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Lipoxin A₄: Anti-Inflammatory and Anti-Angiogenic Impact on Endothelial Cells¹

Nicole Baker, Sarah J. O’Meara, Michael Scannell,² Paola Maderna, and Catherine Godson³

Lipoxins (LX) are a class of eicosanoid that possesses a wide spectrum of antiinflammatory and proresolutive bioactions. The term LX refers to the provenance of these mediators, that is, lipoxygenase interaction products. The anti-inflammatory bioactions of endogenously produced LX₄₄, aspirin-triggered lipoxins (ATL), and stable synthetic LX analogs have been described in both in vivo and in vitro systems (1, 2). LX bioactions include inhibition of chemotactic responses of polymophonuclear neutrophils and the activation of monocytes and macrophages (reviewed in Ref. 3). LX generation plays a pivotal role in both physiologic and pathologic responses (4). More recent evidence has emerged that LX and other lipid mediators, including the resolvins and neuroprotectins, whose biosynthesis is linked in space and time to the resolution phase of an inflammatory response, may dynamically regulate the resolution of inflammation through bioactions that include stimulation of macrophage clearance of apoptotic polymophonuclear neutrophils from an inflammatory focus, modulation of cytokine-stimulated metalloproteinase activity, and inhibition of cellular proliferation (5–8). In addition to the anti-inflammatory and proresolutive bioactions of LX, there is a growing appreciation that LX₄₄ may possess important fibrosuppressant activities preventing fibroblast activation (9, 10), growth factor-induced mesangial matrix production, and the progression of renal disease characterized by epithelial to mesenchymal transformation (10).

The bioactions of LX₄₄, the major physiological LX formed in mammalian systems during inflammation, are primarily elicited through binding and activation of the LX₁ receptor (ALXR/FPRL-1), a distinct G protein-coupled receptor of the formyl peptide receptor superfamily (11). The ALXR binds pleiotropic agonists both lipid and peptide (12). LXs inhibit the proinflammatory actions of a variety of inflammatory mediators, including the cysteinyl leukotriene leukotriene D₄ (LTD₄) (13, 14). LX₄₄ antagonism of LTD₄ actions on renal function have been demonstrated in vivo (13), and we previously demonstrated that LX₄₄ inhibits LTD₄-induced mesangial cell proliferation in vitro (7, 15) through a novel mechanism of action, namely the modulation of growth factor receptor phosphorylation (15).

In noninflamed tissues vascular endothelial cells regulate blood flow, control vascular permeability, and are agnostic to circulating leukocytes through the sequestration of leukocyte-interacting molecules such as selectins, chemokines, and ICAM-1. In inflammatory conditions endothelial cell activation is manifested through chemokine production and up-regulation of surface adhesion molecules that facilitate leukocyte adhesion (reviewed in Ref. 16). Progression of the endothelial inflammatory response without resolution is coupled to angiogenesis and neovascularization. Indeed, the plasticity of the endothelial cell response to chronic inflammation has recently been highlighted by evidence of endothelial to mesenchymal transition as a contributor to cardiac fibrosis (17). In addition to the vital role of angiogenesis in development and in tissue remodeling and repair, angiogenesis also plays a pivotal role in aberrant vascularization seen in conditions such as rheumatoid arthritis, diabetic retinopathy, and tumor growth (18). In this context, there is a growing appreciation of the role of endothelial cells in inflammation. Inhibition of factors that promote angiogenesis may reduce inflammation and prevent disease progression (16).

One of the most important factors regulating angiogenesis is vascular endothelial growth factor (VEGF) (19). VEGF is found in multiple isoforms (VEGF-Å, -B, -C, -D, and -E) that are derived from the alternative splicing of the same gene (19). These isoforms transduce their signals to the nucleus mainly through three tyrosine kinase receptors, VEGFR-1, -2, and -3 (20). Of these receptors,
VEGF-2 appears to play a critical role in the regulation of angiogenesis (20). Whereas VEGFR-1 also seems to participate in pathological angiogenesis (21), VEGFR-3 is responsible for lymphangiogenesis (22).

A number of studies have shown that VEGFR-2 is the principal mediator of several physiological and pathological effects of VEGF-A. These include proliferation (23, 24), migration (25), survival (26), and permeability (27). Activation of VEGFR-2 is coupled to proliferation and survival via the phospholipase C-γ (PLC-γ)/protein kinase C/Raf-MAPK pathway and activation of PI3K and Akt (26).

LX have previously been shown to modulate responses of endothelial cells including stimulation of prostacyclin production by HUVECs (28). Work by Fierro and colleagues has shown the stable synthetic analog of ATL-1, 15-epi-16-(para-fluoro)-phenoxy-LXA4 (ATL-1), inhibits VEGF- and LTD4-stimulated angiogenesis in vitro and in vivo (29–31). Additionally, ATL-1 has been shown to regulate the proteolytic activity necessary to digest basement membrane (30) and to modulate endothelial cell migration (31), key events in the angiogenic process (32).

We have previously demonstrated that LXA4 can block primary human mesangial cell proliferation in response to LTD4 and growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) by means of “receptor trans-inhibition” (7, 33), whereby activation of the ALXR by LXA4 is coupled to dephosphorylation of growth factor receptors and inhibition of downstream mitogenic signaling (15). Herein, we have focused on the activation of VEGFR-2 in response to VEGF and the signaling mechanisms by which LXA4 can subsequently inhibit VEGFR-2 phosphorylation. These data will allow us to elaborate on the profile of cell signaling events that underscore a supporting role for these novel lipid mediators as modulators of angiogenesis.

Herein we present evidence that the endogenously produced LX, LXA4, can modulate VEGFR 2 signaling by inhibiting proinflammatory cytokine responses, endothelial cell proliferation, and tube formation, and that this response is coupled to inhibition of VEGFR-2 phosphorylation. In addition to directly affecting VEGF receptor activation, we also demonstrate attenuation of LTD4-stimulated proliferation and tube formation.

Materials and Methods

Cell culture

HUVECs were obtained from PromoCell and were cultured in endothelial cell growth medium, supplemented with 0.4% endothelial cell growth supplement/H, 2% FCS, 0.1 mg/ml EGF, 1 μg/ml hydrocortisone, and 1 μg/ml basic fibroblast factor, also from PromoCell. All culture vessels were precoated with 0.2% gelatin for 1 h at 37°C and were placed at 4°C for 5 min. The gelatin was removed and cells were seeded at the appropriate density. HUVECs (passage 3–6) were cultured at 37°C, 5% CO2, and cells were serum starved for 24 h before experiments unless stated otherwise.

Immunofluorescence

HUVECs were plated on gelatin-coated coverslips in 12-well plates and cultured in complete medium for 48 h and serum starved for 24 h. The cells were treated with vehicle (ethanol) or LXA4 (10 nM; BIOMOL) for 30 min at 37°C, 5% CO2. The cells were washed with PBS, fixed with 3.7% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and blocked with 1% BSA in PBS for 30 min. For staining of the receptor, not permeabilized and permeabilized cells were incubated with ALXR Ab (C1508, a kind gift from Dr. J. F. Parkinson, Berlex) at a concentration of 1 μg/ml in 1% BSA/PBS for 1 h at room temperature, followed by Alexa Fluor 568 goat anti-rabbit Ab (Molecular Probes; 1/100 dilution in 1% BSA/PBS) for 1 h at room temperature. Cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Excess DAPI was removed by washing before mounting coverslips with Probing Antifade medium (Molecular Probes). Images were obtained using a Zeiss LSM 510 META scanning confocal microscope (×63 oil immersion objective) and analyzed by Zeiss LSM imaging software.

For F-actin rearrangement, permeabilized cells were stained with Oregon Green phallloidin (0.33 μM; Molecular Probes) for 30 min at room temperature. Cells were viewed on an Axiovert 200M fluorescent microscope (Zeiss) using Axiosvision image analysis software (Imaging Associates).

RT-PCR detection of VEGFR-2, ALXR, and CysLT receptors

HUVEC RNA was extracted using TRIzol reagent (Invitrogen) and cDNA synthesized with SuperScript II (Invitrogen) by reverse transcription. Primers against various genes were constructed from their respective published sequences using a web-based software program (Primer3, available at frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi (Whitehead Institute for Biomedical Research)). cDNA (1–10 ng), dNTPs (2.5 mM), MgCl2 (25 mM), 10× buffer, 30% glycerol, sense and antisense primers (20 μM), TaqDNA polymerase (1 μl/μg), and dH2O were added and mixed together to a final volume of 50 μl. A negative control with no DNA and a positive control (GAPDH) were also subjected to PCR amplification. Amplification reactions were performed on a Peltier Thermal Cycler 200 at 94°C for 60 s, followed by 50°C for 30 s and, finally, 72°C for 60 s of product sequence for 35–40 cycles. For the detection of ALXR, a nested PCR was required in human mesangial cells and HUVECs. A detailed list of primers used within this study can be found in supplemental Table 1.

Proliferation assay

For analysis of proliferation, HUVECs were grown to ~60% confluence on 24-well plates before serum restricted in 0.2% FCS in endothelial cell growth medium for 24 h. After this period, cells were stimulated with various agents in duplicate wells for 24 h as indicated. Proliferation was measured by determining [3H]thymidine incorporation; 1 μCi [3H]thymidine (90–120 Ci/mmol; Amersham Biosciences) was added to each well and [3H]thymidine incorporation was measured using a Packard Tri-Carb 2900TR liquid scintillation analyzer. As CPM [3H]thymidine incorporation varied between experiments, HUVEC proliferation rates were expressed as fold basal CPM-treated cells relative to vehicle-treated cells.

Endothelial capillary/tube-like network formation assessed using Matrigel matrix

One volume of reduced growth factor Matrigel (8.4 mg/ml; BD Biosciences) was mixed with two volumes of serum-free EGM media. Aliquots (250 μl) of reconstituted Matrigel solution were placed in each well of 24-well culture plates and polymerized at 37°C for 1 h. The gel-solidified HUVECs (1.5 × 104 cells/ml) were added to the well and pretreated with LXA4 (10 nM) for 30 min before stimulation with VEGF 165 (R&D Systems) (10 ng/ml) and LTD4 (10 nM) (Sigma-Aldrich). Thereafter, cells were incubated at 37°C for 20 h. Images were captured using a Zeiss microscope equipped with digital camera and Matrox Intelllicam imaging software. Tube number/formation was quantified using AngioSys software (TCS CellWorks) as per the manufacturer’s instructions.

Endothelial capillary/tube-like network formation assessed using AngioKit

Human endothelial cells were cocultured with human diploid fibroblasts in a specially designed medium in a 24-well plate (AngioKit ZHA-1000; TCS CellWorks). Every 3 days, LXA4 (10 nM) was added 30 min before the addition of VEGF (2 ng/ml). Suramin (20 μM) and VEGF (2 ng/ml) were used as negative and positive controls, respectively. On the 11th day, media were aspirated from cells. Each well was washed with 1 ml PBS. Ice-cold (1 ml) fixative (70% ethanol) was added to each well and incubated at room temperature for 30 min. The fixative was decanted and 0.5 ml of CD31 (PECAM-1)/primary Ab (1/400 dilution) from BD Biosciences in blocking buffer (PBS supplemented with 1% BSA) was added to the wells for 1 h at 37°C. The primary Ab solution was removed and the cells washed three times with 1 ml of blocking buffer at room temperature for 10 min. The secondary Ab (goat anti-mouse IgG alkaline phosphatase conjugate from Cell Signaling Technology; 1/500 in blocking buffer) was added and incubated for 60 min at 37°C. Thereafter, wells were washed three times with 1 ml of blocking buffer at room temperature for 10 min, followed by washing with 1 ml of D2O for 10 min. BCP/NBT substrate (0.5 ml) was added and per AngioKit well and incubated at 37°C until tubules develop a dark purple color (usually 5–15 min). Cells were then washed carefully three times with 1 ml of D2O for 10 min at room temperature. The cells were

5 The online version of this article contains supplemental material.
Na3VO4). After 10 min of incubation on a rocking platform at 4°C, lysates were clarified (14,000 × g for 5 min). The supernatant was retained and protein assay performed by Bradford assay, and the concentration dependence of LXA4 (0.01–100 nM) on IL-10 production was investigated. The potential impact of inhibition of NO synthase on LXA4-stimulated IL-10 production was investigated in cells pretreated with L-NAME (Nω-nitro-L-arginine methyl ester) (Calbiochem) for 30 min. Accordingly, supernatants harvested after 18 h for cytokine assays. Absorbance was determined using a microplate reader (SpectraMax M2; Molecular Devices) set to 450 nm. The concentration dependence of LXA4 (0.01–100 nM) on IL-10 production was investigated in cells pretreated with L-NAME (Nω-nitro-L-arginine methyl ester) (Calbiochem) for 30 min.

Flow cytometry for ICAM-1 expression

The expression of adhesion molecule ICAM-1 (CD54 FITC; Dako) was measured by flow cytometry (CyAn ADP; Dako) and analyzed using FlowJo software from Tree Star. The results are expressed as stimulation index, which was calculated using the median fluorescence intensity (MFI) of each group/MFI of control.

Data analysis

Statistical analysis was conducted using the unpaired Student’s t test. All values are expressed as either the mean ± SD or the mean ± SEM. Values of p ≤ 0.05 were considered to indicate a statistically significant difference.

Results

Lipoxin A4 modulates VEGF- and LTD4-stimulated endothelial cell proliferation

In preliminary experiments we investigated expression of the ALXR/FPRL-1 and the cysteinyl leukotriene receptors (CysLT1

Quantitation of VEGFR-2 phosphorylation

Phosphorylated VEGFR-2 in cell lysates was measured by sandwich ELISA following the manufacturer’s instructions (R&D Systems). Briefly, serum-starved HUVECs were preincubated with LXA4 (10 nM) for 30 min before stimulation with VEGF (10 ng/ml) for 10 min. Cells were washed with ice-cold PBS and lysed in buffer 9 (1% Nonidet P-40, 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM activated sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Quantification of sample protein concentration was performed by Bradford assay, and 50 μg of protein per well was used in duplicate wells. Absorbance was determined using a microplate reader (SpectraMax M2; Molecular Devices) set to 450 nm.

Immunoblotting

Serum-starved HUVECs were preincubated with LXA4 (10 nM) for 30 min before stimulation with VEGF (10 ng/ml) for 10 min. Cells were washed with ice-cold PBS and lysed in 100 μl of RIPA lysis buffer containing protease and phosphatase inhibitors (20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% Na-deoxycholate, 0.1% SDS, 5 mM NaF, 1 mM PMSF, 1 μM leupeptin, 0.3 μM aprotinin, and 1 mM Na3VO4). After 10 min of incubation on a rocking platform at 4°C, lysates were clarified (14,000 × g for 5 min). The supernatant was retained and protein assay performed by Bradford analysis. Thereafter, lysates were denatured in sample buffer (10% 2-ME (v/v), 2% SDS (w/v), 30% glycerol (v/v), 0.025% bromophenol blue (w/v), 50 mM Tris (pH 6.8)), resolved by SDS/PAGE, electrophoretically transferred onto polyvinyldiene difluoride membrane and blocked in blocking buffer (5% (w/v) nonfat milk PBST). Phosphorylated and total VEGFR-2, Akt, ERK1/2, and PLC-γ (Cell Signaling Technology) were detected using specific rabbit polyclonal Abs and an anti-rabbit peroxidase-conjugated secondary Ab (Cell Signaling Technology), followed by detection using a chemiluminescence-based system. All primary Abs were used at 1/1000 dilution and secondary Abs used at 1/2000 dilution unless stated otherwise.

Cytokine immunoassays

IL-6, IL-8, IL-10, TNF-α, and IFN-γ were measured by sandwich ELISA following the manufacturer’s instructions (R&D Systems). Briefly, serum-starved HUVECs were treated with LXA4 (10 nM, 30 min) or otherwise, before stimulation with VEGF (10 ng/ml), and supernatants harvested after 18 h for cytokine assays. Absorbance was determined using a microplate reader (SpectraMax M2; Molecular Devices) set to 450 nm. The concentration dependence of LXA4 (0.01–100 nM) on IL-10 production was investigated. The potential impact of inhibition of NO synthase on LXA4-stimulated IL-10 production was investigated in cells treated with 100 μM t-NAME (Nω-nitro-arginine methyl ester) (Calbiochem) for 30 min.

Air dried and photographed immediately. Images were captured using a Zeiss microscope equipped with digital camera and Matrox Intellimac imaging software.

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Results

Lipoxin A4 modulates VEGF- and LTD4-stimulated endothelial cell proliferation

In preliminary experiments we investigated expression of the ALXR/FPRL-1 and the cysteinyl leukotriene receptors (CysLT1
and CysLT2) using RT-PCR. ALXR/FPR1-1 receptor expression was detected (Fig. 1A; HEK-293 ALXR stably transfected and primary human mesangial cells were used as positive control) (15). CysLT2 expression was also detected, and consistent with Gronert et al. we found no expression of CysLT1 (data not shown) (14). We also investigated ALXR expression by immunofluorescence and confocal microscopy. In resting HUVECs, ALXR is predominantly expressed on the cell surface in non-permeabilized cells with evidence of receptor in the cytosol in permeabilized cells (Fig. 1B). Stimulation of HUVECs with LXA4 (10 nM) for 30 min led to a significant decrease in the expression of the ALXR detected at the cell surface under

**FIGURE 3.** Effect of LXA4 on tube assay formation in HUVECs. HUVECs were plated on Matrigel with or without LXA4 (10 nM) for 30 min. Thereafter, cells were stimulated with VEGF (10 ng/ml) or with LTD4 (10 nM) for 20 h. A, Images were captured a Matrox Intellicam software package. B, Tube number/formation was quantified using AngioSys software (TCS CellWorks, U.K.). *, p < 0.05 and **, p < 0.01. C, HUVECs were cocultured with human diploid fibroblasts in a specially designed medium in a 24-well plate. Every 3 days, LXA4 (10 nM) was added 30 min before the addition of VEGF (2 ng/ml). Suramine (20 μM) and VEGF (2 ng/ml) were used as negative and positive controls, respectively. On the 11th day, cells were fixed with ice-cold 70% ethanol. Tubule formation was visualized by staining for anti-human CD31 (PECAM-1). D, HUVECs plated on gelatin-coated coverslips were treated with vehicle (ethanol) or LXA4 (10 min or 30 min). F-actin was detected using Oregon Green-conjugated phalloidin.
nonpermeabilizing conditions. As shown in cells under permeabilizing conditions, there was a substantial internalization of the receptor that seems to localize to the perinuclear region (Fig. 1B). The ALXR Ab specificity was determined in HeLa cells transiently transfected with ALXR and appropriate nontransfected controls (data not shown).

Using [3H]thymidine incorporation we measured HUVEC proliferation in response to both VEGF and to LTD4. As show in Fig. 2A, LXA4 inhibited VEGF-stimulated proliferation of HUVECs in a concentration-dependent manner (Fig. 2A). As shown in Fig. 2B, VEGF stimulated HUVEC proliferation ~4-fold relative to vehicle, and this was consistently inhibited by pretreatment with 10 nM LXA4 by ~50% ($p < 0.005$). LTD4-stimulated proliferation was reduced to basal levels by pretreatment with LXA4 as seen in Fig. 2 ($p < 0.005$). Whereas CPM [3H]Thymidine incorporation varied between experiments the ratio of CPM stimulated to basal was consistent across all experimental conditions (e.g., vehicle 13,336 CPM [3H] incorporated, VEGF 56,811 CPM, VEGF + 10 nM LXA4 38,008 CPM).

LXA4 stimulates actin rearrangement and inhibits VEGF- and LTD4-stimulated angiogenesis of HUVECs

Capillary formation starts with endothelial cell differentiation and tube formation in vitro, and it is also a consequence of endothelial cell differentiation. We demonstrate herein that LXA4 decreased the formation of tubes in VEGF-stimulated HUVECs in Matrigel in vitro. It was also confirmed by staining HUVECs cocultured with human diploid fibroblast with CD31. Tube formation was quantified using an AngioSys software (Fig. 3B). Vehicle-treated HUVECs did not form tubes, but VEGF- (10 ng/ml) or LTD4 (10 nM)-stimulated HUVECs formed visible tubes after 20 h. Pretreatment with 10 nM LXA4 significantly inhibited tube formation and prevented mesh formation as observed in VEGF- and LTD4-treated HUVECs (Fig. 3).

It has been shown that ATL-1 inhibits the VEGF-induced formation of actin stress fibers, but ATL-1 alone does not have the same effect (30). On the contrary, in our experimental conditions, native LXA4 causes a profound change in actin rearrangement. Untreated HUVECs displayed a well-organized bundle of stress fibers with a ring of polymerized actin on the membrane. After LXA4 treatment (10 nM for 30 min) stress fibers disappeared and filaments accumulated within ruffles (Fig. 3D).

LXA4 inhibits phosphorylation of VEGFR-2 in HUVEC stimulated by VEGF

The angiogenic activity of VEGF is thought to be mainly mediated by VEGFR-2 (KDR or flk-1/kinase domain region) present on
endothelial cells. Pretreatment with LXA₄ significantly decreased subsequent VEGF-stimulated phosphorylation of VEGFR-2 on HUVECs, as measured by ELISA with a phospho-specific Ab (Fig. 4A) and immunoblotting of crude membrane fractions (Fig. 4B). As controls we verified levels of VEGFR-2 expression by RT-PCR, and it was noted that there were no differences in the

FIGURE 6. LXA₄ down-regulates VEGF-induced ICAM-1, IL-8, IL-6, TNF-α, and IFN-γ and up-regulates IL-10 production. A and B. Serum-starved HUVECs were preincubated with LXA₄ (10 nM) for 30 min before stimulation with VEGF (10 ng/ml) for 18 h. A, IL-6, IL-8, IL-10, TNF-α, and IFN-γ were measured by sandwich ELISA following the manufacturer’s instructions (R&D Systems). C and D. Serum-starved HUVECs were incubated with LXA₄ (0.001–100 nM) or preincubated with (D) L-NAME (100 μM) for 30 min. Supernatants were harvested after 18 h and IL-10 production was measured by sandwich ELISA following the manufacturer’s instructions (R&D Systems). Absorbance was determined using a microplate reader (SpectraMax M2; Molecular Devices) set to 450 nm. B, The expression of adhesion molecule ICAM-1 was measured by flow cytometry (CyAn ADP; Dako) and analyzed using FlowJo software from Tree Star. The results are expressed in stimulation index, which was calculated using the median fluorescence intensity (MFI) of each group/MFI of control. **, p < 0.001 and ***, p < 0.0001 relative to VEGF-treated cells.
expression of VEGFR-2 when HUVECs were pretreated with LXA₄ following stimulation with VEGF or LTD₄ (Fig. 4C).

**LXA₄ inhibits VEGF-induced phosphorylation of p42/44 MAPK, Akt, and PLC-γ1**

VEGF receptor binding triggers a signaling cascade that results in tyrosine phosphorylation of PLC-γ1, leading to increases in intracellular levels of inositol 1,4,5-trisphosphate and elevation of intracellular calcium (34), as well as activation of the MAPKs that leads to proliferation of endothelial cells (35). VEGF also induces survival of endothelial cells mainly via activation of Akt (26). We assessed the effect of LXA₄ on these pathways in VEGF-stimulated HUVECs. As expected, VEGF enhanced the phosphorylation status of Akt, p42/44 MAPK, and PLC-γ1. However, pretreatment with LXA₄ significantly reduced VEGF-stimulated phosphorylation of Akt, p42/44 MAPK, and PLC-γ1 (Fig. 5).

**LXA₄ down-regulates VEGF-induced ICAM-1, IL-8, IL-6, TNF-α, and IFN-γ and up-regulates IL-10 production**

In inflammatory conditions VEGFR activation is coupled to cytokine release, adhesion molecule activation, and leukocyte endothelial interactions, which exacerbate the inflammatory response (16). We investigated whether LXA₄ might affect endothelial cell cytokine release. As shown, HUVECs treated for 18 h with VEGF up-regulate the secretion of IL-10, IL-8, IL-6, IFN-γ, and TNF-α as observed by ELISA (Fig. 6A), as well as the expression of ICAM-1 as observed by flow cytometry (Fig. 6B). However, if endothelial cells were pretreated with LXA₄ (10 nM) for 30 min before the addition of VEGF (10 ng/ml), the increased levels of cytokine secretion IL-8, IL-6, IFN-γ, and TNF-α observed were attenuated. VEGF up-regulation of expression of ICAM-1 was attenuated by pretreatment with LXA₄, consistent with its role as a modulator of leukocyte trafficking in inflammation (36).

Interestingly, in the case of IL-10, whose secretion was up-regulated by VEGF, when pretreated with LXA₄ this was further increased. LXA₄ up-regulated IL-10 secretion in a dose concentration-dependent manner (0.01–100 nM) with maximal effect at 10 nM (Fig. 6C). The potential role of NO in LXA₄-mediated IL-10 production was investigated in cells pretreated with l-NAME, an inhibitor of NO synthase. These data suggest a partial dependence of LXA₄-stimulated IL-10 production on NO, as L-NAME inhibited IL-10 production by 33% at 10 nM LXA₄.

**Discussion**

In adults, new vessels are produced through angiogenesis in response to an appropriate stimulus, as the quiescent vasculature can be activated to grow new capillaries. The angiogenic process is controlled by the net balance between molecules that have positive and negative regulatory activity, and this concept has led to the notion of the “angiogenic switch,” depending on an increased production of one or more of the positive regulators of angiogenesis (38). Numerous factors can impact this balance, including mitogenic factors such as fibroblast growth factors 1 and 2, TGF-β, VEGF, and LTD₄ (39). Nonmitogenic regulators include selected cytokines, CXC chemokines, and angiotensin, and, more recently, internal peptide fragments of larger polypeptides, angiotatin and endostatin, have been identified as potent angiogenesis inhibitors (32).

LXA₄ has been shown to be among the family of endogenously produced potent (nanomolar) antiinflammatory and proresolutive lipid mediators (for recent reviews, see Refs. 41–43); here, we present data showing that LXA₄ prevents proliferation and angiogenesis in endothelial cells in vitro. Effects of LXA₄ on endothelial cells may be of particular relevance given the biosynthesis of this agent within the inflamed vasculature. It is noteworthy that the modulation of endothelial cell proliferation and VEGF receptor signal transduction that we report closely parallels the bioactions of the synthetic aspirin-triggered 15R LX eicosanoid ATL-1. ATL-1 has been reported to inhibit VEGF-stimulated proliferation of HUVECs with a maximal effect of ~50% at 10 nM (29), suggesting similar efficacy to LXA₄. However, it appears that ATL-1 may be more potent than LXA₄, as 0.1 nM ATL-1 modulated VEGF-stimulated proliferation, a concentration at which LXA₄ was not consistently effective in our study.

We report that LXA₄ pretreatment of HUVECs reduced phosphorylation of VEGFR-2 in response to subsequent exposure to VEGF. This is noteworthy given our previous data demonstrating activation of a tyrosine phosphatase associated with dephosphorylation of the EGF and PDGF receptor tyrosine kinases. By analogy we propose that trafficking of stimulated VEGFR-2 to lipid rafts promotes association with ALXR-activated phosphatases (15). Furthermore, the ability of LTD₄ to transactivate growth factor receptor tyrosine kinases and initiate mitogenic signaling is well established (7), and we propose that the VEGF receptor is the locus of the effect of LXA₄ via ALXR on HUVEC proliferation in response to LTD₄. Analogous conclusions implicating ALXR have been made by others based on mimicry by other ALXR agonists, for example, ATL, the annexin-derived peptide Ac2-26, and relative sensitivity to the ALXR antagonists and the CysLT antagonists (15, 31, 43, 44). The consequences of such modulation of VEGF receptor activation are seen proximally in the modulation of PLC-γ1, MAPK, and Akt activation and physiologically in modulation of cell proliferation and angiogenesis. Interestingly, the serine/threonine kinase Akt/PKB is activated downstream of PI3K and mediates survival of endothelial cells (45). We also show that in HUVECs pretreated with LXA₄ followed by VEGF stimulation, Akt phosphorylation was down-regulated.

It was reported that stable LXA₄ analogs can affect the inflammatory response by modulating the surface expression of adhesion molecules on resting and immunostimulated leukocytes, and that they also inhibit neutrophil adhesion to the activated endothelium (36) through a mechanism involving NO biosynthesis exerting an antiinflammatory effect on the endothelium (46). In this regard, our data show that l-NAME partially preventing LXA₄-stimulated IL-10 production may be important, with LXA₄ and stable analogs (36) targeting both leukocytes and endothelial cells (3, 36).

The activated endothelial cell perpetuates an inflammatory milieu through the production of proinflammatory cytokines. It is interesting that LXA₄ inhibits VEGF-stimulated up-regulation of IL-6, TNF-α, and IFN-γ, consistent with its role in promoting quiescence. Consistent with this function is the modulation of ICAM-1 expression by activated endothelial cells. VEGF-stimulated HUVECs showed increased IL-10 production, which was further amplified by LXA₄. Souza et al. (44) found that there was a marked elevation of the production of IL-10 when ATL-1 was administered to reperfused mice, which demonstrates that LXA₄ plays an important role in inducing IL-10 production and in modulating inflammation in an IL-10-dependent manner at physiologically relevant (nanomolar) concentrations.

The inflammatory response is a critical determinant of effective host defense and its dynamic regulation is important to maintain homeostasis. The recent appreciation of the role of endogenously produced mediators as actively driving an inflammatory phenotype toward resolution (42) represents a paradigm shift in our understanding of innate immunity. In this context we demonstrate that LXA₄, an endogenously produced eicosanoid typically formed by...
Transcellular metabolism within an inflamed vasculature, can potentially regulate endothelial cell responses to prototypic proinflammatory stimuli such as LTD4 and VEGF.

Disclosures

The authors have no financial conflicts of interest.

References