Leukotriene A_4 Metabolites Are Endogenous Ligands for the Ah Receptor[†]

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ABSTRACT: In addition to orchestrating an adaptive metabolic response to xenobiotic compounds, the aryl hydrocarbon receptor (AHR) also plays a necessary role in the normal physiology of mice. The AHR is activated by a structurally diverse group of chemicals ranging from carcinogenic environmental pollutants to dietary metabolites and a number of endogenous molecules. Leukotriene A_4 (5,6-LTA_4) metabolites were identified in DRE-driven luciferase reporter assays as activators of AHR signaling. Various LTA_4 metabolites, including several 5,6- and 5,12-DiHETE products, were screened for AHR activity with 6-*trans*-LTB_4, 6-*trans*-12-*epi*-LTB_4, 5(*S*),6(*S*)-DiHETE, and 5(*S*),6(*R*)-DiHETE eliciting a significant level of AHR transcriptional activity. However, electrophoretic mobility shift assays (EMSAs) revealed that only 5,6-DiHETE isomers were capable of directly binding and activating the AHR to a DNA-binding species in vitro. Furthermore, ligand competition binding experiments confirm the ability of these compounds to directly bind to the AHR. Interestingly, "aged" preparations of 5,6-DiHETE isomers produced an enhanced level of AHR activation while demonstrating an increase in binding affinity for the receptor. Although the reason for this has not been fully determined, the formation of geometric isomers in the conjugated triene region of these molecules may play a role in the observed increase in AHR-mediated transcriptional activity. This work suggests a connection between AHR activation and inflammatory signaling molecules produced by the 5-lipoxygenase pathway.

The aryl hydrocarbon receptor (AHR)[1] is a ligand activated transcription factor belonging to the basic helix−loop−helix/Per-ARNT-Sim (bHLH-PAS) family of DNA-binding proteins (*1*). In the absence of ligand, the AHR remains localized in the cytoplasm as part of a core complex containing dimeric Hsp90 and X-associated protein 2 (also termed ARA9 or AIP). When ligand binds, the activated AHR translocates to the nucleus and, through a poorly understood process, heterodimerizes with the ARNT (aryl hydrocarbon receptor nuclear translocator) to form a functional DNA-binding regulatory complex (*2, 3*). This active heterodimeric AHR−ARNT complex is capable of regulating target gene expression through selective interaction with specific xenobiotic regulatory sequences, known as dioxin response elements (DREs), typically contained in the upstream enhancer region of AHR target genes (*4, 5*). Functioning like a chemosensor, the AHR is a cellular regulatory protein that can be activated by a structurally diverse group of chemicals, ranging from carcinogenic environmental pollutants to dietary metabolites to endogenously formed bioactive lipid molecules (*6*). Historically, the study of AHR biology has centered on the ability of this soluble receptor to mediate the adaptive metabolic response to xenobiotic compounds. By modulating the induction of cytochrome P450s from the CYP1A and CYP1B families, the AHR is able to effectively induce the metabolism of many foreign chemical insults, decreasing their biological half-life by facilitating their excretion (*7*). Recently, through the study of AHR null mice, the receptor has been identified as being critical in the proper development of fetal vasculature (*8*) in addition to occupying an important, yet still undefined, role in cardiac physiology (*9, 10*). Thus, with the AHR emerging as an important regulator of normal physiologic and developmental processes, the identification of key endogenous modulators of receptor activity becomes an area of keen interest.

Here we report the discovery that several eicosanoid molecules, in particular, 5,6-dihydroxyeicosatetraenoic acid isomers (5,6-DiHETEs), are endogenous activators of the AHR. Produced during lipoxygenase metabolism of arachidonic acid, 5,6-DiHETE isomers are formed upon hydrolysis of leukotriene A_4 (LTA_4), an unstable reactive allylic epoxide intermediate (*11, 12*). With an extremely short half-life of approximately 3 s under physiological conditions (*13*), LTA_4 is a transient molecule that, if not further metabolized, will rapidly decompose via nonenzymatic pathways. Enzymatic metabolism of LTA_4 includes the GST-catalyzed conjugation of reduced glutathione (GSH) via a sulfether linkage, resulting in the opening of the epoxide ring and the subsequent formation of the cysteinyl leukotrienes, LTC_4, LTD_4, and LTE_4. These peptide-conjugated bioactive lipids are the potent inflammatory mediators comprising the slow reacting substance of anaphylaxis (*14, 15*). Additional LTA_4 metabolites are produced through hydrolysis of the strained

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[1] Abbreviations: AHR, aryl hydrocarbon receptor; bHLH, basic helix−loop−helix; PAS, Per-ARNT-Sim; ARNT, aryl hydrocarbon receptor nuclear translocator; DiHETE, dihydroxyeicosatetraenoic acid; DRE, dioxin responsive element; LTA_4, leukotriene A_4; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DMSO, dimethyl sulfoxide; TSDS−PAGE, tricine sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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oxirane ring, a reaction capable of proceeding through both enzyme-catalyzed and nonenzymatic reaction mechanisms, ultimately resulting in the formation of various dihydroxy products. For example, LTA_4 hydrolase catalyzes the formation of leukotriene B_4 (LTB_4) (*16, 17*), while the generation of 5(*S*),6(*R*)-DiHETE is mediated by a soluble epoxide hydrolase (*18, 19*). Although the enzyme-catalyzed reactions are stereospecific, producing only a single reaction product, the nonenzymatic addition of water to LTA_4 results in the formation of multiple dihydroxy positional and stereoisomers. The products generated include 5(*S*),6(*R*)-dihydroxy-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid [5(*S*),6(*R*)-DiHETE] and 5(*S*),6(*S*)-dihydroxy-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid [5(*S*),6(*S*)-DiHETE], a pair of dihydroxy fatty acid enantiomers epimeric at position C-6. In addition, nonenzymatic hydrolysis of LTA_4 also generates the more prevalent 5(*S*),12(*R*)-dihydroxy-6(*E*),8(*E*),10(*E*),14(*Z*)-eicosatetraenoic acid (6-*trans*-LTB4) and 5(*S*),12(*S*)-dihydroxy-6(*E*),8(*E*),10(*E*),14(*Z*)-eicosatetraenoic acid (6-*trans*-12-*epi*-LTB4), isomers of enzymatically produced LTB_4 (*11, 12, 20*).

Preliminary findings suggested that LTA_4 mediated significant AHR activity but appeared to be too labile to serve as the active metabolite; thus, we screened various 5,6- and 5,12-DiHETE isomers for their ability to bind and activate the AHR. Although several metabolites were identified as activators of AHR transcriptional activity, only the 5,6-DiHETE isomers demonstrated the ability to function as ligands for the receptor. Thus, a number of LTA_4 metabolites are formed on the nuclear membrane, which could under the appropriate conditions lead to AHR activation.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes. Preparations of methyl-5(*S*),6(*S*)-oxido-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid (LTA_4 methyl ester) were obtained through the generous contributions of C. Channa Reddy (The Pennsylvania State University). Additional amounts of LTA_4 methyl ester along with racemic 5-HETE were purchased from BIOMOL (Plymouth Meeting, PA). 5(*S*),6(*R*)-Dihydroxy-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid [5(*S*),6(*R*)-DiHETE] and 5(*S*),6(*S*)-dihydroxy-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid [5(*S*),6(*S*)-DiHETE] in addition to 5-oxo-6(*E*),8(*Z*),11(*Z*),14(*Z*)-eicosatetraenoic acid (5-KETE) and additional quantities of LTA_4 methyl ester were obtained from Cayman Chemical Co. (Ann Arbor, MI). Optima grade high-purity organic solvents were purchased from Fisher Scientific (Pittsburgh, PA) and 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 S. Safe (Texas A&M University, College Station, TX). [γ-^{32}P]ATP was purchased from PerkinElmer (Boston, MA), while T4 polynucleotide kinase was from Promega (Madison, WI). PolydI:dC was purchased from Amersham Biosciences (Piscataway, NJ), and precast 6% nondenaturing polyacrylamide gels were from Invitrogen (Carlsbad, CA).

Cell Lines and Cell Culture. The HepG2 40/6 reporter cell line (*21*) was generated as described previously, while the Hepa 1.1 reporter cell line (*22*) was a kind gift from M. S. Denison (University of California, Davis, CA). Trypsin-EDTA, PBS, α-MEM, penicillin, and streptomycin were all obtained from Sigma (St. Louis, MO). FBS was purchased from HyClone Laboratories (Logan, UT). Reporter cell lines were grown in α-MEM supplemented with 10% fetal bovine serum (v/v), 100 IU/mL penicillin, and 0.1 mg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% room air. Clonal selection of reporter cell lines was maintained through the use of 300 μg/mL G418 (GibcoBRL, Carlsbad, CA). HaCaT cells were maintained in DMEM supplemented with 4% fetal bovine serum, 1 mM sodium pyruvate, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin.

Cell-Based Reporter Assays. Reporter cell lines were plated into 24-well tissue culture plates at a density of 5.0×10^5 cells/well and allowed to recover for 18 h before a 6 h treatment was begun with increasing amounts of LTA_4 methyl ester, LTA_4 metabolite (5,6- or 5,12-DiHETE), other HETEs, TCDD, or carrier solvent (DMSO). Upon completion of the dosing regiment, cells were rinsed with PBS prior to addition of cell culture lysis buffer (2 mM CDTA, 2 mM DTT, 10% glycerol, and 1% Triton X-100). After being frozen overnight at −80 °C, the lysates were then thawed and centrifuged at 18000*g* for 15 min. The resulting supernatant was assayed for luciferase activity using the Promega luciferase assay system (Promega Corp., Madison, WI) as specified by the manufacturer. Light production was measured using a TD-20e luminometer (Turner Designs, Inc., Sunnyvale, CA). The cytosolic protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Luciferase activity was expressed relative to protein concentration.

Electrophoretic Mobility Shift Assay. DRE-specific electrophoretic mobility shift assays (EMSAs) were performed using in vitro-translated AHR and ARNT proteins. Expression vectors for the murine AHR (mAHR) and ARNT proteins were translated separately using a TNT-coupled transcription and translation rabbit reticulocyte lysate kit (Promega Corp.). In those instances where EMSA analysis was performed using the human AHR (hAHR), the expression vectors for these proteins were translated using a modified version of the standard TNT-coupled transcription and translation protocol, the modification being that sodium molybdate was added to the reaction mixture at a final concentration of 1.25 mM to enhance the stability of the AHR−hsp90 complex. All eicosanoid preparations were evaporated under argon gas and resolubilized in fresh DMSO to achieve stock solutions at the appropriate concentration. Proteins for the transformation reactions were mixed together at a 1:1 molar ratio in HEDG buffer, followed by addition of either 0.5 μL of DMSO-solubilized lipid metabolite or TCDD. All transformation assays were incubated for 90 min at room temperature, followed by the addition of oligonucleotide binding buffer (42 mM HEPES, 0.33 M KCl, 50% glycerol, 16.7 mM DTT, 8.3 mM EDTA, 0.125 mg/mL CHAPS, and 42 ng/μL polydI:dC). Following a 15 min incubation in binding buffer, 200000 cpm of ^{32}P-labeled wild-type DRE was added to each reaction mixture. The samples were mixed with an appropriate amount of 5× loading dye and resolved on a 6% nondenaturing polyacrylamide gel at 100 V for 90 min. Dried gels were visualized for formation of the AHR−ARNT−DRE complex using autoradiography. Complementary synthetic oligonucleotides containing the CYP1A1 DRE3 AHR DNA binding site (5′-GATCTG-GCTCTTCTCACGCAACTCCG-3′ and 3′-ACCGAGAA-

GAGTGCGTTGAGGCCTAG-5′) were a generous gift from M. S. Denison.

AHR Ligand Competition Binding Assay. 2-Azido-3-[^{125}I]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 (*23*). Hepa-1 cell cytosol, a source of the 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 Hepa-1 cytosolic protein incubated with increasing concentrations of competitor 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; i.e., 4×10^5 cpm), was added and incubated for an additional 30 min at 23 °C to achieve equilibrium binding. The samples were photolyzed at >302 nm for 4 min at a distance of 8 cm using two 15 W UV lamps (Dazor Manufacturing Corp., St. Louis, MO). After irradiation, each sample was mixed with an equal volume of 2× tricine sample buffer (TSB) [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−PAGE gel and subjected to denaturing electrophoresis overnight at 15 mA/gel. Proteins were transferred to a PVDF membrane. The membrane was then exposed overnight at −80 °C to X-OMAT-Blue film (Eastman Kodak Co.).

Real-Time PCR Analysis. Isolation of total mRNA was performed with TRI Reagent (Sigma) and amplified using the High Capacity cDNA Archive Kit from Applied Biosystems (Foster City, CA) utilizing the manufacturer's protocol. The levels of human CYP1A1 and GAPDH mRNA were assessed by real-time qPCR using the MyIQ single-color PCR detection system (Bio-Rad, Hercules, CA) and the iQ SYBR Green supermix (Bio-Rad). Experimental analysis was performed in triplicate with error bars representing the standard deviation.

RESULTS

Leukotriene A_4 (LTA_4) Isomers Activate AHR Signaling. Having previously demonstrated that an elevated level of constitutive AHR activity exists in the CV-1 cell line (*24, 25*), due to the presence of a putative endogenous AHR ligand (*25*), and because several nonselective lipoxygenase inhibitors were found to squelch this activity (data not shown), we initiated a methodical examination of lipoxygenase products to search for potential AHR activators. Screening for AHR-mediated transcriptional activity in the human derived HepG2 40/6 reporter cell line, we first identified positional isomers of LTA_4 as the first LOX products possessing AHR activity. Preparations of both 5,6-LTA_4 and 14,15-LTA_4, obtained from the laboratory of C. Channa Reddy, activated AHR signaling in a dose-dependent manner, with the maximum observed induction for each isomer equal to or greater than that observed with 1 nM TCDD (Figure 1A). However, preparations of 5,6-LTA_4 synthesized in our laboratory, in addition to those purchased from commercially available sources, varied greatly in their ability to activate the AHR (data not shown). Thus, due to the extreme

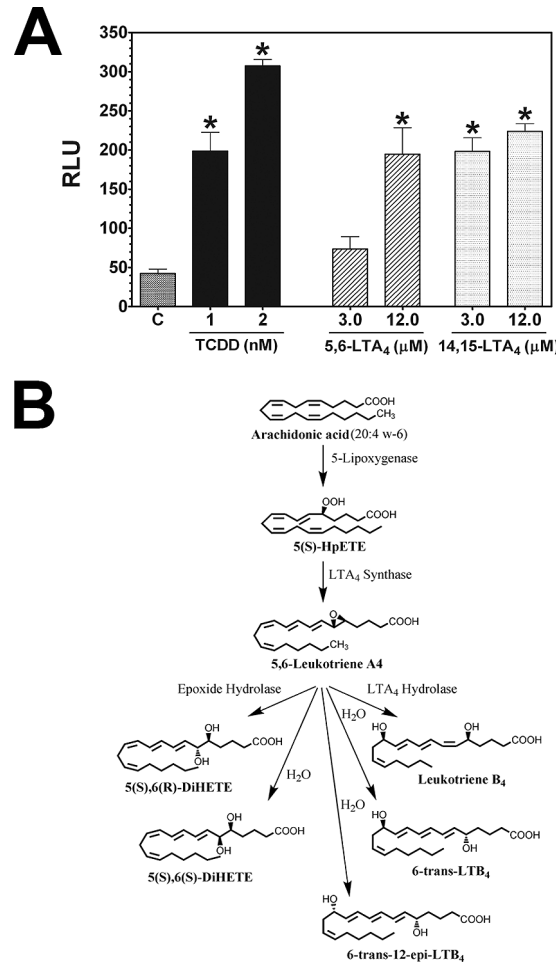

FIGURE 1: Leukotriene A_4 (LTA_4) isomers are capable of activating the AHR in cell-based transcriptional reporter assays. (A) Positional isomers of LTA_4, 5,6-LTA_4 and 14,15-LTA_4, were screened for their ability to activate the AHR in the HepG2 40/6 reporter cell line. The solvent (C) control is a negative control comprised of vehicle (DMSO) treatment only. Values are presented as relative luciferase units and have been normalized to protein concentration. Each data point represents the mean ± standard deviation of three separate determinations. Statistical analysis of treatments was performed using Dunnett's multiple-comparison test ($\alpha = 0.05$). TCDD treatments were compared in manner independent of LTA_4 treatments. Values determined as being statistically significant from the solvent (S) control are indicated by the presence of an asterisk. (B) The 5-LOX metabolic pathway is outlined.

instability and short half-life of these reactive allylic epoxide intermediates, downstream metabolites were immediately suspected as being responsible for the observed induction of AHR transcriptional activity. Because 5,6-LTA_4 is the better characterized of the leukotriene A_4 isomers, our efforts focused on screening the 5-LOX pathway for potential endogenous AHR ligands.

At the center of the 5-LOX pathway is 5,6-LTA_4, a key intermediate formed through 5-LOX-catalyzed insertion of molecular oxygen into arachidonic acid followed by subsequent enzyme-catalyzed dehydration and rearrangement of the hydroperoxy intermediate. As 5,6-LTA_4 is capable of being further metabolized via both enzymatic and nonenzymatic pathways, its cellular metabolism can result in the production of various DiHETE positional isomers and stereoisomers as well as in the formation of leukotriene B_4 (LTB_4) and cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) (Figure 1B).

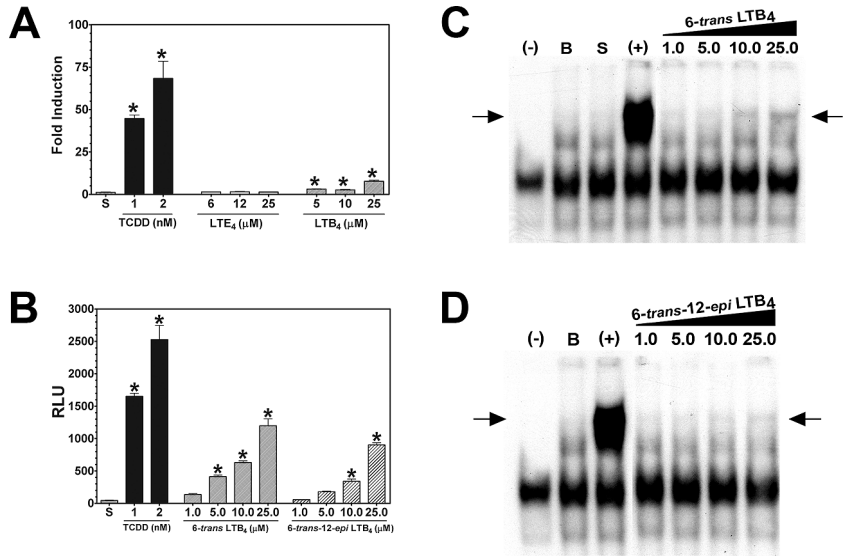

FIGURE 2: Enzymatically vs nonenzymatically derived metabolites of leukotriene A_4 differ in their ability to activate the AHR. (A) Leukotriene B_4 (LTB_4), a dihydroxy metabolite of LTA_4, and leukotriene E_4 (LTE_4), a sulfodipeptide leukotriene, were screened via a bioassay in the human-derived HepG2 40/6 reporter cell line for their ability to activate the AHR. The solvent (S) control is a negative control comprised of vehicle (DMSO) treatment only. (B) 6-*trans*-LTB_4 and 6-*trans*-12-*epi*-LTB_4, a pair of stereoisomers produced through nonenzymatic hydrolysis of LTA_4, were screened via a bioassay in the human-derived HepG2 40/6 reporter cell line for their ability to activate the AHR. Luciferase values have been normalized to protein concentration and are presented as fold induction compared to solvent control. Each data point represents the mean ± standard deviation of three separate determinations. Statistical analysis of treatments was performed using Dunnett's multiple-comparison test ($\alpha = 0.05$). Comparisons of TCDD treatments with control were made in a manner independent of other treatments. Values determined as being statistically significant from solvent (S) control are indicated by the presence of an asterisk. (C and D) Both 6-*trans*-LTB_4 and 6-*trans*-12-*epi*-LTB_4 isomers were tested in an EMSA for their ability to bind and transform the AHR and compared to 20 nM TCDD (+). Controls included a negative (−) control containing no ARNT, a control labeled B comprised of only AHR and ARNT to control for background heterodimerization, and a solvent (S) control to assess the effects of vehicle on heterodimer formation.

Conventional Enzymatic Metabolites of Leukotriene A_4 Fail To Produce Significant Activation of AHR-Mediated Transcription. 5(*S*),12(*R*)-Dihydroxy-6(*Z*),8(*E*),10(*E*),14(*Z*)-eicosatetraenoic acid, more commonly known as LTB_4, is a dihydroxy metabolite of LTA_4, formed enzymatically by the action of LTA_4 hydrolase. Also formed through enzymatic metabolism of LTA_4 is the cysteinyl leukotriene (cysLT) LTE_4 or 5(*S*)-hydroxy-6(*R*)-(*S*-cysteinyl)-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid. Formed from LTD_4 through the action of dipeptidase (*26*), LTE_4 was chosen for testing because of the relatively small size of its conjugated peptide moiety. However, LTE_4 failed to mediate activation of AHR signaling when screened via a bioassay in the human-derived HepG2 40/6 reporter cell line, while LTB_4 treatment yielded only a minor level of AHR-driven transcriptional activity (Figure 2A). To be thorough, all available cysLT's, including LTC_4, LTD_4, LTE_4, and 11-*trans*-LTE_4, were examined for their ability to bind and activate the AHR via DNA electrophoretic mobility shift assays, but none of these peptide-conjugated leukotriene metabolites demonstrated any ability to activate the receptor (data not shown).

All-trans Isomers of LTB_4 Activate the AHR in Cell-Based Activity Assays. Having identified minimal AHR-mediated activity among the common enzymatic metabolites of LTA_4, we analyzed various nonenzymatically formed DiHETEs for the potential to activate the AHR. 6-*trans*-LTB_4 and 6-*trans*-12-*epi*-LTB_4, a pair of C-12 stereoisomers produced through nonenzymatic hydrolysis of LTA_4, demonstrated the ability to induce DRE-driven luciferase reporter gene activity in a dose-dependent manner when administered to the HepG2 40/6 reporter cell line. Although both of these nonenzymatically formed isomers of LTB_4 resulted in significant induction of AHR activity, the effect seen with 6-*trans*-LTB_4 [5(*S*),12(*R*)-dihydroxy-6(*E*),8(*E*),10(*E*),14(*E*)-eicosatetraenoic acid] was more pronounced. This isomer produced a 3-fold induction in reporter gene activity at 1 μM and a maximum 27-fold induction in response to 25 μM treatment, approximately 75% of the AHR induction observed with 1 nM TCDD (Figure 2B). Treatment with 6-*trans*-12-*epi*-LTB_4, an identical molecule except for the opposite stereochemical orientation of the C-12 hydroxyl group, produced a less robust response (Figure 2B). Although these molecules are considered to form primarily as the result of nonenzymatic metabolism of LTA_4, it is important to mention that 6-*trans*-LTB_4 can also be produced through at least two additional biochemical pathways. Although the physiological relevance is not clear, oxidative decomposition of cysteinyl leukotrienes in the presence of myeloperoxidase and hypochlorous acid can also result in the formation of 6-*trans*-LTB_4 (*27*). Furthermore, a cellular factor with double bond isomerase activity has been identified in rat kidney homogenates and shown to be capable of enzymatically converting LTB_4 into 6-*trans*-LTB_4 (*28*).

All-trans Isomers of LTB_4 Fail To Directly Transform the AHR to Its DRE-Binding Form. Despite their ability to activate the AHR pathway in cell culture, neither of the 6-*trans*-LTB_4 isomers was able to induce significant DRE binding activity when analyzed by and EMSA and compared with 20 nM TCDD (Figure 2C,D). Overexposure of the autoradiogram revealed only a minimal amount of heterodimer complex that formed in response to 6-*trans*-LTB_4 and 6-*trans*-12-*epi*-LTB_4 treatment at both 10 and 25 μM. Because neither isomer induced any significant level of AHR transformation into a DNA-binding form in vitro, it was

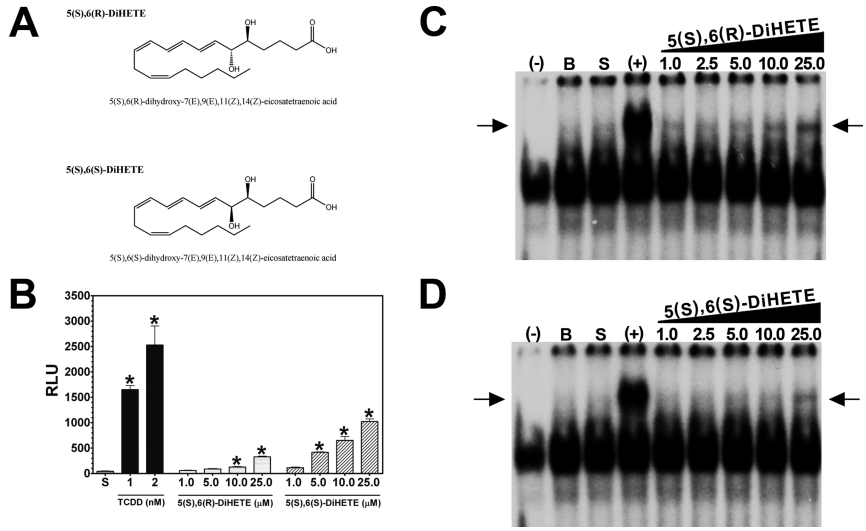

FIGURE 3: 5,6-DiHETE epimers activate the AHR. (A) The structures of both 5,6-DiHETE isomers are depicted. (B) Treatment of the HepG2 40/6 reporter cell line with 5(*S*),6(*R*)-DiHETE or 5(*S*),6(*S*)-DiHETE. The solvent (S) control is a negative control comprised of vehicle (DMSO) treatment only. Values are presented as relative luciferase units and have been normalized to protein concentration. Each data point represents the mean ± standard deviation of three separate determinations. Statistical analysis of treatments was performed using Dunnett's multiple-comparison test ($\alpha = 0.05$). Comparisons of TCDD treatments with control were made in a manner independent of DiHETE treatments. Values determined as being statistically significant from solvent (S) control are indicated by the presence of an asterisk. (C and D) The 5,6-DiHETE isomers were tested for their ability to activate the AHR to its DNA-binding form in a dose-dependent manner by EMSAs. Gel shift controls included 20 nM TCDD (+), a negative (−) control containing no ARNT, a background (B) control comprised of only AHR and ARNT, and a solvent (S) control for assessing the effects of vehicle on heterodimer formation.

concluded these compounds may be activating the AHR through an indirect mechanism rather than functioning as a ligand for the receptor. However, it is possible that within the cellular context these lipids may be capable of transforming the AHR−ARNT heterodimer more efficiently.

5,6-DiHETE Epimers Activate AHR Signaling in Cell-Based Reporter Assays. The 5,6-DiHETEs are a pair of dihydroxy metabolites, epimeric at C-6, that are produced through metabolism of LTA_4 (Figure 3A) (*11*). 5(*S*),6(*R*)-Dihydroxy-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid [5(*S*),6(*R*)-DiHETE] is a molecule which can be formed through multiple pathways, including the enzymatic transformation of LTA_4 by liver cytosolic epoxide hydrolase (*29*). Alternatively, nonenzymatic hydrolysis of the unstable epoxide ring contained in LTA_4 can also result in 5(*S*),6(*R*)-DiHETE. Furthermore, 5(*S*),6(*R*)-DiHETE may also result from 5(*S*)-HETE, through the 6(*R*)-oxygenase activity contained in enzymes such as porcine leukocyte 5-LOX (*30*). Alternatively, its C-6 epimer, 5(*S*),6(*S*)-dihydroxy-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid [5(*S*),6(*S*)-DiHETE], is a molecule known only to be formed through nonenzymatic hydrolysis of LTA_4 (*12*). When tested for AHR activity using the human-derived HepG2 40/6 reporter cell line, both 5(*S*),6(*R*)-DiHETE and 5(*S*),6(*S*)-DiHETE demonstrated the ability to induce AHR-mediated transcriptional activity (Figure 3B). Although the response observed with 5(*S*),6(*R*)-DiHETE was greater; treatment with increasing concentrations of either stereoisomer resulted in the dose-dependent activation of AHR-mediated transcription. However, it is important to note that, while AHR activation with both 5,6-DiHETE epimers was routinely observed, activation potential was not always consistent among epimers.

5,6-DiHETE Epimers Induce Transformation of the AHR to the DNA-Binding Form. EMSA analysis was utilized to assess the ability of 5,6-DiHETE isomers to directly form the AHR to its DNA-binding species. The formation of [^{32}P]DRE-AHR−ARNT complexes in response to 5,6-DiHETE treatments was examined and revealed that both 5,6-DiHETE isomers were capable of inducing DRE binding in a dose-dependent manner (Figure 3C,D).

5,6-DiHETE Epimers Compete for AHR Occupancy. To further analyze the ability of the 5,6-DiHETE isomers to bind the AHR, ligand competition binding experiments were performed. Only a minimal displacement of the radiolabeled dioxin analogue could be seen in response to increasing concentrations of 5(*S*),6(*R*)-DiHETE, while the 5(*S*),6(*S*) epimer demonstrated a greater potential to displace the photoaffinity ligand (Figure 4A,B). Racemic 5-HETE served as a negative control, and it demonstrated no significant ability to displace radioligand. A graphical representation of the ligand competition binding data is presented (Figure 4C,D). Quantitation of the binding data indicates an approximately 50% displacement of the photoaffinity ligand by 5(*S*),6(*S*)-DiHETE at the highest concentration that was examined.

Aged[2] *Preparations of 5(S),6(S)-DiHETE Demonstrate an Improved Ability To Activate the AHR in Biological Activity Assays.* Preparations of 5,6-DiHETE, especially the 5(*S*),6(*S*) epimer, experience a time-dependent increase in biological activity when stored for prolonged periods of time under argon at −80 °C. Treatment of the HepG2 40/6 reporter cell line with increasing concentrations of aged preparations of 5(*S*),6(*S*)-DiHETE resulted in a significantly enhanced dose-dependent activation of AHR transcriptional activity. For example, treatment with 10 μM "aged" 5(*S*),6(*S*)-DiHETE produced reporter gene activity which exceeded that observed with 1 nM TCDD (Figure 5). Compared with results obtained in a similar experiment using a freshly purchased preparation

[2] An aged DiHETE preparation is defined as having been stored under argon gas sparge at −80 °C for ~6 months or more before being used, as opposed to a fresh sample which was used immediately.

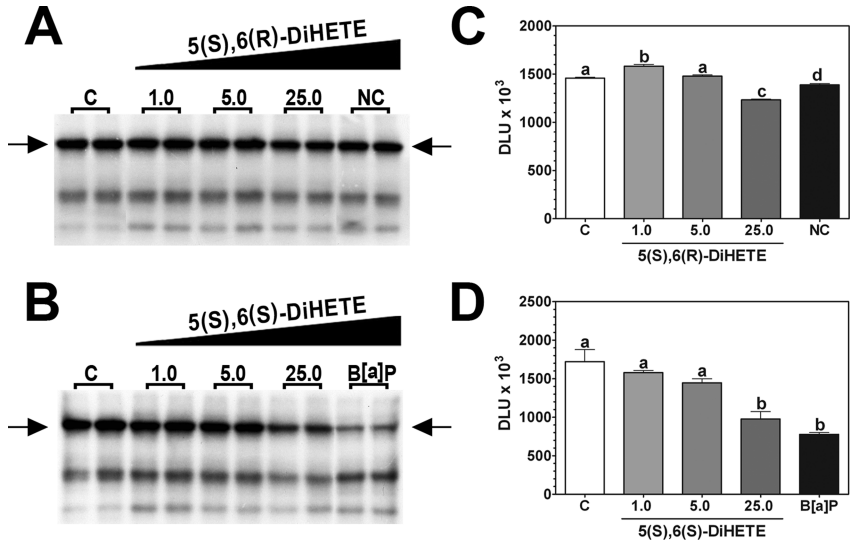

FIGURE 4: Epimer of 5,6-DiHETE that is capable of directly binding the AHR. (A and B) Ability of 5,6-DiHETE isomers to directly compete with an AHR radioactive photoaffinity ligand for AHR ligand binding sites. The AHR−ligand complex is marked with an arrow. Racemic 5-HETE, a negative control (NC), demonstrated no significant ability to displace radioligand at a concentration of 25 μM, while 300 nM benzo[*a*]pyrene (B[a]P) served as the positive control. The solvent (C) control represents vehicle (DMSO) treatment of Ah receptor. (C and D) Quantitative representation of the binding data. Statistical analysis of data was performed using Tukey's multiple-comparison test ($\alpha = 0.05$). Values determined as being statistically significant from each other are denoted with different letters.

of 5(*S*),6(*S*)-DiHETE (Figure 3B), where a 10 μM treatment produced an induction that was only 40% of that seen with 1 nM TCDD, the results obtained here demonstrate the significant increase in biological activity that is gained among aged preparations of 5(*S*),6(*S*)-DiHETE.

Aged Preparations of 5,6-DiHETE Exhibit an Enhanced Ability To Generate the DNA-Binding Form of the AHR. The ability of the 5(*S*),6(*S*)-DiHETE stereoisomer to acquire an enhanced binding affinity for the AHR over time was tested using DNA mobility shift assays. The formation of [^{32}P]DRE-AHR−ARNT complexes in response to treatment with both preparations of 5(*S*),6(*S*)-DiHETE was examined, revealing a significant enhancement in the AHR transformation and DRE binding capability observed with aged 5(*S*),6(*S*)-DiHETE (Figure 5B), compared with fresh 5(*S*),6(*S*)-DiHETE (Figure 3D). Furthermore, the enhancement in AHR activity was observed multiple times over the course of the study using several different DiHETE sample preparations. Interestingly, the same effect could also be expected to occur in a much shorter time by storing the sample sealed under an inert atmosphere at room temperature for several weeks (data not shown).

The Geometric Isomer of 5(S),6(R)-DiHETE Demonstrates Significant AHR-Mediated Transcriptional Activity. Collectively, the observations made with 5,6-LTA$_4$ together with those made with aged preparations of 5,6-DiHETE suggested the possible formation of a geometric isomer in these preparations as potentially being responsible for the observed increase in activity. In support of this rationale is the fact that certain commercial preparations of LTA$_4$ are listed as containing 3−5% of the 11-*trans*-5,6-LTA$_4$ isomer. Furthermore, a slow temperature-dependent isomerization of the C-11 double bond has been observed with such compounds as LTC$_4$, LTD$_4$, LTE$_4$, and 5,6-DiHETEs during low-temperature storage. In addition, the C-11 *trans* isomer of several common leukotrienes and DiHETEs, including 11-*trans*-LTC$_4$, 11-*trans*-LTD$_4$, 11-*trans*-LTE$_4$, and 11-*trans*-5(S),6(R)-DiHETE, have been shown to form in vivo (*31*–*33*). Thus, isomerization of the C-11 double bond in the stereoisomers of 5,6-DiHETE may be responsible for the increase in activity associated with the storage of these molecules. However, with neither of the 11-*trans*-5,6-DiHETE epimers readily available, chemical synthesis was performed and the 11-*trans*-5(*S*),6(*R*)-DiHETE epimer was chosen because its formation in vivo had already been demonstrated (*33*). Using the murine-derived Hepa-1.1 reporter cell line, a direct comparison of the biological activity contained in each of C-11 geometric isomers of 5(*S*),6(*R*)-DiHETE was performed. Under identical experimental conditions, treatment with an increasing concentration of 11-*trans*-5(*S*),6(*R*)-DiHETE resulted in an enhanced dose-dependent activation of AHR signaling, when compared with its C-11 *cis* double bond isomer (Figure 6). While neither isomer produced a strong induction in DRE-driven luciferase reporter gene activity, a significant improvement was achieved solely through isomerization of the C-11 double bond (Figure 6B). This alone provides an important piece of structure−activity relationship information about the compound and potential eicosanoid ligands for the AHR in general, and although the change may appear relatively minor, it has a pronounced effect on the conformation of the molecule (Figure 6A). Double bond isomerization at C-11 should, in theory, allow the olefinic region of the molecule to adopt an altered structural conformation, one more rectangular in shape, which could potentially allow for an increase in the level of AHR binding. Finally, the ability of DiHETE isomers to alter transcription of AHR target gene CYP1A1 was tested in human HaCaT cells. All three DiHETE isomers produced an 8−10-fold induction in CYP1A1 mRNA levels following a 4 h exposure to 4 μM DiHETE (Figure 6C).

All-trans-5(S),6(R)-DiHETE Exhibits Significant Binding Affinity for the AHR. Ligand competition binding experiments confirm that the all-*trans* geometric isomer of 5(*S*),6(*R*)-DiHETE exhibits an increased binding affinity for the AHR relative to the 11-*cis* isomer. Compared with 5(*S*),6(*R*)-DiHETE, which produced minimal radioligand competition

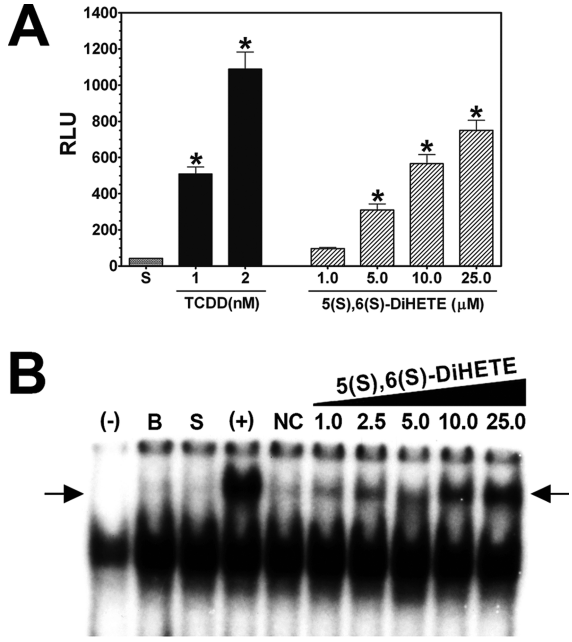

FIGURE 5: Aged preparations of 5(*S*),6(*S*)-DiHETE demonstrate an improved ability to activate the AHR. (A) 5(*S*),6(*S*)-DiHETE preparations experience a time-dependent gain in AHR-mediated transcriptional activity in Hep G2 40/6 reporter cells when stored for prolonged periods of time under argon sparge at −80 °C. The solvent (S) control is a negative control comprised of vehicle (DMSO) treatment only. Values are presented as relative luciferase units and have been normalized to protein concentration. Each data point represents the mean ± standard deviation of three separate determinations. Statistical analysis of treatments was performed using Dunnett's multiple-comparison test ($\alpha = 0.05$). Comparisons of TCDD treatments with control were made in a manner independent of DiHETE treatments. Values determined as being statistically significant from solvent (S) control are indicated by the presence of an asterisk. (B) Ability of aged 5(*S*),6(*S*)-DiHETE to mediate AHR DNA binding in EMSAs. The AHR complex is marked with an arrow. EMSA controls included 20 nM TCDD (+), a negative (−) control containing no ARNT, a background (B) control comprised of only AHR and ARNT which control for background heterodimerization, and a solvent (S) control for assessing the effects of vehicle on heterodimer formation.

(Figure 7A), increasing concentrations of 11-*trans*-5(*S*),6(*R*)-DiHETE produced a more potent displacement, resulting in an ∼70% reduction in photoaffinity ligand occupancy in response to 25 μM treatment (Figure 7B). Meanwhile, 5-oxo-ETE, serving as a negative control, demonstrated no significant ability to displace radioligand. A graphical representation of the ligand competition binding data is presented in Figure 7C,D. The relative ability of 11-*trans*-5(*S*),6(*R*)-DiHETE and 5(*S*),6(*R*)-DiHETE to activate the human AHR to a DNA-binding species was examined using EMSA analysis, where each isomer exhibited the same relative level of activity (Figure 7E).

DISCUSSION

Upon activation, 5-LOX translocates to the nuclear membrane where it interacts with a transmembrane protein known as FLAP (5-LOX-activating protein) producing 5,6-LTA_4 in a perinuclear cellular location (*34, 35*). The results presented in this report demonstrate that certain 5-lipoxygenase products, in particular, 5,6-dihydroxyeicosatetraenoic acid metabolites of 5,6-leukotriene A_4, are capable of activating the AHR. Interestingly, it has been established that the AHR contains both a functional nuclear localization sequence and nuclear export sequence, allowing the unliganded AHR−hsp90 complex to actively undergo nucleocytoplasmic shuttling (*36, 37*). This behavior is also consistent with the perinuclear AHR localization observed during immunohistochemical studies (*38*). These observations suggest that the AHR and activated 5-LOX would exist in the proximity of each other among those cells coexpressing both pathways, such as neutrophils or macrophages. Thus, it is conceivable that localized high concentrations of 5-LOX products could activate the AHR, as depicted in Figure 8. Nonetheless, it remains to be determined whether activation of the AHR in the proposed scenario contributes to inflammatory signaling or, conversely, functions in the programming of cellular events leading to the resolution of inflammation. Studies in the literature can be found supporting both viewpoints with the data suggesting the answer may be quite context-specific (*39*). However, several recent studies firmly support the concept of AHR activation suppressing the expression of certain inflammatory signaling molecules, including IL-6 (*40, 41*). Furthermore, studies in our laboratory (unpublished data) and those of others have shown that expression of *socs2* and *socs3* can be enhanced upon AHR activation (*42, 43*). These proteins function as part of a negative feedback mechanism preventing an excessive cellular response to cytokines and growth factors by repressing signaling through the JAK/STAT pathway, an important cellular signaling cascade generally believed to promote inflammation. A direct comparison of splenic dendritic cells derived from 5-LOX- and AHR-deficient mice revealed impaired induction of *socs2* expression upon exposure to lipoxin A4, suggesting a link between 5-LOX activity and AHR activation in this cell type (*42*). In addition, a link between *CYP1a1/1a2/1b1* expression (genes regulated by the AHR) and a lack of induction of *socs2* expression upon immune stimulation has also been observed (*44*). Interestingly, two recent studies have characterized distinct chemicals identified in antiallergic drug screens that both mediate their effects through the AHR (*41, 45*). Clearly, further studies are needed to assess whether 5-LOX products can mediate similar effects through the AHR.

The identification of bioactive lipid mediators as endogenous ligands and activators of the AHR not only provides additional insight into the potential physiological role(s) for this orphan cellular receptor but also may allow for a better understanding of the toxicity associated with chronic AHR activation. Although the results presented here firmly establish the ability of these lipoxygenase-derived metabolites to function as AHR ligands, it may be argued that the overall concentrations needed to produce a substantial level of AHR-mediated transcriptional activity are higher than those normally achieved under physiological conditions. However, one must consider the very polar nature of these dihydroxy organic acids and their inefficient cellular uptake compared with much more hydrophobic AHR ligands like TCDD, which can efficiently translocate across cellular membranes. Therefore, in actuality, the concentration of DiHETE molecules reaching the cytoplasm of the cell is expected to be far lower than the concentration achieved in the culture medium (*46*). Direct comparison of the activity observed using an endogenous lipid mediator for the AHR with TCDD-mediated gene induction results in a biased compari-

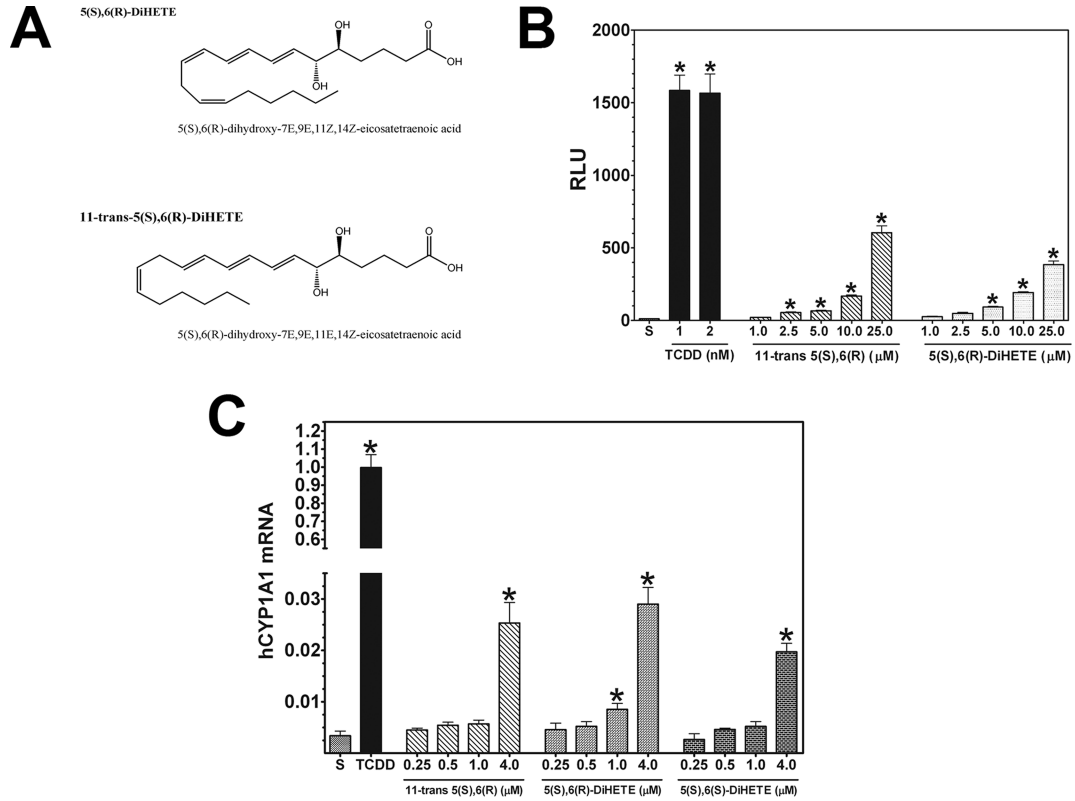

FIGURE 6: All-*trans*-5(*S*),6(*R*)-DiHETE is capable of activating the AHR. (A) The structures of both 5,6-DiHETE isomers are depicted. (B) The Hepa-1.1 reporter cell line was treated with either 11-*trans*-5(*S*),6(*R*)-DiHETE or 5(*S*),6(*R*)-DiHETE and luciferase activity measured. The solvent control (S) is a negative control comprised of vehicle (DMSO) treatment only. Values are presented as relative luciferase units and have been normalized to protein concentration. (C) The ability of three 5,6-DiHETE isomers to enhance *CYP1A1* mRNA levels in HaCaT cells was assessed by real-time PCR. Each data point represents the mean ± standard deviation of three separate determinations. Statistical analysis of treatments was performed using Dunnett's multiple-comparison test ($\alpha = 0.05$). Comparisons of TCDD treatments with control were made in a manner independent of DiHETE treatments. Values determined as being statistically significant from solvent (S) control are indicated by the presence of an asterisk.

son for several reasons. In addition to uptake issues, it is also unlikely that an endogenous activator would need to produce gene induction of a magnitude equivalent to that seen with TCDD, the most potent of the HAHs. Furthermore, it is likely that the extremely high levels of gene induction seen in response to TCDD exposure contribute to the toxicity associated with this compound. Likewise, biologically relevant induction of target genes occurring in response to an endogenous stimulus would probably result in a much smaller increase in the level of gene expression. The selective induction of a target gene(s) is a highly coordinated process in which an endogenous regulator precisely controls the timing, duration, and magnitude of expression levels. Often a biologically significant response requires only several-fold induction in gene expression. Also, unlike TCDD, which is highly resistant to metabolic degradation and thus continues to drive gene expression, metabolism of 5,6-DiHETE compounds would be expected to begin immediately after uptake, thus effectively reducing their cellular concentration and biological activity. Although the metabolic events responsible for the degradation of many hydroxylated polyunsaturated fatty acids is still largely unknown, termination of 5,6-DiHETE biological activity could be expected to occur via metabolic inactivation in a manner similar to that of the 6-*trans* epimers of LTB4 (*47*).

Although the exact mechanism responsible for the increased activity observed among "aged" preparations of 5,6-DiHETE is still not fully understood, double bond isomerization and the formation of geometric isomers may be involved. It appears likely the time-dependent increase in activity, consistently observed among aged preparations of 5,6-DiHETE, results from formation of a highly active geometric isomer. This assumption, however, is solely based on the lack of observed spectral differences between fresh and aged preparations of compound. With no visible changes in the UV spectra for the highly active aged preparations, it is unlikely that any significant portion of molecules have undergone change. Nevertheless, the biological activity of these samples is greatly enhanced. Meticulous HPLC fractionation eventually revealed a pair of subtle differences in the peak profiles of aged preparations when compared with fresh sample (unpublished data). Distinguishing the aged samples was the presence of two minor peaks eluting ahead of the parent DiHETE compound and not present in fresh preparations, at least at detectable levels. These novel HPLC peaks continue to generate UV spectra characteristic in shape of a DiHETE molecule, but with hypsochromically shifted λ_{max} values. Compared with a λ_{max} value of 270 nm for 5,6-DiHETE in the hexane-based HPLC mobile phase, the new peaks displayed λ_{max} values of 268.8 and 264.1 nm, when analyzed in the same manner. The observed shift in λ_{max} values could potentially indicate isomerization of the conjugated triene functionality to the all-*trans* orientation or formation of a conjugated tetraene. Because these two minor peaks made up the only observable difference between aged and fresh preparations of 5,6-DiHETE, it was hypothesized

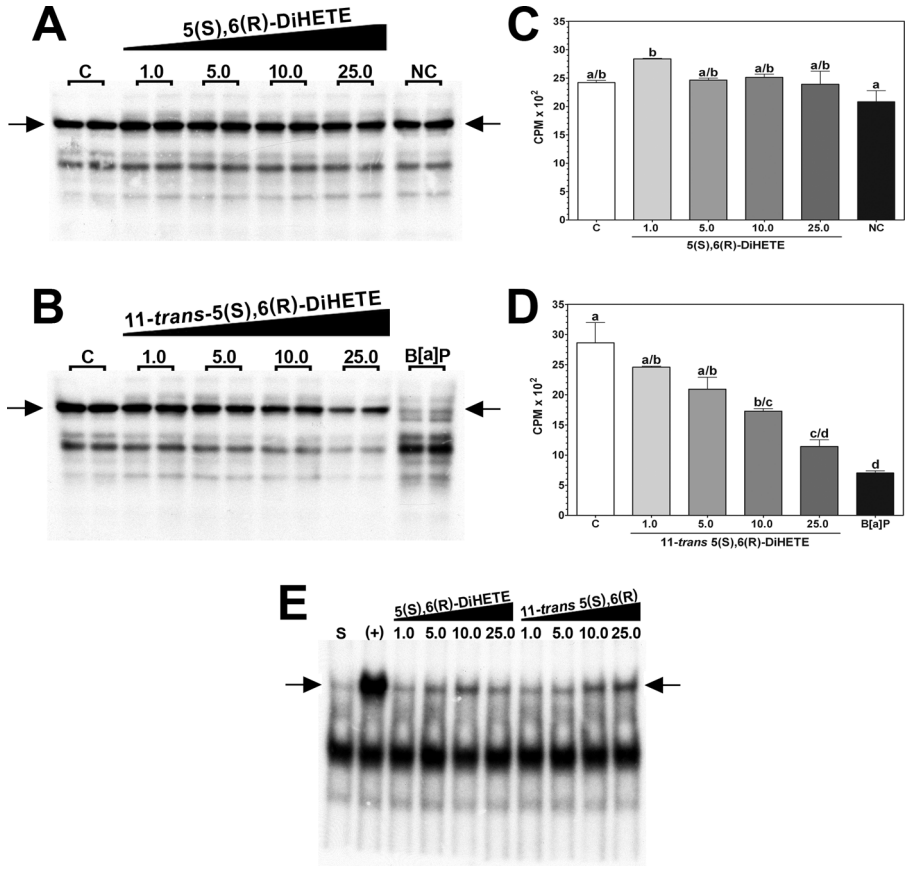

FIGURE 7: All-*trans*-5(*S*),6(*R*)-DiHETE is an AHR ligand. (A and B) Ability of 11-*trans*-5(*S*),6(*R*)-DiHETE or 5(*S*),6(*R*)-DiHETE to compete with the radiolabeled photoaffinity ligand. 5-Oxo-ETE served as a negative control (NC), while a positive control was 300 nM B[a]P. The solvent control (C) represents vehicle (DMSO) treatment of the AHR. (C and D) Quantitative representation of the ligand competition binding data. Statistical analysis of the data was performed using Tukey's multiple-comparison test ($\alpha = 0.05$). Values determined as being statistically significant from each other are denoted with different letters. (E) Ability of 11-*trans*-5(*S*),6(*R*)-DiHETE or 5(*S*),6(*R*)-DiHETE to mediate binding of the human AHR to DNA assessed by EMSAs. Gel shift controls included 20 nM TCDD (+), a negative (−) control containing no ARNT, a background (B) control comprised of only AHR and ARNT which control background heterodimerization, and a solvent (S) control for assessing the effects of vehicle on heterodimer formation.

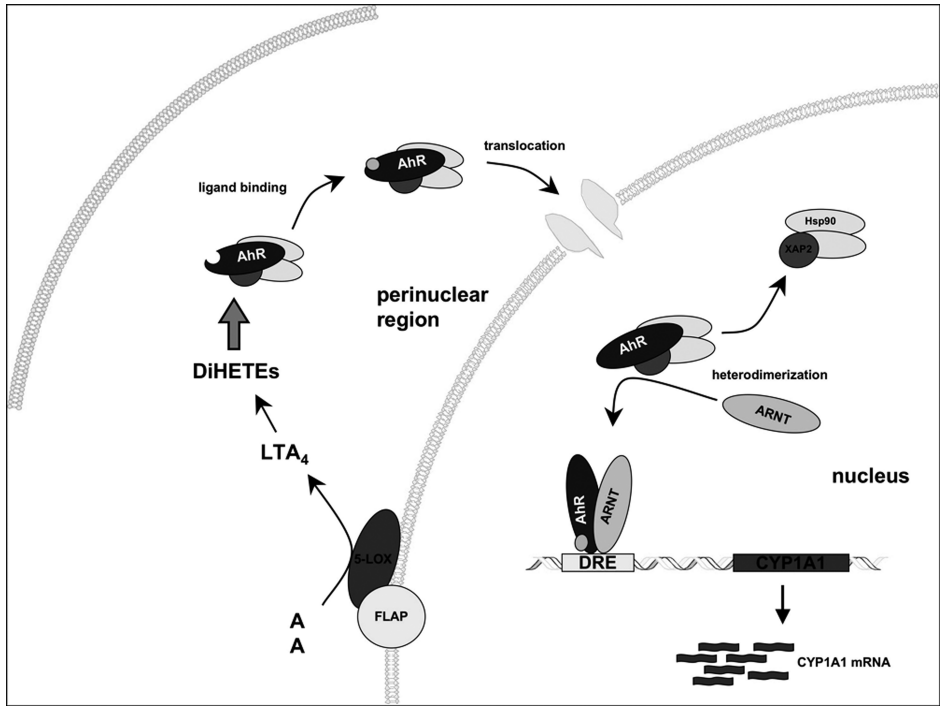

FIGURE 8: Schematic representation of the AHR and 5-LOX pathways in a cell. Upon activation, 5-LOX translocates to the nuclear membrane. The AHR undergoes nucleocytoplasmic shuttling and thus may be in the proximity of the production of 5-LOX products.

that formation of a geometric isomer was responsible for the gain in activity observed among the aged preparations.

To avoid potential issues regarding the biological relevance of the nonenzymatically formed 5(*S*),6(*S*)-DiHETE epimer, only geometric isomers of the epoxide hydrolase-formed 5(*S*),6(*R*)-DiHETE were investigated. Specifically, because its formation in vivo had been previously demonstrated, the 11-*trans* isomer of 5(*S*),6(*R*)-DiHETE was analyzed, and although it displayed improved biological activity, it still produced only modest activation of AHR signaling. However, if indeed the increase in AHR activity of aged DiHETE preparations is resulting from *cis*−*trans* double bond isomerization at C-11, then it is likely that the 11-*trans* isomer of 5(*S*),6(*S*)-DiHETE should produce the greatest level of AHR activation, considering that aged 5(*S*),6(*S*)-DiHETE preparations demonstrated the greatest gain in biological activity during storage.

Interestingly, a similar phenomenon has been observed with positional isomers of LTA_4. Depending on the source, sample preparations exhibited a great deal of variation in the amount of biological activity. For example, commercially available preparations of 5,6-LTA_4, obtained from BIOMOL, consistently demonstrated significant AHR activity even after further purification in our laboratory, while Cayman Chemical Co. preparations exhibited little or no activity. However, samples of Cayman 5,6-LTA_4 stored for extended periods of time at −80 °C under argon sparge demonstrated significant AHR activity upon subsequent EMSA analysis (data not shown). In addition, if the inactive Cayman 5,6-LTA_4 sample preparations were stored at room temperature under argon gas sparge for several days, they acquired the ability to bind and activate the AHR (data not shown), while still retaining the characteristic LTA_4 spectra. Thus, the presence of a highly active geometric isomer may explain the differences in activity observed among different sources of 5,6-LTA_4, while the formation of such an isomer could also explain the relatively rapid gain in activity when inactive LTA_4 preparations are allowed to stand at room temperature under an inert atmosphere.

Conversely, there is still the possibility that a small amount of oxidized, degraded, or rearranged product is responsible for producing the increased AHR activity observed with aged DiHETE samples, which seems unlikely since oxidation and oxidative degradation are minimized in samples stored under an inert atmosphere at −80 °C. Furthermore, protection from light during the storage of samples should minimize rearrangement events. Nevertheless, additional studies are needed to explain the increased activity of the aged DiHETE and LTA_4 preparations.

An important issue to consider in determining the overall importance of DiHETE binding to the AHR is the possible limitations of the assay systems used to assess AHR-mediated transcriptional activity. All three assays used here rely on obtaining a DRE-driven response. However, there are transcriptional responses mediated by the AHR that appear to be gene- and ligand-specific. For example, the gene paraoxonase I is directly regulated by the AHR through binding to a DRE-like element (*48*). Interestingly, transcriptional activation of this gene is not efficiently mediated by TCDD when compared with relatively weak AHR ligands, such as quercetin. Another activity that the AHR exhibits is the ability to repress IL-6 in monomac1 cells (*41*). Whether AHR-mediated gene repression activities also will exhibit ligand selectivity needs to be explored. Thus, it remains to be determined whether 5-LOX products that are capable of activating the AHR can mediate a selective transcriptional response. Overall activation of AHR signaling by pro-inflammatory bioactive lipids could become an issue of significant medical importance, especially considering the number of disease conditions that result from, or are intensified by, a state of chronic inflammation, such as atherosclerosis (*49*), Alzheimer's disease (*50*), and many different autoimmune disorders. Future studies should be directed at determining whether AHR activation during inflammation leads to enhanced or repressed inflammatory signaling and whether this response can be selectively modulated.

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