Lipoxin A_4 Analogues Inhibit Leukocyte Recruitment to *Porphyromonas gingivalis*: A Role for Cyclooxygenase-2 and Lipoxins in Periodontal Disease†

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ABSTRACT: The potential involvement of the inducible cyclooxygenase isoform (COX-2) and the role of novel lipid mediators were investigated in the pathogenesis of periodontal disease. Crevicular fluids from localized juvenile periodontitis (LJP) patients contained prostaglandin (PG)E_2 and 5-lipoxygenase-derived products, leukotriene B_4, and the biosynthesis interaction product, lipoxin (LX)A_4. Neutrophils from peripheral blood of LJP patients, but not from asymptomatic donors, also generated LXA_4, suggesting a role for this immunomodulatory molecule in periodontal disease. To characterize host responses of interest to periodontal pathogens, *Porphyromonas gingivalis* was introduced within murine dorsal air pouches. In the air pouch cavity, *P. gingivalis* elicited leukocyte infiltration, concomitant with elevated PGE_2 levels in the cellular exudates, and upregulated COX-2 expression in infiltrated leukocytes. In addition, human neutrophils exposed to *P. gingivalis* also upregulated COX-2 expression. Blood borne *P. gingivalis* gave significant increases in the murine tissue levels of COX-2 mRNA associated with both heart and lungs, supporting a potential role for this oral pathogen in the evolution of systemic events. The administration of metabolically stable analogues of LX and of aspirin-triggered LX potently blocked neutrophil traffic into the dorsal pouch cavity and lowered PGE_2 levels within exudates. Together, these results identify PMN as an additional and potentially important source of PGE_2 in periodontal tissues. Moreover, they provide evidence for a novel protective role for LX in periodontitis, limiting further PMN recruitment and PMN-mediated tissue injury that can lead to loss of inflammatory barriers that prevent systemic tissue invasion of oral microbial pathogens.

Polymorphonuclear leukocytes (PMNs,[1] neutrophils) play an important role in periodontal disease, the leading cause of tooth loss among adults (for a review, see ref *1*). PMNs are the most abundant immune cells recruited to early inflammatory periodontal lesions and are the most numerous host cells within the periodontal tissues (*2*). The presence of Gram-negative oral pathogens represents the primary etiologic factor; however, the progression of periodontal disease is dependent on the host response to pathogenic bacteria that colonize the tooth surface (*2*). Hence, recruitment of PMNs followed by aberrant release of inflammatory mediators not only contributes to the onset of periodontal disease and is associated with rapid and widespread tissue destruction (*3*), but can also be further amplified by the release of an array of inflammatory mediators by neutrophils within the periodontium.

Several inflammatory mediators such as cytokines, chemokines, and metalloproteases are associated with periodontal disease (*4*−*6*), and prominent among these are the arachidonic acid-derived products, including leukotriene (LT)B_4 and prostaglandin (PG)E_2 (*7*). Indeed, many of the pathophysiological events that occur in periodontal diseases can be explained to a large extent by the activities of lipid mediators (*8*−*12*). For example, LTB_4, a well-appreciated and potent chemoattractant, also initiates the accumulation of leukocytes within inflamed sites, stimulates the release of granule-associated enzymes (*13*), and was recently found to stimulate bone resorption (*14*). Along these lines, PGE_2 is a very potent stimulator of bone loss, which is held to be a hallmark of periodontal disease (*15*). PGE_2 is also well appreciated for its ability to directly mediate vasodilation, increase vascular permeability, enhance pain perception by bradykinin, and histamine, alter connective tissue metabolism, and enhance osteoclastic bone resorption (*16*). The levels of PGE_2 are significantly elevated in the crevicular fluid (CF) of patients with periodontal infections, especially localized juvenile periodontitis, when compared to healthy

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[1] Abbreviations: LX, lipoxin; LXA_4, 5*S*,6*R*,15*S*-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; LXA_4 analogue, 16-phenoxy-LXA_4-methyl ester; ATL, aspirin-triggered lipoxin; ATL analogue, 15*R*/*S*-methyl-LXA_4-methyl ester; CF, crevicular fluid; LT, leukotriene; PG, prostaglandin.

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sites (*17*). These levels correlate with disease severity and aggressiveness and constitute a reliable indicator of ongoing clinical periodontal tissue destruction (*17*). CF-PGE_2 levels can also be used to predict future acute loss of periodontal attachment (*7*).

Prostaglandin endoperoxide synthase (cyclooxygenase, COX) catalyzes two reactions by which arachidonic acid is converted to PGH_2, the common precursor of all prostanoids including PGE_2. To date, two COX isoforms are known (*18*). COX-1 appears to support the levels of prostanoid biosynthesis required for maintaining organ and tissue homeostasis (*18*, *19*), whereas COX-2 expression appears to be restricted in basal conditions within most tissues and is upregulated during inflammation or stress in a wide range of tissues (*20*−*22*). The finding that homogenates of inflamed periodontal tissues display an increased PGE_2 synthetic capacity when compared to homogenates from healthy tissues suggests an increased COX activity is associated with periodontal tissues (*23*−*26*). Moreover, given the clearly deleterious actions of PGE_2 on the integrity of tissues of the periodontal pocket, both the potential involvement of the inducible COX isoform (COX-2) in periodontal disease and potential role of novel lipid mediators are of interest in the pathogenesis of periodontal disease.

Lipoxin (LX) and aspirin-triggered LX (ATL) are arachidonic acid-derived bioactive lipids that are formed by interactions between individual lipoxygenases (LO) and appear to play an important role in downregulating neutrophil responses in inflammation (*27*). In the nanomolar range, LXA_4 and its 15R epimer (15-epi-LXA_4) triggered by aspirin each inhibit fMLP- and LTB_4-stimulated PMN adhesion and transmigration and hence represent potential counterregulatory signals operative in the resolution of inflammatory sites (reviewed in refs *27* and *28*; also see refs *29* and *30*). Like most autacoids and lipid mediators, LX are rapidly generated, act within a local microenvironment, and are rapidly enzymatically inactivated. To advance our understanding of LX and ATL roles in vivo, metabolically stable LX and ATL analogues were designed that resist rapid enzymatic inactivation and mimic the in vitro actions of naturally occurring LX and ATL (*30*). In the present report, LXA_4, PGE_2, and LTB_4 were identified in human CF, and an oral microbe clinically associated with periodontal disease, *Porphyromonas gingivalis*, was examined in an animal model of leukocyte trafficking and activation. This microbe potently attracted leukocytes in vivo and also upregulated the expression of COX-2 from infiltrating leukocytes. Moreover, topical administration of metabolically stable analogues of LX-ATL within the pouch cavity potently blocked *P. gingivalis*-mediated neutrophil infiltration.

EXPERIMENTAL PROCEDURES

Isolation of Human Polymorphonuclear Leukocytes. Human polymorphonuclear leukocytes (PMN) from healthy volunteers and patients with juvenile periodontitis were obtained by gradient centrifugation of heparinized fresh venous blood (*31*). Resulting granulocyte suspensions contained fewer than 0.2% monocytes as determined by esterase staining, and viability was greater than 96% as determined by trypan blue dye exclusion.

Eicosanoid Analyses. Freshly isolated PMN (5×10^6 cells) were suspended in 0.5 mL of Hank's-buffered saline with 1.6 mM Ca^{2+} and incubated with A23187 (4 μM) at 37 °C for 20 min. The incubations were stopped with 2 vol of cold methanol and kept at −20 °C overnight. Protein precipitates were pelleted by centrifugation and washed twice with methanol. The supernatants were pooled and the eicosanoids were extracted with Extract-Clean solid-phase cartridges (500 mg C_{18}, Alltech Associates Inc., Deerfield, IL), using PGB_2 ($[M - H]^- = m/z$ 333) as an internal standard for extraction recovery calculations (*32*). The methyl formate fractions were taken to dryness with a gentle stream of nitrogen and suspended in mobile phase for LC/MS/MS analyses. LC/MS/MS was performed employing an LCQ (Finnigan MAT, San Jose, CA) quadrupole ion trap mass spectrometer system equipped with an electrospray ionization probe. Samples were injected into the HPLC component, comprised of a SpectraSYSTEM P4000 (Thermo Separation Products, San Jose, CA) quaternary gradient pump, a LUNA C18-2 (150 × 2 mm, 5 μm) column, and a SpectraSYSTEM UV2000 (Thermo Separation Products, San Jose, CA) UV−vis absorbance detector. The column was eluted isocratically for 20 min with methanol/water/acetic acid (65:34.99:0.01, v/v/v) at 0.2 mL/min, followed by a 20 min linear gradient to methanol/acetic acid (99.99:0.01, v/v), and into the electrospray probe. The spray voltage was set to 5−6 kV and the heated capillary to 250 °C. Eicosanoids were quantitated by selected ion monitoring (SIM) for analyte molecular anions (e.g., $[M - H]^- = m/z$ 351.5 for LXA_4 and m/z 335.5 for LTB_4). Product ion mass spectra (MS/MS) were also acquired for definitive identification of the compounds.

Gingival CF from juvenile periodontitis patients was collected on periostrips (as in ref *33*). The periostrips were placed in 50 μL of phosphate-buffered saline with 20% Tween 20 and LXA_4, LTB_4, and PGE_2 were quantitated by specific ELISA analyses (Neogen Corporation, Lexington, KY). As determined by LC/MS/MS, recoveries of known amounts of LXA_4, LTB_4, and d_4-LTB_4 from periostrips were linear over a 100 pg to 10 ng range, with 82.7% ($r^2 = 0.996$), 85.6% ($r^2 = 0.999$), and 72.7% ($r^2 = 0.996$) recovery, respectively.

Murine Leukocyte Trafficking: Air Pouches, *P. gingivalis*, *and Local Exudates.* Six to eight week old male BALB/c mice were obtained from Taconic Farms (Germantown, NY). Air pouches were raised on the dorsum by s.c. injection of 3 mL of sterile air on day 0 and day 3, and all experiments were carried out on day 6 (*34*). Individual air pouches (one per mouse) were injected with either vehicle alone (0.1% ethanol), with 10 μg of 15-*R/S*-methyl-LXA_4-me or with 10 μg of 15-epi-16-phenoxy-LXA_4-me, followed by 500 μL of sterile PBS or $\sim 10^5$ cells of *P. gingivalis* strain A7436 ($OD_{600} = 0.9-1.0$) originally obtained from the CF of a patient diagnosed with periodontitis. Mice were sacrificed 4 h postinjection and individual air pouches were lavaged three times with sterile PBS (3 mL for each lavage) as in refs (*34*, *35*). The exudates were centrifuged at 2000 rpm (5 min), and supernatants were taken and stored at −20 °C. Cell pellets were suspended in PBS (200 μL) for enumeration by light microscopy and 50 μL of each cell suspension were mixed with 150 μL of 30% BSA and then centrifuged onto microscope slides at 500 rpm for 5 min using a cytospin centrifuge, air-dried, and stained with Giemsa-Wright to identify individual cell type. Air pouch exudates were assessed for PGE_2 using an enzyme immunoassay (EIA) kit

[Cayman Chemical Co., Ann Arbor, MI (cross-reactivities in the PGE_2 EIA kit were <0.04% for 6-keto $PGF_{1\alpha}$ and <0.01% for LTB_4, thromboxane B_2, and arachidonic acid)]. For intravenous procedures, 100 μL of the same *P. gingivalis* suspension was injected in the orbital plexus.

Northern Blots, RT-PCR. Total RNA isolation and hybridization were performed essentially as in ref *36*. Briefly, filters were hybridized with human or mouse COX-2 cDNA probes which were synthesized by reverse-transcription polymerase chain reaction (RT-PCR). The primers used were 5′-GCT GAC TAT GGC TAC AAA AGC TGG-3′ and 5′-ATG CTC AGG GAC TTG AGG AGG GTA-3′ for human COX-2; 5′-AAC TCC CAT GGG TGT GAA GGG A-3′ and 5′-CCA AAG ATA GCA TCT GGA CGA G-3′ for mouse COX-2. Integrity of the RNA and equal loading on agarose/formaldehyde gels were verified by hybridization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The observed COX-2 mRNA band was approximately 4.6 kb. Autoradiograms were scanned using an Epson 636 scanner (Epson America). For RT-PCR analyses, total RNA was extracted by homogenizing tissues in Trizol (Gibco-BRL, Grand Island, NY), according to the manufacturer's instructions. One microgram of total RNA was used in each reaction using Titan One tube RT-PCR (Roche Molecular Biochemicals, Indianapolis, IN). Reverse transcription (RT) and polymerase chain reaction (PCR) were sequentially performed according to the following profile: 50 °C for 30 min for RT, then 94 °C for 30 s; 60 °C for 30 s; 72 °C for 1 min; repeated 35 times for PCR, followed by a final extension at 72 °C for 10 min. Primers used for mouse COX-2 were identical to those mentioned above and the expected PCR product was 1.0 kb in length. For the detection of *P. gingivalis* in mouse tissues, primers specific for 16S ribosomal RNA of the bacteria (Genbank accession number: L16492) were utilized: 5′-GGC AGG CGG AAT TCG TGG TGT A-3′ and 5′-GAT GTA AGG GCC GTG CTG ATT TGA-3′. PCR products, both for *P. gingivalis* ribosomal RNA and mouse GAPDH, had an expected length of 0.5 kb. Samples were migrated on 1% agarose gel containing ethidium bromide and photographs of the gels were taken under UV illumination. All densitometry analyses were performed using the National Institute of Health Image program (http://rsb.info.nih.gov).

Statistical Analyses. Statistical analyses were performed by Student's unpaired *t*-test (two-tailed), and significance was considered to be attained when P was <0.05.

RESULTS

LX and LT Products of the 5- and 15-LO Pathways: Generation by Peripheral Blood PMN and Presence in CF from Periodontal Disease Patients. It was suggested that localized juvenile periodontitis (LJP) patients present altered lipid metabolism, including the 15-LO pathway (*37*). Therefore, the capacity of peripheral blood PMN from LJP patients and periodontal disease from age, sex, and race matched controls to produce 5- and 15-LO-derived eicosanoids, as well as the LO interaction product LXA_4, was evaluated. PMN from both healthy donors and LJP patients incubated with arachidonic acid produced both 5- and 15-HETE from exogenous substrate, which were identified by LC/MS/MS and chiral-phase HPLC analyses, indicating that the alcohol at carbon 15 position of 15-HETE was in the S configuration, and suggesting involvement of a 15-lipoxygenase (data not shown). On the other hand, activated PMN produced LTB_4 and its ω-oxidation metabolite 20-OH-LTB_4, in addition to 5- and 15-HETE generated from endogenous sources of arachidonic acid (Table 1). Of interest, activated PMN from LJP patients, but not from controls, namely asymptomatic individuals without evidence of clinically documented periodontal disease, also generated LXA_4 (Figure 1). The presence of a number of enzymes involved in the production of lipid mediators, including 5-, 12-, 15-LO, COX-1, and COX-2, in leukocytes from periodontitic patients was also confirmed by RT-PCR (data not shown). CF from periodontal disease, specifically patients with LJP, were analyzed for the presence of key eicosanoids, including LXA_4, PGE_2, and LTB_4 from each major class of eicosanoid mediators. LXA_4 was also present in the crevicular fluid of patients (Table 2), suggesting a potential role for this immunomodulatory molecule (*27*) in the local inflammatory sequelae observed within the periodontium of patients with periodontal diseases. Moreover, both the proinflammatory COX and the 5-LO-derived eicosanoids, PGE_2 and LTB_4, respectively, were also demonstrated in the CF, consistent with a recent report (*16*).

Table 1: Eicosanoids Formed by Activated Peripheral Blood PMN[a]

	LTB_4 (ng)	20-OH-LTB_4 (ng)	5-HETE (ng)	15-HETE (ng)
nonperiodontal disease donor	29	76	0.51	0.11
LJP-001	11	42	0.00	0.46
LJP-002	28	103	0.38	0.04
LJP-003	24	150	0.05	0.52
LJP-004	24	63	4.10	0.65

[a] PMN (5×10^6) of nonperiodontal disease donors and LJP patients were isolated and suspended in 0.5 mL of Hank's with 1.6 mM Ca^{2+} and incubated (20 min, 37 °C) in parallel with A23187 (4 μM). Samples were prepared for LC/MS/MS and eicosanoids were identified by signature MS and MS/MS ions and the quantities were calculated from the recovery of the internal standard (PGB_2). Values for activated PMN from nonperiodontal disease donors are representative and consistent with those obtained for at least $n = 6$ healthy donors.

P. gingivalis Elicits Leukocyte Infiltration and COX-2 Expression in Vivo. PMN recruitment to gingival sites and high PGE_2 levels are associated with periodontal disease (*17*, *37*). We therefore sought to determine the impact that *P. gingivalis* may have in vivo on leukocyte trafficking and COX-2 expression. To this end, we used a murine air pouch model to assess leukocyte infiltration and activation. Six-day air pouches were raised (*34*) and were injected with either *P. gingivalis* or sterile PBS, and lavage exudates were collected for examination (see Experimental Procedures). *P. gingivalis* elicited a massive leukocyte infiltration into the air pouches. Approximately 10 million leukocytes were enumerated in the *P. gingivalis*-injected air pouch exudates (Figure 2). This cell infiltration represents approximately three times more leukocytes than in air pouches injected with murine TNFα (*35*). These inflammatory exudates were comprised predominantly of neutrophilic infiltrate that represented ~80−85% of the total leukocytes recruited at 4 h. The remainder of the recruited leukocytes was mononuclear cell infiltrate ~15−20%, consistent with earlier findings (*35*), yet suggesting that *P. gingivalis* stimulates greater numbers of PMN in this model than murine TNFα.

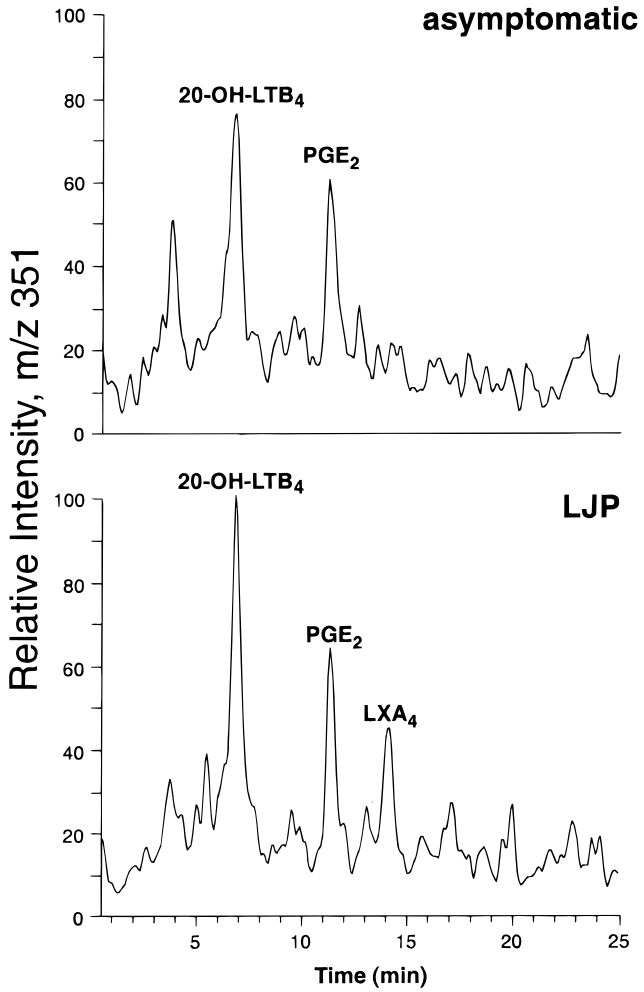

FIGURE 1: Activated peripheral blood PMN from periodontitis patients: LC/MS/MS chromatograms. PMN were freshly isolated from blood of asymptomatic and LJP patients in parallel. Cells were suspended in 0.5 mL of Hank's with 1.6 mM Ca^{2+} and incubated with A23187 (20 min, 37 °C). After stopping with 2 vol of MeOH with PGB_2 as internal standard, samples were prepared for LC/MS/MS analysis by solid-phase extraction. Chromatograms are representative of $n = 4$. Eicosanoids were identified by signature MS and MS/MS ions (see Experimental Procedures).

Table 2: Crevicular Fluids from LJP Patients Contain Eicosanoids[a]

	PGE_2	LTB_4	LXA_4
amount	10.2 ± 0.3	8.7 ± 0.2	1.7 ± 1.0
ratio	6.2	5.1	1

[a] Values represent the mean ± SEM for 10 determinations from 4 LJP patients (pg/μL-CF sample) from specific ELISA analyses. Ratios (relative to LXA_4) are indicated for each eicosanoid. Average CF volume: 0.70 ± 0.16 μL (mean ± SEM; $n = 10$).

Also, in the present experiments, relatively few leukocytes were present in PBS-injected air pouch lavages. These results showed that *P. gingivalis* represents a potent stimulus for the recruitment of leukocytes, predominantly neutrophil infiltrate, within a localized site or cavity (i.e., dorsal pouch).

P. gingivalis Induces COX-2. COX-2 is induced in human PMN by a number of inflammatory mediators, including *Escherichia coli* (*36*). We determined whether *P. gingivalis* directly stimulates COX-2 expression in PMN freshly isolated from peripheral blood. *P. gingivalis* increased the levels of COX-2 mRNA in a time-dependent fashion when compared to that from vehicle-treated PMN (Figure 3). An

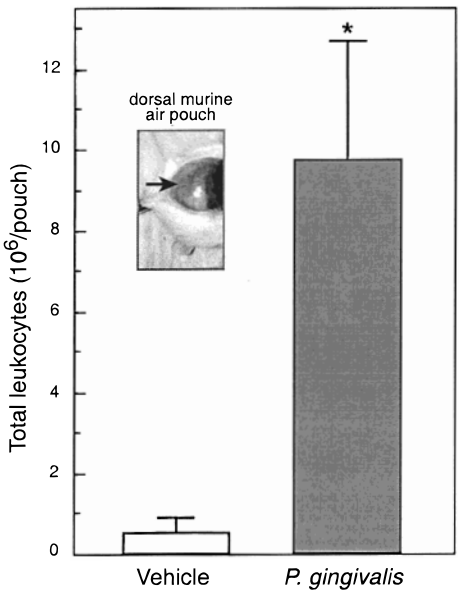

FIGURE 2: *P. gingivalis* elicits leukocyte infiltration in vivo. Six-day air pouches were raised and injected either with sterile PBS (vehicle) or 10^5 bacteria (*P. gingivalis*) and the exudates were collected after 4 h. The total number of leukocytes were enumerated as in Experimental Procedures. Results are expressed as mean ± SEM from three different mice for each group. (*) Statistically significant difference from vehicle-injected air pouches ($P < 0.01$). (Inset) Photographic view of an excised dorsal murine air pouch. Arrow indicates the membranous air pouch cavity formed at 6 days.

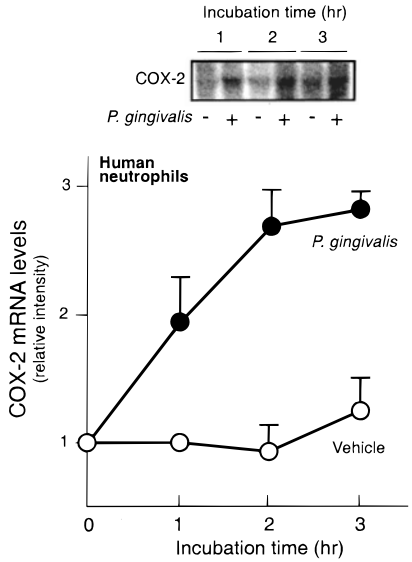

FIGURE 3: *P. gingivalis* induces COX-2 expression in human PMN. Isolated human PMN were incubated for indicated times in the presence of vehicle or 10^5 bacteria (*P. gingivalis*) then processed for RNA extraction; northern blots were performed in order to detect COX-2 mRNA. Densitometric analysis of the bands representing COX-2 mRNA is presented; the intensity of each band was corrected for GAPDH and normalized to vehicle-treated PMN. A representative immunoblot and densitometry results from two separate donors are shown.

increase was first observed at 1 h and COX-2 mRNA levels further increased in the presence of the bacteria, compared to vehicle alone, for up to 3 h. Next, the expression of COX-2 in leukocytes which migrated into the air pouch cavity was determined. As assessed by northern blot, COX-2 expression was upregulated in these activated leukocytes (see Figure 5, inset). These results indicated that oral pathogens associated

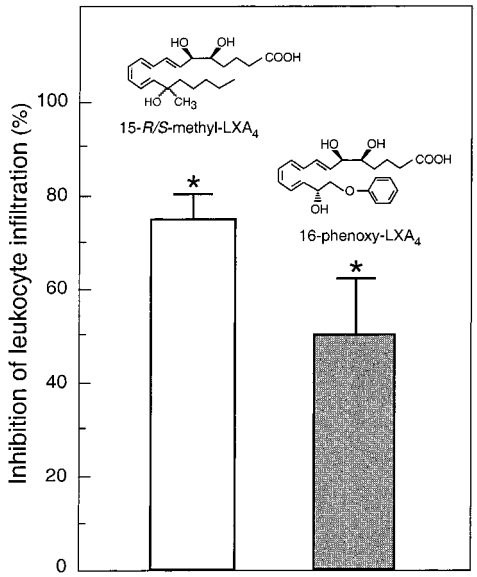

FIGURE 4: LXA_4 analogues inhibit leukocyte infiltration in vivo. Sterile PBS containing either aspirin-triggered analogue (10 μg, 15-*R*/*S*-methyl-LXA_4) or LXA_4 analogue (10 μg, 16-phenoxy-LXA_4) followed by *P. gingivalis* were injected into the 6-day air pouches. After 4 h, the exudates were collected and the total number of leukocytes were enumerated. Results are expressed as mean ± SEM ($n = 3$) from three different mice for each point. (*) Denotes statistically significant difference from air pouches injected with *P. gingivalis* ($P < 0.01$).

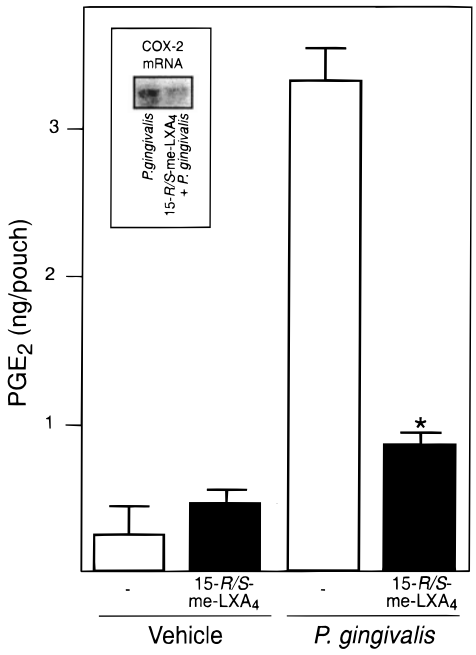

FIGURE 5: Aspirin-triggered LX-analogue, 15-*R*/*S*-methyl-LXA_4, inhibits *P. gingivalis*-induced PGE_2 production in murine air pouch. Sterile PBS containing either vehicle (0.1% ethanol) or 10 μg of 15-*R*/*S*-methyl-LXA_4 (15-*R*/*S*-me-LXA_4), followed by sterile PBS or *P. gingivalis*, was injected into the 6-day air pouches and the exudates were collected at 4 h and PGE_2 levels in the air pouch exudates were determined by ELISA. The results are expressed as mean ± SEM from three different mice for each point. (*) Statistically different from *P. gingivalis*-injected mice without 15*R*/*S*-methyl-LXA_4 ($P < 0.01$).

with periodontal diseases such as *P. gingivalis* upregulate COX-2 expression in human PMN and in infiltrating leukocytes. These results provide support for the hypothesis that neutrophils, which constitute the first and most numerous inflammatory cell type migrating to local gingival tissues in periodontal disease, may constitute a previously unappreciated source of COX-2 derived eicosanoids, including PGE_2, in the periodontium.

LX Analogues Inhibit P. gingivalis-Elicited Leukocyte Infiltration in Vivo. Since LXA_4, an immunomodulatory molecule, was present in CF and was generated in vitro by PMN from periodontal disease (LJP) patients (Tables 1 and 2), evidence was sought for a potential role for lipoxins in the host defense mechanisms evoked by *P. gingivalis*. In view of the finding that LXA_4 and aspirin-triggered LX (LX-ATL) analogues reduce leukocyte trafficking stimulated by TNF-α in the murine air pouch (*35*), and because PMN are the most abundant inflammatory cells recruited to pathogen-infected gingival sites in periodontal disease, we evaluated the impact of the metabolically stable LX-ATL analogues 15-*R*/*S*-methyl LXA_4 and 16-phenoxy LXA_4 on the recruitment of leukocytes into the air pouch. Air pouches were injected either with vehicle, with 15-*R*/*S*-methyl LXA_4 (10 μg/pouch), or with 16-phenoxy LXA_4 (10 μg/pouch) then injected with viable *P. gingivalis*, as in Figure 4. In these experiments, LX-ATL analogues dramatically reduced the recruitment of leukocytes into the air pouches exudate. Both analogues were essentially equipotent inhibitors of leukocyte infiltration, decreasing leukocytes within in the exudates by up to 75% with as little as 10 μg of application (Figure 4).

Since PGE_2 is associated with loss of attachment and bone loss in periodontal disease (*38*), we determined PGE_2 levels in the air pouch exudates. Air pouches injected with *P. gingivalis* contained elevated PGE_2 levels compared to those of vehicle-injected air pouches (Figure 5). *P. gingivalis* stimulated the production of nanogram levels of PGE_2 in the exudates, which paralleled the upregulated expression of COX-2 in infiltrating leukocytes (Figure 5, inset). The ATL analogue 15-*R*/*S*-methyl LXA_4 inhibited PGE_2 production generated in response to the oral pathogen, decreasing PGE_2 levels in the exudates by as much as 75%. This inhibition in PGE_2 levels paralleled the decrement in leukocytes observed within the exudate (Figure 4). Also, the expression of COX-2 was evaluated in exudate leukocytes. 15-*R*/*S*-Methyl LXA_4 within the air pouch decreased the overall expression of COX-2 in exudates (Figure 5, inset). These results indicate that *P. gingivalis* induces the in vivo expression of COX-2 within infiltrating leukocytes as well as the production of PGE_2. Moreover, they indicate that LX-ATLs are potent regulators of PGE_2 production in air pouch exudates as a consequence of inhibiting leukocyte transmigration and reducing COX-2 mRNA levels.

P. gingivalis Elicits Systemic Upregulation of COX-2 Expression. Evidence is accumulating to support the notion that periodontal disease may affect, and worsen, systemic diseases such as coronary heart disease, preterm labor, and diabetes mellitus (*1*) and that brushing or trauma to an inflamed gingival site can lead to septicemia (*39*). Because COX-2 expression is upregulated in acute and chronic inflammatory situations (*40*), we determined the systemic impact that *P. gingivalis* may have by evaluating selected organ-associated levels of COX-2 mRNA. It should be noted that COX-2 was initially identified as an early response gene (*41*). A suspension of *P. gingivalis*, or an equivalent volume of sterile PBS, was injected in the orbital plexus of the mice. After 4 h, animals were sacrificed and COX-2 mRNA levels

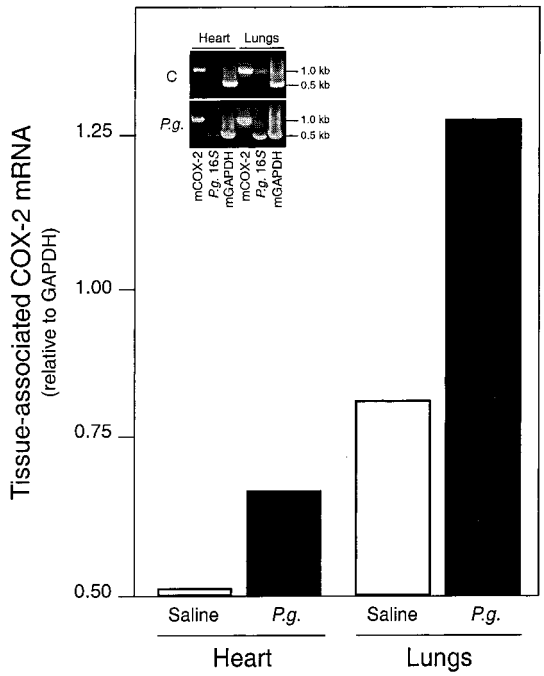

FIGURE 6: *P. gingivalis* causes systemic upregulation of COX-2 mRNA. Mice were intravenously injected as described in Experimental Procedures either with sterile PBS or with *P. gingivalis*. Heart and lungs were collected from sacrificed mice and processed for RT-PCR assessment of COX-2 and GAPDH mRNA levels, as well as for presence of *P. gingivalis* 16S ribosomal RNA (see Experimental Procedures). Results represent expression of COX-2 mRNA relative to that of GAPDH in each sample from two separate experiments. (Inset) Representative RT-PCR for PBS (C)- and *P. gingivalis* (*P.g.*)-injected animals.

were determined in the heart and lungs. Intravenous injection of *P. gingivalis* caused a significant increase in the levels of COX-2 mRNA associated with the heart and lungs (Figure 6). *P. gingivalis*-specific 16*S* ribosomal RNA (see Experimental Procedures) was readily observed in the heart and lungs from mice injected with the oral pathogen and was absent in tissue samples from the PBS-injected animals (Figure 6, inset). These results demonstrate the capacity of *P. gingivalis* to upregulate COX-2 expression systemically in a murine model, supporting a potential role for this oral pathogen in the evolution of systemic events.

DISCUSSION

PMN participate in host defense against bacterial infections and are also involved in noxious inflammatory reactions (*42*, *43*). Recruitment of neutrophils to the periodontium contributes to the progression of periodontal disease and to the destruction of periodontal tissues (*1*, *3*). In the present study, we identified for the first time by LC/MS/MS-based analyses the eicosanoids generated by peripheral blood neutrophils from periodontitis (e.g., LJP) patients. These molecules included LTB_4 and PGE_2, consistent with a recent report (*16*), and we document for the first time the production of LXA_4 by activated neutrophils from LJP patients, as well as the presence of LXA_4 within the crevicular fluid from periodontitis patients with active disease. These results are the first demonstration that LJP peripheral blood neutrophils are in a primed state for LX generation. This in vivo "priming" for upregulated lipoxin profiles was also observed with neutrophils isolated from asthmatic patients (*44*) and can be mimicked in vitro with cytokine priming of neutrophils from healthy donors (*45*).

Results from an earlier report that examined the conversion of exogenous arachidonic acid by peripheral blood neutrophils from LJP donors suggested that LJP PMN may display impaired 15-LO activity since the authors found lower amounts of 15-HETE generated by LJP neutrophils than those from healthy donors (*37*). In the present experiments, activation of intact neutrophils from LJP gave lipoxins from endogenous sources (Figure 1). Impaired 15-LO enzymatic activity was not observed with LJP neutrophils (see Results). Since 15*S*-HETE and 15*S*-hydroperoxy-ETE are precursors for LX biosynthesis by human neutrophils (*27*, *28*), and in view of our present results, it is likely that the lower levels of 15-HETE found with LJP leukocytes examined earlier (*37*) could reflect the in vitro incubation conditions and perhaps further biosynthetic conversion of the substrate 15-HETE to LX.

P. gingivalis rapidly (<4 h) attracted large numbers of leukocytes, primarily neutrophils (>80%), in an in vivo model of leukocyte infiltration. The presence of leukocytes in the air pouch exudates was marked with elevated PGE_2 levels, suggesting that this mediator might originate in part from the infiltrating leukocytes. Of interest, exudate leukocytes express COX-2 in vivo, and exposure of human PMN to *P. gingivalis* also stimulated the expression of COX-2 (Figure 3). These results indicate that periodontal pathogens can attract leukocytes in vivo and induce leukocyte-COX-2, suggesting a role for the neutrophil-derived COX-2 in the production of PGE_2 found in pathogen-infected periodontal tissues.

Our present findings also support the concept that LXA_4, which has an immunomodulatory action, may be involved in the regulation of the local acute inflammatory responses in periodontal disease. Along these lines, we found that LX-ATL analogues, which are topically active (*29*), were potent inhibitors of *P. gingivalis*-elicited leukocyte migration toward a site of infection and concomitantly reduced the overall levels of COX-2 mRNA associated with inflammatory exudates, which was accompanied by a decreased production of PGE_2.

Pathophysiological responses that occur in periodontal diseases, including inflammatory cell recruitment, edema, pain, bone resorption, and collagen destruction can be mediated for the most part by effector molecules originating from the arachidonate cascade (*8*−*12*). In particular, considerable evidence has demonstrated the importance of PGE_2 in the pathogenesis of periodontal diseases. In vitro, PGE_2 increases osteoclast numbers and bone resorption (*46*), decreases proteoglycan synthesis, and increases metalloprotease production by cultured chondrocytes (*47*). Bone resorption in vivo caused by three periodontal pathogens is mediated in part by PGE_2, causing tooth attachment loss and bone loss (*15*). Prior to these findings, PGE_2 was proposed as a reliable molecular indicator of ongoing periodontal tissue destruction that might be used to predict future acute periodontal attachment loss (*7*). In light of the present results, the inducible COX isoform expressed in recruited leukocytes (local exudate) may be a major source responsible for production of PGE_2 found in the CF of periodontal disease patients (Figures 1, 3, and 5). Hence, by their elevated numbers recruited to these sites, the neutrophil can be a

significant source of PGE_2 and a major contributor to PGE_2-mediated bone loss along with macrophages (*48*) and gingival fibroblasts, as recently noted (*49*).

In addition to the deleterious effects of periodontal disease on oral tissues and structural integrity, periodontitis represents a potential risk factor for increased morbidity or mortality for several systemic conditions including cardiovascular diseases, pregnancy complications, and diabetes (*1*, *50*). In this context, we found that the systemic presence of *P. gingivalis* upregulates expression of COX-2 (heart and lungs; Figure 6), a marker of ongoing inflammation (*40*). Also, the concept that a local infection by *P. gingivalis* may have a systemic impact on the status of the immune system was further substantiated by results obtained in pilot studies, where *P. gingivalis* injected in the air pouch upregulated COX-2 mRNA levels in the lung-associated tissues (data not shown). In view of these results, an effective treatment of periodontal conditions is likely to have a beneficial impact on the prognosis of a number of systemic diseases. LXA_4 and ATL analogues reduce leukocyte trafficking stimulated by TNF-α while concomitantly reorientating the cytokine-chemokine axis toward an antiinflammatory profile (*35*). LX-ATL can thus protect host tissues via multilevel regulation of proinflammatory signals. In view of their inhibitory impact on neutrophil recruitment and secondarily on PGE_2 levels, LX-ATL may be beneficial to the host not only in the context of periodontitis, but also in a number of diseases which involve excessive PMN responses that can lead to losses in inflammatory barriers and increase invasion of systemic microbes.

Although bacteria appear to be essential for the causation of periodontitis, progression of periodontal disease is dependent on the host response to pathogens that colonize the tooth surface (*2*). In turn, periodontal disease can be controlled chemotherapeutically by uncoupling host-mediated destruction rather than reducing the etiological load (*51*). Along these lines, a body of evidence has identified the inhibition of PGE_2 formation and its presence at gingival sites as being relevant therapeutic interventions. For example, PGE_2 generation from gingival homogenates is significantly inhibited by flurbiprofen (*26*), and COX-derived eicosanoids in CF are decreased in animals taking flurbiprofen (*52*, *53*). Flurbiprofen also reduced CF-PGE_2 levels, gingival inflammation, tooth attachment loss, and bone loss, and in some cases resulted in bone gain (*54*). In humans, flurbiprofen dramatically decreased the CF-PGE_2 levels (*55*). These findings suggest that NSAIDs may exert their pharmacological action of inhibiting COX-derived proinflammatory eicosanoids within the periodontium and suggest that novel antiinflammatory agents might be useful in managing periodontal diseases.

Given the large number of PMN recruited at inflammatory lesions in periodontal disease, the results of our experiments identify the PMN as a potential cellular primary target for therapeutic intervention. LX-ATLs have the potential of blocking PMN infiltration as well as reducing COX-2 derived PGE_2 present in gingival tissues. In this regard, topical application of novel anti-neutrophil agents (*32*, *35*) as well as new selective COX-2 inhibitors (*22*, *40*, *56*) may prove to be advantageous in this disease and associated pain since it could eliminate potential unwanted side-effects (particularly renal effects in the elderly) associated with systemic delivery of nonsteroidal antiinflammatory drugs (*57*, *58*).

In summary, *P. gingivalis* was found to potently elicit leukocyte infiltration in vivo, concomitant with an upregulation of COX-2 mRNA in recruited leukocytes. These findings indicate a novel role for PMN-associated COX-2 participating in the elevated PGE_2 levels found in CF from periodontal disease patients. Neutrophils from these patients generate LXA_4, and of interest LX-ATL analogues inhibited *P. gingivalis*-stimulated leukocyte recruitment, concomitant with a significant decrease in PGE_2 levels. Hence, the relationship between lipoxin generation and other eicosanoid markers such as PGE_2 and LTB_4 may provide an important disease index in view of the known deleterious effects mediated by PGE_2 in periodontal diseases and the potential protective contributions of LXA_4.

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