

12(*R*)-Hydroxy-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoic Acid [12(*R*)-HETE], an Arachidonic Acid Derivative, Is an Activator of the Aryl Hydrocarbon Receptor^[S]

Christopher. R. Chiaro, Rushang D. Patel, and Gary. H. Perdew

Center for Molecular Toxicology and Carcinogenesis and the Department of Veterinary and Biomedical Sciences, the Pennsylvania State University, University Park, Pennsylvania

Received May 31, 2008; accepted September 8, 2008

ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-regulated transcription factor that can be activated by structurally diverse chemicals, ranging from environmental carcinogens to dietary metabolites. Evidence supporting a necessary role for the AHR in normal biology has been established; however, identification of key endogenous ligand/activator remains to be established. Here, we report the ability of 12(*R*)-hydroxy-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoic acid [12(*R*)-HETE], an arachidonic acid metabolite produced by either a lipoxygenase or cytochrome P-450 pathway, to act as a potent indirect modulator of the AHR pathway. In contrast, structurally similar HETE isomers failed to demonstrate significant activation of the AHR. Electrophoretic mobility shift assays, together with ligand competition binding experiments, have demonstrated the inability of 12(*R*)-HETE to directly bind or directly activate the AHR to a DNA

binding species in vitro. However, cell-based xenobiotic-responsive element-driven luciferase reporter assays indicate the ability of 12(*R*)-HETE to modulate AHR activity, and quantitation of induction of an AHR target gene confirmed 12(*R*)-HETE's ability to activate AHR-mediated transcription, even at high nanomolar concentrations in human hepatoma (HepG2)- and keratinocyte (HaCaT)-derived cell lines. One explanation for these results is that a metabolite of 12(*R*)-HETE is acting as a direct ligand for the AHR. However, several known metabolites failed to exhibit AHR activity. The ability of 12(*R*)-HETE to activate AHR target genes required receptor expression. These results indicate that 12(*R*)-HETE can serve as a potent activator of AHR activity and suggest that in normal and inflammatory disease conditions in skin, 12(*R*)-HETE is produced, perhaps leading to AHR activation.

The aryl hydrocarbon receptor (AHR) is a ligand-activated basic helix-loop-helix/Per-ARNT-Sim transcription factor expressed in most of the cell and tissue types found in vertebrates. In the absence of ligand, the AHR resides in the cytosol in a heterotetrameric protein complex. Contained in this core complex are the AHR ligand-binding subunit, a dimer of the 90-kDa heat shock protein, and a single molecule of the immunophilin-like X-associated protein 2 (also referred to as AIP or ARA9) (Meyer et al., 1998). After ligand binding, the receptor translocates into the nucleus and forms a high-affinity DNA binding complex upon heterodimeriza-

tion with the aryl hydrocarbon receptor nuclear translocator (ARNT). The AHR/ARNT complex can interact with specific DNA target sequences known as xenobiotic-responsive elements (XREs) in the regulatory region of AHR responsive genes, resulting in altered gene expression. Most AHR target genes are involved primarily in foreign chemical metabolism and include the xenobiotic-metabolizing cytochrome P450 enzymes from the CYP1A and CYP1B families, along with NAD(P)H-quinone oxido-reductase 1, an aldehyde dehydrogenase, and several phase II-conjugating enzymes, including GST, Ya, and UDP-glucuronosyltransferase 1A1 (Nebert et al., 2004). In addition, the AHR has been shown to regulate genes involved in growth and cellular homeostasis, such as epiregulin and Hairy and Enhancer Split homolog 1 (Thomson et al., 2004; Patel et al., 2006).

The AHR plays an important role in the adaptive metabolic response to xenobiotic exposure; this response can be modu-

This work was supported by National Institutes of Health grant ES04869 and a grant from Phillip Morris USA Inc. and Philip Morris International.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.108.049379.

[S] The online version of this article (available at <http://molpharm.aspetjournals.org>) contains supplemental material.

ABBREVIATIONS: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; DMSO, dimethyl sulfoxide; HETE, hydroxyeicosatetraenoic acid; 12(*R*)-HETE, 12(*R*)-hydroxy-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoic acid; 12(*S*)-HETE, 12(*S*)-hydroxy-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoic acid; XRE, xenobiotic-responsive element; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HPLC, high-performance liquid chromatography; siRNA, short interfering RNA; 12-KETE, 12-oxo-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoic acid.

lated by a diverse range of compounds, including many potentially toxic manmade environmental contaminants, such as halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons (Wilson and Safe, 1998). In addition to xenobiotic metabolism, the AHR has emerged as playing an important physiological role in vascular development in the liver (Lahvis et al., 2000). However, a key question still remaining unresolved is the identity of important endogenous modulators of AHR activity. Evidence supporting the existence of such endogenous AHR ligands or modulators has been accumulating, with the strongest support resulting from studies with AHR-null mice. These animals displayed multiple hepatic defects, including a decrease in liver size and weight (Fernandez-Salguero et al., 1995), resulting presumably from the presence of a portosystemic shunt (Lahvis et al., 2000), a persistent unresolved remnant of fetal vasculature. Additional aberrations included compromised immune system function (Fernandez-Salguero et al., 1995), altered kidney vasculature, and vascular anomalies in the eye, including the presence of a persistent hyaloid artery. AHR-null animals also exhibited a decrease in constitutive expression of cytochrome P4501A2, along with a complete loss of cytochrome P4501A1, induction normally seen only in response to AHR activation. Together, these observations indicate a crucial biological role(s) for the AHR in normal physiology. Furthermore, assuming that AHR activation requires ligand, these observations provide indirect evidence supporting the existence of one or more high-affinity endogenous AHR ligands, existing to modulate the timing, duration, and magnitude of AHR function in the cell. Identifying such a ligand(s) would enable the biological role(s) for this enigmatic orphan receptor to be more precisely determined, thus allowing the significance of excessive AHR activation by environmental compounds to be more thoroughly evaluated. In the past, several eicosanoid molecules, including lipoxin A₄ (Schaldach et al., 1999), 5,6-DiHETE (Chiaro et al., 2008), and prostaglandins (Seidel et al., 2001), have been reported as AHR ligands. During a screening of eicosanoids, in particular those produced by lipoxygenase or cytochrome P450 metabolic pathways, several molecules possessing AHR activity were identified. In this report, we characterize 12(R)-hydroxy-5(Z),8(Z),10(E),14(Z)-eicosatetraenoic acid [12(R)-HETE], an arachidonic acid metabolite of either lipoxygenase or cytochrome P-450 origin, as an activator of the AHR signal transduction pathway.

Materials and Methods

Chemicals and Enzymes. 12(S)-Hydroxy-5(Z),8(Z),10(E),14(Z)-eicosatetraenoic acid [12(S)-HETE] and 12(R)-HETE were purchased from BIOMOL (Plymouth Meeting, PA). Additional amounts of both 12-HETE isomers along with 12-oxo-5(Z),8(Z),10(E),14(Z)-eicosatetraenoic acid (12-KETE) were purchased from Cayman Chemical Company (Ann Arbor, MI). 12(R)-Hydroxyeicosatrienoic acid [12(R)-HETrE] was a gift from Dr. John R. Falck (University of Texas, Southwest Medical Center, Dallas, TX). Optima-grade high-purity organic solvents were purchased from Fisher Scientific (Pittsburgh, PA) and were used in all chromatographic separations. Anhydrous dimethyl sulfoxide (DMSO) (99.9%⁺ purity) was purchased from Sigma-Aldrich (Milwaukee, WI). TCDD was a generous gift from Dr. Steven Safe (Texas A&M University). [³²P]γATP was purchased from PerkinElmer (Waltham, MA), whereas T4 polynucleotide kinase was from Promega (Madison, WI). Poly(dI:dC) was purchased

from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK), and precast 6% nondenaturing polyacrylamide gels were from Invitrogen (Carlsbad, CA).

Cell Lines and Cell Culture. The HepG2 40/6 reporter cell line was generated as described previously (Long et al., 1998), whereas the Hepa 1.1 reporter cell line was a kind gift from Dr. Michael S. Denison (University of California, Davis, CA). HaCaT cells, a spontaneously immortalized aneuploid human keratinocyte cell line, were obtained from Adam Glick (The Pennsylvania State University, University Park, PA). Trypsin-EDTA, phosphate-buffered saline, α-minimal essential medium, penicillin, and streptomycin were all obtained from Sigma (St. Louis, MO). Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT). Reporter cell lines were grown in α-minimal essential medium supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂/95% room air. Clonal selection of reporter cell lines was maintained through the use of 300 μg/ml G418 (Invitrogen, Carlsbad, CA).

Cell-Based Reporter Assay. Reporter cell lines were plated into 24-well tissue culture plates (Falcon; BD Biosciences Discovery Labware, Bedford, MA) at a density of 5.0×10^5 cells/well and were allowed 18 h of recovery before beginning a 6-h treatment with increasing amounts of 12-HETE or a 12-HETE metabolite. Upon completion of the dosing regiment, cells were rinsed thoroughly with phosphate-buffered saline before the addition of 1× cell culture lysis buffer (2 mM CDTA, 2 mM dithiothreitol, 10% glycerol, and 1% Triton X-100). After being frozen overnight at -80°C, the lysates were then thawed and centrifuged at 18,000g for 15 min. The resulting cytosol was assayed for luciferase activity using the Promega luciferase assay system as specified by the manufacturer. Light production was measured using a TD-20e Luminometer (Turner Designs, Inc., Sunnyvale, CA). Cytosolic protein concentration was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Luciferase activity was expressed relative to protein concentration.

Electrophoretic Mobility Shift Assay. XRE-specific electrophoretic mobility shift assays were performed using *in vitro*-translated AHR and ARNT proteins. Expression vectors for these proteins were translated using a TNT-coupled transcription and translation rabbit reticulocyte lysate kit (Promega). A solution of 12-hydroxyhexadecatetraenoic acid in ethanol was evaporated under argon gas and resolubilized in DMSO to achieve a stock solution of appropriate concentration. Serial dilutions of this stock were made in DMSO to achieve the various working stocks needed to perform a dose-response curve. Proteins for the transformation reactions were mixed together at a 1:1 M ratio in HEPES/EDTA/dithiothreitol/glycerol buffer, followed by the addition of either 0.5 μl of DMSO-solubilized 12-HETE isomer, 12-HETE metabolite, or TCDD. All transformation assays were incubated for 90 min at room temperature, followed by the addition of oligonucleotide buffer [42 mM HEPES, 0.33 M KCl, 50% glycerol, 16.7 mM dithiothreitol, 8.3 mM EDTA, 0.125 mg/ml CHAPS, and 42 ng/μl poly(dI:dC)]. After a 15-min incubation in oligonucleotide buffer, ~200,000 cpm of ³²P-labeled wild-type XRE was added to each reaction. Samples were then mixed with an appropriate amount of 5× loading dye and electrophoresed on a 6% nondenaturing polyacrylamide gel. Wild-type XRE oligonucleotides composed of nucleotide sequences 5'-GATCTGGCTCTTCTCACG-CAACTCCG and 3'-ACCGAGAAGAGTGCCTTGAGGCCTAG were a gift from Dr. M. S. Denison.

AHR Ligand Competition Binding Assay. 2-Azido-3-[¹²⁵I]iodo-7,8-dibromodibenzo-*p*-dioxin was synthesized in our laboratory according to the procedure described previously and was stored in methanol protected from light (Poland et al., 1986). Hepa-1 cell cytosol, a source of mouse AHR, was prepared in MENG buffer (25 mM MOPS, 2 mM EDTA, 0.02% sodium azide, and 10% glycerol, pH 7.4) and diluted to a final protein concentration of 1.0 mg/ml. All binding experiments were carried out in the dark with 150 μg of soluble Hepa-1 cytosolic protein incubated with increasing concentrations of 12-HETE for 30 min. Next, a saturating concentration of

the AHR photoaffinity ligand, 2-azido-3- 125 I]iodo-7,8-dibromodibenzo-*p*-dioxin (0.10 pmol, 4×10^5 cpm) was added and incubated for an additional 30 min at 23°C to achieve equilibrium binding. The samples were photolysed at >302 nm for 4 min at a distance of 8 cm using two 15-W UV lamps (Dazor Mfg. Corp., St. Louis, MO). After irradiation, each sample was mixed with an equal volume of 2× tricine sample buffer (0.9 M Tris, pH 8.45, 24% glycerol, 12% (w/v) SDS, 0.015% (w/v) Coomassie blue G, and 0.005% (w/v) phenol red) and heated to 95°C for 5 min. Equal amounts of each sample were loaded onto a 9% Tricine-SDS-polyacrylamide gel electrophoresis gel and subjected to denaturing electrophoresis overnight at 15 mA/gel. The gels were fixed in destain (25% isopropyl alcohol, 10% acetic acid, and 10% glycerol), dried under vacuum, and exposed at -80°C to a sheet of X-OMAT-Blue film (Carestream Health, Rochester, NY).

HPLC Purification and Spectral Analysis. HPLC purifications were performed using a Waters system, consisting of a Waters 600E multisolvent delivery unit and controller coupled with a Waters 996 photodiode array detector (Waters, Milford, MA). The system was integrated and operated through the use of Waters Millennium³² software. Normal-phase HPLC purification of 12-HETE was performed on a LiChrosphere 5 μm Silica-60 (4.6×250 mm) column (Supelco, Bellefonte, PA) using a hexane/isopropanol/acetic acid solvent system [989:10:1 (v/v/v)] applied at 1 ml/min with a linear gradient increase in isopropanol concentration of 0.8%/min over 30 min. High-resolution accurate mass determination for 12(R)-HETE and the tandem mass spectrometry product ion spectrum for the molecular ion (m/z 319) were both acquired on a Waters QTOF Ultima mass spectrometer using an electrospray ionization source operated in negative ion mode and was performed by Dr. A. Daniel Jones (Michigan State University, East Lansing, MI).

Quantitative Reverse Transcription-Polymerase Chain Reaction analysis. Total RNA was isolated from cells using TRI Reagent (Sigma-Aldrich) and was reverse-transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) according to the respective manufacturer's protocols. The cDNA made from 25 ng of RNA was used for each qPCR reaction. qPCR was performed on a DNA Engine Opticon system using the IQ SYBR Green qPCR Kit purchased from Bio-Rad Laboratories (Hercules, CA). Data were analyzed and plotted using Prism 4 software (GraphPad Software Inc., San Diego, CA). Each bar represents the mean \pm S.D. of three separate determinations. Statistical comparison of treatments was performed using the Student's *t* test ($\alpha = 0.05$). Values determined as being statistically significant from controls are indicated by the presence of an asterisk.

Results

12(R)-HETE Can Activate the AHR in Cell-Based Assays. Preliminary studies with extracts generated from CV-1 cells indicated the possibility of endogenous bioactive lipids as mediators of the AHR activity (C. R. Chiaro and G. H. Perdew, unpublished data). The molecular mass of many known potent AHR ligands is between 250 and 360 Da. The majority of eicosanoids, bioactive lipids formed from arachidonic acid, also fit into this same size range and are capable of adopting a structural conformation that could be accommodated by the AHR ligand binding pocket. Therefore, key metabolites of the 5-, 12-, and 15-lipoxygenase pathways were screened for AHR activity using cell culture-based biological activity assays. Bioassays performed using the HepG2 40/6 cell line, a human liver-derived reporter cell line containing a stably integrated copy of an XRE-driven luciferase reporter construct, were used to screen various hydroxyeicosatetraenoic acid (HETE) metabolites for potential AHR transcriptional activity. This approach led to the discovery of 12(R)-HETE as a modulator of AHR activity in cells, capable of stimulating the AHR signaling pathway in a dose-dependent manner (Fig. 1). Activation of the AHR by 12(R)-HETE, although only moderate in magnitude, seems to be highly specific compared with the lack of activity seen among all other HETE isomers examined. It is interesting that analysis of the isomer activity profile indicates that both position and stereochemical orientation of the hydroxyl group play important roles in the ability of these molecules to modulate AHR activity.

Analysis of the Biochemical Purity of 12(R)-HETE Preparations. Several commercially available preparations of specific eicosanoid molecules produced false-positive results in initial assays, probably because of the presence of hydrophobic contaminants. Therefore, the purity and structural integrity of 12(R)-HETE preparations needed to be analyzed before further experimental use. To remove any possible contaminating impurities, especially the early eluting hydrophobic material identified as possessing significant AHR activity in some commercially prepared HETE preparations (data not shown), 12(R)-HETE preparations were

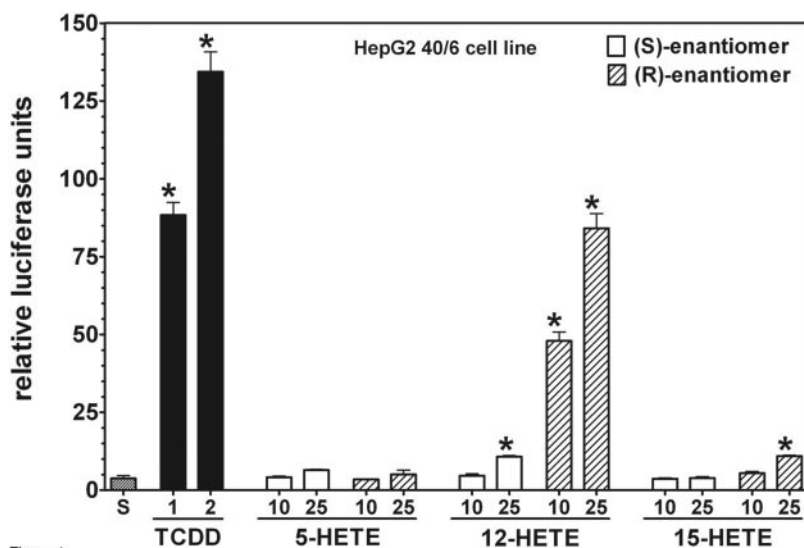


Fig. 1. 12(R)-HETE activates the AHR in cell-based reporter activity assays. Various HETE isomers were screened via bioassay in the HepG2 40/6 reporter cell line for their ability to activate the AHR. Luciferase values are expressed as relative light units (RLU) and corrected for the amount of total protein. S, carrier solvent control. The concentrations of HETEs are given in the micromolar range, and TCDD is in the nanomolar range. Each data point represents the mean \pm S.D. of three separate determinations. Values are presented as relative luciferase units and have been normalized to protein concentration.

subjected to normal-phase HPLC purification (Supplemental Fig. S1A). Eluting with a retention time (T_R) of 12.25 min, the peak representing the purified 12(R)-HETE fraction was collected and further analyzed before being used in any experimental applications. Evaporation of the HPLC mobile phase followed by immediate solubilization in degassed absolute methanol rendered the sample preparation available for subsequent analytical techniques. Spectral analysis of purified 12(R)-HETE preparations generated spectra displaying a characteristic absorbance maximum at 235 nM (Supplemental Fig. S1B), indicating the presence of a conjugated diene functionality. Additional confirmation of the purity and integrity of the 12(R)-HETE sample was achieved via mass spectroscopy analysis using a Waters QTOF Ultima mass spectrometer using electrospray ionization in negative ion mode (ESI⁻). After loss of a proton, sample molecules exhibit the characteristic molecular ion for 12(R)-HETE at a mass-to-charge ratio (m/z) of 319. Furthermore, high-accuracy mass determination of the molecular ion resulted in a measured mass of 319.2272, well in agreement with the theoretical calculated mass for the compound of 319.2273, and confirming an elemental composition of $C_{20}H_{31}O_3$ (Supplemental Fig. S1C). Tandem mass spectrometry analysis of the molecular ion also produced characteristic daughter fragments of $m/z = 301$ and 257, indicating loss of H_2O and the loss of both CO_2 and H_2O , respectively. The peak at $m/z = 179$ is produced through cleavage of the carbon backbone between C-11 and C-12 (Supplemental Fig. S1D). With the purity of 12(R)-HETE preparations being firmly established after repurification, only freshly prepared repurified preparations were used in all subsequent analyses.

12(R)-HETE Is Incapable of Directly Binding to the Ah Receptor. Despite its ability to activate the AHR pathway in cell culture, 12(R)-HETE failed to induce AHR heterodimerization or XRE binding as determined by electrophoretic mobility shift assay. Using 12(S)-HETE as a control, both 12-HETE enantiomers were tested for AHR activity and

were compared against a TCDD-induced AHR-positive control. Neither of the 12-HETE isomers was capable of inducing transformation of the AHR to its DNA binding form, as evident by the complete lack of shifted radiolabeled complex (^{32}P -XRE-AHR:ARNT) after electrophoresis on a nondenaturing polyacrylamide gel. The lack of heterodimer complex formation, even at 10 and 25 μM at which a substantial induction in reporter gene activity was observed, clearly indicates the inability of 12(R)-HETE to serve as a direct AHR ligand (Fig. 2, A and B). Further evidence for lack of 12(R)-HETE binding to the AHR was obtained through the use of in vitro ligand competition binding assays. Using cytosolic preparations containing AHR generated from the Hepa-1.1 cell line, increasing concentrations of 12(R)-HETE failed to demonstrate any significant ability to displace a radiolabeled photoaffinity ligand from the AHR (Fig. 2C), confirming the inability of this compound to serve as a direct AHR ligand. Likewise, 15(R)-HETE, serving as a negative control, also demonstrated no significant ability to displace the radiolabeled dioxin-like compound. In contrast, 300 nM benzo[a]pyrene exhibited strong displacement of the photoaffinity ligand from the AHR (Fig. 2C). Quantitation of the level of radioactivity in each receptor gel band upon displacement with HETE is represented in Fig. 2D. Taken together, the results of these assays demonstrate the inability of 12(R)-HETE to either directly bind to receptor or activate the AHR to its DNA binding form.

Metabolites of 12(R)-HETE Fail to Activate AHR. Because purified 12(R)-HETE was unable to directly bind and activate the AHR in vitro, yet displayed AHR activity in cell-based assay systems, it was hypothesized that a downstream metabolite of 12(R)-HETE may be responsible for the observed activity of this compound. Therefore, in an attempt to identify the active metabolite, several known 12(R)-HETE metabolic pathways were screened for the ability to activate AHR transcriptional activity. In most cells, metabolism of 12(R)-HETE is limited to peroxisomal-mediated β -oxidation

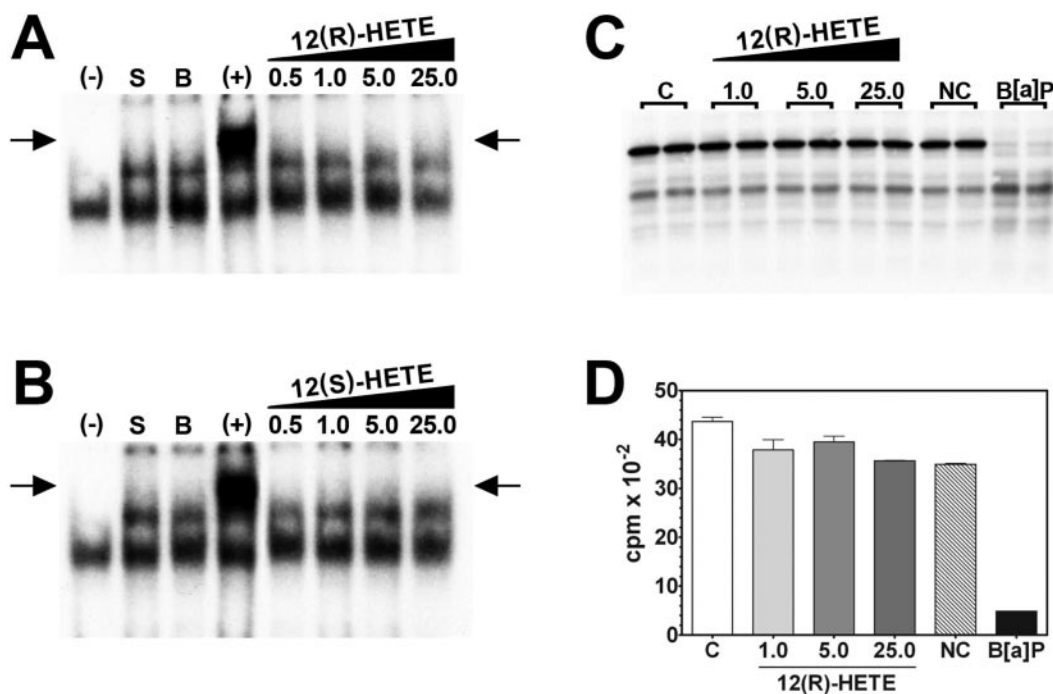


Fig. 2. 12(R)-HETE is incapable of directly binding to the AHR. A and B, both 12-HETE enantiomers were tested for AHR activity in a gel shift assay. TCDD (20 nM) was used as a positive control (+). Additional gel shift controls included a negative (-) control containing no ARNT, a background (B) control composed of only AHR and ARNT to assess background heterodimerization, and a solvent (S) control to assess the effects of vehicle on heterodimer formation. C, 12(R)-HETE was subjected to an AHR ligand competition binding assay. Another monohydroxylated HETE, 15(R)-HETE (25 μM), served as a negative control (NC), whereas a positive control used 300 nM benzo[a]pyrene (B[a]P). The solvent control (DMSO) represents vehicle (DMSO) treatment of AHR. D, a graphic representation of the ligand competition binding data from C is presented.

or cytochrome P450-catalyzed ω -oxidation (Fig. 3A). These reactions will generate chain-shortened metabolites, such as tetranor-12(R)-HETE [8(R)-HHxTrE], or ω -hydroxylated metabolites such as 12(R), 20-DiHETE, respectively (Marcus et al., 1984; Gordon et al., 1989). An additional metabolic pathway involving oxidation of 12-HETE to a keto intermediate (12-oxo-EETE) followed by keto-reduction to 12(R)-HETrE, a 12(S)HETE analog, has been described in porcine neutrophil and bovine corneal epithelial microsomes (Yamamoto et al., 1994). Using the HepG2 40/6 reporter cell line, several 12(R)-HETE metabolites were screened for their ability to modulate AHR transcriptional activity. The results obtained with 8(R)-HHxTrE and 12-oxo-EETE (12-KETE), two common metabolites of 12(R)-HETE, are depicted in Fig. 3B. In addition, the metabolite 12(R)-HETrE failed to activate AHR-mediated transcription (data not shown). Unfortunately, we were unable to obtain 12(R), 20-DiHETE for testing. Nevertheless, the 12(R)-HETE metabolites tested failed to significantly activate the AHR. These results further underscore the high level of specificity in 12(R)-HETE-mediated receptor activation.

12(R)-HETE Activates Transcription of AHR Target Genes in Multiple Human Cell Lines. The ability of

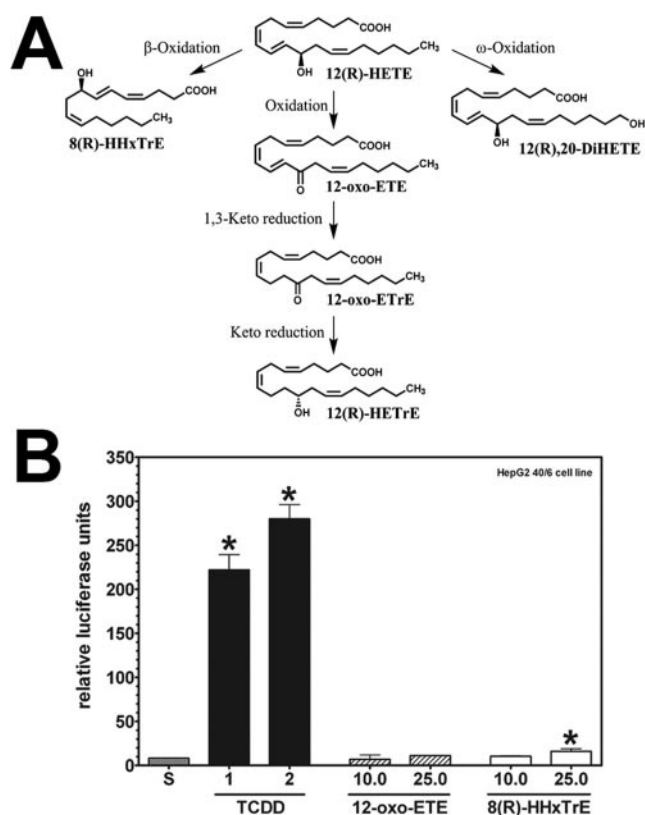


Fig. 3. Metabolites of 12(R)-HETE fail to mediate AHR activity. A, the established biochemical pathways of 12(R)-HETE metabolism in cells. B, using the HepG2 40/6 reporter cell line, several 12(R)-HETE metabolites were screened for their ability to modulate AHR activity. The results obtained with 8(R)-HHxTrE and 12-KETE, two common metabolites of 12(R)-HETE, are shown. Luciferase values are expressed as relative light units (RLU) and corrected for the amount of total protein. S, carrier solvent control. The concentrations of HETEs are given are in the micromolar range, and TCDD is in the nanomolar range. Each data point represents the mean \pm S.D. of three separate determinations. Values are presented as relative luciferase units and have been normalized to protein concentration.

12(R)-HETE to activate AHR target genes was determined using two different human cell lines. *CYP1A1* was chosen because its expression is almost totally dependent on AHR activity. 12(R)-HETE-driven transcription of *CYP1A1*, a classic AHR target gene, was confirmed for both the HepG2 and HaCaT cell lines. In HepG2 cells, 12(R)-HETE treatment resulted in a dose-dependent induction of *CYP1A1* mRNA levels, with a maximal observed induction of approximately 20-fold more than control after 4 μ M 12(R)-HETE treatment. However, statistically significant induction was observed at a much lower concentration, occurring in the nanomolar range, with the 250 nM treatment displaying an approximate 2-fold induction and 500 nM 12(R)-HETE treatment generating an

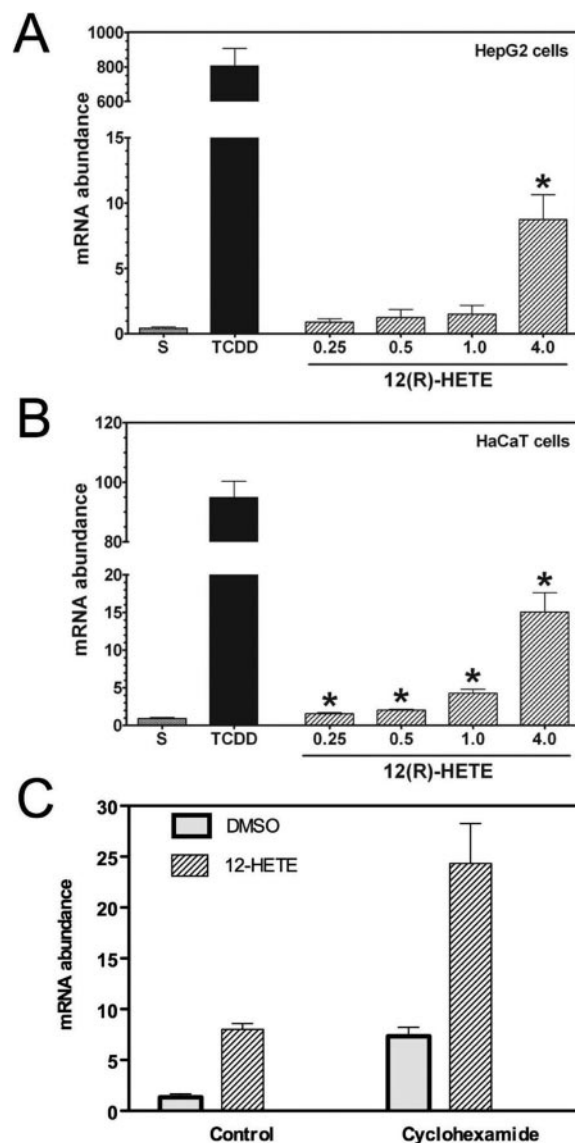


Fig. 4. 12(R)-HETE can activate an AHR target gene in multiple human cell lines and is not blocked by cycloheximide treatment. The ability of 12(R)-HETE to activate AHR-mediated transcription of *CYP1A1* was confirmed in two different human cell lines. A, in HepG2 cells, 12(R)-HETE treatment resulted in a dose-dependent induction of *CYP1A1* mRNA levels. B, a similar result was also obtained with HaCaT cells. S, control composed of vehicle only. C, HepG2 cells were treated with 4 μ M 12(R)-HETE or vehicle for 3 h in the presence or absence of cycloheximide (10 μ g/ml) and *CYP1A1* mRNA measured. Each assay was performed in triplicate. Asterisks indicate a statistically significant difference relative to control ($P < 0.05$).

approximate 3-fold induction in *CYP1A1* mRNA levels (Fig. 4A). Similar results were also obtained with HaCaT cells, which demonstrated a 16-fold induction in response to the 4 μ M dose point and an approximate 2-fold induction as the result of 500 nM treatment (Fig. 4B). Thus, at high nanomolar concentrations, 12(R)-HETE treatment of human cell lines results in the induction of AHR target gene mRNA levels.

12(R)-HETE Directly Activates Transcription of an AHR Target Gene. HepG2 cells were treated with cycloheximide in the presence of 12(R)-HETE to test whether activation of an AHR target gene by 12(R)-HETE requires protein translation. Results in Fig. 4C clearly indicate that 12(R)-HETE does not require protein translation to activate *CYP1A1* transcription. Thus, 12(R)-HETE seems to directly regulate AHR-mediated activation of *CYP1A1*.

12(R)-HETE Modulates AHR-Mediated Transcription in a Receptor-Dependent Manner. Considering that 12(R)-HETE failed to demonstrate direct binding to the AHR, yet retains the ability to regulate AHR target genes in cells, it was necessary to determine whether the observed activation was occurring through an AHR-dependent mechanism. AHR protein expression in HaCaT cells was significantly reduced in response to an siRNA targeted against the AHR (Fig. 5A). Quantitation through filmless autoradiographic analysis revealed that AHR protein expression levels were reduced by ~75% (Fig. 5B). Under suppressed AHR protein levels, we can evaluate whether AHR is required for the effect of 12(R)-HETE to affect gene expression. Quantitative reverse transcription-polymerase chain reaction was used to analyze the expression of three AHR target genes, *CYP1A1*, *CYP1B1*, and Ah receptor repressor, in response to 12(R)-

HETE in both AHR siRNA-treated and control HaCaT cells. 12(R)-HETE-mediated activation of *CYP1A1* is significantly squelched in the AHR siRNA-treated cells, indicating a required role for the receptor in mediating the observed induction in *CYP1A1* mRNA levels (Fig. 5C). Similar results were obtained for *CYP1B1* (Fig. 5D) and Ah receptor repressor (Fig. 5E). These results have definitively demonstrated that 12(R)-HETE activation of AHR target genes is mediated through the AHR.

Discussion

To appreciate the significance of 12(R)-HETE-mediated activation of the AHR requires an understanding of 12(R)-HETE production, which seems to be restricted to specific tissues and disease states. 12(R)-HETE is generated by enzymatic oxidation of arachidonic acid via a 12(R)-lipoxygenase or cytochrome P450-mediated pathway and may serve as a proinflammatory lipid mediator. In addition, this compound can also be formed through enzymatic reduction of a 12-oxo-5(Z),8(Z),10(E),14(Z)-eicosatetraenoic acid (12-oxo-ETE) molecule. 12(R)-HETE is a major eicosanoid metabolite identified in bovine, rabbit, and human ocular tissue, in which corneal epithelial microsomes have been shown to convert arachidonic acid to 12(R)-HETE in the presence of NADPH (Murphy et al., 1988). As the predominant lipoxygenase product in ocular tissue during inflammation, 12(R)-HETE is produced in the cornea after injury most likely by CYP4B1 (Mastyugin et al., 1999). This HETE is also produced upon treatment with vasopressin or after short-term contact lens wear in ocular tissue (Mastyugin et al., 2001). In the cornea, 12(R)-HETE is a potent chemotactic and angio-

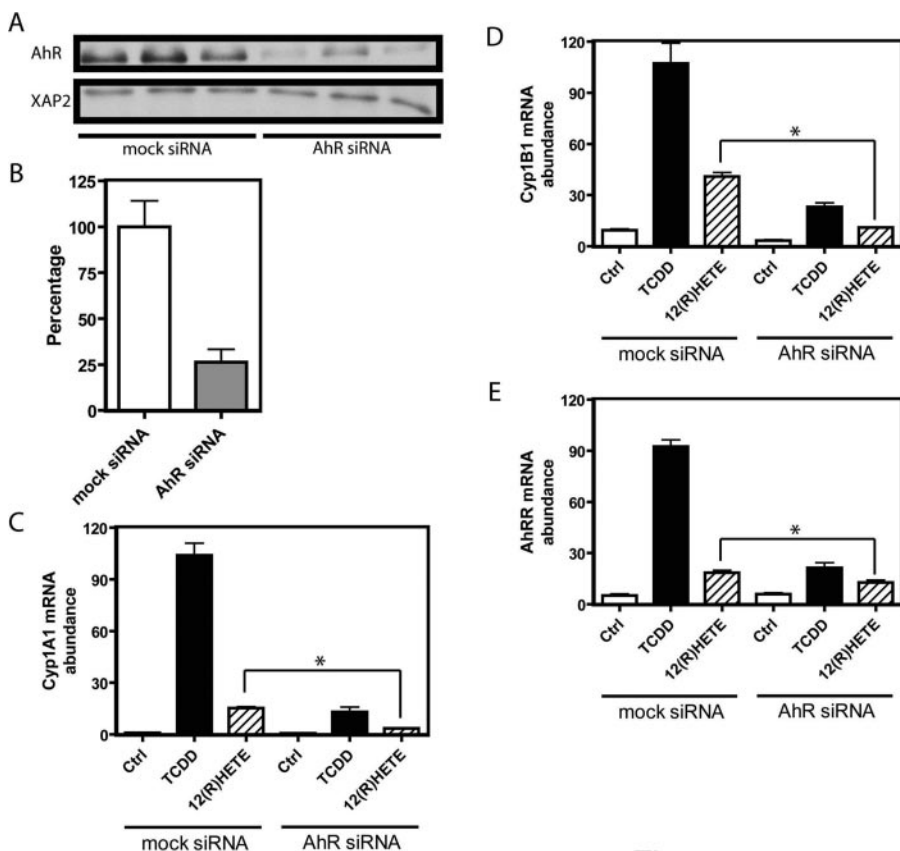


Fig. 5. 12(R)-HETE modulates AHR signaling in a receptor-dependent manner. RNA interference methodology was used to suppress AHR gene expression in HaCaT cells. A, protein blot analysis reveals a significant reduction in AHR expression levels in response to siRNA treatment. B, filmless autoradiographic analysis of the radioactivity in AHR protein bands. C, 12(R)-HETE mediated activation of *CYP1A1* in HaCaT cells in the presence of control or AHR siRNA. Similar results were also obtained for *CYP1B1* (D) and Ah receptor repressor (E), confirming a role for the receptor in the activation of AHR target genes by 12(R)-HETE. Each assay was performed in triplicate. Asterisks indicate a statistically significant difference relative to control ($P < 0.05$).

genic factor that may contribute to the growth of new blood vessels during long-term inflammation (Masferrer et al., 1991). Exposure to 12(R)-HETE also leads to lower intraocular pressure, and this physiological endpoint is related to the inhibition of Na,K-ATPase activity (Delamere et al., 1991). Indeed, this enzymatic activity can be efficiently inhibited by 12(R)-HETE, whereas 12(S)-HETE largely fails to inhibit this enzyme (Masferrer et al., 1990). However, whether the ability of 12(R)-HETE to inhibit Na,K-ATPase plays a role in AHR activation will require further investigation. The presence of the AHR in ocular tissue has not been determined, but *CYP1A1* expression, which is dependent on AHR activity for expression, has been detected in murine, rat, and porcine tissues (McAvoy et al., 1996; Nakamura et al., 2005). It is interesting that a significant constitutive level of *CYP1A1* mRNA was detected in 3- to 5-week-old rat extralenticular tissue (Nakamura et al., 2005). Thus, it is likely that ocular inflammation would lead to AHR activation.

A second site of significant 12(R)-HETE production is in human skin, under both normal conditions (Baer and Green, 1993; Antón et al., 1995) and during psoriasis, as well as other inflammatory dermatoses (Baer et al., 1991). Unlike in the cornea, the enzyme responsible for 12(R)-HETE production is the skin-specific 12(R)-lipoxygenase (Sun et al., 1998). The 12(R)-lipoxygenase is expressed in neonatal skin and during skin development (Sun et al., 1998). In addition, it has been recognized recently that 12(R)-lipoxygenase deficiency in mice disrupts epidermal barrier function and thus establishes the importance of 12(R)-HETE production to normal skin development (Epp et al., 2007; Moran et al., 2007). In humans, a mutation in the 12(R)-lipoxygenase gene has been associated with the genetic disorder nonbullous congenital ichthyosiform erythroderma, further supporting the significance of this lipoxygenase (Jobard et al., 2002). Thus, the AHR may also play a role in normal skin function, although the level of 12(R)-HETE in normal skin seems to be relatively low (Opas et al., 1989). During psoriasis, 12-HETE is highly elevated, and 12(R)-HETE is the predominant form detected (Hammarstrom et al., 1975; Woollard, 1986). Neutrophil infiltration is a common characteristic of psoriasis, and topical application of 12(R)-HETE produces erythema and neutrophil accumulation (Cunningham and Woollard, 1987). These results together support the conclusion that 12(R)-HETE may play a role during skin inflammation. It is interesting that a transgenic mouse expressing a constitutively active form of the AHR in keratinocytes exhibited postnatal inflammatory skin lesions (Tauchi et al., 2005). This suggests that the AHR may play a role in the inflammatory responses observed in human skin diseases.

The actual mechanism of 12(R)-HETE-mediated activation of the AHR remains to be determined, but there are several possibilities. First, 12(R)-HETE might be metabolized to a ligand for the AHR. Attempts to test known 12(R)-HETE metabolites have revealed that the established primary metabolites do not exhibit an ability to activate the AHR. A second possible mechanism is that 12(R)-HETE activates a pathway that leads to the release of an endogenous ligand found in the cell. A third mechanism that is plausible is that 12(R)-HETE or a metabolite activates a cell-signaling pathway that mediates ligand-independent activation of the AHR. We believe that it is most likely that the AHR is activated by 12(R)-HETE through an indirect mechanism.

Clearly, whatever the actual mechanism of AHR activation mediated by 12(R)-HETE, the level of specificity is remarkable, with other monohydroxylated HETEs and 12(R)-HETE metabolites exhibiting essentially no potential to activate the AHR. Further investigations are warranted to explore the unique properties of 12(R)-HETE. The only specific biochemical mechanism of action observed with 12(R)-HETE is the inhibition of Na,K-ATPase activity. Future studies will focus on determining the mechanism of AHR activation.

The AHR is considered an orphan receptor despite the demonstration that several endogenous compounds have been found to be ligands, because the potential endogenous ligands are fairly weak ligands or have not really been demonstrated to have a physiological role. A relatively high-affinity compound, 2-(1'-H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester, was isolated from acid-treated cytosolic lung extracts (Song et al., 2002). However, it was never demonstrated that this apparent tryptophan and cysteine product actually exists in tissue extracts under physiological conditions. A tryptophan photoproduct, 6-formylindolo[3,2b]carbazole, has been shown to form within cultured cells but has not been shown to form in vivo (Fritsche et al., 2007). Indirubin isolated from human urine has been found to be a relatively potent AHR ligand (Adachi et al., 2001). However, although indirubin can be formed from indole by cytochrome P450 activity and subsequent dimerization, whether it plays a role in normal physiological processes is unknown (Gillam et al., 2000). Both bilirubin and 7-ketocholesterol, two physiologically relevant compounds, have been demonstrated to be ligands for the AHR (Phelan et al., 1998; Savouret et al., 2001). However, because these compounds are relatively weak receptor ligands and occur only under very restrictive in vivo conditions, they are less likely to be important AHR ligands. It is interesting that treatment with arachidonic acid in combination with hydrodynamic shear stress resulted in a ~3-fold increase in *CYP1A1* activation compared with stress alone (Mufti and Shuler, 1996). This suggests that an arachidonic acid metabolite might be involved in the activation of *CYP1A1*. The data presented here, coupled with a recent report from our laboratory demonstrating that 5,6-DiHETE is a ligand for the AHR (Chiaro et al., 2008), suggest that the AHR may be activated under inflammatory conditions. This provides a possible link between inflammation or differentiation, production of certain lipoxygenase products, and AHR activation within a variety of tissues. This work provides important clues as to a possible physiological function for the AHR in inflammatory signaling.

Acknowledgments

We thank Drs. Steven H. Safe, John R. Falck, and Mike S. Denison for reagents. Finally, we thank Dr. A. Daniel Jones for his help with the mass spectral analysis of 12(R)-HETE.

References

- Adachi J, Mori Y, Matsui S, Takigami H, Fujino J, Kitagawa H, Miller CA 3rd, Kato T, Saeki K, and Matsuda T (2001) Indirubin and indigo are potent aryl hydrocarbon receptor ligands present in human urine. *J Biol Chem* **276**:31475–31478.
- Antón R, Abián J, and Vila L (1995) *Rapid Commun Mass Spectrom* **S169**–S182.
- Baer AN, Costello PB, and Green FA (1991) Stereospecificity of the products of the fatty acid oxygenases derived from psoriatic scales. *J Lipid Res* **32**:341–347.
- Baer AN and Green FA (1993) Fatty acid oxygenase activity of human hair roots. *J Lipid Res* **34**:1505–1514.
- Chiaro CR, Morales JL, Prabhu KS, and Perdew GH (2008) Leukotriene A4 metabolites are endogenous ligands for the Ah receptor. *Biochemistry* **47**:8445–8455.
- Cunningham FM and Woollard PM (1987) 12(R)-hydroxy-5, 8,10,14-eicosatetraenoic acid is a chemoattractant for human polymorphonuclear leucocytes in vitro. *Prostaglandins* **34**:71–78.

- Delamere NA, Socci RR, King KL, and Bhattacharjee P (1991) The influence of 12(R)-hydroxyeicosatetraenoic acid on ciliary epithelial sodium, potassium-adenosine triphosphatase activity and intraocular pressure in the rabbit. *Invest Ophthalmol Vis Sci* **32**:2511–2514.
- Epp N, Fürstenberger G, Müller K, de Juanes S, Leitges M, Hausser I, Thieme F, Liebisch G, Schmitz G, and Krieg P (2007) 12R-lipoxygenase deficiency disrupts epidermal barrier function. *J Cell Biol* **177**:173–182.
- Fernandez-Salguero P, Pineau T, Hilbert DM, McPhail T, Lee SS, Kimura S, Nebert DW, Rudikoff S, Ward JM, and Gonzalez FJ (1995) Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. *Science* **268**:722–726.
- Fritsche E, Schäfer C, Calles C, Bernsmann T, Bernshausen T, Wurm M, Hübenthal U, Cline JE, Hajimiragha H, Schroeder P, et al. (2007) Lightening up the UV response by identification of the arylhydrocarbon receptor as a cytoplasmic target for ultraviolet B radiation. *Proc Natl Acad Sci U S A* **104**:8851–8856.
- Gillam EM, Notley LM, Cai H, De Voss JJ, and Guengerich FP (2000) Oxidation of indole by cytochrome P450 enzymes. *Biochemistry* **39**:13817–13824.
- Gordon JA, Figard PH, and Spector AA (1989) Identification of the major metabolite of 12-HETE produced by renal tubular epithelial cells. *J Lipid Res* **30**:731–738.
- Hammarström S, Hamberg M, Samuelsson B, Duell EA, Stawiski M, and Voorhees JJ (1975) Increased concentrations of nonesterified arachidonic acid, 12L-hydroxy-5,8,10,14-eicosatetraenoic acid, prostaglandin E₂, and prostaglandin F_{2α} in epidermis of psoriasis. *Proc Natl Acad Sci U S A* **72**:5130–5134.
- Jobard F, Lefèvre C, Karaduman A, Blanchet-Bardon C, Emre S, Weissenbach J, Özgüç M, Lathrop M, Prud'homme JF, and Fischer J (2002) Lipoxygenase-3 (ALOXE3) and 12(R)-lipoxygenase (ALOX12B) are mutated in non-bullous congenital ichthyosiform erythroderma (NCIE) linked to chromosome 17p13.1. *Hum Mol Genet* **11**:107–113.
- Lahvis GP, Lindell SL, Thomas RS, McCuskey RS, Murphy C, Glover E, Bentz M, Southard J, and Bradfield CA (2000) Portosystemic shunting and persistent fetal vascular structures in aryl hydrocarbon receptor-deficient mice. *Proc Natl Acad Sci U S A* **97**:10442–10447.
- Long WP, Pray-Grant M, Tsai JC, and Perdew GH (1998) Protein kinase C activity is required for aryl hydrocarbon receptor pathway-mediated signal transduction. *Mol Pharmacol* **53**:691–700.
- Marcus AJ, Safier LB, Ullman HL, Broekman MJ, Islam N, Oglesby TD, and Gorman RR (1984) 12S,20-dihydroxyeicosatetraenoic acid: a new icosanoid synthesized by neutrophils from 12S-hydroxyeicosatetraenoic acid produced by thrombin- or collagen-stimulated platelets. *Proc Natl Acad Sci U S A* **81**:903–907.
- Masferrer JL, Rimarachin JA, Gerritsen ME, Falck JR, Yadagiri P, Dunn MW, and Laniado-Schwartzman M (1991) 12(R)-hydroxyeicosatrienoic acid, a potent chemotactic and angiogenic factor produced by the cornea. *Exp Eye Res* **52**:417–424.
- Masferrer JL, Rios AP, and Schwartzman ML (1990) Inhibition of renal, cardiac and corneal (Na⁺-K⁺)ATPase by 12(R)-hydroxyeicosatetraenoic acid. *Biochem Pharmacol* **39**:1971–1974.
- Mastyugin V, Aversa E, Bonazzi A, Vafaes C, Mieyal P, and Schwartzman ML (1999) Hypoxia-induced production of 12-hydroxyeicosanoids in the corneal epithelium: involvement of a cytochrome P-4504B1 isoform. *J Pharmacol Exp Ther* **289**:1611–1619.
- Mastyugin V, Mosaed S, Bonazzi A, Dunn MW, and Schwartzman ML (2001) Corneal epithelial VEGF and cytochrome P450 4B1 expression in a rabbit model of closed eye contact lens wear. *Current Eye Res* **23**:1–10.
- McAvoy M, Singh AK, and Shichi H (1996) In Situ Hybridization of Cyp1a1, Cyp1a2 and Ah receptor mRNAs expressed in murine ocular tissues. *Exp Eye Res* **62**:449–452.
- Meyer BK, Pray-Grant MG, Vanden Heuvel JP, and Perdew GH (1998) Hepatitis B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity. *Mol Cell Biol* **18**:978–988.
- Moran JL, Qiu H, Turbe-Doan A, Yun Y, Boeglin WE, Brash AR, and Beier DR (2007) A mouse mutation in the 12R-lipoxygenase, Alox12b, disrupts formation of the epidermal permeability barrier. *J Invest Dermatol* **127**:1893–1897.
- Mufti NA and Shuler ML (1996) Possible role of arachidonic acid in stress-induced cytochrome P4501A1 activity. *Biotechnol Prog* **12**:847–854.
- Murphy RC, Falck JR, Lumin S, Yadagiri P, Zirrolli JA, Balazy M, Masferrer JL, Abraham NG, and Schwartzman ML (1988) 12(R)-hydroxyeicosatrienoic acid: a vasodilator cytochrome P-450-dependent arachidonate metabolite from the bovine corneal epithelium. *J Biol Chem* **263**:17197–17202.
- Nakamura K, Fujiki T, and Tamura HO (2005) Age, gender and region-specific differences in drug metabolising enzymes in rat ocular tissues. *Exp Eye Res* **81**:710–715.
- Nebert DW, Dalton TP, Okey AB, and Gonzalez FJ (2004) Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J Biol Chem* **279**:23847–23850.
- Opas EE, Argenbright LW, and Humes JL (1989) An enzymatic method for distinguishing the stereoisomers of 12-hydroxyeicosatetraenoic acid in human epidermis and psoriatic scale. *Br J Dermatol* **120**:49–58.
- Patel RD, Kim DJ, Peters JM, and Perdew GH (2006) The aryl hydrocarbon receptor directly regulates expression of the potent mitogen ephregulin. *Toxicol Sci* **89**:75–82.
- Phelan D, Winter GM, Rogers WJ, Lam JC, and Denison MS (1998) Activation of the Ah receptor signal transduction pathway by bilirubin and biliverdin. *Arch Biochem Biophys* **357**:155–163.
- Poland A, Glover E, Ebetino FH, and Kende AS (1986) Photoaffinity labeling of the Ah receptor. *J Biol Chem* **261**:6352–6365.
- Savouret JF, Antenos M, Quesne M, Xu J, Milgrom E, and Casper RF (2001) 7-Ketocholesterol is an endogenous modulator for the aryl hydrocarbon receptor. *J Biol Chem* **276**:3054–3059.
- Schaldach CM, Riby J, and Bjeldanes LF (1999) Lipoxin A4: a new class of ligand for the Ah receptor. *Biochem* **38**:7594–7600.
- Seidel SD, Winters GM, Rogers WJ, Ziccardi MH, Li V, Keser B, and Denison MS (2001) Activation of the Ah receptor signaling pathway by prostaglandins. *J Biochem Mol Toxicol* **15**:187–196.
- Song J, Clagett-Dame M, Peterson RE, Hahn ME, Westler WM, Sicinski RR, and DeLuca HF (2002) A ligand for the aryl hydrocarbon receptor isolated from lung. *Proc Natl Acad Sci U S A* **99**:14694–14699.
- Sun D, McDonnell M, Chen XS, Lakkis MM, Li H, Isaacs SN, Elsea SH, Patel PI, and Funk CD (1998) Human 12(R)-lipoxygenase and the mouse ortholog. Molecular cloning, expression, and gene chromosomal assignment. *J Biol Chem* **273**:33540–33547.
- Tauchi M, Hida A, Negishi T, Katsuoka F, Noda S, Mimura J, Hosoya T, Yanaka A, Aburatani H, Fujii-Kuriyama Y, et al. (2005) Constitutive expression of aryl hydrocarbon receptor in keratinocytes causes inflammatory skin lesions. *Mol Cell Biol* **25**:9360–9368.
- Thomsen JS, Kietz S, Ström A, and Gustafsson JA (2004) HES-1, a novel target gene for the aryl hydrocarbon receptor. *Mol Pharmacol* **65**:165–171.
- Wilson CL and Safe S (1998) Mechanisms of ligand-induced aryl hydrocarbon receptor-mediated biochemical and toxic responses. *Toxicol Pathol* **26**:657–671.
- Woollard PM (1986) Stereochemical difference between 12-hydroxy-5,8,10,14-eicosatetraenoic acid in platelets and psoriatic lesions. *Biochem Biophys Res Commun* **136**:169–176.
- Yamamoto S, Nishimura M, Connors MS, Stoltz RA, Falck JR, Chauhan K, and Laniado-Schwartzman M (1994) Oxidation and keto reduction of 12-hydroxy-5,8,10,14-eicosatetraenoic acids in bovine corneal epithelial microsomes. *Biochim Biophys Acta* **1210**:217–225.

Address correspondence to: Dr. Gary H. Perdew, Center for Molecular Toxicology and Carcinogenesis, 309 Life Science Building, The Pennsylvania State University, University Park, PA 16802. E-mail: ghp2@psu.edu