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Lipid A-associated proteins from *Porphyromonas gingivalis* stimulate release of nitric oxide by inducing expression of inducible nitric oxide synthase

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Choi E-Y, Hwang Y-M, Lee J-Y, Choi J-I, Choi I-S, Jin J-Y, Ko JS, Kim S-J. Lipid A-associated proteins from Porphyromonas gingivalis *stimulate release of nitric oxide by inducing expression of inducible nitric oxide synthase. J Periodont Res 2007; 42: 350–360. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard*

Background and Objective: The purpose of this study was to examine the effects of lipid A-associated proteins from *Porphyromonas gingivalis*, a major cause of inflammatory periodontal disease, on the production of nitric oxide and expression of inducible nitric oxide synthase in the murine macrophage cell line, RAW264.7. We also attempted to throw light on the signaling mechanisms involved in *P. gingivalis* lipid A-associated protein-induced nitric oxide production.

Material and Methods: The lipid A-associated proteins from *P. gingivalis* 381 were prepared by standard hot phenol-water extraction of endotoxin isolated by the butanol method. Nitric oxide production was assayed by measuring the accumulation of nitrite in culture supernatants. Western blot analysis of inducible nitric oxide synthase and analysis of reverse transcription-polymerase chain reaction products were carried out.

Results: We found that *P. gingivalis* lipid A-associated proteins can induce inducible nitric oxide synthase expression and stimulate the release of nitric oxide without additional stimuli, and we demonstrated that multiple signaling pathways, such as nuclear factor-κB, microtubule polymerization, protein tyrosine kinase, protein kinase C, and mitogen-activated protein kinase cascades, are involved in *P. gingivalis* lipid A-associated protein-stimulated nitric oxide production. The production of nitric oxide required L-arginine.

Conclusion: The present study clearly shows that *P. gingivalis* lipid A-associated proteins fully induced inducible nitric oxide synthase expression and nitric oxide production in RAW264.7 cells in the absence of other stimuli. The ability of *P. gingivalis* lipid A-associated proteins to promote the production of nitric oxide may be important in the pathogenesis of inflammatory periodontal disease.

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The proteinaceous components of endotoxin, known as lipid A-associated proteins or endotoxin-associated proteins, are a family of proteins that bind to lipopolysaccharide in the cell wall of gram-negative bacteria (1). The lipid A-associated proteins were first recognized when it was discovered that the B-cell mitogenic activity of

Escherichia coli endotoxin in the lipopolysaccharide-unresponsive C3H/HeJ mouse depended on the protein content of the endotoxin (2). The lipid A-associated proteins may be co-extracted with lipopolysaccharide, depending on the method used to extract lipopolysaccharide, and may contribute to its biological action. Given the potent immunomodulatory activities of the enteric bacterial lipid A-associated proteins and their extreme stability to heat and proteases, it is possible that such proteins from periodontopathogenic bacteria may play an important role in mediating the tissue destruction characteristic of inflammatory periodontal disease.

Reddi *et al*. (3) showed that the lipid A-associated proteins of *Porphyromonas gingivalis* and *Prevotella intermedia* were able to stimulate human gingival fibroblasts or the human myelomonocytic cell line, Mono-Mac-6, to produce interleukin-6. Comparison of the activities of the lipid A-associated proteins and lipopolysaccharide from *P. gingivalis* on human gingival fibroblasts revealed that the lipopolysaccharide was less potent than lipid A-associated proteins at inducing interleukin-6 release from the fibroblasts (3). Comparison of the lipopolysaccharide and lipid A-associated proteins of *Actinobacillus actinomycetemcomitans* also demonstrated that the lipid A-associated proteins were significantly more active inducers of cytokine synthesis than the lipopolysaccharide (4). Sharp *et al*. (5) have characterized the lipid A-associated proteins from the major periodontopathogen *P. gingivalis* and have identified the active cytokine-inducing component in this population of proteins. In addition, the lipid A-associated proteins of *A. actinomycetemcomitans*, *P. gingivalis*, *and Eikenella corrodens* were able to stimulate the breakdown of cultured bone (6). These findings add weight to the hypothesis that the lipid A-associated proteins from periodontopathogens play a major part in the pathology of the chronic lesion accompanied by osseous tissue destruction observed in inflammatory periodontal disease.

Nitric oxide is a short-lived bioactive molecule produced by immunocompetent cells, such as macrophages, and serves as a messenger molecule for various physiological and pathological processes (7). It is synthesized from L-arginine by nitric oxide synthase, which is present in various tissues (8). Three distinct isoforms of nitric oxide synthase – neural, endothelial and inducible – have been reported in mammalian tissues (8). Expression of inducible nitric oxide synthase, also commonly called NOS-2, is induced by inflammatory stimuli, such as bacterial lipopolysaccharide, and by pro-inflammatory cytokines, such as tumor necrosis factor-α, interleukin-1β, and interferon-γ, in a variety of cell types (including macrophages) following bacterial infection (9,10). Inducible nitric oxide synthase, once expressed, can generate large amounts of nitric oxide for extended periods of time, and is believed to be involved in cytotoxic effects following inflammation (7). Nitric oxide has recently received considerable attention as a novel type of mediator (7); inhibition of nitric oxide synthase activity and nitric oxide production frequently limits the progression and severity of experimental inflammatory diseases such as osteoarthritis, glomerulonephritis, and colitis (11,12).

Periodontal disease is a chronic inflammatory process accompanied by destruction of surrounding connective tissue and alveolar bone, and sometimes loss of teeth (13). The primary causative agents of periodontal disease are particular gram-negative anaerobic bacteria that accumulate in the gingival sulcus. Nitric oxide is thought to have an important role in the pathogenesis of inflammatory periodontal disease as it does in other inflammatory diseases. Enhanced production of nitric oxide has been demonstrated in periodontal disease (14), and gingival tissues from patients with chronic periodontitis have higher levels of inducible nitric oxide synthase protein and mRNA than healthy tissue (15–18). Macrophages, polymorphonuclear cells, and fibroblasts are the sources of inducible nitric oxide synthase in periodontal tissues, with endothelial cells also contributing (15–18). Moreover, lipopolysaccharide from *A. actinomycetemcomitans*, a major pathogen of early-onset periodontitis, has been demonstrated to induce significant production of nitric oxide in macrophages (19,20), and lipopolysaccharides from *P. intermedia* and *P. nigrescens*, the causative agents of inflammatory periodontal disease, fully induced inducible nitric oxide synthase expression and nitric oxide production in the murine macrophage cell line, RAW264.7, in the absence of other stimuli (21,22).

P. gingivalis is a major periodontal pathogen that is dominant in the periodontal pockets of patients with adult periodontitis (23,24). The lipid A-associated proteins of *P. gingivalis* may play a key role as a virulence factor in the development and progression of chronic inflammatory periodontal disease, stimulating the host cells to produce and release nitric oxide. The purpose of this study was to investigate the effects of *P. gingivalis* lipid A-associated proteins on the production of nitric oxide and the expression of inducible nitric oxide synthase protein and mRNA in RAW264.7 cells, a murine macrophage cell line. We also attempted to throw light on the signaling pathway involved in the stimulation of nitric oxide production.

Material and methods

Bacteria and culture conditions

P. gingivalis 381 was used throughout. It was grown anaerobically on the surface of enriched Trypticase soy agar containing 5% (v/v) sheep blood, or in general anaerobic medium broth (Nissui, Tokyo, Japan) supplemented with 1 μg/mL of menadione and 5 μg/mL of hemin. Plate-grown cultures were routinely incubated for 4 d and used as the inoculum for liquid growth. Liquid-grown cells were incubated for $\approx$24 h, to late-exponential growth phase. They were collected by centrifugation at 12,000 *g* for 20 min at 4°C, washed three times with phosphate-buffered saline (pH 7.2), and lyophilized. Culture purity was assessed by gram staining and plating on solid medium.

Preparation of lipid A-associated proteins

To remove bioactive extracellular material, the bacteria were suspended in saline, stirred gently for 1 h at 4°C, and harvested by centrifugation. This process was repeated, and the endotoxin was extracted from the surface-washed *P. gingivalis* by using the butanol extraction method of Morrison & Leive (25). Briefly, cells were suspended in 0.15 M NaCl at 4°C and an equal volume of butanol was added. The suspension was mixed thoroughly at 4°C for 10 min and centrifuged at 35,000 *g* for 20 min. The aqueous phase was removed, and the butanol, together with the insoluble residue, was further extracted twice with approximately half of the initial volume of saline for each extraction. The combined aqueous phases were centrifuged to remove any insoluble residues, dialysed against distilled water at 4°C for 48 h, and lyophilized. This crude preparation, which contains both lipid A-associated proteins and lipopolysaccharide, was resuspended in water and ultracentrifuged at 105,000 *g* for 3 h. This procedure was repeated once more and the endotoxin lyophilized. The lipid A-associated proteins were prepared from lyophilized endotoxin by the standard hot phenol-water method (26). Briefly, 90% phenol was added to the endotoxin suspended in pyrogen-free distilled water and the mixture was extracted at 68°C for 20 min. After cooling, the phenol phase, which contains the lipid A-associated proteins, was separated by centrifugation at 35,000 *g* and the aqueous phase was removed. The phenol phase was extracted twice more with water, before being dialysed against distilled water for 96 h. The lipid A-associated proteins were then lyophilized and stored at −70°C until required.

Analysis of lipid A-associated proteins

The protein content of the purified lipid A-associated proteins was determined by the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard. The carbohydrate content was estimated by the method of Dubois *et al.* (27), using glucose as a standard. The lipopolysaccharide content in the lipid A-associated proteins preparation was determined by a commercial chromogenic *Limulus* amoebocyte lysate assay (QCL-1000; Cambrex, Walkersville, MD, USA) with *E. coli* lipopolysaccharide as a standard. The discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis procedure of Laemmli (28) was employed for determination of protein distribution. Acrylamide separating gels of 12% were used. Gels were run with a 4% acrylamide stacking gel in a Bio-Rad mini-Protean II system (Bio-Rad, Richmond, VA, USA) and proteins were visualized by Coomassie Brilliant Blue-R-250 stain (Sigma Co., St Louis, MO, USA).

Cell cultures

The murine macrophage cell line, RAW264.7 (American Type Culture Collection, Rockville, MD, USA), was grown in Nunc flasks containing Dulbecco's modified Eagle's medium supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, 10 mM HEPES, 2 mM L-glutamine, 0.2% $NaHCO_3$, 1 mM sodium pyruvate, and 10% (v/v) heat-inactivated fetal bovine serum, in a humidified chamber with 5% CO_2/95% air at 37°C. At confluence, the medium and nonadherent cells were removed and replaced with fresh culture medium. After an additional 24 h of culture, the cells were harvested by gentle scraping with a rubber policeman, washed three times, and viable cells counted. The cells were seeded into 24-well culture plates at a density of 5×10^5 cells/well and incubated for at least 2 h to allow them to adhere to the plates. After washing three times with medium, various concentrations of lipid A-associated proteins were added and the cells were cultured for the indicated times, after which culture supernatants were collected and assayed for nitric oxide.

Cytotoxicity assay

The cellular toxicity of several inhibitors was assessed by the MTT assay, which is based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan by mitochondrial dehydrogenases (29). Cells were pretreated with the indicated concentrations of each inhibitor before incubation for 24 h with *P. gingivalis* lipid A-associated proteins (10 μg/mL). MTT was added to the cultures to a final concentration of 0.5 mg/mL after 24 h. After incubation at 37°C in 5% CO_2 for 2 h, the supernatant was removed and the cells were solubilized in dimethylsulfoxide. The extent of reduction of MTT to formazan within the cells was quantified by measuring absorbance at 570 nm with a Spectra Max 250 ELISA Reader (Molecular Devices, Palo Alto, CA, USA). Cell viability is expressed as a percentage of the control value.

Measurement of nitric oxide production

Nitric oxide production was assayed by measuring the accumulation of the stable oxidative metabolite, nitrite (NO_2^-), in culture supernatants (30). Briefly, 5×10^5 cells/well were stimulated in 24-well tissue culture plates for the indicated periods of time, and 100 μL of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, and 2.5% phosphoric acid) (Sigma) was added to equal volumes of culture supernatants in a 96-well flat-bottomed microtiter plate and left at room temperature for 10 min. Optical densities at 540 nm were read with a Spectra Max 250 ELISA Reader (Molecular Devices), and nitrite concentrations were calculated from a standard curve established with serial dilutions of $NaNO_2$ (Sigma) in culture medium.

Western blot analysis of inducible nitric oxide synthase

Cells were plated in 60-mm tissue culture dishes, at a density of 2×10^6 cells per dish, and treated with various

concentrations of *P. gingivalis* lipid A-associated proteins for the indicated periods of time. After incubation, they were washed three times with ice-cold phosphate-buffered saline and lysed by incubating for 30 min on ice with 200 μL of lysis buffer (50 mM Tris·Cl, pH 8.0, 150 mM NaCl, 0.002% sodium azide, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40) containing protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 5 mg/mL of aprotinin, 5 mg/mL of pepstatin A, and 5 mg/mL of leupeptin). The cell lysates were centrifuged at 10,000 *g* for 10 min to remove insoluble material, and their protein concentrations determined with the bicinchoninic acid protein assay reagents (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The same amount of protein (50 μg) from each supernatant was then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% acrylamide gels with 3% stacking gels. The resolved proteins were transferred to a nitrocellulose membrane by electroblotting, and the blots were blocked for 1 h in phosphate-buffered saline containing 0.1% Tween-20 and 3% nonfat dry milk, followed by incubation with polyclonal antibody against inducible nitric oxide synthase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted (1 : 50) in phosphate-buffered saline containing 1.5% goat serum for 1 h at room temperature. They were then washed three times, for 10 min each, with phosphate-buffered saline containing 0.1% Tween-20, incubated with a 1 : 2000 dilution of horseradish peroxidase-conjugated goat antirabbit secondary antibody (Santa Cruz Biotechnology) at room temperature for 1 h, and visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK), as recommended. The intensity of each protein-specific band was quantified by densitometry by using densitometric software.

Reverse transcription–polymerase chain reaction and analysis of PCR products

Cells were plated in 100-mm tissue culture dishes at a density of 2×10^7 cells per dish and treated with 10 μg/mL of *P. gingivalis* lipid A-associated proteins for the indicated periods of time. Following incubation, they were washed twice with phosphate-buffered saline and collected by centrifugation. Total RNA was isolated with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Synthesis of cDNA from the extracted RNA, and subsequent amplification of the cDNA by reverse transcription-polymerase chain reaction (RT-PCR), were carried out with an AccuPower RT/PCR Premix kit (Bioneer, Daejeon, Korea) and thermal cycler (GeneAmp PCR system 2400; PE Applied Biosystems, Foster City, CA, USA) (31). β-actin served as an internal control. The number of cycles that ensured nonsaturating PCR conditions was established in preliminary experiments. PCR amplification of inducible nitric oxide synthase was carried out for 35 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min. The oligonucleotide primers were as follows: inducible nitric oxide synthase, 5′-TCACTGGGACAGCACAGAAT-3′ (sense) and 5′-TGTGTCTGCAGATGTGCTGA-3′ (antisense) (corresponding to positions 348–367 and 857–838, respectively, of the published mouse inducible nitric oxide synthase mRNA sequence), yielding a 510-bp product; β-actin, 5′-TCCTTCGTTGCCGGTCCACA-3′ (sense) and 5′-CGTCTCCGGAGTCCATCACA-3′ (antisense) (corresponding to positions 44–63 and 553–534, respectively, of the published mouse actin mRNA sequence), yielding a 508-bp product. The PCR-amplified products were run on a 1.5% agarose gel containing ethidium bromide and visualized with ultraviolet light. The intensities of the PCR bands on gel photographs were quantified by densitometry, and expression of inducible nitric oxide synthase mRNA was calculated as the ratio of the densities of the inducible nitric oxide synthase and actin bands. Preliminary experiments established that there was a linear relationship between total RNA levels in cell extracts (after 24 h of stimulation with lipid A-associated proteins) and the density of PCR products from inducible nitric oxide synthase and β-actin mRNA (data not shown).

Statistical analysis

Statistical analysis was performed using the Student's paired *t*-test, with a *p*-value of < 0.05 considered statistically significant. Data are expressed as means ± standard deviation of four independent experiments.

Results

Composition of *P. gingivalis* lipid A-associated proteins

The protein and carbohydrate contents of *P. gingivalis* lipid A-associated proteins were 15.5% and 15.6%, respectively. The lipopolysaccharide content of the lipid A-associated proteins preparation, determined by the *Limulus* amoebocyte lysate assay, was 0.026 EU/ng. When fractionated on a 12% gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the lipid A-associated proteins from *P. gingivalis* revealed four very prominent bands at approximate molecular weights of 52, 47, 32, and 29 kDa, together with other faint bands (Fig. 1).

Nitric oxide induction by *P. gingivalis* lipid A-associated proteins

Concentrations of nitrite, an indicator of nitric oxide production, were measured 24 h after adding various concentrations of isolated *P. gingivalis* 381 lipid A-associated proteins to RAW264.7 cells. *P. gingivalis* lipid A-associated proteins induced nitric oxide release from the RAW264.7 cells over the range of 1 to 100 μg/mL (Fig. 2). Basal nitrite release was ≈1 μM. It was effective at a concentration as low as 1 μg/mL, and maximum nitric oxide production (≈ 47 μM) was achieved at a concentration of 100 μg/mL.

RAW264.7 cells were challenged with 10 μg/mL of *P. gingivalis* lipid A-associated proteins, and production of nitric oxide in the culture supernatant was measured at various

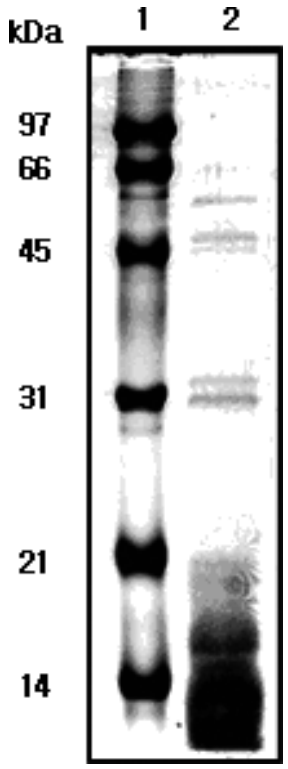

Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of lipid A-associated proteins from *Porphyromonas gingivalis* 381. Each lane contains 30 μg of protein. Lane 1, molecular weight standards; Lane 2, *P. gingivalis* lipid A-associated proteins.

time-points thereafter. After an initial lag of 4 h, nitric oxide secretion increased linearly from 4 to 24 h and plateaued thereafter. Nitrite accumulation reached 25 μM (Fig. 3).

Lipid A-associated proteins were diluted to a concentration of 10 μg/mL and either heated to 100°C for 1 h or digested with 0.25% trypsin (Sigma) for 30 min prior to addition to cells. The heat- or trypsin-treated lipid A-associated proteins were added and the cells were cultured for 24 h, after which culture supernatants were collected and assayed for nitric oxide. Exposure to heat or trypsin inhibited the nitric oxide-stimulating activity of the *P. gingivalis* lipid A-associated proteins by 21.1% and 39.2%, respectively (Fig. 4). *P. gingivalis* lipid A-associated proteins (10 μg/mL) were also incubated with 40 μg/mL of polymyxin B at 37°C for 60 min before adding RAW264.7 cells to the wells and then incubated for a further 24 h. Polymyxin B inhibited the nitric oxide-inducing effect of *P. gingivalis* lipid A-associated proteins by 49.5% (Fig. 4).

To determine whether bacterial surface components other than lipid A-associated proteins could stimulate nitric oxide synthesis by RAW264.7 cells, cells were incubated with 100 μg/mL of saline-extractable surface-associated material (SAM), lipopolysaccharide or lipid A-associated proteins from *P. gingivalis*. Lipopolysaccharide extracted from this bacterium was significantly more potent than the other surface extracts in stimulating release of nitric oxide (Fig. 5). The activity of *P. gingivalis* lipopolysaccharide was ≈86% of *Salmonella typhimurium* lipopolysaccharide treatment values, as seen in Fig. 5. The SAM and lipid A-associated proteins were approximately equipotent (Fig. 5).

Expression of inducible nitric oxide synthase protein and mRNA

To determine whether the elevated nitric oxide production was a result of the up-regulation of the inducible nitric oxide synthase protein, we examined the effect of *P. gingivalis* lipid A-associated proteins on the expression of inducible nitric oxide synthase protein and demonstrated an increase in inducible nitric oxide synthase protein by immunoblotting. Cells stimulated with *P. gingivalis* lipid A-associated proteins expressed a protein of ≈130 kDa,

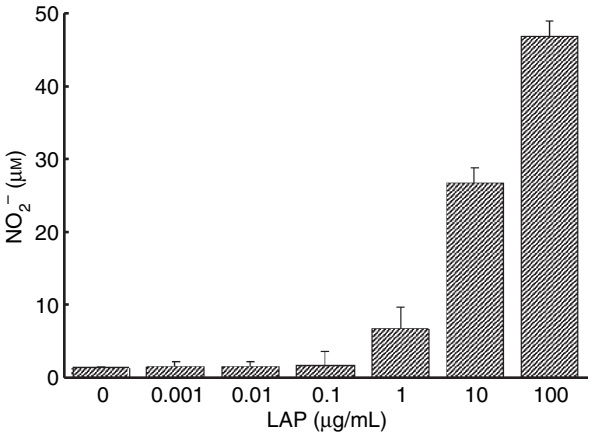

Fig. 2. Dose–response of nitric oxide production by RAW264.7 cells stimulated with *Porphyromonas gingivalis* lipid A-associated proteins. Cells were incubated with increasing concentrations of lipid A-associated proteins and supernatants were removed after 24 h and assayed for nitric oxide. The results are the means ± standard deviation of four experiments. LAP, lipid A-associated proteins.

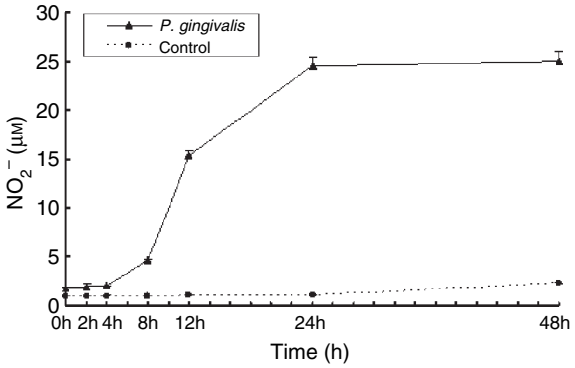

Fig. 3. Time course of nitric oxide production by RAW264.7 cells stimulated with *Porphyromonas gingivalis* lipid A-associated proteins. Cells were incubated with 10 μg/mL of lipid A-associated proteins. Other details as in Fig. 2.

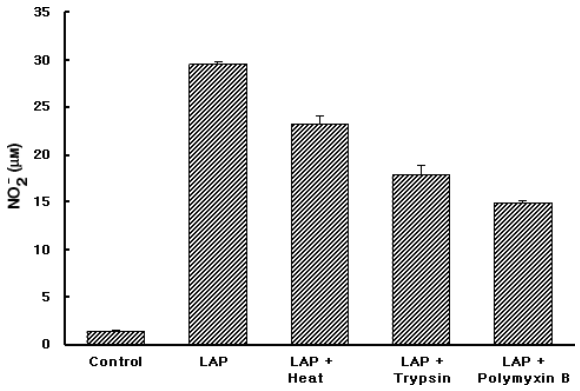

Fig. 4. Inhibition of nitric oxide-inducing effects of *Porphyromonas gingivalis* lipid A-associated proteins with heat, trypsin, or polymyxin B. Lipid A-associated proteins (10 μg/mL) were either heated to 100°C for 1 h or digested with 0.25% trypsin for 30 min prior to addition to cells. *P. gingivalis* lipid A-associated proteins (10 μg/mL) were also incubated with 40 μg/mL of polymyxin B at 37°C for 60 min before adding RAW264.7 cells to the wells and then incubated for an additional 24 h. LAP, lipid A-associated proteins.

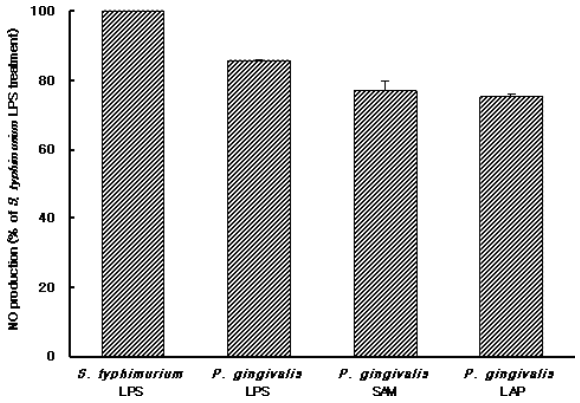

Fig. 5. Ability of surface components of *Porphyromonas gingivalis* to stimulate RAW264.7 cells to synthesize nitric oxide. Cells were incubated with 100 μg/mL of surface-associated material, lipopolysaccharide or lipid A-associated proteins from *P. gingivalis*. The nitric oxide production shown is a percentage of *Salmonella typhimurium* lipopolysaccharide treatment values. Other details as in Fig. 2. LPS, lipopolysaccharide; NO, nitric oxide; SAM, surface-associated material.

recognized by specific antibody to inducible nitric oxide synthase (Fig. 6). When RAW264.7 cells were exposed to increasing concentrations of *P. gingivalis* lipid A-associated proteins, there was a concentration-dependent accumulation of inducible nitric oxide synthase (Fig. 6A). Inducible nitric oxide synthase protein was detectable with a concentration of *P. gingivalis* lipid A-associated proteins as low as 0.1 μg/mL and reached a maximum at a concentration of 100 μg/mL. Control cells did not produce a detectable inducible nitric oxide synthase band, as seen in Fig. 6A. Figure 6B shows the time course of changes in inducible nitric oxide synthase protein expression induced by 10 μg/mL of *P. gingivalis* lipid A-associated proteins. Inducible nitric oxide synthase protein showed detectable signal at 4 h, and maximum expression was achieved at 24 h.

The effect of *P. gingivalis* lipid A-associated proteins on inducible nitric oxide synthase transcription and accumulation of inducible nitric oxide synthase mRNA was confirmed by RT-PCR. Exposure of cells to *P. gingivalis* lipid A-associated proteins enhanced inducible nitric oxide synthase mRNA expression (Fig. 7). Figure 7 shows the time course of changes in inducible nitric oxide synthase mRNA expression induced by 10 μg/mL of *P. gingivalis* lipid A-associated proteins. Inducible nitric oxide synthase mRNA showed detectable signal at 2 h, and maximum expression was achieved at 8 h. Unstimulated RAW264.7 cells did not contain detectable amounts of inducible nitric oxide synthase mRNA.

Effects of various inhibitors on nitric oxide production

N^G-monomethyl-L-arginine, an L-arginine analogue, is a specific inhibitor of nitric oxide production in the L-arginine-dependent pathway (32). To determine if the signaling mechanism of *P. gingivalis* lipid A-associated proteins-induced nitric oxide production involves this pathway, RAW264.7 cells were pretreated with the indicated concentrations of N^G-monomethyl-L-arginine for 1 h before incubation with *P. gingivalis* lipid A-associated proteins. Addition of N^G-monomethyl-L-arginine inhibited nitric oxide production in a concentration-dependent manner (Fig. 8A).

The effect of nitric oxide synthase inhibitor on nitric oxide synthesis induced by *P. gingivalis* lipid A-associated proteins was tested. Cells were pretreated with the indicated concentrations of nitro-L-arginine methyl ester, a nonspecific nitric oxide synthase inhibitor, for 1 h before incubation with *P. gingivalis* lipid A-associated proteins. Nitro-L-arginine methyl ester suppressed *P. gingivalis* lipid A-associated proteins-induced nitric oxide production by RAW264.7 cells (Fig. 8B).

To elucidate the role of nuclear factor-κB in *P. gingivalis* lipid A-associated proteins-induced nitric oxide production, RAW264.7 cells were pretreated with pyrrolidine dithiocarbamate, an antioxidant that acts as a specific inhibitor of nuclear factor-κB activation, for 1 h before incubation with *P. gingivalis* lipid A-associated proteins. Pretreatment with pyrrolidine

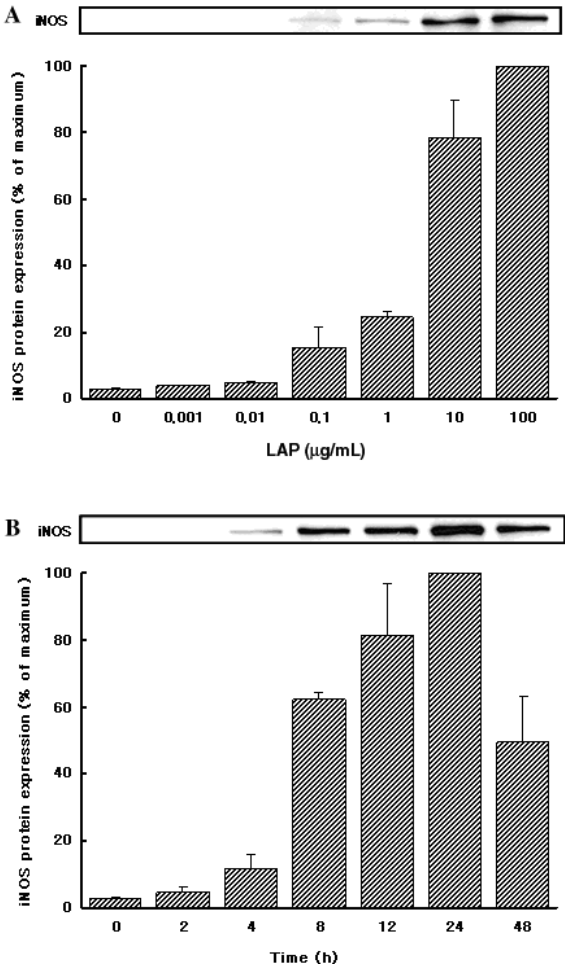

Fig. 6. Dose–response (A) and time course (B) of inducible nitric oxide synthase protein expression in RAW264.7 cells stimulated with *Porphyromonas gingivalis* lipid A-associated proteins. Inducible nitric oxide synthase protein synthesis was measured by immunoblot analysis of cell lysates using inducible nitric oxide synthase-specific antibody, and inducible nitric oxide synthase protein levels were quantified by densitometry. A representative immunoblot from two separate experiments with similar results is shown. (A) Cells were incubated with different concentrations of *P. gingivalis* lipid A-associated proteins for 24 h. (B) Cells were incubated in the presence of 10 μg/mL of *P. gingivalis* lipid A-associated proteins for different periods of time. iNOS, inducible nitric oxide synthase; LAP, lipid A-associated proteins.

dithiocarbamate produced substantial inhibition of nitric oxide production (Fig. 8C).

We also assessed whether production of nitric oxide by *P. gingivalis* lipid A-associated proteins stimulation depends on microtubule polymerization. RAW264.7 cells were pretreated with various concentrations of colchicine, a mitosis inhibitor, for 30 min before incubation with *P. gingivalis* lipid A-associated proteins. *P. gingivalis* lipid A-associated proteins-induced nitric oxide production was found to be suppressed by colchicine pretreatment (Fig. 8D).

To investigate the possible involvement of signaling kinases (e.g. protein tyrosine kinase and protein kinase C) in signaling nitric oxide induction, cells were pretreated with inhibitors of these kinases for 30 min before incubation with *P. gingivalis* lipid A-associated proteins. As shown in Fig. 8E, the specific protein tyrosine kinase inhibitor, genistein, reduced *P. gingivalis* lipid A-associated proteins-induced nitrite production. The protein kinase C inhibitor, bisindolylmaleimide, also reduced *P. gingivalis* lipid A-associated proteins-induced nitric oxide production (Fig. 8F). However, the depressant effects of bisindolylmaleimide at high concentrations were caused by its cytotoxic action, as demonstrated by the MTT assay.

We next examined the involvement of the mitogen-activated protein kinases (MAPK) [c-Jun N-terminal kinase, extracellular signal-regulated kinase (ERK), and p38] in the *P. gingivalis* lipid A-associated proteins-induced nitric oxide production. To investigate which MAPK pathway is involved in the *P. gingivalis* lipid A-associated proteins-induced production of nitric oxide, cells were pretreated with specific inhibitors of MAPK for 30 min before incubation with *P. gingivalis* lipid A-associated proteins. Pretreatment with SP600125 and SB203580, specific inhibitors of c-Jun N-terminal kinase and p38 pathways, respectively, markedly attenuated the stimulation of nitric oxide secretion by *P. gingivalis* lipid A-associated proteins (Figs 8G,H). However, the ERK inhibitor, PD98059, was less potent in the suppression of lipid A-associated proteins-induced nitric oxide production than SP600125 and SB203580, as shown in Fig. 8I.

Discussion

Because production of nitric oxide has been recognized as a marker in a variety of human diseases associated with inflammation (33,34), we studied the effects of the lipid A-associated proteins of *P. gingivalis*, a major cause of inflammatory periodontal disease, on the production of nitric oxide and the expression of inducible nitric oxide synthase in the murine macrophage cell line, RAW264.7. Macrophages are known to be the main source of inducible nitric oxide synthase in periodontal tissues (18).

P. gingivalis lipid A-associated proteins stimulated the release of nitric oxide from the RAW264.7 cells. The exposure of *P. gingivalis* lipid A-associated proteins to heat or trypsin resulted in significant, but not total, loss of activity. The failure to block the

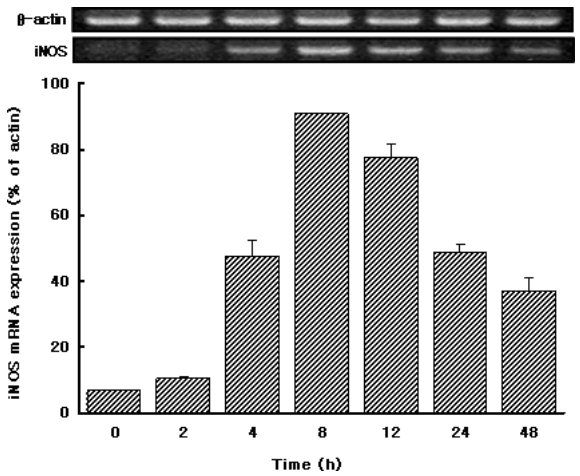

Fig. 7. Time course of inducible nitric oxide synthase mRNA expression in RAW264.7 cells stimulated with *Porphyromonas gingivalis* lipid A-associated proteins. See Materials and methods for further details. The bands obtained after polymerase chain reaction amplification, in one of two separate experiments yielding similar results, are shown on a gel photograph. Cells were incubated in the presence of 10 μg/mL of *P. gingivalis* lipid A-associated proteins for different periods of time. iNOS, inducible nitric oxide synthase.

nitric oxide-stimulating activity of lipid A-associated proteins completely may be explained by the known extreme stability of the lipid A-associated proteins to heat and proteases (1,5,35,36). Interestingly, polymyxin B was also capable of inhibiting *P. gingivalis* lipid A-associated proteins-induced nitric oxide production from RAW264.7 cells. Polymyxin B has other actions on cells independently of lipopolysaccharide, such as the inhibition of protein kinase (37,38). When it was taken into account that protein kinase C has a direct role in nitric oxide synthesis (39), polymyxin B might have a direct inhibitory effect on the nitric oxide pathway. Based upon the measured lipopolysaccharide content of *P. gingivalis* lipid A-associated proteins and the inhibition of activity by heat and trypsin, we conclude that the nitric oxide-inducing activity of the lipid A-associated proteins of *P. gingivalis* is caused by these proteins and not by lipopolysaccharide contamination.

It is important to determine the relative contribution that surface components of pathogenic bacteria make towards the pathogenesis of periodontal disease in terms of their pro-inflammatory mediator-stimulating activities. In this study we have compared the ability of the SAM, lipid A-associated proteins and lipopolysaccharide from *P. gingivalis* to stimulate RAW264.7 cells to synthesize nitric oxide. The results of this study have demonstrated that *P. gingivalis* lipopolysaccharide was significantly more potent than the other surface components in stimulating release of nitric oxide. In the case of *A. actinomycetemcomitans*, the lipopolysaccharide was a very weak cytokine-stimulating agonist, while the lipid A-associated proteins and SAM were capable of inducing cytokine release from mesenchymal and myelomonocytic cells (4).

There are strain-dependent variations in the ability of *P. gingivalis* lipopolysaccharide to induce nitric oxide production. Lipopolysaccharide from *P. gingivalis* ATCC 33277 failed to induce nitric oxide production by RAW264.7 cells, similar to that of W50 (40,41). In contrast, nitric oxide could be produced by murine macrophages when stimulated with lipopolysaccharide preparations from two strains of *P. gingivalis*, A7436 and 381; however, these lipopolysaccharides were far less potent than lipopolysaccharide from enteric bacteria (41) (and unpublished observations).

Because inducible nitric oxide synthase is the catalytic enzyme of nitric oxide production (42), we examined the effect of *P. gingivalis* lipid A-associated proteins on the expression of inducible nitric oxide synthase protein and demonstrated an increase in inducible nitric oxide synthase protein by immunoblotting. Inducible nitric oxide synthase is controlled mainly at the transcriptional level and we confirmed, in this study, that *P. gingivalis* lipid A-associated proteins induces inducible nitric oxide synthase expression predominantly at the transcriptional level. It is of interest to note that there was some delay between inducible nitric oxide synthase mRNA expression and nitric oxide production. Inducible nitric oxide synthase mRNA showed detectable signals at 2 h, whereas nitric oxide production increased above the control level only at 8 h. Moreover, maximum inducible nitric oxide synthase mRNA expression was achieved at 8 h. Evidently, in the RAW264.7 cells, inducible nitric oxide synthase mRNA is produced at high levels, but decreases before nitric oxide reaches its maximum, and high levels of nitric oxide are found long after expression of inducible nitric oxide synthase mRNA had started to decline.

We demonstrated an absolute requirement for endogenous L-arginine in nitric oxide production. Nitro-L-arginine methyl ester, a nonspecific nitric oxide synthase inhibitor, attenuated *P. gingivalis* lipid A-associated proteins-induced nitric oxide production by RAW264.7 cells, indicating that the presence of nitric oxide synthase is a prerequisite for nitric oxide production. We also tested whether pyrrolidine dithiocarbamate, an antioxidant that acts as a specific inhibitor of nuclear factor-κB activation, would affect the induction of nitric oxide by *P. gingivalis* lipid A-associated proteins. Nuclear factor-κB, a ubiquitous transcription factor, is known to regulate the transcription of a variety of genes involved in the inflammatory process (43), and an increasing body of evidence has suggested that the expression of inducible nitric oxide synthase is dependent on the activation of nuclear factor-κB (10,44,45). Binding of nuclear factor-κB to the specific binding nucleotide sequences in the promoter region of the inducible nitric

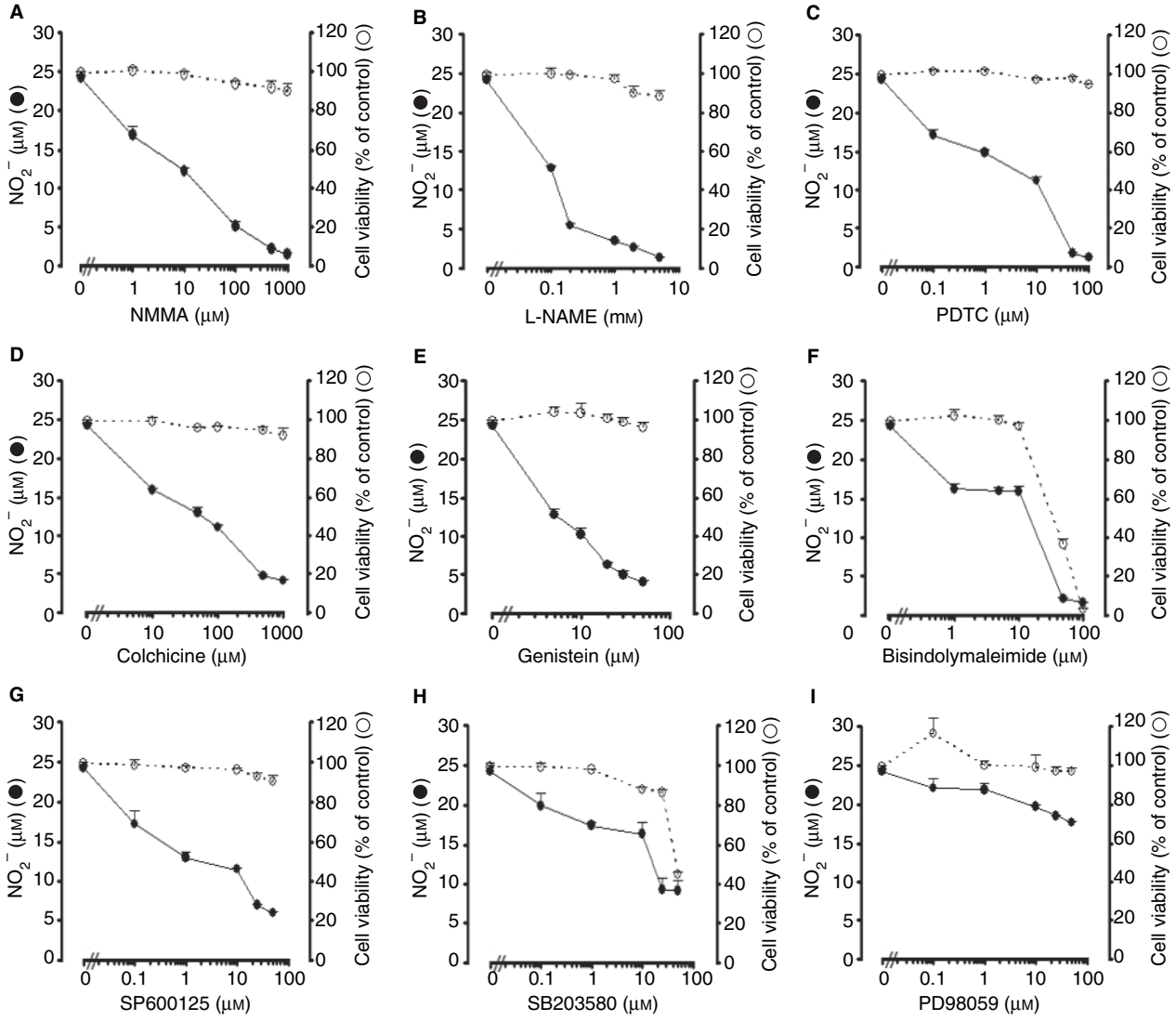

Fig. 8. Effects of various inhibitors on *Porphyromonas gingivalis* lipid A-associated proteins-induced nitric oxide production. RAW264.7 cells were pretreated with the indicated concentrations of each inhibitor before incubation for 24 h with *P. gingivalis* lipid A-associated proteins (10 μg/mL). Supernatants were removed after 24 h and assayed for nitric oxide. Viability of RAW264.7 cells was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are expressed as means ± standard deviation of four experiments. NMMA, N^G-monomethyl-L-arginine; L-NAME, nitro-L-arginine methyl ester; PDTC, pyrrolidine dithiocarbamate.

oxide synthase gene results in rapid and effective transcription of this gene (45). In the present study, the addition of nuclear factor-κB inhibitor, pyrrolidine dithiocarbamate, markedly suppressed nitric oxide synthesis, indicating that nuclear factor-κB mediates *P. gingivalis* lipid A-associated proteins-induced nitric oxide production in RAW264.7 cells.

We demonstrated that *P. gingivalis* lipid A-associated proteins-induced nitric oxide production by RAW264.7 cells was inhibited by the microtubule-disrupting agent, colchicine. Our findings suggest that microtubules are involved in nitric oxide production by macrophages activated with *P. gingivalis* lipid A-associated proteins. We also examined the effect of signaling kinases. Activation of protein tyrosine kinase and protein kinase C appears to be necessary for *P. gingivalis* lipid A-associated proteins-induced nitric oxide production in murine macrophages.

Various members of the MAPK family are considered to play roles in inflammatory responses (46,47) and may modulate the production of nitric oxide in stimulated monocytes/macrophages. To elucidate which MAPK pathway is involved in the *P. gingivalis* lipid A-associated proteins-induced production of nitric oxide, we also performed blocking studies using specific inhibitors for MAPK pathways. Our results showed that the c-Jun N-terminal kinase inhibitor, SP600125,

and the p38 MAPK inhibitor, SB203580, markedly suppressed *P. gingivalis* lipid A-associated proteins-mediated nitric oxide production in RAW264.7 cells. The ERK inhibitor, PD98059, had a much smaller effect on the lipid A-associated proteins-induced nitric oxide production than did SP600125 and SB203580. These data thus provided good evidence that the MAPK signaling pathways, including c-Jun N-terminal kinase, ERK, and p38, are also involved in the *P. gingivalis* lipid A-associated proteins-induced nitric oxide production.

Taken together, these results indicate that multiple signaling pathways, such as nuclear factor-κB, protein tyrosine kinase, protein kinase C, and MAPK cascades, are involved in *P. gingivalis* lipid A-associated proteins-stimulated nitric oxide production. It would be interesting to find out how these pathways coordinate for nitric oxide production. The inhibitors of nitric oxide production have been considered as potential anti-inflammatory agents. In this study, we evaluated the effects of various inhibitors on *P. gingivalis* lipid A-associated proteins-induced nitric oxide production. As a result, pyrrolidine dithiocarbamate showed potent inhibition of nitric oxide production without affecting cell viability (≈ 93% inhibition at the test concentration of 50 μM). The inhibition of nitric oxide production by pyrrolidine dithiocarbamate may be useful in the therapy of inflammatory diseases, such as periodontitis. This hypothesis, however, remains to be tested.

Nitric oxide synthesis is increased in periodontal disease, as a result of macrophage infiltration in the periodontal tissues (14,48). There are no previous reports of the ability of the lipid A-associated proteins from periodontal pathogens to stimulate the release of nitric oxide by inducing inducible nitric oxide synthase expression, and the present study clearly shows, for the first time, that the lipid A-associated proteins of *P. gingivalis*, a major periodontal pathogen, fully induced inducible nitric oxide synthase expression and nitric oxide production in the murine macrophage cell line, RAW264.7, in the absence of other stimuli. Analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis has shown that *P. gingivalis* lipid A-associated proteins comprise four very prominent bands at approximate molecular weights of 52, 47, 32, and 29 kDa, together with other faint bands. It would be necessary to determine which of the components in this population of proteins is responsible for the stimulation of nitric oxide induction.

Nitric oxide might play a role in the pathogenesis of both periodontitis and subsequent bone loss, either directly or indirectly, by modulating the production of other pro-inflammatory cytokines (49). The ability of *P. gingivalis* lipid A-associated proteins to promote the production of nitric oxide may be important in the establishment of the chronic lesion observed in inflammatory periodontal disease. The precise mechanism by which *P. gingivalis* lipid A-associated proteins induces nitric oxide production remains to be elucidated.

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