Lipoxin A₄ inhibits Porphyromonas gingivalis-induced aggregation and ROS production - role of neutrophil/platelet interaction and CD11b expression

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Abstract

*Porphyromonas gingivalis* is an etiological agent strongly associated with periodontal disease and 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 pro-resolving 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 lumi-aggregometry and fluorescence microscopy. Integrin activity was studied by flow cytometry, detecting surface expression of CD11b/CD18 as well as exposure of high affinity integrin epitope, whereas activation of Rac2/Cdc42 was examined using a GST-pulldown assay. The study shows that *P. gingivalis* activates Rac2 and Cdc42 and up-regulates 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, respectively, LXA4 was unable to inhibit either aggregation or ROS production. 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 inhibition of Rho GTPase-signalling and down-regulation of CD11b/CD18. These findings may contribute to new strategies in the prevention and treatment of periodontitis-induced inflammatory disorders, such as atherosclerosis.
Introduction

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 is not only involved in tooth loss, but may also 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 hyper-responsive 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 oxidation of low-density lipoprotein (LDL) (31, 38, 57).

We have recently reported that exposure of human blood to P. gingivalis causes formation of atherogenic LDL through a gingipain-mediated cleavage of apoB-100 (5). Furthermore, P. gingivalis has, unlike other periodontopathic bacteria, 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, we have previously shown 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 signalling to expose and activate its high affinity epitope and enable ligand binding (6). *P. gingivalis* has been shown to induce inside-out activation of CD11b/CD18 in monocytes/macrophages (25, 29), and to up-regulate 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 signalling pathways have been implicated. Firstly, the CD14-mediated binding of fimbriae and LPS to toll-like receptor 2 (TLR-2) stimulates CD11b/CD18 activation through a Rac1- and PI3K-mediated pathway (26, 28, 29). Secondly, 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 & Hajishengallis (29) have previously shown 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 signalling 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 binding of GTP, Rac as well as 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 repeatedly been demonstrated (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 pro-resolving effects (41, 63). Merched et al. (44) proposed that a failure in the endogenous synthesis of LXA₄ may underlie the unremitting inflammation that fuels atherosclerosis. LXA₄ functions mainly through the G-protein coupled receptor ALXR (18), and has repeatedly been shown to be protective in periodontal disease (36, 37, 61). Mouse models demonstrate that 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 control animals (64). Lipoxins have also been shown to down-regulate CD11b/CD18 expression on whole blood leukocytes (17). We have previously shown that LXA₄ modulates inside-out activation of CD11b/CD18 in neutrophils (51), and Godson and co-workers showed that LXA₄ may influence 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 bacterial-induced expression and function of CD11b/CD18 on neutrophils, possibly by inhibiting Rac2 and...
Cdc42 signalling 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 as follows: (S),6(R),15,Trihydroxyeicosa-7E,9E,11Z,13E-tetraenoic acid (LXA₄) (BioMol Research Lab inc., Plymouth Meeting, PA, USA); 5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol) and horseradish peroxidase (HRP); fMet-Leu-Phe (fMLF); Ethylenediaminetetraacetic acid (EDTA), Tween-20, fluorescein isothiocyanate (FITC) (Sigma Chemical Co., St. Louis, MO, USA); rhodamine phalloidin (Molecular Probes, Eugene, OR, USA); phenylmethanesulfonyl fluoride (PMSF), leupeptin, aprotenin, guanosine 5'-0-(thio)triphosphate (GTP₇S), dithiothreitol (DTT), Nonidet P-40 (NP-40), Pefablock SC [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF)] (Roche Diagnostics Corporation, Indianapolis, IN, USA); Ficoll-Paque, glutathione-Sepharose beads, and enhanced chemiluminescence (ECL) (GE-Healthcare GmbH, Uppsala, Sweden); Polymorphprep™ (Axis-Shield PoC AS, Oslo, Norway).

Buffers. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 6.7 mM Na₂HPO₄ × 2 H₂O, pH 7.3; Krebs-Ringer glucose buffer (KRG): 120 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 1.7 mM KH₂PO₄, 8.3 mM Na₂HPO₄, 10 mM glucose, pH 7.3; Acid citrate dextrose (ACD): 85 mM trisodiumcitrate × 2 H₂O, 71 mM citric acid and 111 mM glucose; Tris-buffer saline (TBS): 25 mM Tris base, 0.15 M NaCl, pH 7.4; TEDG-buffer: 50 mM Tris pH 7.4, 1.5 mM EDTA, 10% glycerol, 0.4% NaCl, 0.2 M dithiothreitol (DTT), 1 mM MgCl₂, 0.5% phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, and 1 µg/mL aprotenin; Lysis buffer for pulldown: 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 µg/mL leupeptin, 20 µg/mL aprotenin, 2.8 µg/mL pepstatin, and 2 mM Na₃VO₄.
Porphyromonas gingivalis (ATCC 33277, American Type Culture Collection, Manassas, VA 20108 USA) 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, UK). The bacteria was cultured for 3-4 days in fastidious anaerobe broth (29.7 g/L, pH 7.2), before being washed and resuspended in KRG supplemented with 1.1 mM CaCl₂. To estimate the bacterial concentration, optical density (OD) was measured at 600 nm in a Hitachi U2000 Spectrophotometer (KEBO Lab AB, Stockholm, Sweden) and set to approximately OD 2. Through viable count, where the bacteria were grown on fastidious anaerobe agar (46.0 g/L supplemented with L-tryptophan 0.1 g/L, pH 7.2; Lab M, Lancashire, UK) for 5-7 days, OD 2 was determined to correlate with approximately 10⁹ colony forming units (CFU)/mL.

Labelling of P. gingivalis with FITC. Bacteria, suspended in KRG supplemented with 1.1 mM CaCl₂, were incubated with 10 µg/mL FITC for 1 hour at room temperature (RT) during gentle agitation. The bacteria were washed in PBS-Tween (1% v/v), resuspended in KRG supplemented with 1.1 mM CaCl₂, and stored at -70°C. The bacteria were sonicated before further usage.

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 substituted with the same volume of 0.9 % NaCl. Heparin, which was used as an anti-coagulant, 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 Bexnorn et al. (8) 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/L CaCl₂ and kept on ice until further usage.

**Preparation of platelets.** Platelets were isolated from heparinised whole blood as previously described (25). Briefly, blood was mixed with RT ACD in 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 Bürker chamber and the suspension was kept in plastic tubes at RT until used. 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, in order to avoid any pre-activation. Previous evaluation has shown that platelets isolated in this manner are not activated and that contamination of other blood cells is negligible.

**Whole blood lumi-aggregometry.** Aggregation and ROS production was measured in heparinized whole or platelet-depleted blood using a lumi-aggregometer model 560 (Chrono-Log Corp., Havertown, PA, USA). Cell aggregation was measured as increased impedance (Ω).
between two platinum electrodes, and ROS production was simultaneously determined 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 pre-incubated 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) were subsequently added and incubated for 25 min, initiating cellular aggregation and ROS production. The instrument was calibrated before each experiment so that a 5-Ohm 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., Wildbaden, Germany). Briefly, PMN (1×10^6 cells/mL) were suspended in KRG containing 50 µM 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 nM, 100 nM), before being stimulated with *P. gingivalis* (1×10^7 CFU/mL) for one hour, 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 control. Care was taken to change the position of the samples between different experiments, so that the channels were used randomly.

**Platelet aggregation.** Aggregation of isolated platelets was analyzed under stirring conditions using a calibrated two-sample Lumi-Aggregometer 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, either in the presence or absence of LXA₄ (1 nM, 100 nM), before and then stimulated with *P. gingivalis* (1×10⁷ CFU/mL). Aggregation was measured as change in light transmission, where the unstimulated platelet suspension was set to 0% and the buffer (KRG) to 100%.

Fluorescence microscopy. Whole blood was pre-incubated for 15 min in the presence or absence of 500 nM LXA₄, prior to incubation with FITC-labelled *P. gingivalis* (1×10⁷ CFU/mL blood) for 25 min, and fixation overnight in 4% paraformaldehyde (PFA) at 4°C. In order 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 were after further washing mounted on a cover slip. 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 cover slip) using Scion Image software.

Flow cytometry. Immunolabeling was performed as described previously (51, 60). In short, whole blood was incubated at 37°C in 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, a R-Phycoerythrin (RPE)-conjugated anti-human CD11b antibody (mouse monoclonal ab, Clone 2LPM19c; DAKO, Glostrup, Denmark) was added 5 min after the onset of bacterial stimulation. To detect the high affinity epitope of CD11b, a FITC-conjugated anti-human CD11b antibody (mouse monoclonal ab, Clone CBRM1/5 (12)) diluted 1:13 was added 1 min prior to addition of the bacteria. The antibodies were added to separate samples in order to avoid sterical 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 was determined, representing roughly 7000 granulocytes, 4000 lymphocytes and 700 monocytes.

**Pull-down and Western blot.** 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 67-150 of PAK1 were cloned into the expression vector pGEX-4T3, kindly provided by the late Dr. Gary M. Bokoch (Scripps Research Institute, La Jolla, CA, USA) and expressed in *Escherichia coli*. Neutrophils (5×10⁶/sample) were pre-incubated in the presence or absence of 5 nM LXA₄ for 5 min at 37°C, and stimulated with *P. gingivalis* (6×10⁵ CFU/mL) for 10 min. The cells were subsequently lysed in ice-cold lysis buffer for 15 min at 4°C during gentle agitation. Cellular debris was cleared by centrifugation (10,000 × g, 10 min, 4°C) after which the supernatants were incubated for 45 min at 4°C with glutathione-Sepharose beads, which had been pre-coupled with GST-PBD. The beads were washed three times in lysis buffer and boiled in Laemmli sample buffer (98°C for 5 min) in order 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 1h, at RT), prior to incubation with the mouse monoclonal anti-Cdc42 antibody (1:250; Clone 44; BD Transduction Laboratories, San Diego, CA, USA), or a rabbit polyclonal anti-Rac2 antibody (1:200; Clone C-11; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1h at RT. The membranes were thereafter incubated with a HRP-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology), or a HRP-conjugated donkey anti-rabbit antibody (GE-Healthcare GmbH), respectively. To ascertain the specificity of the assay, 100 µM GTPγS (a non-hydrolysable GTP analogue, that arrests Cdc42 and Rac2 in their active state), and 1 mM EDTA were added in the cell lysate of fMLF (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-test, or by one-way ANOVA followed by Bonferroni Post Hoc test. Data were expressed as mean ± SEM, and $P<0.05$ were considered to be statistically significant.
Results

The effect of LXA4 on P. gingivalis-induced aggregation and ROS production in whole blood. Using lumi-aggregometry, P. gingivalis-induced aggregation and ROS production was examined in heparinized whole blood. No spontaneous aggregation or ROS production was seen when whole blood was incubated for 15 min at 37°C, nor did incubation with LXA4 per se induce any detectable responses (Figure 1A). Addition of P. gingivalis potently triggered both aggregation and ROS production of whole blood cells, as measured by changes in impedance and luminol-amplified chemiluminescence (Figure 1). The bacterial response was initiated after 5-6 min and reached an irreversible maximum after approximately 15 min. LXA4 inhibited the aggregation and ROS generation in a concentration-dependent manner (Figure 1B). Pre-incubation with 500 nM LXA4 significantly decreased both the P. gingivalis-induced aggregation (63%; P<0.01), and ROS production (31%; P<0.01) (Figure 1B). At a cellular level, LXA4 is usually potent at concentrations ranging between 1-10 nM. In our experimental set-up, 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 metabolic inactivation of the bacteria since we found that LXA4 did not per se affect P. gingivalis viability in buffer (data not shown).

The 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-labelled P. gingivalis and rhodamine phalloidin-staining of F-actin in leukocytes and platelets. Stimulation of whole blood with P. gingivalis induced the formation of large mixed aggregates of platelets, leukocytes and bacteria (Figure 2).
A majority of the leukocytes had internalised a high number of bacteria (Figure 2C, E). Pre-incubation 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 (Figure 2D, F, G). There was no spontaneous formation of aggregates in whole blood when incubated at 37°C, neither after incubation with LXA4 per se (Figure 2A, B). Accumulations of rhodamine-stained neutrophils and platelets, together with FITC-labelled bacteria, appear as yellow when the colours merge.

**The 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 over the course of one hour. *P. gingivalis* induced a marked increase of ROS production. However, preincubation with LXA4, at either 1 nM or 100 nM concentrations, did not alter the *P. gingivalis*-induced ROS production (Figure 3). Unstimulated neutrophils did not produce any significant levels of ROS, nor did pre-incubation with LXA4 per se induce any detectable ROS (data now shown).

**The 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) (Figure 4). Unstimulated platelets did not spontaneously aggregate, nor did LXA4 induce an aggregatory response by itself (data not shown).

**The 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 lumi-aggregometry. 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 (Figure 5).

**The effect of LXA₄ on *P. gingivalis*-induced up-regulation of CD11b and exposure of the high affinity integrin epitope on leukocytes.** The *P. gingivalis*-induced up-regulation of CD11b, and exposure of the high affinity epitope on neutrophils, was analyzed by flow cytometry. Expression of CD11b on the cell surface of neutrophils was increased by 25% upon stimulation with *P. gingivalis*, and was significantly decreased to basal level when the cells were pre-incubated with 500 nM LXA₄ (*P*<0.05) (Figure 6A, 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 pre-incubation with 500 nM LXA₄ (*P*<0.01) (Figure 6B, D). A small and possibly dose-dependent reduction in CD11b expression and high affinity epitope exposure was seen when incubating cells with merely LXA₄. However, this reduction was not statistically significant and was therefore disregarded. It is noteworthy that a small subset of the PMN population appeared to express reduced CD11b when stimulated with *P. gingivalis* (Figure 6C). A possible explanation to this could be 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 was therefore inaccessible to the antibody. *P. gingivalis* also induced surface expression of CD11b/CD18 in the monocytic population, which furthermore was inhibited by LXA₄ (supplemental Figure 1). Lymphocytes expressed very low levels of CD11b/CD18, and neither *P. gingivalis* nor PMA (which was used as positive control) affected the expression in our protocol setup (data not shown).
The effect of LXA$_4$ on *P. gingivalis*-induced activation of Rac2 and Cdc42. *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). Activation of both Rac2 and Cdc42 from isolated neutrophils was detected as early as 0.5 min after addition of *P. gingivalis* and persisted up to 10 min (Figure 7A). Pre-incubation with 5 nM LXA$_4$ for 5 min markedly inhibited the *P. gingivalis*-induced activation of both Rac2 and Cdc42 (Figure 7A). No unspecific binding using uncoupled GST beads was detected (Figure 7B).
Discussion

In the present study, the anti-inflammatory and pro-resolving eicosanoid LXA$_4$ is shown to effectively inhibit *P. gingivalis*-induced aggregation and ROS production in whole blood. Our data suggest that this effect is mediated by a LXA$_4$-induced impediment of *P. gingivalis*-triggered activation of the small GTPases Rac2 and Cdc42 in neutrophils, and the up-regulation of CD11b/CD18 and its high affinity epitope. Interestingly the effect of LXA$_4$ 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 LXA$_4$. *P. gingivalis*-induced surface expression of CD11b/CD18 in monocytes was also inhibited by LXA$_4$, although the high affinity epitope remained unaffected. This is noteworthy since *P. gingivalis*-induced CD11b/CD18 up-regulation on monocytes has been shown to have a crucial role in stimulating monocyte adhesion to endothelial cells and transendothelial migration (28). Possibly LXA$_4$ could thereby also 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 signalling to expose and activate its high affinity epitope and enable ligand binding (7). 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 previously shown that ligation of CD11b/CD18 activates Rac2 and Cdc42 (21). Lipoxins have been shown to modulate inside-out activation of CD11b/CD18 (48), and to down-regulate CD11b/CD18 expression in neutrophils (18). In monocytes/macrophages, LXA₄ has also 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 up-regulation and activation. Another possible mechanism of how LXA₄ modulates CD11b/CD18 integrins could be through impeding Akt activation, since CD11b up-regulation 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 pro-resolving and anti-inflammatory properties of lipoxins (8, 62). 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 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 in order 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 is therefore possible that LXA₄ inhibits *P. gingivalis*-induced ROS via intercepting neutrophil/platelet interaction through the down-regulation of CD11b/CD18.

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 internalised a high number of *P. gingivalis*. Hajishengallis *et al.* (26) have shown that fimbriae of *P. gingivalis* induce CD11b/CD18-binding and internalisation of the bacteria within macrophages through CD14/TLR2 signalling. Internalised *P. gingivalis* replicates within the host cell by activating cellular autophagy, while suppressing apoptosis (2), which is a mechanism of bacterial survival and spreading of the infection. Thus, our findings 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 down-regulation of the *P. gingivalis*-induced Rac2 and Cdc42 activation and the inside-out signalling of CD11b/CD18. Consequently, we suggest that a supplement of exogenous LXA₄ may facilitate anti-inflammatory actions and reduce the progression of chronic inflammatory disorders, such as periodontitis and atherosclerosis.
Acknowledgement

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References


inflammation and osteoclast-mediated bone destruction in periodontitis. Faseb J 20:401-403.


Figure 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; 1-500 nM) prior to stimulation with *P. gingivalis* (P.g; 1×10⁷ CFU/mL blood) for 25 min. Aggregation was measured as changes in impedance and reactive oxygen species (ROS) production was detected by luminol-amplified chemiluminescence. Figure (A) shows a representative experiment (500 nM lipoxin A₄) and figure (B) shows *P. gingivalis*-induced aggregation and ROS production presented as fold induction. Data are presented as mean ± SEM of n=7. *P < 0.05 and **P < 0.01.
P. gingivalis

Vehicle LXA₄

Aggregate size (µm²)

G

* 20µm 100µm 20µm 20µm 20µm

100µm

P. g. P.g. + LXA₄

Aggregate size (µm²)

0 1000 2000 3000 4000 5000 6000

P. g. P.g. + LXA₄

*
Figure 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 (1×10⁷ 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-B) or presence (C-F) of P. gingivalis and pre-incubated with (B, D, F) or without (A, C, E) LXA₄ as indicated. Rhodamine phalloidin-labelled leukocytes and platelets appear as red, and FITC-labelled bacteria as green. Figure (A) shows unstimulated whole blood and (B) whole blood in the presence of LXA₄. (A-D) shows magnification ×63 and (E-F) magnification ×10. To analyze the microscopy data, the size of 30 aggregates over a set area was assessed using Scion Image software (G). Paired student t-test was used for statistical analysis and data are presented as representative images (A-F) or mean ± SEM of n=6. *P < 0.05.
Isolated polymorphonuclear cells (PMN), consisting predominantly of neutrophils, were preincubated for 15 minutes 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 over the course of one hour. PMN stimulated with vehicle did not produce any detectable ROS nor did LXA₄ induce a chemiluminescence response. ANOVA was used for statistical analysis and data are presented as fold induction mean ± SEM of n=5.
Platelet Aggregation
(% of P.g.-stimulated control)

P.g.

P.g. + LXA4 (1 nM)

P.g. + LXA4 (100 nM)

Light transmission (aggregation, %)

P. gingivalis

Control

P. gingivalis

Lipoxin A4 (1 nM)

P. gingivalis

Lipoxin A4 (100 nM)
Figure 4. LXA₄ does not affect P. gingivalis-induced aggregation of isolated platelets. Isolated platelets were pre-incubated for 15 minutes 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). Figure (A) shows representative aggregation traces of P. gingivalis with or without LXA₄ (1 nM or 100 nM) and figure (B) shows P. gingivalis-induced aggregation presented as fold induction. The vehicle did not cause platelet aggregation nor did LXA₄ induce an aggregatory response. Data are presented as fold induction mean ± SEM of n=3.
Figure 5. The effect of LXA$_4$ 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 LXA$_4$ (500 nM). Aggregation was measured as changes in impedance and ROS production as luminol-amplified chemiluminescence during 25 min incubation with P. gingivalis (1.5×10$^7$ CFU/ml blood). Figure (A) shows a representative experiment and figure (B) shows P. gingivalis-induced ROS production presented as fold induction. Paired student t-test was used for statistical analysis. Data is presented as mean ± SEM of n=3. AU = arbitrary units.
Figure 6. LXA₄ inhibits P. gingivalis-induced up-regulation 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; 1×10⁷ CFU/mL blood) for 10 min. Following lysis of erythrocytes, the samples were fixed in 0.1% PFA and analyzed by flow cytometry. The granulocyte population (consisting of predominantly neutrophils) was gated by plotting forward scatter (FSC) versus side scatter (SSC) and the 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, C) and the high affinity domain was recognized through the use of a FITC-conjugated mouse anti-human CD11b antibody clone CBRM1/5 (B, D). ANOVA was used for statistical analysis and data are presented as mean ± SEM of n=5. *P < 0.05 and **P < 0.01.
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A. **P. gingivalis (min)**

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B. **Pos.** **Wc.** **Neg.**

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Figure 7. LXA₄ inhibits P. gingivalis-induced activation of Rac2 and Cdc42 in neutrophils. Neutrophils (5×10⁶/sample) were pre-incubated in the absence or presence of 5 nM LXA₄ for 5 min at 37°C, and stimulated with P. gingivalis (6×10⁵ CFU/mL) for various time points as indicated in the figure. The stimulation was stopped, and activated forms of Rac2 and Cdc42 were precipitated from the lysates using GST-pulldown assay. (A) Neutrophils stimulated by P. gingivalis for 0.5, 3 and 10 min with and without pre-incubation with LXA₄, GST-pulldown assay performed with beads lacking the GST-PBD (Neg; negative control) or neutrophils incubated at 0°C or 37°C (basal level). (B) Neutrophils stimulated with fMLF + GTPγS (Pos; positive control), whole cell lysate (Wc; positive control), or GST-pulldown assay 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 blot technique using a polyclonal rabbit anti-human Rac2 antibody (1:200) and a mouse monoclonal anti-human Cdc42 antibody (1:250) Clone 44.