Novel lipid mediator aspirin-triggered lipoxin A₄ induces heme oxygenase-1 in endothelial cells

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Silva, V. Nascimento, M. A. Arruda, C. Barja-Fidalgo, C. G. Villela, and I. M. Fierro. Novel lipid mediator aspirin-triggered lipoxin A₄ induces heme oxygenase-1 in endothelial cells. Am J Physiol Cell Physiol 289: C557–C563, 2005. First published May 18, 2005; doi:10.1152/ajpcell.00045.2005.—Lipoxins (LX) and aspirin-triggered LX (ATL) are eicosanoids generated during inflammation via transcellular biosynthetic routes that elicit distinct anti-inflammatory and proresolution bioactivities, including inhibition of leukocyte-mediated injury, stimulation of macrophage clearance of apoptotic neutrophils, repression of proinflammatory cytokine production, and inhibition of cell proliferation and migration. Recently, it was reported that aspirin induces heme oxygenase-1 (HO-1) expression on endothelial cells (EC) in a COX-independent manner, what confers protection against prooxidant insults. However, the underlying mechanisms remain unclear. In this study, we investigated whether an aspirin-triggered lipoxin A₄ stable analog, 15-epi-16-(para-fluoro)phenoxy-lipoxin A₄ (ATL-1) was able to induce endothelial HO-1. Western blot analysis showed that ATL-1 increased HO-1 protein expression associated with increased mRNA levels on EC in a time- and concentration-dependent fashion. This phenomenon appears to be mediated by the activation of the G protein-coupled LX₄ receptor because pertussis toxin and Boc-2, a receptor antagonist, significantly inhibited ATL-1-induced HO-1 expression. We demonstrate that treatment of EC with ATL-1 inhibited VCAM and E-selectin expression induced by TNF-α or IL-1β. This inhibitory effect of the analog is modulated by HO-1 because it was blocked by SnPPIX, a competitive inhibitor that blocks HO-1 activity. Our results establish that ATL-1 induces HO-1 in human EC, revealing an undescribed mechanism for the anti-inflammatory activity of these lipid mediators.

signaling transduction; resolution of inflammation

LIPOXINS (LX) are endogenous lipid mediators that dampen the host response and orchestrate resolution of inflammation. In humans, three main biosynthetic pathways are described for LX formation, each involves transcellular biosynthetic use of intermediates between distinct cell types that are in close proximity during inflammatory responses (43). Monocytes, eosinophils, and airway-epithelial cells can convert arachidonate into 15-hydroxyicosatetraenoic acid (15S-HETE) by a 15-lipooxygenase (LO) catalyzed reaction. 15S-HETE is rapidly taken up by neutrophils and converted to lipoxin A₄ by a 5-LO-catalyzed reaction (43). The second pathway for lipoxin biosynthesis was determined for interactions that occur predominantly within the vasculature between 5-LO, present in myeloid cells, and 12-LO, which is present in platelets. The 5-LO product leukotriene A₄ is converted in a transcellular manner by platelet 12-LO to lipoxins (43). More recently, a third major pathway for lipoxin generation was discovered that involves aspirin and the action of cyclooxygenase (COX)-2 and 5-LO (11). Endothelial and epithelial cells express COX-2 in response to diverse stimuli such as cytokines, hypoxia, and bacterial infections. Aspirin acetylates COX-2 and switches its catalytic activity for conversion of arachidonic acid to 15R-HETE in lieu of prostanooid biosynthesis. 15R-HETE is released from endothelial and epithelial cells and transformed by leukocyte 5-LO, via transcellular routes, to 15-epimer lipoxins (aspirin-triggered lipoxins or ATL). A recent study (9) that reported that daily treatment with low dose of aspirin triggers the formation of 15-epi-LXA₄ in healthy individuals, which may account for aspirin’s anti-inflammatory actions in vivo.

Lipoxins and ATL have diverse bioactions on individual leukocytes, while inhibiting the activation of polymorphonuclear neutrophils (PMN) (12, 15, 36, 41), eosinophils (6), and lymphocytes (5), and stimulating the activation of monocytes and macrophages (30, 31). In addition, LX have been shown to modulate the activity of cells of nonmyeloid origin, including fibroblast cells (45), endothelial cells (14, 38), renal mesangial cells (34), and splenic dendritic cells (3). It is now appreciated that lipoxins, LXA₄ in particular, are potent counterregulatory signals in vitro and in vivo for endogenous proinflammatory mediators, including lipids (leukotrienes and platelet-activating factors) and cytokines (TNF-α and IL-6), resulting in inhibition of leukocyte-dependent inflammation (13).

LX exert their bioactions through distinct G protein-coupled receptors (43). However, our current understanding of the mechanism by which LXA₄ and its receptor, namely ALXR, activate anti-inflammatory signals remains incomplete. In neutrophils, for example, lipoxins do not promote a sustained mobilization of intracellular calcium or generation of cAMP. However, LXA₄ binding to its receptor triggers the activation of GTPase, phospholipase A₂, and phospholipase D, responses that are inhibited by pretreatment of the cells with pertussis toxin (PTX) (42). In addition, lipoxins are not receptor-level antagonists for inflammatory stimuli such as fMLP and LTβ₄. It may be considered that the bioactions of LX are due to the generation of either anti-inflammatory intermediates or antagonism at the receptors coupled to inflammatory responses. It is probable that a combination of both mechanisms applies.

It was recently demonstrated that aspirin, in a cultured endothelial cell lineage derived from human umbilical vein, was able to increase heme oxygenase-1 (HO-1) protein levels (22). This enzyme performs the seemingly lackluster function of catalyzing heme to generate bilirubin, carbon monoxide,
and free iron. The activity of the enzyme results in profound changes in the cells’ abilities to protect themselves against oxidative injury (35). Under normal physiological conditions, most cells express low or undetectable levels of HO-1 protein. The gene expression is highly inducible by diverse agents or conditions that increase oxidative stress and redox signaling plays a crucial role in its regulation. These stimuli include bacterial lipopolysaccharides, hypoxia, ischemia, cytokines, nitric oxide, stimuli that deplete cellular glutathione stores, and its own substrate heme (53).

Consistent with the diversity of signaling cascades involved in HO-1 induction, the promoter region of HO-1 contains a wide variety of regulatory elements (1). It includes DNA-binding sites for oxidative stress-responsive transcription factors, such as nuclear factor (NF)-κB, NF-E2-related factor 2 (Nrf2), and AP-1 (10, 26). HO-1 has been shown to have anti-inflammatory, antiapoptotic, and antiatherogenic effects (26), and a recent study by Soares et al. (44) demonstrated that the overexpression of HO-1 in cultured endothelial cells (EC) inhibits proinflammatory cytokine-induced expression of adhesion molecules associated with EC activation.

It is noteworthy, despite all of the studies focusing on the pathophysiologic implications of both LX and HO-1 pathways on the inflammatory response, that direct evidence showing the synergy between these routes remained unrevealed. A better knowledge of the convergence of these pillars of the resolution of inflammation is quite challenging and could lead to new approaches to control inflammation, particularly chronic inflammatory states.

Our group shows for the first time that 15-epi-16-parafluoro)-phenoxy-lipoxin A4 (ATL-1), an aspirin-triggered lipoxin A4 stable analog, is able to induce HO-1 expression in EC. ATL-1 induction of HO-1 gene and protein expression correlates with the downregulation of adhesion molecules, including vascular cell adhesion molecule (VCAM) and E-selectin. This phenomenon appears to be mediated by the activation of ALXR because PTX and a receptor antagonist including vascular cell adhesion molecule (VCAM) and E-

**MATERIALS AND METHODS**

**Reagents.** Hemin, HEPES, trypsin, EDTA, bovine serum albumin (BSA), PMSF, benzamidine, leupeptin, and soybean trypsin inhibitor were from Sigma-Aldrich (St. Louis, MO). Boc-2 (Boc-Phe-Leu-Phe-Leu-Phe) was obtained from Phoenix Pharmaceuticals. Tin protoporphyrin IX (SnPPix) was from Porphyrin Products (Logan, UT). Fetal calf serum (FCS) was obtained from GIBCO-BRL (Grand Island, NY). Monoclonal mouse anti-VCAM-1 antibody (Ab) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). TRIzol reagent was obtained from Invitrogen (Carlsbad, CA). Moloney murine leukemia virus reverse transcriptase, oligo (dT)15 primer, RQ1 RNase-Free DNase, the set of dNTP and RNasin RNase inhibitor were from Promega (Madison, WI). ATL-1, the stable 15-epi-Lipoxin A4 analog, was a generous gift from Brigham and Women’s Hospital (Harvard Medical School, Boston, MA).

**Cell culture.** Endothelial cells were isolated from human umbilical vein (HUVEC) as previously described (25) and cultured in medium 199 containing 20% FCS, 50 U/ml penicillin, 15 μg/ml streptomycin, 8 U/ml heparin, and 6 g/l HEPES. The umbilical vein EC cell line ECV 304 was cultured in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cultures were incubated at 37°C in humidified 5% CO2-95% air atmosphere. The medium was changed 24 h after being plated and the cells were passaged at confluence after dissociation with 0.1% 0.01% trypsin/EDTA and seeded into new culture flasks (for a maximum of three passages).

**Immunofluorescence microscopy.** HUVEC grown on gelatin-coated glass coverslips were stimulated with ATL-1 (100 nM) or heme (10 μM) in the absence or in the presence of tin protoporphyrin IX (SnPPix; 50 μM) at 37°C. After 18 h, the cells were stimulated with human rTNFα (10 ng/ml) for 6 h. The monolayers were washed twice with PBS, then immunofluorescence studies were performed as previously described (17). Briefly, the cells were fixed with 4% paraformaldehyde/4% sucrose in PBS for 20 min and blocked with 5% BSA in PBS for 30 min. The cells were then incubated with monoclonal mouse anti-VCAM-1 Ab (1:500) overnight at 4°C. Subsequently, the cells were washed three times with PBS and incubated with biotin-conjugated anti-rabbit IgG (1:50), followed by incubation with FITC-conjugated streptavidin (1:50) for 1 h at room temperature. The cells were covered with 4,6-diamidino-2-phenylindole for 1 min and washed three times with PBS. Coverslips were mounted on a slide with the use of a 20% N-propylgallate and 80% glycerol solution in PBS before examination under an microscope (model BX40 Olympus, Tokyo, Japan) equipped for epifluorescence. The images were analyzed using Photoshop software (Adobe Systems, San Jose, CA).

**Western blot analysis.** ECV304 were cultured on 24-well microtiter plates for 24 h before incubation with medium, ATL-1 (3–300 nM), heme (10 μM), or aspirin (300 nM). The cells were then washed three times with ice-cold PBS and lysed in lysis buffer (50 mM HEPES, pH 6.4, 1 mM MgCl2, 10 mM EDTA, 1% Triton X-100, 1 μg/ml DNase, 0.5 μg/ml RNase) that contained the following protease inhibitors: 1 mM PMSF, 1 mM benzamidine, 1 μM leupeptin, and 1 μM soybean trypsin inhibitor. The total protein content in the cell extracts was determined by the Bradford method (8). Cell lysates were denatured in sample buffer (50 mM Tris·HCl, pH 6.8, 1% SDS, 5% 2-ME, 10% glycerol, and 0.001% bromophenol blue) and heated in a boiling water bath for 3 min. Samples (50 μg total protein) were resolved by 12% SDS-PAGE and proteins were transfected to polyvinylidinedifluoride membranes. Rainbow markers were run in parallel to estimate molecular weights. Membranes were blocked with Tween-TBS (20 mM Tris·HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20) containing 1% BSA and probed with polyclonal anti-HO-1 Ab (1:500) overnight at 4°C. The membranes were rinsed with T-TBS and incubated for 1 h at room temperature with biotin-conjugated anti-IgG Ab (1:1,000), followed by incubation with HRP-conjugated streptavidin (1:1,000). Immunoreactive proteins were visualized by 3’,3’-diaminobenzidine staining. The bands were quantified by densitometry with the use of Image Software (Scion, Frederick, MD).

**RNA isolation and RT-PCR.** ECV304 were grown in 100-mm tissue culture dishes until they were ∼80% confluent. One hour after vehicle or ATL-1 (100 nM) or heme (10 μM) addition to the cells, total RNA from endothelial cell was isolated using TRIZol reagent. After DNase treatment (RQ1 RNase-Free DNase), the mRNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase and oligo (dT)15 primer. Primers based on the sequence of human HO-1 (GeneBank accession no. NM002133). The following primers were used to amplify HO-1 cDNA, sense, 5’-TGGAGGAGGAGATGGCGGC-3’ (262–281), and antisense, 5’-TGTTGAGCGAGAACGGTGC-3’ (692–711). The expected size of the HO-1 PCR product was 450 bp. PCR was performed with the following parameters: 95°C for 2 min for 1 cycle and 35 cycles of denaturation at 95°C for 45 s, annealing at 56°C for 1 min, and elongation at 72°C for 1 min. GAPDH primers were used to validate the cDNA in each reaction. PCR products were separated by 2% agarose gel electrophoresis and visualized by UV exposure on a transilluminator. PCR
products were obtained using a GeneAmp PCR System 2400 (Perkin Elmer).

Statistical analysis. Statistical significance was assessed by ANOVA, followed by Bonferroni’s t-test, and P < 0.05 was taken as statistically significant.

RESULTS

Effect of ATL-1 on HO-1 protein and mRNA expression on endothelial cells. Various pro- and anti-inflammatory agents promote HO-1 expression on EC. Recent work (22) revealed that aspirin, but not other anti-inflammatory drugs, is able to increase HO-1 expression on ECV304 cells. This effect could be due to the unique property of aspirin to generate ATL, endogenous mimetics of natural lipoxin A4. To address this question, we first investigated whether the treatment with ATL-1 could induce HO-1 protein expression on EC. ECV304 were pretreated for 18 h with ATL-1 (100 nM) leading to an increase on HO-1 protein as assessed by Western blot analysis, a result similar to that observed with aspirin (300 nM) (Fig. 1A). This result was confirmed by RT-PCR after treatment of the cells with ATL-1 and heme (10 μM), a known inducer of HO-1, used as a positive control (Fig. 1B).

Concentration-dependent effect of ATL-1 on HO-1 protein expression. To determine the effective concentration of ATL-1 required to induce HO-1 expression, ECV304 were exposed to increasing concentrations (3–300 nM) of ATL-1 for 18 h. HO-1 protein expression was evident at all concentration used with a maximum effect seen at 300 nM (Fig. 2).

Kinetics of ATL-1-induced HO-1 expression. We next examined the time course of ATL-1-induced HO-1 expression in ECV304. As shown in Fig. 3, protein expression was slightly detected in cells immediately after their dissociation from culture plates (fresh cells). When cells were treated with 100 nM of ATL-1 for different periods of time (3–36 h), HO-1 expression was significantly increased already at 3 h, remaining elevated until 18 h, declining thereafter and returning to basal levels by 24 h.

Inhibitory effect of PTX and Boc-2 on ATL-1-induced HO-1 protein expression. LXA₄ and ATL exert their bioactions through a high-affinity, seven-transmembrane G protein-linked receptor named ALXR, coupled in some cell types to a PTX-sensitive G protein. As shown in Fig. 4A, ECV304 exposure to PTX (0.1 μg/ml) for 30 min reduced by ~70% ATL-1-induced HO-1 expression. These results were confirmed by pretreatment of EC with Boc-2 (100 μM), an effective antagonist of ALXR (18), which ablated ATL-1-induced HO-1 protein expression (Fig. 4B). Together, these data indicate that ATL-1 effect on EC involves ALXR.

Effect of HO-1 induced by ATL-1 on adhesion protein expression. The overexpression of HO-1 in EC significantly inhibits TNF-α-induced expression of proinflammatory adhesion molecules associated with EC activation, E-selectin, and VCAM-1 (53). Because ATL-1 induces high levels of HO-1 protein in EC, we decided to investigate the physiological relevance of this effect examining adhesion molecules expression. As illustrated in Fig. 5, the expression of VCAM-1 induced by TNF-α in HUVEC was significantly inhibited in the presence of ATL-1 (100 nM) (Fig. 5D), an effect completely reversed when the cells were coincubated with SnPPIX (50 μM), a competitive inhibitor that blocks HO-1 activity (Fig. 5F). As expected, treatment of EC with heme (10 μM) abrogated VCAM-1 expression (Fig. 5H). Similar results were obtained with E-selectin expression when EC were exposed to IL-1β (data not shown).

DISCUSSION

Inflammation is a beneficial host response to foreign challenge or tissue injury that leads ultimately to the restoration of tissue structure and function. However, prolonged inflammation can cease to be a beneficial event and contribute to the pathogenesis of many pathological states. The resolution of acute inflammation is a highly coordinated and active process that is controlled by endogenous “pro-resolving” mediators. Recent studies have shown that certain lipids might have a
crucial role in the resolution of inflammation rather than promoting an inflammatory response (27), and among these molecules, the lipoxins have been highlighted as a major component. Lipoxins are generated through cell-cell interactions via a transcellular metabolism of arachidonic acid by LO/LO interaction in pathways (40, 42). It has been shown that nanomolar concentrations of lipoxins inhibit neutrophil and eosinophil chemotaxis (6, 32) and lipoxin A4 blocks neutrophil migration across postcapillary venules and inhibits neutrophil entry into inflamed tissue on animal models (12, 47). In contrast to their inhibitory effects on neutrophil and eosinophil recruitment, lipoxins are potent chemoattractant for monocytes (30, 31) and potentiate apoptotic neutrophil phagocytosis by monocyte-derived macrophages (19), two crucial events during the resolution process.

Lipoxins and their 15-epimers, ATL, have proved to be highly effective in a range of experimental disease models, including renal ischemia-reperfusion injury (29), in skin diseases (39), and in gastritis (46).
The stress-inducible enzyme HO-1 has also been shown to play important roles in the modulation of both innate and adaptative immunity. HO-1 has been identified as one of the endogenous factors that play a crucial role in the resolution of acute pleurisy. Current studies indicate that this enzyme might also have a role during the onset phase of acute inflammation by regulating PMN trafficking. The evolving paradigm of HO-1-mediated protection of cells and tissues is supported by several animal models of oxidant injury (endo-toxic shock, ischemia, and hyperoxia) and acute inflammation. In these models, HO-1 elevation confers potent resistance to stress, cell injury, and lipopolysaccharide-induced death, whereas blocking of HO activity abrogates cytoprotection, resulting in severe tissue damage. In addition, increased HO-1 expression levels have clinically been demonstrated in a wide variety of inflammatory conditions, such as ischemia-reperfusion injury, atherosclerosis, asthma, Alzheimer’s disease, and acute renal failure. Recently, it has been suggested that IL-10, a key molecule for controlling inflammation, mediates many of its anti-inflammatory effects via upregulation of HO-1.

Collectively, these studies indicate that lipoxins, ATL, and HO-1 are tightly associated with the resolution of acute inflammation, but the convergence of these pathways had never been investigated. In the present study, we demonstrated for the first time that an ATL synthetic analog was able to trigger HO-1 expression on endothelial cells. The human HO-1 promoter has been shown to bear functionally active response elements to various inducers, such as UV irradiation, heavy metals, iron, hyperoxia, hypoxia, and heme. Here, we demonstrate that in EC, the HO-1 gene is also potently induced by ATL-1, confirming de novo HO-1 synthesis.

Neutrophils, monocytes, and cells from nonmyeloid origin, such as epithelial cells express high-affinity receptors for LXA4, namely ALXR, that had been cloned and characterized and appear to be a member of the superfamily of membrane-bound G protein-coupled receptors. LXA4 and ATL, as well as their stable analogs, bind to this receptor also referred to as formyl peptide receptor-like 1 (FPRL1). Maderna et al. showed that tissue factor expression in HUVEC and ECV 304 is markedly enhanced via an ALXR-mediated signaling pathway. The latter observations extend the role of ALXR activation to resident cells in the vascular tissue compartment. There is increasing evidence that nonhematogenous cells, such as endothelial cells and glomerular mesangial cells, express at least two classes of membrane surface receptors for LXA4, one subtype that is specific for LXA4 and another shared by cysteinyl-LTs, such as ATL.
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C562 ATL INDUCES HO-1 EXPRESSION

expression significantly inhibits TNF-
HO-1 expression. We found that ATL-1-triggered HO-1 ex-
esized whether ATL-1 would have additional functions that
activity via bilirubin and Fe. In the present study, we hypoth-
reports (44) have shown that E-selectin and VCAM expression
upregulation abrogates adhesion molecule expression and leu-
creases heme-induced ICAM-1 expression and leukocyte in-
therefore crucial in determining the outcome of the inflamma-
mmatory cells to sites of acute and chronic inflammation and
the analog actions.

adhesion molecule expression in a concentration-and time-dependent manner. This phenomenon appears to be mediated via the ALXR/FPRL-1. The pathophysiologic importance of this finding is reflec-
expression on HUVEC, what may contribute to the impairment of
lymocyte influx during the resolution phase of inflammation.
Taken together, these data suggest that ATL-1-induced HO-1 expression in human cells by pharmacological means could be
a promising strategy for the treatment of various diseases and
resolution of inflammation.

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