

Lipoxin A₄ Inhibits *Porphyromonas gingivalis*-Induced Aggregation and Reactive Oxygen Species Production by Modulating Neutrophil-Platelet Interaction and CD11b Expression^{∇†‡}

Emma Börgeson,^{1,2#} Johanna Lönn,^{2,3,4#*} Ida Bergström,⁵ Veronika Patcha Brodin,⁶ Sofia Ramström,⁷ Fariba Nayeri,⁴ Eva Särndahl,³ and Torbjörn Bengtsson^{2,3}

School of Medicine and Medical Science, University College Dublin Diabetes Research Centre, Conway Institute, University College Dublin, Belfield, Dublin, Ireland¹; Division of Drug Research² and Division of Cardiovascular Medicine,⁵ Department of Medical and Health Sciences, and Division of Medical Microbiology⁶ and Division of Clinical Chemistry,⁷ Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Linköping, Sweden; Division of Clinical Medicine, School of Health and Medical Sciences, Örebro University, Örebro, Sweden³; and PEAS Institute, Linköping, Sweden⁴

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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 β_2 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).

* Corresponding author. Mailing address: Division of Clinical Medicine, School of Health and Medical Sciences, Örebro University, Örebro, Sweden. Phone: 46 19 602 6664. Fax: 46 19 602 6650. E-mail: johanna.lonn@oru.se.

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These authors contributed equally.

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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 monocytes/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 most extensively studied in monocytes/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 P13K/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 oxidase 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 unremitting 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 LXA₄ analogues significantly inhibits *P. gingivalis*-induced neutrophil influx, cyclooxygenase-2 expression, and prostaglandin E₂ secretion, which is done without promoting any further spreading of the infection (56). Moreover, 15-lipoxygenase transgenic rabbits, overexpressing LXA₄, show significantly diminished bone loss upon infection with *P. gingivalis* compared to the bone loss of control animals (64). Lipoxins also have been shown to downregulate CD11b/CD18 expression on whole-blood leukocytes (17). We have shown previously that LXA₄ modulates the inside-out activation of CD11b/CD18 in neutrophils (51), and Godson and coworkers showed that LXA₄ may influence the activation of integrins in monocytes/macrophages by modulating Rho GTPases (40, 59).

In this study, we demonstrate that LXA₄ inhibits *P. gingivalis*-induced platelet/leukocyte aggregation and ROS production in whole blood. Furthermore, LXA₄ blocks the bacterium-induced expression and function of CD11b/CD18 on neutrophils, possibly by inhibiting Rac2 and Cdc42 signaling pathways. Interestingly, the effects of LXA₄ in *P. gingivalis*-

induced cellular responses appear to be dependent on platelet-neutrophil interactions.

MATERIALS AND METHODS

Materials and chemicals. The materials and their sources were (S),6(R),15-trihydroxyeicosa-7E,9E,11Z,13E-tetraenoic acid (LXA₄) (BioMol Research Lab Inc., Plymouth Meeting, PA); 5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol) and horseradish peroxidase (HRP); fMet-Leu-Phe (fMLF); EDTA, Tween 20, and fluorescein isothiocyanate (FITC) (Sigma Chemical Co., St. Louis, MO); rhodamine phalloidin (Molecular Probes, Eugene, OR); phenylmethanesulfonyl fluoride (PMSF), leupeptin, aprotinin, guanosine 5'-0-(thio)triphosphate (GTP γ S), dithiothreitol (DTT), Nonidet P-40 (NP-40), and Pefablock SC [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBF)] (Roche Diagnostics Corporation, Indianapolis, IN); Ficoll-Paque, glutathione-Sepharose beads, and enhanced chemiluminescence (ECL) (GE-Healthcare GmbH, Uppsala, Sweden); and Polymorphprep (Axis-Shield PoC AS, Oslo, Norway).

Buffers. Phosphate-buffered saline (PBS) consisted of 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 6.7 mM Na₂HPO₄ × 2H₂O, pH 7.3; Krebs-Ringer glucose buffer (KRG) consisted of 120 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 1.7 mM KH₂PO₄, 8.3 mM Na₂HPO₄, and 10 mM glucose, pH 7.3; acid citrate dextrose (ACD) contained 85 mM trisodiumcitrate × 2H₂O, 71 mM citric acid, and 111 mM glucose; Tris-buffer saline (TBS) consisted of 25 mM Tris base, 0.15 M NaCl, pH 7.4; TEDG buffer contained 50 mM Tris, pH 7.4, 1.5 mM EDTA, 10% glycerol, 0.4% NaCl, 0.2 M DTT, 1 mM MgCl₂, 0.5% PMSF, 1 mM benzamidine, and 1 μ g/ml aprotinin; and lysis buffer for pulldown consisted of 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 200 mM NaCl, 2% NP-40, 10% glycerol, 1 mM DTT, 2 mM PMSF, 20 μ g/ml leupeptin, 20 μ g/ml aprotinin, 2.8 μ g/ml pepstatin, and 2 mM Na₃VO₄.

Porphyromonas gingivalis. ATCC 33277 (American Type Culture Collection, Manassas, VA) was grown under anaerobic conditions (80% N₂, 10% CO₂, and 10% H₂) at 37°C in an anaerobic chamber (Concept 400 Anaerobic Workstation; Ruskinn Technology Ltd., Leeds, United Kingdom). The bacteria were cultured for 3 to 4 days in fastidious anaerobe broth (29.7 g/liter, pH 7.2) before being washed and resuspended in KRG supplemented with 1.1 mM CaCl₂. To estimate the bacterial concentration, the optical density (OD) was measured at 600 nm in a Hitachi U2000 spectrophotometer (KEBO Lab AB, Stockholm, Sweden) and set to an OD of approximately 2. Through viable count, where the bacteria were grown on fastidious anaerobe agar (46.0 g/liter supplemented with L-tryptophan 0.1 g/liter, pH 7.2; Lab M, Lancashire, United Kingdom) for 5 to 7 days, an OD of 2 was determined to correlate with approximately 10⁹ CFU/ml.

Labeling of *P. gingivalis* with FITC. Bacteria, suspended in KRG supplemented with 1.1 mM CaCl₂, were incubated with 10 μ g/ml FITC for 1 h at room temperature (RT) during gentle agitation. The bacteria were washed in PBS-Tween (1%, vol/vol), resuspended in KRG supplemented with 1.1 mM CaCl₂, and stored at -70°C. The bacteria were sonicated before further use.

Whole and platelet-depleted blood. Peripheral venous blood was drawn from healthy donors and collected in heparin-containing (20 U/ml) Vacutainer tubes. The donors had no allergies and were not undergoing any medical treatment. To obtain platelet-depleted blood, whole blood was centrifuged at 220 × g for 20 min, after which platelet-rich plasma (PRP) was removed and replaced with the same volume of 0.9% NaCl.

Heparin, which was used as an anticoagulant, has been reported to inhibit complement activation. This effect of heparin may have a role in our study, since different factors of the complement system stimulate ROS production in neutrophils, e.g., through CD11b/CD18. However, work by Bexborn et al. (6a) shows that although heparin activates the complement system, the activation is relatively mild.

Preparation of neutrophils. Polymorphonuclear cells (PMN) were isolated from whole blood by density centrifugation as previously described (24). Briefly, whole blood was layered upon Polymorphprep, and PMN were isolated through density centrifugation (480 × g, 40 min, RT). Contaminating erythrocytes were lysed using hypotonic water, after which the osmotic pressure was restored. The cells were washed (400 × g, 5 min, 4°C), counted, and resuspended in KRG supplemented with 1.1 mmol/liter CaCl₂ and kept on ice until further use.

Preparation of platelets. Platelets were isolated from heparinized whole blood as previously described (25). Briefly, blood was mixed with RT ACD at a ratio of 5:1. Following centrifugation (220 × g, 20 min, 22°C), PRP was obtained and carefully collected. A final centrifugation (400 × g, 20 min, 22°C) resulted in a platelet-rich pellet, which was carefully washed before being resuspended in KRG. The cell concentration was calculated by using a Bürker chamber, and the suspension was kept in plastic tubes at RT until use. Immediately before an experiment, the extracellular calcium concentration was set to 1 mM. Since no

platelet inhibitors 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 lumiaggregation. Aggregation and ROS production was measured in heparinized whole or platelet-depleted blood using a lumiaggregometer 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 μ M 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* (1×10^7 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- Ω 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., Wildbad, Germany). Briefly, PMN (1×10^6 cells/ml) were suspended in KRG containing 50 μ M luminol and 4 U/ml 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* (1×10^7 CFU/ml) for 1 h, during which time chemiluminescence was registered. The mean integral value of the chemiluminescence curve was taken as a measurement of ROS production and calculated as fold induction compared to levels for the control. Care was taken to change the position of the samples between different experiments so that the channels were used randomly.

Platelet aggregation. The aggregation of isolated platelets was analyzed under stirring conditions using a calibrated two-sample lumiaggregometer model 560 (ChronoLog Corp., Havertown, PA). Briefly, platelets (2×10^8 cells/ml) were suspended in KRG with extracellular calcium set to 1 mM, incubated for 15 min at 37°C in either the presence or absence of LXA₄ (1 or 100 nM), and then stimulated with *P. gingivalis* (1×10^7 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* (1×10^7 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 μ g/ml rhodamine phalloidin and 100 μ g/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, 60). 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* (1×10^7 CFU/ml blood) for 10 min. To detect the total CD11b expression, an R-phycoerythrin (RPE)-conjugated anti-human CD11b antibody (mouse monoclonal antibody; clone 2LPM19c; 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 μ M 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-*

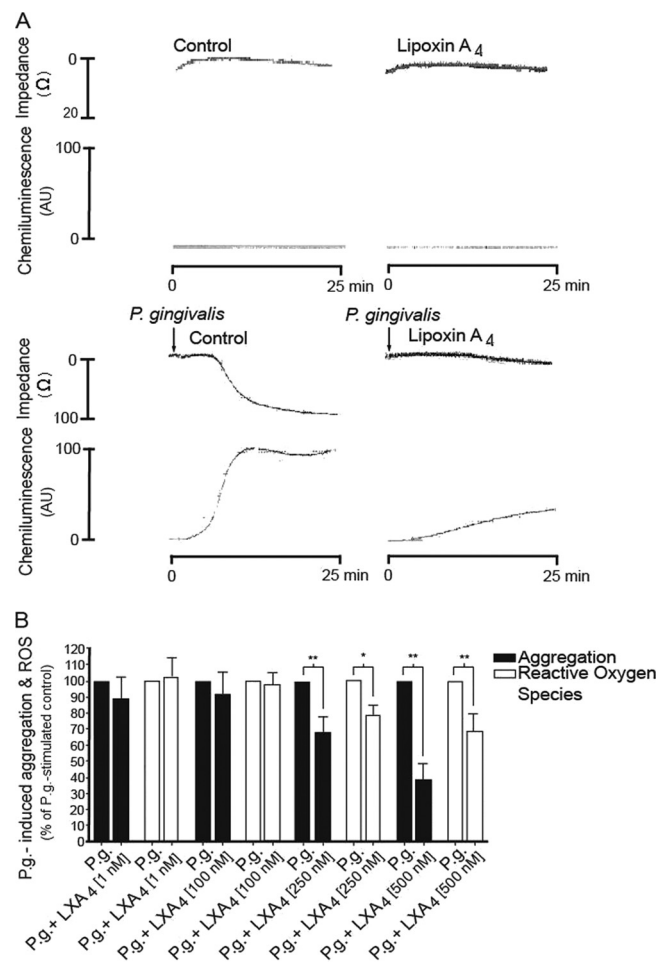


FIG. 1. LXA₄ inhibits *P. gingivalis*-induced aggregation and ROS production in whole blood. Whole blood was incubated for 15 min in the absence or presence of lipoxin A₄ (LX; 1 to 500 nM) prior to stimulation with *P. gingivalis* (P.g; 1×10^7 CFU/ml blood) for 25 min. Aggregation was measured as changes in impedance, and ROS production was detected by luminol-amplified chemiluminescence. (A) Representative experiment (500 nM lipoxin A₄). (B) *P. gingivalis*-induced aggregation and ROS production, presented as percentage of *P. gingivalis*-stimulated control. Data are presented as means \pm SEM of $n = 7$. *, $P < 0.05$; **, $P < 0.01$.

richia coli. Neutrophils (5×10^6 /sample) were preincubated in the presence or absence of 5 nM LXA₄ for 5 min at 37°C and stimulated with *P. gingivalis* (6×10^5 CFU/ml) for 10 min. The cells subsequently were lysed in ice-cold lysis buffer for 15 min at 4°C during gentle agitation. Cellular debris was cleared by centrifugation ($10,000 \times g$, 10 min, 4°C), after which the supernatants were incubated for 45 min at 4°C with glutathione-Sepharose beads, which had been precoupled with GST-PBD. The beads were washed three times in lysis buffer and boiled in Laemmli sample buffer (98°C for 5 min) to extract the proteins. The proteins were separated using standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a polyvinylidene difluoride membrane.

Unspecific binding was blocked by incubating the membranes in 5% milk-TBS (for 1 h at RT) prior to incubation with the mouse monoclonal anti-Cdc42 antibody (1:250; clone 44; BD Transduction Laboratories, San Diego, CA) or a rabbit polyclonal anti-Rac2 antibody (1:200; clone C-11; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at RT. The membranes were thereafter incubated with an HRP-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology) or an HRP-conjugated donkey anti-rabbit antibody (GE-Healthcare GmbH), respectively. To ascertain the specificity of the assay, 100 μ M GTP γ S (a nonhydrolyzable GTP analogue that arrests Cdc42 and Rac2 in their active state) and

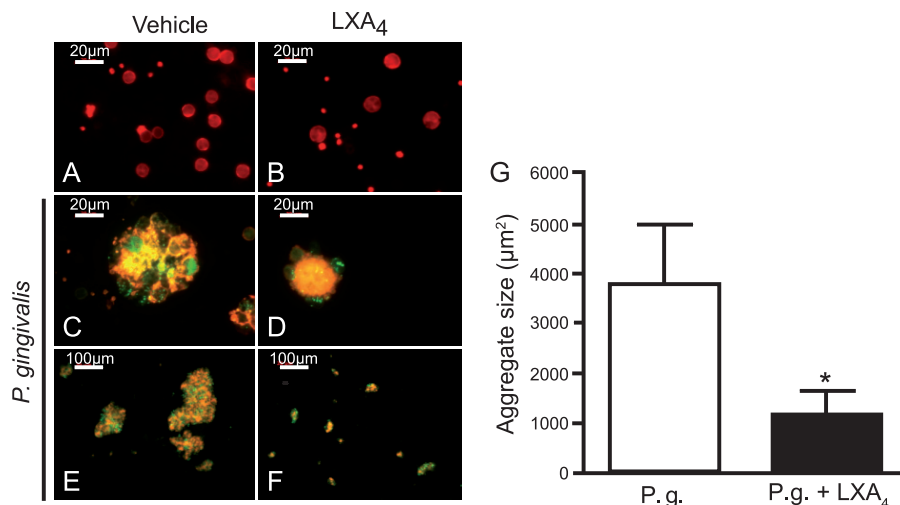


FIG. 2. LXA₄ inhibits *P. gingivalis*-induced formation of cellular aggregates. Whole blood was incubated in the absence or presence of 500 nM LXA₄ for 15 min before being stimulated with *P. gingivalis* (1×10^7 CFU/ml blood) for 25 min. An aliquot of each sample was fixed in 4% PFA overnight and analyzed by fluorescence microscopy. The figure shows samples in the absence (A and B) or presence (C to F) of *P. gingivalis* and preincubated with (B, D, and F) or without (A, C, and E) LXA₄ as indicated. Rhodamine phalloidin-labeled leukocytes and platelets appear red, and FITC-labeled bacteria appear green. (A) Unstimulated whole blood. (B) Whole blood in the presence of LXA₄. Magnifications are $\times 63$ (A to D) and $\times 10$ (E and F). To analyze the microscopy data, the size of 30 aggregates over a set area was assessed using Scion Image software (G). A paired Student *t* test was used for statistical analysis, and data are presented as representative images (A to F) or means \pm SEM of $n = 6$. *, $P < 0.05$.

1 mM EDTA were added to the cell lysate of fMLF (1 μ M)-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 \pm standard errors of the means (SEM), and $P < 0.05$ was considered statistically significant.

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 phalloidin 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 significant levels of ROS, and preincubation with LXA₄ did not *per se* induce any detectable ROS (data now shown).

Effect of LXA₄ on *P. gingivalis*-induced aggregation of isolated platelets. Platelet aggregation was measured to clarify the effects of *P. gingivalis* 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

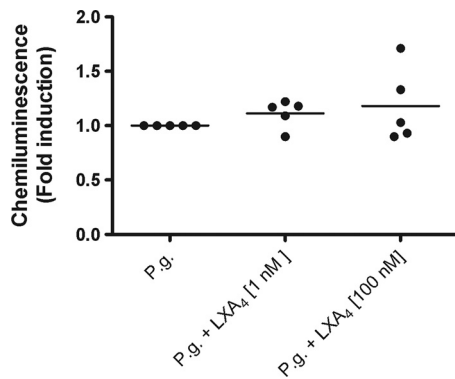


FIG. 3. LXA₄ does not affect *P. gingivalis*-induced ROS production in isolated neutrophils. Isolated polymorphonuclear cells (PMN), consisting predominantly of neutrophils, were preincubated for 15 min at 37°C in the presence or absence of LXA₄ (1 or 100 nM). Following subsequent stimulation with *P. gingivalis* (1 × 10⁷ CFU/ml), luminol-amplified chemiluminescence was measured during the course of 1 h. PMN stimulated with vehicle did not produce any detectable ROS, and LXA₄ did not induce a chemiluminescence response. ANOVA was used for statistical analysis, and data are presented as fold induction (means ± SEM of n = 5).

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*-in-

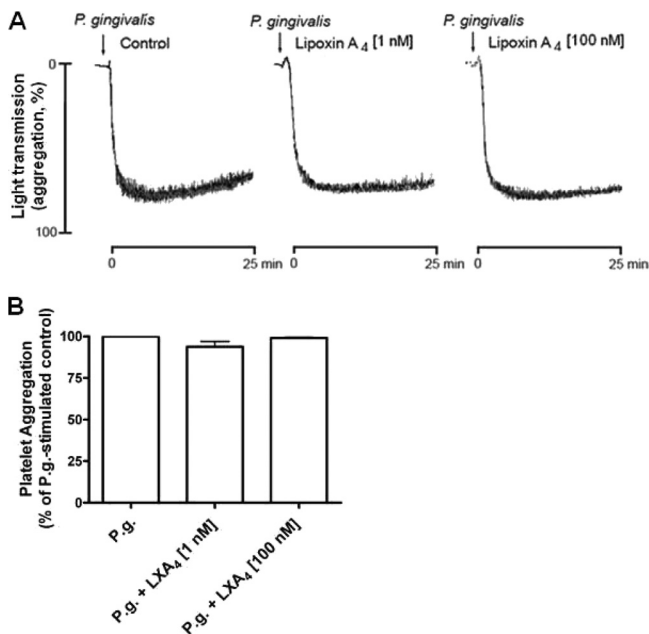


FIG. 4. LXA₄ does not affect *P. gingivalis*-induced aggregation of isolated platelets. Isolated platelets were preincubated for 15 min at 37°C in the presence or absence of LXA₄ (1 or 100 nM) and then monitored for aggregation upon stimulation with *P. gingivalis* (P.g; 1 × 10⁷ CFU/ml). (A) Representative aggregation traces of *P. gingivalis* with or without LXA₄ (1 or 100 nM). (B) *P. gingivalis*-induced aggregation presented as fold induction. The vehicle did not cause platelet aggregation, and LXA₄ did not induce an aggregatory response. Data are presented as percentage of *P. gingivalis*-stimulated control (means ± SEM of n = 3).

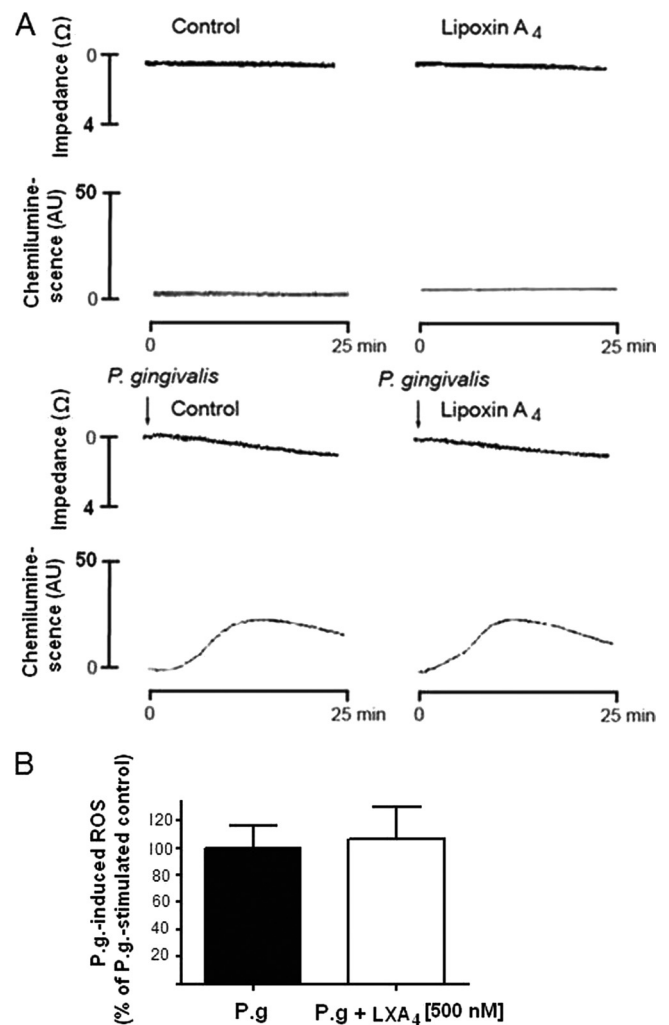


FIG. 5. Effect of LXA₄ on *P. gingivalis*-induced aggregation and ROS production in platelet-depleted whole blood. Platelet-depleted whole blood was incubated in the absence or presence of LXA₄ (500 nM). Aggregation was measured as changes in impedance and ROS production as luminol-amplified chemiluminescence during 25 min of incubation with *P. gingivalis* (1.5 × 10⁷ CFU/ml blood). (A) Representative experiment. (B) *P. gingivalis*-induced ROS production presented as percentage of *P. gingivalis*-stimulated control. Paired student *t* test was used for statistical analysis. Data are presented as means ± SEM of n = 3. AU, arbitrary units.

duced 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 were 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

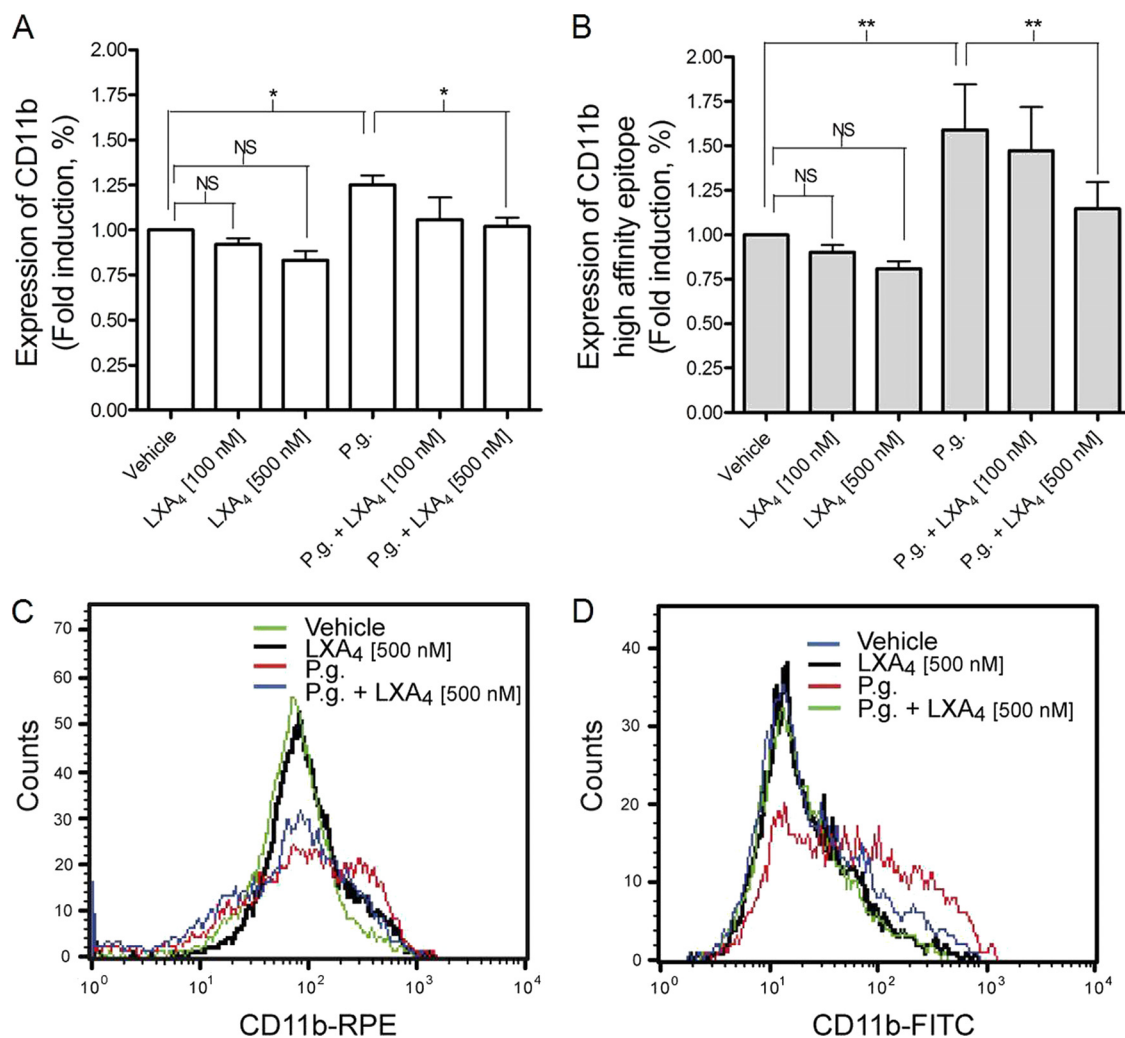


FIG. 6. LXA₄ inhibits *P. gingivalis*-induced upregulation of CD11b/CD18 and exposure of the CD11b/CD18 high-affinity epitope on neutrophils. Whole blood was incubated in the presence or absence of LXA₄ (100 or 500 nM) for 15 min and subsequently stimulated with *P. gingivalis* (P.g; 1×10^7 CFU/ml blood) for 10 min. Following the lysis of erythrocytes, the samples were fixed in 0.1% PFA and analyzed by flow cytometry. The granulocyte population (consisting predominantly of neutrophils) was gated by plotting forward scatter (FSC) versus side scatter (SSC), and CD11b/CD18 expression was determined by mean fluorescence intensity (MFI). Total CD11b surface expression was detected by use of RPE-conjugated mouse anti-human CD11b clone 2LPM19c (A and C), and the high-affinity domain was recognized through the use of an FITC-conjugated mouse anti-human CD11b antibody, clone CBRM1/5 (B and D). ANOVA was used for statistical analysis, and data are presented as means \pm SEM of $n = 5$. *, $P < 0.05$; **, $P < 0.01$.

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 $10,000 \times g$ 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*-

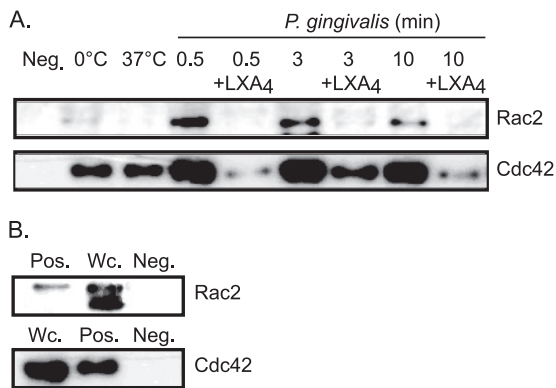


FIG. 7. LXA₄ inhibits *P. gingivalis*-induced activation of Rac2 and Cdc42 in neutrophils. Neutrophils (5×10^6 /sample) were preincubated in the absence or presence of 5 nM LXA₄ for 5 min at 37°C and stimulated with *P. gingivalis* (6×10^5 CFU/ml) for various times as indicated in the figure. The stimulation was stopped, and activated forms of Rac2 and Cdc42 were precipitated from the lysates using a GST pull-down assay. (A) Neutrophils were stimulated by *P. gingivalis* for 0.5, 3, and 10 min with and without preincubation with LXA₄, a GST pull-down assay was performed with beads lacking the GST-PBD (Neg; negative control), or neutrophils were incubated at 0 or 37°C (basal level). (B) Neutrophils were stimulated with fMLF plus GTPγS (Pos; positive control) or whole-cell lysate (Wc; positive control), or a GST-pull-down assay was performed with beads lacking the GST-PBD (Neg; negative control). An equivalent volume was loaded in each lane, and the electrophoretically separated proteins were detected with Western blotting using a polyclonal rabbit anti-human Rac2 antibody (1:200) and a mouse monoclonal anti-human Cdc42 antibody (1:250), clone 44.

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 impediment 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, the effect of LXA₄ on *P. gingivalis*-induced responses is dependent on platelet-neutrophil interaction.

To investigate the effect of *P. gingivalis* on cell activation, we examined 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 fimbriae 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 bacteremias, where periodontal pathogens, including *P. gingivalis*, interact with blood cells, form neutrophil-platelets 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 of lipoxin in the 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.

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