring the wild-type hIGFBP-1 · 500/· 169-bp proximal promoter (pGL3basic/hIGFBP-1^{PRO} WT-Luc) or the · DRE promoter (pGL3basic/hIGFBP-1^{PRO} ΔDRE-Luc), in which the AhR-binding half-site has been mutated. Transfected cells were transferred to 0.5% reduced serum media. After overnight incubation, cells were exposed, as indicated to vehicle (DMSO), 10 nM TCDD, or 300 ·M omeprazole for 24 h. Cells were lysed, and luciferase activity was determined. Luciferase output was normalized to ·-galactosidase activity to control for transfection efficiency. Data represent mean ·fold luciferase induction · S.E.M. over vehicle-treated control. Data were subjected to ANOVA, and P · 0.05 values were deemed statistically significant and are indicated by an asterisk.

Fig. 6. siRNA-mediated knockdown of hAhR ablates TCDD and omeprazole-mediated induction of hIGFBP-1 mRNA. HepG2 cells were transiently transfected with 100 nM siRNA oligonucleotides directed against control GFP (siRNA-GFP Ctrl) or hAhR (siRNA-hAhR). Transfected cells were transferred to 0.5% reduced serum media. After overnight incubation, cells were exposed, as indicated to vehicle (DMSO), 10 nM TCDD, or 300 μ M omeprazole for 24 h. Total RNA and protein were isolated using TRIzol reagent. A, protein (50 μ g) were resolved by Tricine/SDS-polyacrylamide gel electrophoresis, transferred to PVDF membrane, and probed as indicated for AhR. Ribosomal protein S6 (hrS6) served as a loading control. B, siRNA-mediated knockdown of hAhR protein expression visualized and illustrated in A was

quantified by phosphorimaging. Data are expressed as mean percentage change $\pm$ S.E.M. relative to vehicle-treated siRNA-GFP control, arbitrarily set at 100%. C and D, total RNA was isolated and reverse transcribed. cDNA were amplified by quantitative PCR using hCYP1A1 (C) and hIGFBP-1 (D)-specific primer sets (listed in Table 1). hCYP1A1 and hIGFBP-1 mRNA levels were normalized against hrL13a mRNA. Data are expressed as mean -fold hCYP1A1/hIGFBP-1 mRNA induction $\pm$ S.E.M. over vehicle-treated siRNA-GFP control. Data were subjected to ANOVA, and $P < 0.05$ values were deemed statistically significant and are indicated by an asterisk.

Fig. 7.

Omeprazole enhances hIGFBP-1 expression and secretion by HepG2 cells. HepG2 cells were maintained in routine culture media to 80% confluence, and then they were transferred to 0.5% reduced serum media. After overnight incubation, cells were exposed, as indicated to vehicle (DMSO), 3 ·M omeprazole, or 30 ·M omeprazole for 24 h, and the culture media were collected. A, 75 ·l of vehicle- or omeprazole-conditioned media were resolved by Tricine/SDS-polyacrylamide gel electrophoresis, transferred to PVDF membrane, and probed as indicated for hIGFBP-1. Albumin served as a loading control. B, hIGFBP-1 expression/secretion visualized and illustrated in A was quantified by phosphorimaging. Data are expressed as mean percentage of change in hIGFBP-1 protein · S.E.M. relative to vehicle-treated control, arbitrarily set at 100%. Data were subjected to ANOVA, and $P < 0.05$ values were deemed statistically significant and are indicated by an asterisk.

TABLE 1
Application and nucleotide sequences of oligonucleotides

Oligonucleotide	Application	Nucleotide Sequence (5'-3')
hrL13a (F)	RTq-PCR	CCTGGAGGAGAAAGAGAAAGAGA
hrL13a (R)	RTq-PCR	GAGACCTCTGTGTATTTGTCAA
hGFBP-1 (F)	RTq-PCR	CCCAGAGAGACACGGAGATTA
hGFBP-1 (R)	RTq-PCR	TATCTGGCAGTTGGGGTCTC
hCYP1A1 (F)	RTq-PCR	TCTTCCTTCGTCCCTTCAC
hCYP1A1 (R)	RTq-PCR	TGGTTGATCTGCCACTGGTT
hGFBP-1 ^{Pro} (F)	Subcloning	AACTCGAG-GGGCTTTTCCACAGATAACC
hGFBP-1 ^{Pro} (R)	Subcloning	TTAAGCTTCTCCAGGGCGGAGCAGCAG
hGFBP-1 ^{Pro} , DRE (F)	Mutagenesis	TGAACACTCAGCTCCGACGTGCGCGCTGCCA
hGFBP-1 ^{Pro} , DRE (R)	Mutagenesis	TGGCAGCGCGCACGTCGGGAGCTGAGTGTCA
hAhr (sense)	siRNA	GCAACAAGAGAGUCUAUUUU
hAhr (antisense)	siRNA	AAUAGACUCUAUCUUGUGCUU
GFP	siRNA	Proprietary heterogeneous oligonucleotide mix

F, forward; and R, reverse.