8-hydroxyhexadecatrienoic acid, a predominant metabolite of 12-HETE, is an endogenous ligand for the Ah receptor

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Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated basic helix-loop-helix (bHLH) transcription factor expressed in most cell types. Ligand binding transforms the receptor, resulting in its dissociation and subsequent translocation into the nucleus. In the nucleus the ligand activated AhR can dimerize with ARNT (aryl hydrocarbon receptor nuclear translocator) forming a high affinity complex capable of binding specific DNA sequences known as dioxin responsive elements (DRE), in the regulatory region of responsive genes[1]. Genes transcriptionally regulated by the AhR are primarily involved in foreign chemical metabolism and include the xenobiotic metabolizing cytochrome P450 enzymes from the 1A and 1B families[2].

Halogenated aromatic hydrocarbons (HAH) and polycyclic aromatic hydrocarbons (PAH) are two of the most potent classes of AhR ligands known and include carcinogenic compounds such as polychlorinated dibenzo-p-dioxins and benzo[a]pyrene[3,4]. Nevertheless, a key question that remains unresolved is the identity of true endogenous ligands of AhR activity. Evidence supporting the existence of such endogenous ligands has been accumulating and strong evidence supporting a physiological role for the receptor has been derived from studies with AhR knockout mice. These mice displayed multiple hepatic defects, including a decrease in liver size and weight[5]. Other aberrations included compromised immune system function[5], reproductive defects[6] and persistent neonatal vascular structures[7]. In addition mice harboring AhR null alleles exhibited decreased constitutive expression of the xenobiotic metabolizing enzyme cytochrome P4501A2, along with a complete loss of cytochrome P4501A1 induction, normally seen in response to dioxin exposure[7]. Taken together, these observations provide support for the existence of a high affinity endogenous AhR ligand(s) and it is conceivable that the AhR plays an important biological role(s) in normal cellular metabolism with one or more high affinity endogenous ligand existing to modulate the timing, duration and magnitude of its function in the cell. Elucidating the structure of such a ligand(s) would enable a precise determination of a biological role for this enigmatic orphan receptor.

Materials and Methods

Chemicals and Enzymes 8-hydroxyhexadecatrienoic acid (tetranor 12(R)-HETE) was purchased from BIOMOL (Plymouth Meeting, PA).

Cell Lines and Cell Culture The HepG2 40/6 reporter cell line[13] was generated in our lab while the Hepa1.1 reporter cell line was a gift from Dr. M.S. Denison. Trypsin-EDTA, PBS, a-MEM, penicillin, and streptomycin were obtained from Sigma (St. Louis, MO). Both cell lines were grown in a- modified minimal essential media (a-MEM) supplemented with 10% fetal bovine serum (v/v), 100IU/ml penicillin, and 0.1 mg/ml streptomycin at 37 ^{oC} in a humidified atmosphere containing 5% CO_2 / 94% room air. Clonal selection of reporter cell lines was maintained through the use of 300 µg/ml of G418 (GibcoBRL, Carlsbad, CA).

Luciferase and BCA Assays HepG2 40/6 cells or Hepa1.1 cells were treated in 24-well tissue culture plates (Falcon,) with an increasing amount of tetranor 12(R)-HETE for 6 h. Upon completion of the dosing cells were rinsed with PBS followed by the addition of 1X cell culture lysis buffer (2 mM CDTA, 2 mM DTT, 10% Glycerol, 1% Triton X-100). Lysates were centrifuged at 18,000 x g for 15 min. and the resulting cytosol was assayed for luciferase activity using the Promega luciferase assay system as specified by the manufacturer. Cytosolic protein concentration was determined using the bicinchoninic acid (BCA) assay. Luciferase activity was expressed relative to protein concentration.

Electrophoretic Mobility Shift Assays DRE-specific EMSAs were performed using *in-vitro* translated AhR and ARNT proteins. Expression vectors for these proteins were translated using a Promega TNT coupled transcription and translation rabbit reticulocytelysate kit. A solution of 8-hydroxyhexadecatrienoic acid was evaporated under argon gas and resolubilized in Me_2SO to achieve a stock solution of appropriate concentration. Proteins for the transformation reactions were mixed together at a 1:1 molar ratio in HEDGE buffer, followed by addition of either 0.5 µl Me_2SO solubilizedtetranor 12(R)-HETE 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 DTT, 8.3 mM EDTA, 0.125 mg/ml CHAPS, 42 ng/ul poly dI:dC). After a 15 min incubation, 200,000 cpm of ^{32}P-labeled wild type DRE was added to each reaction. Following an additional 15 min incubation, samples were mixed with 6X loading dye and electrophoresed on a 6% non-denaturing polyacrylamide gel.

Real Time PCR Real time qPCR was performed on the DNA Engine Opticon (MJ Research, Inc.) using DyNAmo Hot Start SYBR Green qPCR kit purchased from MJ Research, Inc. cDNA synthesis was carried out using High Capacity cDNA Archive Kit from Applied Biosystems. cDNA synthesized from 50 ng of total RNA was used per qPCR reaction.

Results and Discussion

Preliminary studies utilizing extracts from cells that exhibited a high level of AhR activity, suggested the possibility of endogenous bioactive lipids as mediators of the AhR. Additionally, the use of various cyclooxygenase, lipoxygenase and cytochrome P450 monooxygenase inhibitors indicated the likelihood of eicosanoids, in particular lipoxygenase metabolites, as potential regulators of the AhR. Therefore, key metabolites of the 5, 12 and 15-lipoxygenase pathways were screened for AhR activity via cell culture based assays. It was discovered that both isomers of 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) could activate the AhR pathway in cell culture. While a lipoxygenase reaction is responsible for producing 12(S)-HETE, 12(R)-HETEis produced either by a cytochrome P450 mediated reaction or by 12(R)-lipoxygenase. However, despite their ability to activate the AhR pathway in cells neither enantiomer of this lipid molecule could directly activate the AhR as determined by electrophoretic mobility shift assays (EMSA). This suggested that 12-HETE undergoes further *in-vivo* metabolism to an AhR ligand. In an attempt to identify the active molecule, various metabolites of 12-HETE were screened for AhR activity.

In most cells the metabolism of 12-HETE is limited to peroxisome-mediated β-oxidation, cytochrome P450 catalyzed ω-oxidation or further lipoxygenase metabolism. These reactions will generate chain shortened metabolites, such as tetranor 12(R)-HETE, or omega hydroxylated metabolites such as 12,20-diHETE respectively [8,9]. Stimulated nuetrophils can also convert 12-HETE into various 5,12-diHETE metabolites. In particular 12(R)-HETE can serve as a substrate for 5-lipoxygenase with the resulting product being 5(S),12(R)-dihydroxy-6(E),8(E),10(E),14(Z)-eicosatetraenoic acid (6-trans-LTB4)[10]. Conversely 12(S)-HETE can be converted into 5(S),12(S)-dihydroxy-6(Z),8(E),10(E),14(Z)-eicosatetraenoic acid, a stereoisomer of leukotriene B_4[11]. An additional metabolic pathway involving oxidation of 12-HETE to a keto intermediate (12-oxo-EET) followed by keto-reduction to the dihydro metabolite, 12-hydroxy-5,8,14-eicosatrienoic acid (12-HETrE) has been described in porcine neutrophil and bovine corneal epithelial microsomes[12]. These metabolites were screened for their ability to bind and activate the AhR and one compound emerged as a direct endogenous lipid modulator of AhR activity.

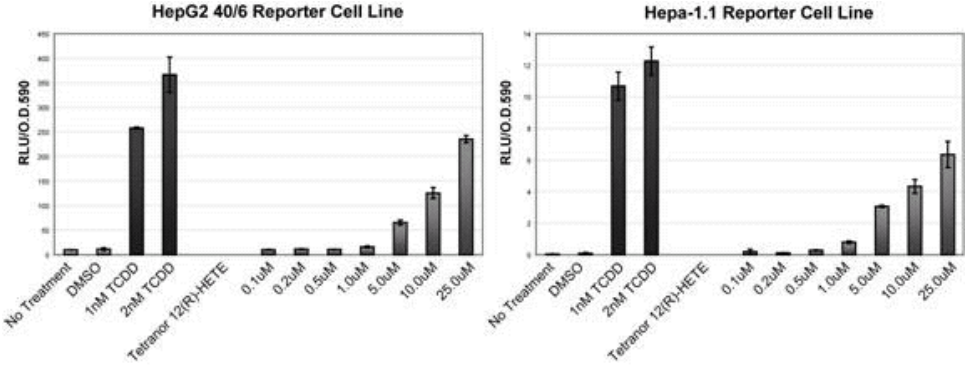
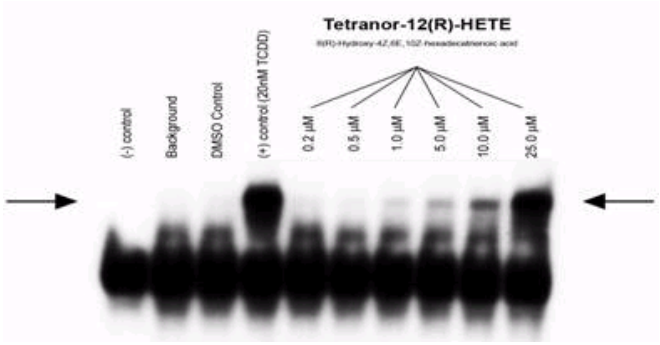


Figure 1: Biological Activity Assays: The ability of tetranor 12(R)-HETE to activate the Ah receptor was tested in two stable reporter cell lines each containing an integrated version of the pGudLuc DRE-driven luciferase reporter construct. Cells were treated with freshly prepared tetranor 12(R)-HETE at various concentrations for 6 h.

Bioassays performed in HepG2 40/6 and Hepa 1.1 cells, DRE-driven reporter cell lines of human and murine origin respectively, revealed that 8-hydroxy-6(Z),8(E),10(Z)-hexadecatrienoic acid (tetranor 12(R)-HETE) could activate the AhR in both cell lines (Fig.1). Electrophoretic mobility shift assays (EMSA) confirmed this activation to be via direct binding of tetranor 12(R)-HETE to the Ah receptor (Fig. 2). Furthermore, results from real time PCR indicated that tetranor 12(R)-HETE can enhance AhR target gene expression (e.g. CYP1A1) at nanomolar concentrations in Hepa 1 cells.

Figure 2: Electrophoretic mobility shift assay



These results clearly demonstrate thattetranor 12(R)-HETE, the predominant β-oxidation product of 12(R)-HETE, is an endogenous ligand for the Ah receptor. Formation of the tetranor metabolite from 12-HETE has been shown to occur in various tissue/cell types, including; smooth muscle cells, peritoneal macrophages and ocular tissue. Taken together these results suggest a role for the AhR in inflammatory responses.

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