Lipoxin A₄ Inhibits Porphyromonas gingivalis-Induced Aggregation and Reactive Oxygen Species Production by Modulating Neutrophil-Platelet Interaction and CD11b Expression

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Received 19 July 2010/Returned for modification 20 August 2010/Accepted 23 December 2010

Porphyromonas gingivalis is an etiological agent that is strongly associated with periodontal disease, and it correlates with numerous inflammatory disorders, such as cardiovascular disease. Circulating bacteria may contribute to atherogenesis by promoting CD11b/CD18-mediated interactions between neutrophils and platelets, causing reactive oxygen species (ROS) production and aggregation. Lipoxin A₄ (LXA₄) is an endogenous anti-inflammatory and proresolving mediator that is protective of inflammatory disorders. The aim of this study was to investigate the effect of LXA₄ on the P. gingivalis-induced activation of neutrophils and platelets and the possible involvement of Rho GTPases and CD11b/CD18 integrins. Platelet/leukocyte aggregation and ROS production was examined by lumiaggregometry and fluorescence microscopy. Integrin activity was studied by flow cytometry, detecting the surface expression of CD11b/CD18 as well as the exposure of the high-affinity integrin epitope, whereas the activation of Rac2/Cdc42 was examined using a glutathione S-transferase pulldown assay. The study shows that P. gingivalis activates Rac2 and Cdc42 and upregulates CD11b/CD18 and its high-affinity epitope on neutrophils, and that these effects are diminished by LXA₄. Furthermore, we found that LXA₄ significantly inhibits P. gingivalis-induced aggregation and ROS generation in whole blood. However, in platelet-depleted blood and in isolated neutrophils and platelets, LXA₄ was unable to inhibit either aggregation or ROS production, respectively. In conclusion, this study suggests that LXA₄ antagonizes P. gingivalis-induced cell activation in a manner that is dependent on leukocyte-platelet interaction, likely via the inhibition of Rho GTPase signaling and the downregulation of CD11b/CD18. These findings may contribute to new strategies in the prevention and treatment of periodontitis-induced inflammatory disorders, such as atherosclerosis.

Periodontitis is one of the most prevalent inflammatory diseases in humans, the key etiologic agent being the Gram-negative anaerobic rod Porphyromonas gingivalis (54). This bacterium not only is involved in tooth loss but also may cause recurrent bacteremias and contribute to systemic disorders, such as cardiovascular disease (10, 22, 23, 39, 46, 65). P. gingivalis expresses a broad range of virulence factors, such as cysteine proteinases (gingipains), fimbriae, lipopolysaccharide (LPS), and capsular polysaccharide. Infection with the bacterium may lead to chronic inflammation in which hyperresponsive neutrophils contribute to host-mediated tissue destruction. P. gingivalis has been found in human atherosclerotic plaques (15, 27) and has been shown to promote the phenotypic switch of murine monocytes into foam cells, e.g., by inducing reactive oxygen species (ROS) generation and the oxidation of low-density lipoprotein (LDL) (31, 38, 57).

We have recently reported that the exposure of human blood to P. gingivalis causes the formation of atherogenic LDL through a gingipain-mediated cleavage of apoB-100 (5). Furthermore, P. gingivalis, unlike other periodontopathic bacteria, has been shown to trigger platelet aggregation in vitro (55, 66), mainly through the interaction between bacterial gingipains and protease-activating receptors (PARs) on the platelets (49). Since platelet aggregation precedes thromboembolic events, this is an important pathogenic feature of the bacterium (1, 32).

CD11b/CD18 (complement receptor 3 or Mac-1), the main β₂ integrin expressed on leukocytes, plays an important role in inflammation by promoting leukocyte adhesion and transmigration to sites of infection and by stimulating iC3b-mediated phagocytosis and cytokine production (21). In neutrophils, CD11b/CD18 binds to the platelet GPIIb/IIIa receptor via fibrinogen, thereby mediating neutrophil-platelet interaction and ROS production (11). In accordance with this, we have shown previously that platelet-leukocyte aggregation and ROS production in whole blood are mediated through selectin- and integrin-dependent interactions involving P-selectin and CD11b/CD18 (4).
CD11b/CD18 requires inside-out signaling to expose and activate its high-affinity epitope and to enable ligand binding (6). *P. gingivalis* has been shown to induce inside-out activation of CD11b/CD18 in monocyes/macrophages (25, 29) and to upregulate the CD11b/CD18 receptors on human neutrophils via LPS (68). The *P. gingivalis*-induced activation of CD11b/CD18 has been extensively studied in monocyes/macrophages, where two main signaling pathways have been implicated. First, the CD14-mediated binding of fimbriae and LPS to toll-like receptor 2 (TLR-2) stimulates CD11b/CD18 activation through a Rac1- and phosphatidylinositol 3-kinase (PI3K)-mediated pathway (26, 28, 29). Second, the bacteria can bind and activate PAR2 via gingipains, which induces CD11b/CD18 activation (34), possibly via a Rho-dependent pathway (69). Interestingly, these two pathways have been suggested to work synergistically (67). *P. gingivalis* also mediates platelet and neutrophil activation by acting on platelet TLR-2 and the PI3K/Akt pathway (7, 35). Harokopakis and Hajishengallis (29) have shown previously that fimbriae of *P. gingivalis* induce CD11b/CD18 activation in human neutrophils; however, the mechanisms by which the whole bacterium interacts with CD11b/CD18 and the associated intracellular signaling in neutrophils need to be clarified.

In neutrophils, Rac2 accounts for >96% of the Rac protein expressed (33, 58) and is involved in oxidative activity (13). Upon the binding of GTP, Rac and the closely related Rho GTPase Cdc42 interact with the downstream effector p21-activated kinase (PAK) (43). In human neutrophils, CD11b/CD18-mediated adhesion and phagocytosis activates Rac2 as well as Cdc42, which correlates with ROS production (9). The involvement of Rac2 in ROS generation has been demonstrated repeatedly (13), whereas Cdc42 is suggested to have an antagonistic role in oxidative activation (14).

Lipoxins (LXs) are endogenously produced eicosanoids with potent anti-inflammatory and proresolving effects (41, 63). Merched et al. (44) proposed that a failure in the endogenous synthesis of LXA4 underlies the unmitigating inflammation that fuels atherosclerosis. LXA4 functions mainly through the G protein-coupled receptor ALXR (18) and has been shown repeatedly to be protective in periodontal disease (36, 37, 61). Mouse models demonstrate that the administration of stable LXs repeatedly to be protective in periodontal disease (36, 37, 61).

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platelets were used during the preparation, extra care was taken when handling the cells to avoid any preactivation. Previous evaluation has shown that platelets isolated in this manner are not activated, and that the contamination of other blood cells is negligible.

Whole-blood lumiaaggregometry. Aggregation and ROS production was measured in heparinized whole or platelet-depleted blood using a lumiaaggregometer model 560 (Chrono-Log Corp., Havertown, PA). Cell aggregation was measured as increased impedance (Ω) between two platinum electrodes, and ROS production was determined simultaneously through luminol-amplified chemiluminescence, as previously described (5). Briefly, heparinized whole or platelet-depleted blood, drawn no more than 20 min before the onset of an experiment, was diluted in a ratio of 1:1 in physiological sodium chloride (0.9% NaCl) containing 100 μM luminol. The samples were preincubated for 15 min at 37°C, either in the presence or absence of 500 nM LXA4, in plastic cuvettes with siliconized stirring bars rotating at 800 rpm. P. gingivalis (1 × 107 CFU/ml blood) subsequently was added and incubated for 25 min, initiating cellular aggregation and ROS production. The instrument was calibrated before each experiment so that a 5-s change in impedance determined 7.5 mm deflection.

Neutrophil ROS production. ROS production in isolated neutrophils was analyzed using a six-channel Biolumat LB 9505 (Berthold Co., Wildbadken, Germany). Briefly, PMN (1 × 106 cells/ml) were suspended in KRG containing 50 μM luminol and 4 Units HRP, and extracellular calcium was set to 1 mM. The cells were incubated for 15 min at 37°C, either in the presence or absence of LXA4 (1 or 100 nM, respectively) before being stimulated with P. gingivalis (1 × 107 CFU/ml for 25 min), and then stimulated with P. gingivalis (1 × 107 CFU/ml). Aggregation was measured as the change in light transmission, where the unstimulated platelet suspension was set to 0% and the buffer (KRG) to 100%.

Fluorescence microscopy. Whole blood was preincubated for 15 min in the presence or absence of 500 nM LXA4 prior to incubation with FITC-labeled P. gingivalis (1 × 107 CFU/ml blood) for 25 min and fixation overnight in 4% paraformaldehyde (PFA) at 4°C. To visualize F-actin in leukocytes and platelets, the cells were washed and incubated in a mixture of 600 μg/ml rhodamine phalloidin and 100 μg/ml lysophosphatidylcholine in darkness for 30 min and, after further washes, were mounted on a coverslip. The samples were analyzed by inverted fluorescence microscopy (Axiovert 200; Carl Zeiss, Germany), where the number and the size of aggregates on a set area were counted (aggregates were included if located on a cross line drawn in the center of the coverslip) using Scion Image software.

Flow cytometry. Immunolabeling was performed as described previously (51, 50). In short, whole blood was incubated at 37°C in the presence or absence of LXA4 (1, 100, 250, or 500 nM) for 15 min and subsequently stimulated with P. gingivalis (1 × 107 CFU/ml blood) for 10 min. To detect the total CD11b expression, an R-phycocerythrin (RPE)-conjugated anti-human CD11b antibody (mouse monoclonal antibody; clone 2LM19c; Dako, Glostrup, Denmark) was added 5 min after the onset of bacterial stimulation. To detect the high-affinity epitope of CD11b, an FITC-conjugated anti-human CD11b antibody (mouse monoclonal antibody; clone CBRM1/5 [12]) diluted 1:13 was added 1 min prior to the addition of the bacteria. The antibodies were added to separate samples to avoid steric hindrance. Stimulation was stopped by incubation for 30 min on ice, after which erythrocytes were lysed for 5 min at 15°C using lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 100 μM EDTA), and the remaining cells were kept in 0.1% PFA until flow cytometry analysis. Granulocytes (predominantly neutrophils), monocytes, and lymphocytes were identified and gated by plotting forward scatter (FSC) versus side scatter (SSC), excluding cell debris. Unspecific binding was determined through the use of isotypic antibodies. For each sample, the mean fluorescence intensity (MFI) values of 15,000 events were determined, representing roughly 7,000 granulocytes, 4,000 lymphocytes, and 700 monocytes.

Pulldown and Western blotting. To assess the activation of Rac2 and Cdc42, a pulldown assay was used, employing a fusion protein of glutathione-S-transferase (GST) and the p21-binding domain (PBD) of Pak, as described previously (3, 19, 42). Briefly, the cDNA encoding residues 67 to 150 of PAK1 was cloned into the expression vector pGEX-4T3, kindly provided by the late Gary M. Bokoch (Scripps Research Institute, La Jolla, CA) and expressed in Esche-
RESULTS

Effect of LXA4 on P. gingivalis-induced aggregation and ROS production in whole blood. Using lumiaggregometry, P. gingivalis-induced aggregation and ROS production were examined in heparinized whole blood. No spontaneous aggregation or ROS production was seen when whole blood was incubated for 15 min at 37°C, and incubation with LXA4 did not per se induce any detectable responses (Fig. 1A). The addition of P. gingivalis potently triggered both the aggregation and ROS production of whole-blood cells, as measured by changes in impedance and luminol-amplified chemiluminescence (Fig. 1). The bacterial response was initiated after 5 to 6 min and reached an irreversible maximum after approximately 15 min. LXA4 inhibited the aggregation and ROS generation in a concentration-dependent manner (Fig. 1B). Preincubation with 500 nM LXA4 significantly decreased both the P. gingivalis-induced aggregation (63%; P < 0.01) and ROS production (31%; P < 0.01) (Fig. 1B). At a cellular level, LXA4 usually is potent at concentrations ranging from 1 to 10 nM. In our experimental setup, the use of whole blood required a higher concentration of LXA4 in concordance with previous findings (16), most likely due to the interaction of LXA4 with blood components, e.g., albumin. The effect of LXA4 is not due to the metabolic inactivation of the bacteria, since we found that LXA4 did not per se affect P. gingivalis viability in buffer (data not shown).

Effect of LXA4 on P. gingivalis-induced formation of cellular and bacterial aggregates. The effect of LXA4 on P. gingivalis-induced aggregate formation in whole blood was visualized with fluorescence microscopy by using FITC-labeled P. gingivalis and the rhodamine phallodin staining of F-actin in leukocytes and platelets. The stimulation of whole blood with P. gingivalis induced the formation of large mixed aggregates of platelets, leukocytes, and bacteria (Fig. 2). The majority of the leukocytes had internalized a high number of bacteria (Fig. 2C and E). The preincubation of whole blood with 500 nM LXA4 decreased the size of aggregates by 67% (P = 0.018) after 20 min of stimulation with the bacteria (Fig. 2D, F, and G). There was no spontaneous formation of aggregates in whole blood when incubated at 37°C and after incubation with LXA4 per se (Fig. 2A and B). Accumulations of rhodamine-stained neutrophils and platelets, together with FITC-labeled bacteria, appear yellow when the colors are merged.

Effect of LXA4 on P. gingivalis-induced ROS production in isolated neutrophils. P. gingivalis-induced ROS production in isolated neutrophils was determined through luminol-amplified chemiluminescence during the course of 1 h. P. gingivalis induced a marked increase in ROS production. However, preincubation with LXA4, at either 1 or 100 nM concentration, did not alter the P. gingivalis-induced ROS production (Fig. 3). Unstimulated neutrophils did not produce any detectable levels of ROS, and preincubation with LXA4 did not per se induce any detectable ROS (data not shown).

Effect of LXA4 on P. gingivalis-induced aggregation of isolated platelets. Platelet aggregation was measured to clarify the effects of LXA4 on the activation of isolated platelets. We found that P. gingivalis caused an extensive platelet aggregation, and that this response was not inhibited by LXA4 (1 or 100 nM) (Fig. 4). Unstimulated platelets did not spontaneously

1 mM EDTA were added to the cell lysate of MLF (1 µM)-stimulated cells (15 min, 30°C), thereby serving as a positive control. Unspecific binding was excluded by the use of uncoupled GST beads (beads without the PBD domain of PAK1).

Statistical analysis. Data were statistically analyzed by paired Student t tests or by one-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test. Data were expressed as means ± standard errors of the means (SEM), and P < 0.05 was considered statistically significant.
aggregate, and LXA4 did not induce an aggregatory response by itself (data not shown).

**Effect of LXA4 on P. gingivalis-induced aggregation and ROS production in platelet-depleted whole blood.** *P. gingivalis*-induced aggregation and ROS production was studied in platelet-depleted whole blood using lumiaggregometry. The addition of *P. gingivalis* triggered a considerable production of ROS but just a marginal aggregatory response. Neither the *P. gingivalis*-induced ROS production nor the minor *P. gingivalis*-induced aggregation was affected by preincubation with LXA4 (Fig. 5).

**Effect of LXA4 on P. gingivalis-induced upregulation of CD11b and exposure of the high-affinity integrin epitope on leukocytes.** The *P. gingivalis*-induced upregulation of CD11b and exposure of the high-affinity epitope on neutrophils was analyzed by flow cytometry. The expression of CD11b on the cell surface of neutrophils was increased by 25% upon stimulation with *P. gingivalis* and was significantly decreased to basal
levels when the cells were preincubated with 500 nM LXA4 (P < 0.05) (Fig. 6A and C). Similarly, the surface expression of the CD11b high-affinity epitope increased by 59% upon stimulation with *P. gingivalis* and was significantly reduced by 28% by preincubation with 500 nM LXA4 (P < 0.01) (Fig. 6B and D). A small and possibly dose-dependent reduction in CD11b expression and high-affinity epitope exposure was seen when cells were incubated with only LXA4. However, this reduction was not statistically significant and therefore was disregarded.

It is noteworthy that a small subset of the PMN population appeared to express reduced CD11b when stimulated with *P. gingivalis* (Fig. 6C). A possible explanation for this is that the RPE-conjugated antibody was added after bacterial stimulation, unlike the FITC-conjugated antibody, and that a portion of the CD11b receptor had already been internalized and therefore was inaccessible to the antibody. *P. gingivalis* also induced the surface expression of CD11b/CD18 in the monocytic population, which furthermore was inhibited by LXA4 (see Fig. S1 in the supplemental material). Lymphocytes expressed very low levels of CD11b/CD18, and neither *P. gingivalis* nor PMA (which was used as a positive control) affected the expression in our protocol setup (data not shown).

**Effect of LXA4 on *P. gingivalis*-induced activation of Rac2 and Cdc42.** The *P. gingivalis* activation of Rac2 and Cdc42 in neutrophils was analyzed by GST pulldown and immunoblotting. GTP-bound forms of Rac2 and Cdc42 were precipitated from the 10,000 × g fraction of lysed cells using a fusion protein consisting of the PBD of PAK1 and GST (GST-PBD). The activation of both Rac2 and Cdc42 from isolated neutrophils was detected as early as 0.5 min after the addition of *P. gingivalis* and persisted up to 10 min (Fig. 7A). Preincubation with 5 nM LXA4 for 5 min markedly inhibited the *P. gingivalis*
induced activation of both Rac2 and Cdc42 (Fig. 7A). No unspecific binding using uncoupled GST beads was detected (Fig. 7B).

DISCUSSION

In the present study, the anti-inflammatory and proresolving eicosanoid LXA₄ is shown to effectively inhibit P. gingivalis-induced aggregation and ROS production in whole blood. Our data suggest that this effect is mediated by an LXA₄-induced impairment of P. gingivalis-triggered activation of the small GTPases Rac2 and Cdc42 in neutrophils and the upregulation of CD11b/CD18 and its high-affinity epitope. Interestingly, we found that the expression of CD11b/CD18 integrins on the cell surface of neutrophils. Interestingly, we found that P. gingivalis not only increased the surface expression of CD11b/CD18 but also enhanced the expression of integrins in the high-affinity state. Furthermore, both of these effects were significantly suppressed by LXA₄. The P. gingivalis-induced surface expression of CD11b/CD18 in monocytes also was inhibited by LXA₄, although the high-affinity epitope remained unaffected. This is noteworthy, since P. gingivalis-induced CD11b/CD18 upregulation on monocytes has been shown to have a crucial role in stimulating monocyte adhesion to endothelial cells and transendothelial migration (28). LXA₄ thereby could play an important role in inhibiting integrin activation on monocytes and limiting P. gingivalis infection. Lymphocytes, on the other hand, expressed very low levels of this integrin, and consequently we were not able, in our system, to observe any effects of P. gingivalis on lymphocyte CD11b/CD18 expression. CD11b/CD18 requires inside-out signaling to expose and activate its high-affinity epitope and enable ligand binding (7). The activation of CD11b/CD18 by P. gingivalis has been shown in monocytes and macrophages, through a Rac1 and PI3K-mediated (28, 30, 31), and possibly a Rho-dependent, pathway (64), and we have shown previously that the ligation of CD11b/CD18 activates Rac2 and Cdc42 (21). Lipoxins have been shown to modulate the inside-out activation of CD11b/CD18 (48) and to downregulate CD11b/CD18 expression in neutrophils (18). In monocytes/macrophages, LXA₄ also has been shown to influence integrins by modulating the functions of Rho GTPases (40, 54). Our study demonstrates that LXA₄ modifies P. gingivalis-induced Rac2 and/or Cdc42 activation, which could be the mechanism by which it inhibits CD11b/CD18 upregulation and activation. Another possible mechanism of how LXA₄ modulates CD11b/CD18 integrins is through impeding Akt activation, since CD11b upregulation in PMN is Akt dependent (47) and LXA₄ has been shown to modulate PI3K/Akt activation (45).

Resolvins and protectins are mediators endogenously produced from ω-3 polyunsaturated fatty acids that share many of the proresolving and anti-inflammatory properties of lipoxins (8, 62). The topical application of resolvins protects against inflammation-induced tissue and bone loss associated with periodontitis in vivo (30). Thus, to include these compounds in future experiments and investigate their effects on the P. gingivalis-induced modulation of CD11b integrins would be of great interest. Activated CD11b/CD18 has a crucial role in platelet-leukocyte aggregate formation and the associated ROS production by the binding of the integrin to the platelet GPIIb/IIIa receptor via fibrinogen (5, 62). Our data clearly demonstrate that neutrophil-platelet interaction is essential for LXA₄ to inhibit P. gingivalis-induced effects. We found that P. gingivalis-induced ROS production in platelet-depleted blood is unaffected by LXA₄. The crucial role of interplay between platelets and neutrophils is further strengthened by our finding that neither P. gingivalis-induced ROS production in isolated neutrophils nor P. gingivalis-induced aggregation in isolated platelets was affected by LXA₄. It therefore is possible that LXA₄ inhibits P. gingivalis-induced ROS via intercepting neutrophil-platelet interaction through the downregulation of CD11b/CD18.

The microscopic evaluation of the aggregates in whole blood formed by P. gingivalis infection revealed that they contained a mixture of platelets, leukocytes, and bacteria, and that many leukocytes had internalized a high number of P. gingivalis. Hajishengallis et al. (26) have shown that fimbiae of P. gingivalis induce CD11b/CD18 binding and the internalization of the bacteria within macrophages through CD14/TLR2 signaling. Internalized P. gingivalis replicates within the host cell by activating cellular autophagy while suppressing apoptosis (2), which is a mechanism of bacterial survival and the spreading of the infection. Thus, our finding that LXA₄ inhibits CD11b/CD18 expression as well as the formation of aggregates in whole blood suggests a role of LXA₄ in inhibiting the propagation of the infection induced by the bacteria.
Patients with stable and unstable angina pectoris show an increased number of circulating neutrophil-platelet aggregates, which are suggested as an early and sensitive marker of myocardial infarction and an important component of systemic inflammation (20, 48, 50, 52, 53). Periodontitis may contribute to cardiovascular disease by recurrent and transient bacteria, where periodontal pathogens, including P. gingivalis, interact with blood cells, form neutrophil-platelet conjugates, and trigger inflammatory processes in the vessels. In this study, we show that LXA4 effectively inhibits neutrophil-platelet interaction and the formation of aggregates, thus supporting a role in prevention and treatment of cardiovascular disease.

In summary, we have demonstrated that P. gingivalis-induced ROS generation and aggregation in whole blood is inhibited by LXA4, possibly by mechanisms dependent on leukocyte-platelet interaction and involving the downregulation of the P. gingivalis-induced Rac2 and Cdc42 activation and the inside-out signaling of CD11b/CD18. Consequently, we suggest that a supplement of exogenous LXA4 facilitates anti-inflammatory actions and reduces the progression of chronic inflammatory disorders, such as periodontitis and atherosclerosis.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Research Council, the Swedish Heart-Lung Foundation, the Swedish Fund for Research without Animal Experiments, the Swedish Heart and Lung Association, the Foundation of Ölle Engkvist, the Emil & Maria Palm Foundation, and the Filip Lundberg Foundation.

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VOL. 79, 2011 LIPOXINS AND P. GINGIVALIS-INDUCED CELL ACTIVATION 1497


Editor: J. N. Weiser