Lipoxin A4 Induces Hyperadhesiveness in Human Endothelial Cells for Neutrophils

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Lipoxin A4 (LXA4), but not lipoxin B4, induced in vitro a dose-dependent, slowly emerging hyperadhesiveness in human umbilical vein endothelial cells (HUVEC), leading to a 1.9-fold increase in the binding of neutrophils (polymorphonuclear neutrophil granulocytes [PMN]). The maximal response to LXA4 occurred at 1 nmol/L and after 30 minutes of treatment of HUVEC. These response kinetics were intermediate in comparison with those of fast-acting inducers of HUVEC adhesivity (eg, thrombin, leukotriene B4 [LTB4] or platelet activating factor [PAF]), needing 5 to 15 minutes, or to the slow inducer interleukin-1 (IL-1β), which requires hours. The maximal LXA4 effect was slightly lower than that of LTB4 (100 nmol/L) and thrombin (1 U/mL), and less than that of PAF (100 nmol/L) or IL-1β (2.5 U/ml) (2.2-, 2.0-, 2.4-, or 13.6-fold increases, respectively). The LXA4 effect was inhibited by the PAF receptor antagonist WEB-2086; however, it could not be blocked by pertussis toxin. LXA4 conferred a slow, sustained increase in HUVEC cytosolic calcium ion concentrations, whereas thrombin did so rapidly and transiently. LXA4 also caused PMN to become hyperadhesive. Thus, this novel effect of LXA4 on HUVEC appears to be associated with endogenous PAF expression and slow increases of cytosolic calcium concentrations but not pertussis-sensitive G proteins.

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ENDOTHELIAL cells provide adhesive structures, thereby enhancing the emigration of polymorphonuclear neutrophil granulocytes (PMN) to the tissues. These structures can be activated by means of several inflammatory mediators. One such class of stimuli, eg, leukotriene B4 (LTB4) and platelet activating factor (PAF), confers a rapidly emerging hyperadhesiveness that is evident within minutes.1,2 Also belonging to this class are thrombin and leukotriene C4 (LTC4), which are dependent on the endogenous human umbilical vein endothelial cells (HUVEC) generation and the expression of PAF.3 Thrombin also confers an increase in the cytosolic concentration of calcium, [Ca2+]i,4 though the relation of [Ca2+]i increases to hyperadherence is, however, unclear. A second class of stimuli, leading to a slowly emerging hyperadhesiveness of endothelial cells that becomes evident after hours, is represented by lipopolysaccharide (LPS) and cytokines (eg, interleukin-1β [IL-1β], tumor necrosis factor [TNF]).5

We recently described that LTB4 induced a very rapidly emerging and transient hyperadhesive state in cultured HUVEC that was half the magnitude of what equimolar concentrations of PAF conferred but that was at least as potent as that of thrombin.2,5,6 This was highly stereospecific for LTB4,2,4 and was dependent on the availability of CD18 and 54,10 whereas at the same time it could not be ascribed to the passive retention of LTB4.

In this report we describe that another lipoxygenase product, lipoxin A4 (LXA4), induced hyperadhesiveness in HUVEC. Lipoxins, eg, LXA4 and LXB4, are the products of the concerted action of the 5 and 15 lipoxygenases in PMN and platelets, as well as in eosinophils, and have recently been shown to be formed during angioplasty.11-14 LXA4 is a potent modulator of vascular tone and bronchoconstriction,15 an inhibitor of natural killer (NK) cytotoxicity,16 a weak promoter of PMN chemotaxis17 and oxidative responses18 but not of homotypic aggregation,19,20 and an inducer of prostacyclin generation21 and of myelopoesis.22 We also show here that this endothelial-dependent hyperadhesiveness exhibited response kinetics intermediate to those of fast and slow HUVEC adhesion agonists, and that it was associated with slowly occurring [Ca2+]i increases in HUVEC.

MATERIALS AND METHODS

Chemicals. Formyl-methionyl-leucyl-phenylalanine (fMLP), human serum albumin (HSA, essential fatty acid free), PAF, thrombin, IL-1β, pertussis toxin, phospholipid myristate acetate (PMA), and sodium dodecyl sulphate (SDS) were obtained from Sigma Chemical Co (St Louis, MO). LTB4, dissolved in ethanol, and WEB-208622,23 were kind gifts from Drs J. Rokach (Merck-Frosst Inc, Dorval, Canada), and S.E. Dahlén (Karolinska Institute, Stockholm, Sweden), respectively. LXA4 and LXB4, produced by total synthesis, as described by Nicolaou et al24 and stored in ethanol from which fresh dilutions were made up for each experiment, were a generous gift from Dr J.A. Lindgren (Karolinska Institute) or purchased from Sigma; the biologic activity of these two preparations were similar. Hanks' balanced salt solution (HBSS) and other tissue culture media and chemicals were from Gibco (Paisley, Scotland, UK). Fura-2/AM was from Calbiochem (La Jolla, CA), BCECF/AM from Molecular Probes (Eugene, OR), tissue culture plastic ware from Nunc (Roskilde, Denmark), 96-well microtiter plates (Immunolon) from Dynatech Laboratories (Chantilly, VA), Percoll from Pharmacia (Uppsala, Sweden) and EDTA was from Merck (Darmstadt, Germany).

Culture of HUVEC. Endothelial cells were obtained from human umbilical veins as described.2,5,8,25 Cells were grown in a culture medium in tissue culture flasks precoated with gelatin. HU-
VEC were trypsinized when confluent growth was obtained, resuspended in medium and either seeded into new culture flasks (for a maximum of two passages, once per week) or seeded into 16-mm culture wells or 96-well microtiter plates. When new monolayers were established in the culture wells, they were then used for adherence assessments. Thus, in this study HUVEC had never been passed more than twice. We have reported that hyperadhesiveness to LTB4, as well as to PAF, PMA, and thrombin, declines with an increasing number of passages. Monolayer adherence of HUVEC by using an indirect immunofluorescence staining for factor VII-related antigen. These HUVEC did not express CD18 upon immunofluorescence staining with the monoclonal antibody (MoAb) 60.3 to the common β integrin.

Changes of HUVEC [Ca²⁺], was assessed by treating HUVEC, grown on coverslips, with 3 μmol/L Fura-2/AM in HBSS with 10 mmol/L HEPES and 1% HSA for 40 minutes, as described by Wickham et al.7 Those coverslips were positioned at 45° to the exciting and emitted light (at 340 and 500 nm, respectively) in a cuvette with HBSS. Agonist-induced fluorescence changes were followed in a Hitachi F-3000 spectrofluorimeter (Tokyo, Japan) for at least 30 minutes at 37°C with continuous stirring of the buffer. Stained smears showed that only PMNs were counted and corresponding adherence was thus calculated. Calibration of the system and calculations of the changes of cytosolic calcium levels were performed as described.26

Neutrophil preparations. PMNs were obtained from healthy donors by a one step discontinuous Percoll gradient centrifugation as described. The purified neutrophils (>95% purity and viability) were resuspended in HBSS with 0.4% HSA. PMNs, to be stained with the fluorescent probe BCECF/AM, were resuspended in Ca²⁺- and Mg²⁺-free HBSS, incubated with BCECF/AM (4 μmol/L) for 20 minutes at 37°C. PMN were washed twice and transferred to regular HBSS (with Ca²⁺ and Mg²⁺) with 0.4% HSA and 25 mmol/L HEPES buffer (assay buffer). Adherence of PMN to an albumin-coated plastic surface or to HUVEC monolayers was assessed as described.3,10 Three protocols for adherence were used in this report. In the first, the HUVEC monolayers were stimulated by an agonist (or by buffer alone for the assessment of spontaneous adherence) for 5 to 240 minutes, as indicated, and washed. Subsequently, PMN (0.5 mL) with approximately 2.5 × 10⁶ cells/mL, warmed at 37°C for 10 minutes) were added. In the second and third protocols PMN were allowed to settle onto the HUVEC monolayers or onto an albumin-coated plastic surface. The stimulus (eg, LTB4) was then added to one well and an equal amount of HBSS to the adjacent control well. These assays were used in conjunction with evaluation of pertussis toxin (PT) and WEB-2086 effects on HUVEC and PMN, respectively. Common to all three protocols was the incubation of the dishes at 37°C and 5% CO₂. At the end of the incubation period (which ranged between 5 and 30 minutes the fluids were aspirated and the wells washed manually, using a highly standardized technique. PMN numbers in the combined aspirates and washing fluids were subsequently counted and corresponding adherence was thus calculated.

Fig 1. The effect of the PAF receptor antagonist WEB-2086 on PMN adherence to HBSS-treated HUVEC for 30 minutes, or hyperadherence (ie, the increase of adherence above that of HBSS-treated HUVEC) induced by LXA4 (1 nmol/L) or by PMNs treated with buffer alone whereas the black bars (m) represent cells treated with WEB-2086. The number of separate experiments, run in duplicates, are given at the base of the bar. Statistical symbols as in Fig 2.
pretrial experiments the concentration of 10 μM was chosen, as it was the lowest concentration conferring maximal inhibition of PAF-induced hyperadherence of PMN.15,23

PT (750 ng/mL) was used to study G-protein-mediated events. HUVEC were treated for 2 hours at 37°C and subsequently washed. This treatment did not affect the spontaneous binding of neutrophils to HUVEC (11.6% ± 1.5% adherence for pertussis-treated HUVEC and 8.5% ± 1.0% adherence for controls; n = 19). Similar treatment of PMN for 2 hours conferred an inability in these cells to either respond to LTB4 with superoxide ion generation (>90% inhibition) or to adhere to HUVEC when LTB4 was added after PMN had settled onto the monolayer (+3.9% increase of adherence, when controls without pertussis toxin but otherwise similarly treated showed +19.0% hyperadherence).

Statistical analyses were performed with Student's t-test for paired samples.

RESULTS

HUVEC monolayers treated with LXA4 concentrations from 0.01 to 100 nmol/L exhibited a dose-dependent increased binding of PMNs with a peak activity at 1 nmol/L (Fig 2). At 10 pmol/L no additional PMN binding relative to HBSS-treated HUVEC was observed. Hyperadhesiveness was also reduced at concentrations higher than 1 nmol/L. Because the response kinetics are of significance for detection of the LTB4 and LXA4 effect in PMN,2,3,16,17,18 we followed HUVEC-adhesive responses for 5 to 120 minutes. We found that a 30-minute LXA4 treatment of HUVEC was optimal for the promotion of HUVEC adhesive properties, whereas 5-, 15-, 60-, and 120-minute incubation periods were associated with significantly less adherence (Fig 2 and data not shown). Similarly, during the subsequent incubation period with neutrophils, the highest adherence values were found after a 30-minute incubation, whereas 5-, 15-, and 60-minute incubations conferred significantly lower adherence (Figure 2 and data not shown). For the subsequent experiments described here we chose an LXA4 treatment time of HUVEC of 30 minutes and an incubation with PMN for 30 minutes. Attempts to see whether LXA4 was passively bound and still present after washings were hampered by a significant detachment of monolayers from the substratum after prolonged incubations at +4°C.

The stereospecificity of the LXA4 response was tested by using LXB4. When assessed at various time points of stimulation and adherence no significant changes from buffer-treated controls were observed (Fig 2 and data not shown).

The response to LXA4 was compared with that of a number of other fast- or slow-acting inducers of HUVEC adherence (Fig 2, Table 1). Treatment with LTB4 and PAF at 0.1 μmol/L and thrombin at 1 U/mL conferred maximal HUVEC hyperadhesiveness, as we and others have reported1,3,16 (Palmblad and Lerner, unpublished results, 1992). The maximal responses to LTB4 and thrombin, which were slightly higher in magnitude than that of LXA4, occurred at 5 minutes of treatment of HUVEC and after 5 minutes of PMN adherence. PMA responses were maximal at 1 μmol/L. PAF and PMA responses were faster than that observed for LXA4, requiring 15 minutes of treatment and 5 to 15 minutes of PMN adherence.19,30 IL-1β required incubation periods of 2 to 4 hours before marked hyperadhesiveness became apparent (Table 1, Fig 2).

We also studied whether the adhesive response of endothelial cells to LXA4 might be mediated by PAF, generated and

Fig 2. HUVEC hyperadhesivity, induced by LXA4 (.), LXB4 (■), LTB4 (□), and IL-1β (■). (A) depicts effects of treatment for 5 minutes of HUVEC with agonists and subsequent adherence of PMN for 5 minutes. (B) shows dose-response curves for treatment with agonists for 30 minutes and adherence of PMN for the same time period. (C) exhibits effects of a 120-minute period of agonist treatment and 10 minutes of PMN adherence. The data are based on the number of experiments given in Table 1 (for the optimal agonist concentrations) or for at least 3 separate experiments, run in duplicates. *P < .05, **P < .01, and ***P < .001 when compared with buffer-treated controls.
Thrombin, 1 U/mL +10.3 * 1.9 18 5+5
fMLP, 100 nmol/L +0.3

ence above HBSS-treated controls). HUVEC monolayers were treated were added. Hyperadherence (or spontaneous adherence) was assessed 5 to 30 minutes later. The maximal response is given. The IL-1 responses gradually increased over time; the values for incubation for 2 hours were +27.6% ± 2.3%. Spontaneous adherence did not vary significantly over the here used time periods, being 5% to 10% of added neutrophils. n = number of separate experiments, run in duplicate or triplicate.

expressed by HUVEC as a result of the exposure to LXA4, in analogy to what has been observed after treatment of HUVEC with LTC4 and LTD4.6 When the PAF antagonist WEB-2086 was used at 10 μmol/L, HUVEC-adhesive response to LXA4 was inhibited (Fig 1). The same concentration of WEB-2086 also abolished (or partly inhibited) the binding of PMN induced by PAF (or thrombin) in HUVEC (Fig 1) and the PMN adherence to an albumin-coated plastic surface (+2.8% ± 2.8%, relative to controls without WEB-2086: +15.3% ± 4.4%; n = 4). WEB-2086 did not affect spontaneous adherence of HUVEC for PMN (Fig 1).

The effect of PT on induction of HUVEC hyperadherence by LXA4 and IL-1β was then assessed. Using the same protocol, where neutrophil adhesive and oxidative responses to LT4 were completely inhibited, we were only able to observe modest reductions of HUVEC hyperadherence (Table 2), thereby indicating that the stimulus-response coupling for these stimuli did not involve PT-sensitive G-proteins.

Previous studies have suggested a reduction of the PMN chemotactic response to LT429 when the cells had been treated with LXA4, possibly indicating that these stimuli share common second-messenger reactions. Against this background we sought to test if a similar reaction could be detected in HUVEC. When monolayers had been treated with 1 nmol/L LXA4 (or HBSS) for 30 minutes, subsequently washed and exposed to 100 nmol/L LT4 (or HBSS) for 5 minutes, PMN were added and adhesion was assessed 5 minutes later. Pretreatment with LXA4 abolished the upregulation of HUVEC adhesivity that LT4 conferred (+8.3% for HUVEC pretreated with HBSS, and +1% for cells pretreated with LXA4, mean of two separate experiments).

Because increases of [Ca2+]i is an early event in the stimulus-response coupling of HUVEC to, eg, thrombin and other stimuli,28,31 we loaded HUVEC, grown on coverslips, with Fura-2/AM, and assessed fluorescence after administration of agonists. Thrombin induced a rapid and transient, dose-response-related increase of fluorescence, that was observed at LXA4 concentrations of 100 nmol/L (but not at lower) was 193 ± 29 nmol/L (n = 5) above the basal level.

Finally, because LXA4 can activate the PMN to chemotaxis37 we assessed if LXA4 causes hyperadhesivity of PMN to an albumin-coated plastic surface. When PMN had been
treated for 30 minutes no hyperadhesivity was detected, but with further incubation such a reaction became evident. After 1 hour of incubation a significant increase of adherence to +9.2% ± 3.1% (n = 10; P < .02) was found, that still persisted after 4 hours (+9.3% ± 4.2%; n = 7; P < .05) when the LXA4 concentration was 0.1 μmol/L. No consistent increases of PMN adherence were observed at lower LXA4 concentrations.

**DISCUSSION**

This study has shown that LXA4 is able to induce a slowly occurring though transient hyperadhesive state in HUVEC monolayers as well as in PMN. The effect of LXA4 on HUVEC was stereospecific, as the structural analogue LXB4, which differs with regard to stereochemistry and that has no PMN activating activity,13,14 did not confer HUVEC hyperadhesiveness. The mechanisms behind this ligand recognition, eg, whether HUVEC express cell-surface receptors for LXA4, are not known. Receptors for LXA4 have been suggested to be expressed on PMN and mesangial cells, where the signal transduction appeared to involve pertussis toxin-sensitive G-proteins.9,30,31 This is in contrast to the insensitivity to the pertussis toxin, as described here for HUVEC. The relationship to receptors for other lipid oxygenase substances is at this time unclear. However, available data indicate that LXA4 and LTD4 receptors may share common features.30,31 Moreover, LTβ and LXA4 may share common steps of the stimulus response coupling, as indicated by the inhibitory effect conferred by LXA4 on LTβ4-induced PMN chemotaxis29 and, as shown here, on HUVEC hyperadhesiveness.

LXA4 is not as efficacious as PAF or PMA in the induction of endothelial adhesiveness, with the maximal effect being approximately half that of the two latter agents, but similar to that of thrombin and LTδ4.2,3,10 The LXA4-induced hyperadhesiveness appeared more slowly than it did for LTδ4, PMA, PAF, and thrombin, but was faster than seen for IL-1β. All these LXA4 characteristics resemble other functional effects of LXA4, eg, the kinetics and potency of activation of neutrophils and smooth muscle cells.1,13,16,17,18,25

Another unique feature of LXA4 is the dose-response relationship for functional reactions. The peak of activity for HUVEC, as noted here, occurred at 1 nmol/L, whereas lower and higher concentrations were shown to be less efficient. An identical dose-dependency has been observed for PMN chemotaxis,4 whereas activation of adherence to a plastic surface (as shown here), arachidonate release, and the NADPH-dependent oxidase of PMN was observable at 0.1 to 1 μmol/L.19 A biphasic response pattern of human PMN phospholipase D to LXA4 has recently been described, with one peak of activity at 0.1 nmol/L and the other at 1 μmol/L.22 Thus, it appears that there exists functional and cell-specific concentration requirements for LXA4.

Our results also suggest that the adherence-promoting effect of LXA4 on HUVEC may be related to endogenously formed and expressed PAF. This suggestion is based on the finding that the PAF receptor antagonist WEB-208622,23 inhibited the binding of PMN to the LXA4-treated HUVEC. Moreover, it abrogated the adherence-promoting effect of PAF on PMN, documenting the efficacy of the protocol. It also partly impaired the thrombin-induced binding of PMN to HUVEC, which is in agreement with Toothill et al.23 Thus, the signal transduction for LXA4 effects in HUVEC might share common features with LTC4 and LTD4, which also appear to require HUVEC formation of PAF to sustain adherence of PMN.5

The HUVEC cytosolic calcium changes to thrombin were as immediate as the adhesive response, which is in agreement with findings of others.6,7 When using LXA4 we were able to detect a slow calcium increase that persisted for the 30-minute time period that was technically possible to monitor Fura-2-fluorescence. This suggests that LXA4 mobilizes calcium by a mechanism that differs from that of thrombin, at least kinetically.

Thus, LXA4, which may be formed during vascular injury, eg, angioplasty,13 and which is involved in vascular tone regulation11,12 and HUVEC prostacyclin production,20 may also be one of several agents that participates in the inflammatory process by mediating adhesive endothelial structures for neutrophils. The kinetics of the response is between that of fast (eg, LTδ4, thrombin) and slow (eg, IL-1β, TNF) inducers of endothelial adhesiveness. This may explain why no PMN adherence to the vascular bed has been previously observed.13,19,33 The interaction between LXA4, HUVEC, and PMN may also confer endothelial cell injury.34 This process is completely abrogated by MoAbs to CD11b/CD18 and CD54, and partly inhibited by antibodies to P-selectin (J Bratt, B Ringertz, R Lerner and J Palmblad, unpublished observations, 1992-3).

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