Lipoxin A_4: A New Class of Ligand for the Ah Receptor

C. M. Schaldach, Jacques Riby, and Leonard F. Bjeldanes*

Division of Nutritional Sciences and Toxicology, University of California, Berkeley, California 94720-3104

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ABSTRACT: The Ah receptor is a ligand-activated transcription factor that mediates many of the biological actions of a large class of environmental compounds. Support for a role of the Ah receptor in normal physiology also has been reported, but an endogenous regulating ligand has not been identified. We have examined candidate endogenous lipophilic substances and report here the ability of the arachidonic acid metabolite, lipoxin A_4, to bind to and activate the Ah receptor in Hepa-1 cells. Lipoxin A_4 produced a concentration-dependent response in a DRE-driven CAT reporter construct, with a greater than 10-fold increase in CAT activity at 0.3 μM. Lipoxin A_4 transformed the Ah receptor to an active DRE-binding form in a concentration-dependent manner as indicated by gel mobility shift analysis. Results of Ah receptor competitive binding experiments indicated that at a concentration of 100 nM, lipoxin A_4 produced a half-maximum displacement (EC_{50}) of [^{3}H]TCDD binding. Results of Northern blot analyses indicated a transient increase in mRNA levels of the Ah receptor-responsive gene *CYP1A1*, which peaked at 4 h, consistent with the kinetics observed for lipoxin A_4-induced CYP1A1 enzyme activity. Further, lipoxin A_4 was found to be a competitive inhibitor for the CYP1A1 enzyme, with a calculated $K_i = 1.1$ μM. These results establish lipoxin A_4 as a new class of Ah receptor ligand, one that differs dramatically from classical Ah receptor ligands.

The aryl hydrocarbon receptor (AhR)[1] is a ligand-activated transcription factor and a member of the basic helix-loop-helix superfamily of DNA-binding proteins (*1*). Upon ligand binding, the AhR is transformed, dissociates from a heat shock protein dimer with which it is complexed in the cytosol, and enters the nucleus where it joins with a nuclear protein, the AhR nuclear translocator (ARNT). Interaction of this AhR/ARNT heterodimer with specific DNA sequences, called dioxin response elements (DREs), in the regulatory regions of responsive genes can result in activation of certain genes, most of which are involved primarily in xenobiotic metabolism, including CYP1A1, CYP1A2, CYP1B1, the glutathione S-transferase Ya subunit, quinone oxidoreductase, UDP-glucuronosyltransferase, and aldehyde-3-dehydrogenase (*2*−*8*).

Despite several decades of research on the function and mode of action of the AhR, the central role of the AhR in cell function remains controversial. Certainly, the most obvious effect of the AhR is to induce enzymes that can accelerate the metabolism and clearance of planar lipophilic substances, including the polycyclic aromatic hydrocarbons, which comprise a large class of environmental carcinogens. It is not clear, however, that this group of substances has occurred in the environment in the quantities and for the periods of time needed to exert the selective pressure required for the evolution of this widely occurring and highly specialized metabolic system.

In addition, persistent activation of the AhR can result in seriously deleterious biological effects. Thus, the adverse acute and chronic effects of TCDD, which include induction of teratogenesis, inhibition of gluconeogenesis, immunosuppression, vitamin A depletion, wasting, and cancer promotion, among other negative effects, are thought to arise from the unmodulated activation of the AhR pathway (*9*, *10*). Thus, the AhR apparently can affect a host of systems with no obvious relation to the xenobiotic metabolizing pathways.

Support of the notion that the AhR plays a key role in normal physiology independent of its responses to xenobiotic toxins comes from studies in AhR null mice and in cultured AhR-deficient hepatoma cells. The most consistently observed phenotypic abnormality in the two reports on AhR null mice is a drastic reduction (50%) in liver weight with increased fatty infiltration (*11*). One report also cited a high fetal mortality and severely depressed immune function (*12*). Results of studies of murine hepatoma cell mutants deficient in AhR expression indicated a prolongation of the cell cycle that was associated with a partial block in the G1 phase and morphology and biochemical characteristics of a less differentiated state (*13*).

If the AhR has important functions in normal growth and differentiation, independent of exposures to planar exogenous substances, what endogenous ligand(s) regulates its function? To explore this question, we have begun to examine candidate endogenous lipophilic substances as AhR ligands. This exploration, resulting in the discovery of LXA_4 as an endogenous ligand, was not entirely serendipitous. Over the

* To whom correspondence should be addressed. Tel: 510-642-1601. Fax: 510-642-0535.

[1] Abbreviations: AhR, aryl hydrocarbon receptor; CAT, chloramphenicol acyltransferase; CYP, cytochrome P450 protein or mRNA; DRE, dioxin response element; EROD, ethoxyresorufin *O*-deethylase; HPLC, high-performance liquid chromatography; ICZ, indolo[3,2-*b*]-carbazole; LXA_4, lipoxin A_4; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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past 30 years, it has been experimentally established that the cytochrome P450 family of enzymes metabolizes arachidonic acid, and that the resulting metabolites have important physiological roles (*14*). Sadek and Allen-Hoffmann (*15*) found that cell suspension activates the AhR, possibly through production or release of an endogenous ligand, thus suggesting the importance of the cell membrane and cell shape. Additionally, hydrodynamic shear has been found to transiently increase CYP1A1 activity, resulting in observations consistent with a mechanism in which an arachidonic acid metabolite was responsible for the CYP1A1 induction (*16*). Most recently, Puga et al. (*17*) reported the essential involvement of the AhR in cyclooxygenase-2 expression, thus establishing a role for the AhR in arachidonic acid metabolism.

We report here that lipoxin A_4, an acyclic, negatively charged, immunomodulatory metabolite of arachidonic acid is a new class of ligand for this enigmatic receptor.

EXPERIMENTAL PROCEDURES

Materials. Indolo[3,2-*b*]carbazole (ICZ) was prepared by K. Grose (University of California, Berkeley, CA) according to the procedure of Robinson (*18*). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and [^{3}H]TCDD (30 Ci/mmol) samples were obtained from B. Ames (University of California, Berkeley, CA). [γ-^{32}P]ATP (3000 Ci/mmol) and [^{3}H]-chloramphenicol (250 μCi/mmol) were purchased from DuPont NEN. Ethoxyresorufin was purchased from ICN Biochemical Co., and resorufin was from Aldrich. Lipoxin A_4 (LXA_4) was purchased from Sigma (purity 95%) and CalBioChem (purity >97%). All cell culture and molecular biological grade chemicals were purchased from Boehringer-Mannheim, Fisher, Sigma, or Life Technologies, Inc. Other compounds included lipoxin B_4, 12-hydroxyeicosatetraenoic acid, 15(*S*)-hydroxyeicosatetraenoic acid, 5(*S*),15(*S*)-dihydroxyeicosatetraenoic acid, 5(*S*),6(*S*)-dihydroxyeicosatetraenoic acid (purity 90%), leukotriene B_4, and leukotriene C_4 (all purchased from Sigma, all with purity >97% except 5,6-diHETE), and 5(*S*)-hydroperoxyeicosatrienoic acid, 5(*S*)-hydroxyeicosatetraenoic acid, 5-hydroxyeicosatetraenoic acid lactone, and leukotriene A_4 (all purchased from BioMol, all with purity >98%).

Cell Culture. The murine hepatoma cell line, Hepa-1c1c7 (Hepa-1), cells were a gift from O. Hankinson (University of California, Los Angeles, CA). A mutant derivative, c37 cells (a nonfunctional CYP1A1 mutant), was a gift from A. Puga (University of Cincinnati, Cincinnati, OH). The cells were grown as monolayers at 37 °C in 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Solutions of LXA_4 dissolved in 100% ethanol or ICZ dissolved in HPLC grade Me_2SO (DMSO) were added to the medium as a final vehicle concentration of 0.1% (v/v).

Stable Transfection of Hepa-1 Cells with DRE/CAT Reporter. The Hepa-1 cells were cotransfected with both the pcDNA 3 plasmid as a transfection marker that confers resistance to the antibiotic derivative G418 (Geneticin) and the pMcat 5.9 plasmid, which is a chimeric CAT reporter under the control of the *Cyp1a1* enhancer/murine mammary tumor virus promoter. The pMcat plasmid was a kind gift from J. P. Whitlock, Jr. (Stanford University, Palo Alto, CA). Transfection was done by the calcium phosphate coprecipitation method followed by a glycerol shock. Each 100 mm culture dish at 1:15 confluence was transfected with 10 μg of pcDNA 3 and 30 μg of pMcat 5.9. After 2 days of growth in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, cells were split 1:15 in the same medium containing G418 (500 μg/mL). Cells were re-fed every 4 days with the same selection medium for 2 weeks, and individual colonies were subcloned and tested for inducible CAT activity.

Assay of CAT Activity. We assayed chloramphenicol acyltransferase activity by the phase extraction assay (*19*). Near confluent plates ($0.8-1.0 \times 10^7$ cells) were treated with various concentrations of inducer for up to 18 h to induce CAT expression. Cells were harvested by trypsinization, resuspension in culture medium, and centrifugation. Cell pellets were resuspended in hypotonic Tris buffer, incubated at room temperature for 5 min, and recentrifuged. The cell pellets were resuspended in Tris buffer containing 0.1% Triton X-100 and incubated at room temperature for 5 min, and the lysates were centrifuged to remove nuclei. Aliquots of cell lysates were incubated at 65 °C for 10 min to inactivate inhibitors. Substrates ([^{3}H]chloramphenicol and butyryl-CoA) were added, and the reaction mixture was incubated at 37 °C for 30 min. The reactions were stopped by the addition of tetramethylpentadecane/xylenes (2:1) and vigorous vortexing. The organic phase containing the ^{3}H-butylated chloramphenicol was counted for radioactivity.

Induction and Assay of EROD Activity. Confluent Hepa-1 cells were treated with LXA_4 (0.3 μM) for the durations specified under Results, washed in phosphate-buffered saline, and harvested by trypsinization, and ethoxyresorufin *O*-deethylase (EROD) activity was determined by fluorometric assay as described previously (*20*). The production of fluorescent resorufin was recorded at 37 °C with 586 nm emission and 510 nm excitation and slit width of 20 nm using a Perkin-Elmer 650-10W spectrofluorometer (Perkin-Elmer). For inhibition experiments, cells were preincubated with ICZ and the LXA_4 competitor was added at the end of incubation for enzyme analysis. Cells were counted using a Coulter Cell Counter, and activities are expressed as pmol of resorufin/min/10^6 cells.

RNA Isolation and Analysis. Total RNA was isolated from the Hepa-1 wild-type and c37 nonfunctional CYP1A1 mutant cells by the acid guanidinium thiocyanate/phenol/chloroform procedure (*21*). RNA samples (10 μg/lane) were electrophoresed through formaldehyde-agarose gels, transferred to a nylon membrane, and probed as described elsewhere (*22*). The *Cyp1a1* cDNA in PBR322 plasmid used as a probe to detect the CYP1A1 mRNA band at 2.9 kilobases was kindly provided by D. Nebert (University of Cincinnati, OH). The GAPDH cDNA probe was kindly provided by G. Firestone (University of California, Berkeley, CA). Both probes were labeled with biotin by labeling kit (NEBlot Phototope, New England BioLabs). The nylon membrane blot was prehybridized for 2 h, hybridized overnight (0.5 mM sodium phosphate buffer, 0.1 mM EDTA, 7% SDS, 0.7% bovine serum albumin, and 65 °C), and washed, and signals were detected as described (Phototope-Star Detection, New England BioLabs). mRNA was quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Gel Mobility Retardation Assay. Hepa-1 cells were incubated with inducers for 1 h, and nuclear extracts were prepared as described previously (*23*, *24*). A complementary pair of synthetic oligonucleotides containing the sequence 5′-GATCTGGCTCTTCTCACGCAACTCCG-3′ and 5′-GATCCGGAGTTGCGTGAGAAGAGCCA-3′ (corresponding to the Ah receptor-binding site of DRE3 and designated the wild-type DRE oligonucleotide) and 5′-GATCTGGCTCTTCTCACACAACTCCG-3′ and 5′-GATCCGGAGTTGTGTGAGAAGAGCCA-3′ (identical to the wild-type DRE oligonucleotide but containing a single substitution (underlined) within the DRE core consensus sequence which eliminates binding of the transformed ligand·AhR complex and designated as the mutant DRE oligonucleotide) were synthesized, purified, annealed, and radiolabeled with [γ-^{32}P]-ATP as described previously (*25*).

Competitive Binding Assay. We assayed Ah receptor-binding affinity by the method of Gasiewicz and Neal (*26*). Protein concentration of guinea pig liver cytosol, kindly provided by M. Denison (University of California, Davis, CA), was 3 mg/mL, as measured by the Bradford assay using bovine serum albumin as the standard. Briefly, for each competitive binding assay, 2.0 mL of liver cytosol was incubated with 0.3 nM [^{3}H]TCDD in *p*-dioxane (a volume of 5 μL/ml of cytosol); unlabeled competitive ligands were added in 10 μL immediately after the addition of [^{3}H]TCDD. The samples were mixed and incubated in a 20 °C shaker water bath for 2 h. The proteins were precipitated by addition of 0.25 mL of hydroxylapatite suspension to 0.2 mL of the cytosolic mixture and incubated on ice for 30 min, vortexing every 10 min to resuspend the hydroxylapatite. The pellet was washed four times with buffer containing 1% Triton X-100, following which the final pellet was resuspended in 1.0 mL of ethanol and transferred to a scintillation vial. The tubes were washed with an additional 1.0 mL of ethanol and added to the scintillation vial, along with 10.0 mL of scintillation fluid. Nonspecific binding was determined using 100-fold excess unlabeled TCDD.

RESULTS

For our screen of cellular lipids that can activate the aryl hydrocarbon receptor (AhR) pathway, we determined the effect of substances on a dioxin response element (DRE)-driven CAT reporter gene stably transfected in Hepa-1 cells. The results (for chemical structures see Figure 1) showed that lipoxin A_4 (LXA_4) produced a concentration-dependent response in this reporter construct (Figure 2). The maximum available concentration of 0.3 μM resulted in a greater than 10-fold increase over control in CAT enzyme activity in these wild-type cells with an EC_{50} of approximately 60 nM. This result indicates that LXA_4 regulates CYP1A1 at the level of transcription as do other AhR ligands. Other lipids were tested in a concentration-dependent manner (the highest molar concentration is indicated), including lipoxin B_4 (3×10^{-7}), 12-HETE (3×10^{-7}), 5(*S*)-HETE (10^{-7}), 5(*S*)-HPETE (10^{-7}), 5-HETE lactone (2×10^{-7}), 15(*S*)-HETE (5×10^{-7}), 5(*S*),15(*S*)-DiHETE (3×10^{-7}), 5(*S*),6(*S*)-DiHETE (3×10^{-7}), leukotriene A_4 (2×10^{-7}), leukotriene B_4 (3×10^{-7}), and leukotriene C_4 (10^{-7}), but none was active. For comparison, cells were also treated with indolo[3,2-*b*]carbazole (ICZ), which resulted in a 30-fold increase over control at the highest concentration of 1 μM.

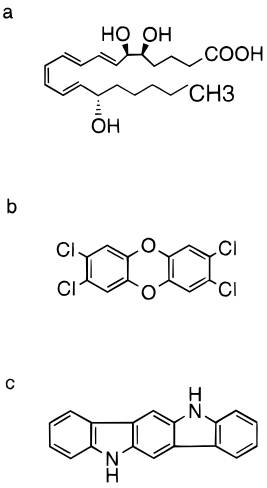


FIGURE 1: Structures of AhR ligands: (a) lipoxin A_4 (LXA_4), (b) 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD), and (c) indolo-[3,2-*b*]carbazole (ICZ).

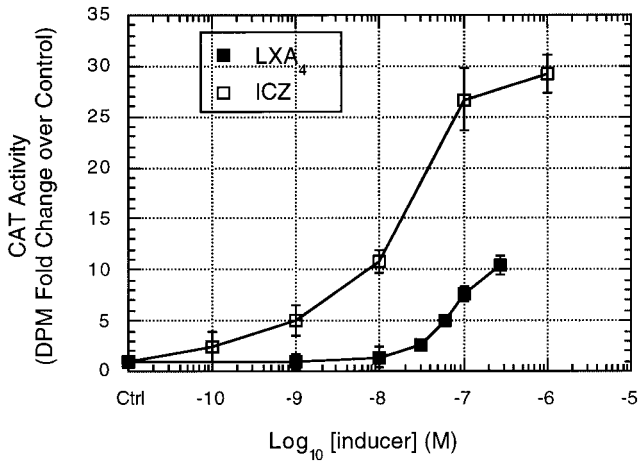


FIGURE 2: Concentration-dependent effect of LXA_4 as an inducer of DRE-driven CAT reporter gene activity in Hepa-1 cells. Cells, stably transfected with the reporter gene construct, were incubated with either LXA_4, ICZ, or vehicle for 18 h before being assayed. Values are the means ±sd of three determinations.

To examine whether LXA_4 can activate an endogenous AhR-responsive gene and to examine the kinetics of this response, we determined the kinetics of LXA_4-induced CYP1A1 enzyme activity. As indicated in Figure 3, EROD activity in wild-type Hepa-1 cells was observed to increase to a maximum at 8 h and decrease to basal levels by 48 h. In sharp contrast, ICZ-induced EROD activity reached a maximum at 8 h and remained elevated over the entire 48 h period.

To confirm the transcriptional control and to examine the possible role of CYP1A1 in LXA_4 metabolism, we determined the kinetics of LXA_4-induced CYP1A1 mRNA expression in both Hepa-1 wild-type and c37 mutant cells. c37 cells generate CYP1A1 mRNA but do not produce a functional CYP1A1 enzyme (*27*). Results of the Northern blot analyses, shown in Figure 4, indicate a transient increase in CYP1A1 mRNA in wild-type cells, consistent with the kinetics observed for EROD activity. In contrast, CYP1A1 mRNA levels in cells deficient in this enzyme activity remained elevated over the 24 h following treatment. For comparison, treatment of both cell types with ICZ, which is not a substrate for CYP1A1, resulted in CYP1A1 mRNA

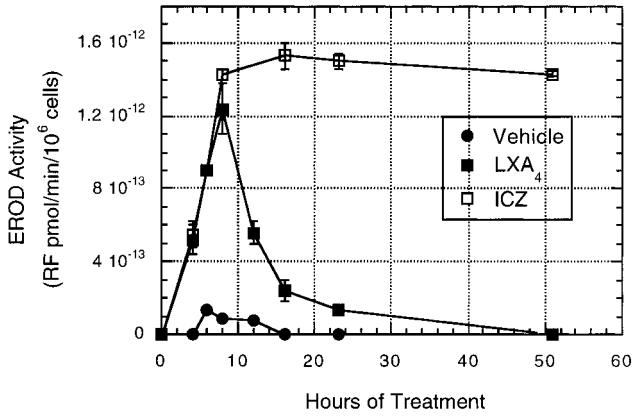
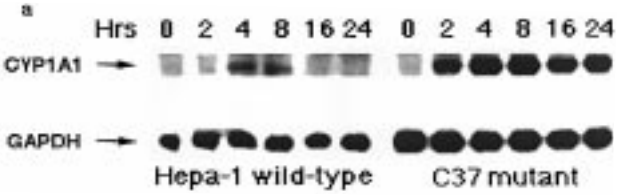


FIGURE 3: Kinetics of EROD induction by LXA_4 in Hepa-1 cells. Wild-type cells were incubated with LXA_4 (0.3 μM) or ICZ (1.0 μM) from 0 to 50 h prior to harvesting. Controls received only vehicle. Experiments were conducted three times with similar results. Values are the means ±sd of three determinations.



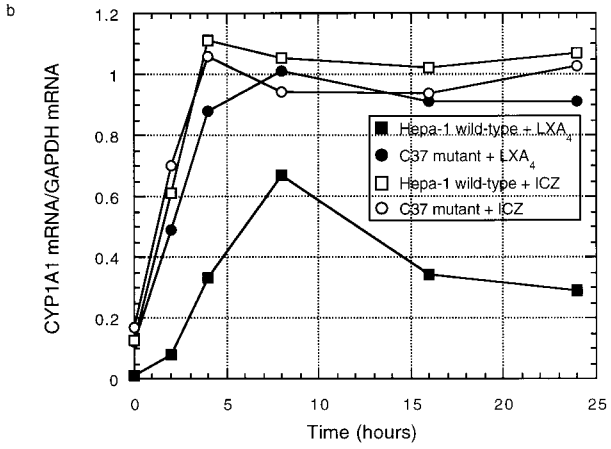


FIGURE 4: Effect of LXA_4 on the kinetics of CYP1A1 mRNA levels in Hepa-1 and c37 cells. Both wild-type Hepa-1 and c37 mutant cells were incubated with LXA_4 (0.3 μM) or ICZ (1.0 μM) from 0 to 24 h prior to RNA isolation. mRNA signals from LXA_4, as represented in a representative Northern blot (a), are normalized to the control of GAPDH signals, and data represented in panel b are the average of at least two experiments.

levels that were virtually identical over the 24 h period: mRNA levels rose to a maximal level at 4 h and remained elevated.

To investigate the ability of LXA_4 to transform the AhR to an active DRE-binding form, a key step in the activation of the *Cyp1a1* gene, we performed a gel mobility shift assay. Results shown in Figure 5 indicate a concentration-dependent AhR activation, with an EC_{50} of 50 nM. A similar AhR activation was observed with ICZ, resulting in an EC_{50} of 10 nM. No inducible protein·DNA complex was observed when 200× cold DRE was added, but the complex was seen when 200× mutant DRE was used in addition to the ^{32}P-labeled DRE sequence (data not shown). This latter result establishes the DNA-binding specificity of the LXA_4- or ICZ-

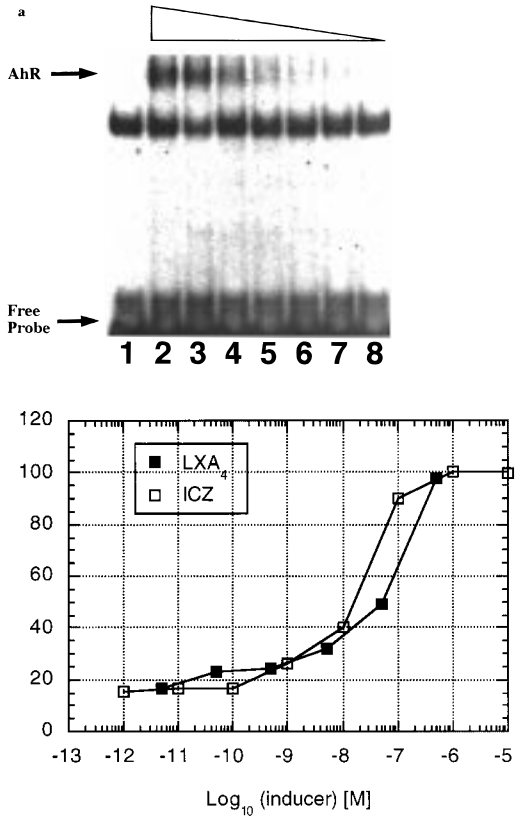


FIGURE 5: Concentration-dependent formation of the transformed Ah receptor by LXA_4. Hepa-1 cells were incubated for 1 h with vehicle or the indicated concentrations of LXA_4 or ICZ. Nuclear extracts were mixed with ^{32}P-labeled DRE3 oligonucleotide, and the formation of protein·DNA complexes was analyzed by gel retardation assay and quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA). (a) Treatments were as follows: lane 1, ethanol (negative control); lane 2, 10 μM ICZ (positive control); lane 3, 100 nM LXA_4; lane 4, 10 nM; lane 5, 1 nM; lane 6, 100 pM; lane 7, 10 pM; lane 8, 1 pM. Note the concentration-dependent AhR activation, with a resultant EC_{50} for LXA_4 at ~50 nM. The experiment was repeated twice with similar results.

activated AhR and implies that this complex represents the inducer·AhR·DRE complex.

To confirm that LXA_4 binds to the AhR and can displace an established polycyclic aromatic ligand, we conducted a competitive binding assay for this receptor. As shown in Figure 6, LXA_4 competes with [^{3}H]TCDD (0.3 nM) for AhR binding, with a concentration of 10 μM required for maximum displacement and an EC_{50} of 100 nM. This concentration agrees well with the EC_{50} values found for both AhR transformation and induction of CAT activity. Our results with 3-methylcholanthrene, another well-established AhR ligand, are consistent with literature values (*26*).

To examine further the nature of the interaction of LXA_4 with CYP1A1, we determined the inhibitory effects of LXA_4 on EROD activity. The results, included as a double-reciprocal plot in Figure 7, demonstrate that LXA_4 is a competitive inhibitor of EROD activity in Hepa-1 cells with the calculated $K_i = 1.1$ μM. This finding also suggests that the EROD activity presented in Figure 3 may be somewhat underestimated.

DISCUSSION

Our results indicate that LXA_4 is a strong ligand for the Ah receptor that can mediate the transformation of this

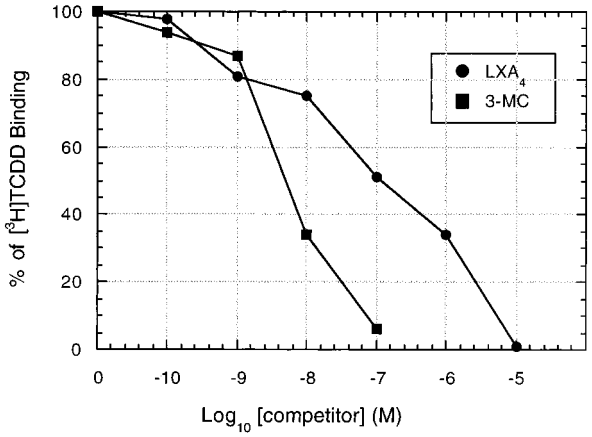


FIGURE 6: Competitive AhR binding of [^{3}H]TCDD and LXA_4. By using [^{3}H]TCDD (final concentration of 0.3 nM), we added competitive ligands and incubated them for 2 h. Proteins were precipitated by the addition of hydroxylapatite which was resuspended in ethanol and counted by liquid scintillation. Data are the average of two experiments. 3-Methylcholanthrene was used as a control ligand. Note that LXA_4 is found to have an EC_{50} of 0.1 μM.

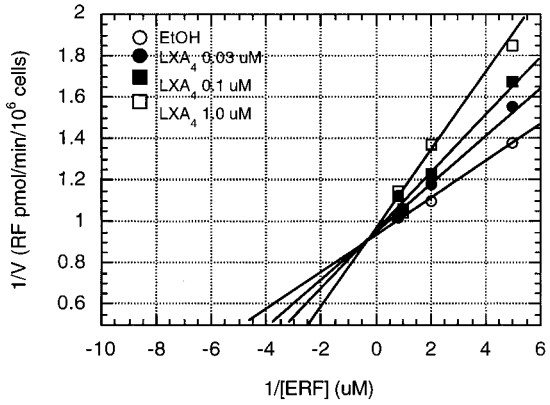


FIGURE 7: Inhibition of EROD activity by LXA_4. Wild-type Hepa-1 cells were preincubated with ICZ (0.5 μM), and LXA_4 or ethanol (control) was added prior to assay. The double-reciprocal plot suggests that LXA_4 is a competitive inhibitor of EROD activity with a calculated $K_i = 1.1$ μM.

receptor to a form that binds to the cognate DNA response element (DRE) and activates transcription of the associated gene, *CYP1A1*. In addition, we provide evidence that LXA_4 is a substrate for CYP1A1. Thus, since the expression of CYP1A1 is regulated by the activated Ah receptor, the metabolism of LXA_4 is autoregulated by this system. The autoregulated expression of CYP1A1 in response to certain other AhR ligands is well-established (*27*).

Most known AhR ligands are exogenous molecules that are neutral, planar, polycyclic substances, generally with a hydrocarbon framework. Well-established ligands include aryl hydrocarbons such as benzo[*a*]pyrene and 3-methylcholanthrene, and heterocyclic aromatic compounds such as the plant-derived product, ICZ, and, of course, the potent environmental toxin, TCDD. Compounds of this type with binding affinities for the AhR similar to that of LXA_4 include 1,2,4-trichloro-dibenzo-*p*-dioxin (*26*) and 5*H*-benzo[2,3-*a*]-carbazole (*28*). Other researchers have identified the tryptophan metabolites tryptamine and indole acetic acid (*29*) and the heme metabolite bilirubin (*30*) as more hydrophilic AhR ligands.

To our knowledge, no acyclic substance has been shown previously to exhibit appreciable affinity for the Ah receptor. LXA_4 contains neither rings nor fully planar geometry, and at physiological pH, it possesses an overall net charge of -1. Thus, our results establish LXA_4 as a new class of AhR ligand, one that differs dramatically from classical AhR ligands. That lipoxin B_4, the very close structural relative of LXA_4, exhibited no appreciable affinity for the AhR indicates very specific structural requirements for the binding of this class of ligand.

LXA_4 is an immunomodulatory molecule generated through the arachidonic acid cascade that functions in an anti-inflammatory role, eliciting responses in an autocoid manner. Lipoxins are generated in micromolar quantities by single-cell types such as activated macrophages of rainbow trout (*31*) or in nanomolar levels by transcellular biosynthetic routes documented during cell−cell interactions, in particular those observed during human neutrophil-platelet biosynthesis of eicosanoids (*32*). Our EC_{50} results in the physiological range of 100 nM make this interaction potentially relevant biologically.

Metabolism of LXA_4 in rat liver microsomes has been shown to involve an NADPH-dependent P450 monooxygenase (*33*), and using PMA-differentiated HL-60 and adherent monocytes, the NAD-dependent 15-hydroxyprostaglandin dehydrogenase has been suggested as the metabolic enzyme (*34*). Recently, purified recombinant human neutrophil LTB_4 omega-hydroxylase was shown to catalyze the omega-hydroxylation of LXA_4 (*35*). Consistent with these findings, our results demonstrate the transcriptional control of CYP1A1 expression by LXA_4 and, taken together with the results of our EROD induction experiments, provide support for CYP1A1 metabolism of LXA_4 in murine hepatoma cells. That the LXA_4-induced CYP1A1 activity was transient indicates that the effect may be autoregulated, suggesting that LXA_4 can stimulate its own metabolism, a physiologically relevant feature common to many AhR ligands (*36*). Recent detection of the mRNA for a high-affinity LXA_4 receptor in mouse liver suggests the possible generation and metabolism of the corresponding molecule there also (*37*). Although the physiological role of the AhR in LXA_4 metabolism is not established, our results are consistent with the notion that the AhR/CYP1A1 system may function under conditions of high release of LXA_4.

The possibility of a contaminant causing this effect was addressed by several techniques. Samples were obtained from two independent sources, one of which reported a 95% purity level, the other >97%. This would require an extremely high potency of a possible contaminant for activation of the AhR. Furthermore, we obtained these samples over a period of ~18 months, arguing against batch contamination. Additionally, other lipids arriving in identical packaging did not result in similar induction, thus eliminating packaging contamination. Finally, we ran UV absorption spectroscopic scans in the wavelength region of 250−400 nm on samples from both sources, finding only the characteristic LXA_4 signature of peaks at 287, 302, and 315 nm (*34*). Taken together, these considerations make unlikely the presence of a high-affinity contaminant in the LXA_4 preparations.

Several researchers have offered physical explanations for the differences in binding of molecules to the Ah receptor. Gillner et al. (*28*), on the basis of a ball-and-stick model,

suggested a geometrical connection; specifically, that all potent receptor ligands could fit into a rectangle of 6.8 × 13.7 Å. In a later work, Gillner et al. (*38*) performed quantum chemical calculations of the free energy of solvation of the relevant molecules surrounded by hundreds of explicit water molecules (but zero ionic strength). These authors believe a steric restriction is the most plausible explanation for the binding of heterocyclic ligands, although they recognized that other properties such as charge distribution and polarizability are also important and require further investigation. Others have suggested a more sophisticated electrostatic model. McKinney and Singh (*39*) have indicated that the net polarizability of the molecule and the preferential dipole distribution of the molecule accounted for differences in molecular binding. Murray et al. (*40*) suggested a recognition process involving the electrostatic potential that the molecule creates in its surrounding space. Specific characteristics of this potential, such as the drastic weakening of the oxygen negative potential in a plane above the molecule, were found to be significant. More recently, Bonati et al. (*41*) proposed that the molecular electrostatic potential (MEP) in relevant planes around the molecule is important. These authors found that the overall MEP topology, rather than simply minimum values at specific points, is involved. They also established that it was mostly the noncovalent electrostatic forces that gave rise to the binding. Most recently, Rabinowitz et al. (*42*) introduced calculations analogous to the MEP, but which take into account the influence of positive charge on the receptor, the probe interaction potential. Such positive charge had been uncovered by X-ray crystallographic measurements on the retinoic acid receptor (*43*). The positively charged probe was found to polarize the charge distribution of the ligand.

From a steric point of view, the entire hairpin-shaped lipoxin A_4 molecule does not fit into a 6.8 × 13.7 Å rectangle. However, it has been established that the most stable conformation of all biphenyls with or without ortho substituents is nonplanar and that neither planarity nor symmetry is an inherent requirement for receptor binding (*39*). Clearly there are other considerations important in controlling the binding of this ligand to the Ah receptor; neither the molecular electrostatic potential nor the free energy of solvation for LXA_4 in a physiological environment is known.

In our initial efforts to characterize the mechanism of binding of widely diverse molecules, including acyclic substances, to the Ah receptor, we have employed extensions of the calculations discussed above. The preliminary results of these studies suggest that the pocket might be modeled as an elliptic paraboloid whose surface incorporates the electrostatic charges of the various amino acids of the Ah receptor. Positive charges are included at the entrance of the binding pocket, consistent with the known crystallographic structure of the retinoic acid receptor (*43*). The binding of a great number of ligands, ring as well as hairpin structures, can be accommodated by this model.

Taken together, our results indicate that, whereas the AhR is an unusually promiscuous receptor with appreciable affinity for a fatty acid-derived substance, in addition to a large group of planar polycyclic ligands, it is also highly selective in its binding among different substances in this former class of ligands. Whether binding to this class of substance is unique to LXA_4 and plays a significant physiological role remains to be seen.

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