Nitric oxide production and inducible nitric oxide synthase expression induced by *Prevotella nigrescens* lipopolysaccharide

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Abstract

We have examined the effects of lipopolysaccharide (LPS) from *Prevotella nigrescens*, one of the causative agents of inflammatory periodontal disease and endodontic infections, on the production of nitric oxide (NO) and expression of inducible nitric oxide synthase (iNOS) in the murine macrophage cell line RAW264.7. We also attempted to throw light on the signaling mechanisms involved in *P. nigrescens* LPS-induced NO production. We found that *P. nigrescens* LPS can induce iNOS expression and stimulate the release of NO without additional stimuli and demonstrated an important role of the transcription factor NF-κB and microtubule polymerization in NO production. The production of NO required L-arginine and protein tyrosine kinase but not activation of protein kinase C. The ability of *P. nigrescens* LPS to promote the production of NO may be important in the pathogenesis of inflammatory periodontal disease and endodontic infections.

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1. Introduction

Nitric oxide (NO) is a short-lived bioactive molecule produced by immunocompetent cells such as macrophages that serves as a messenger molecule for various physiological and pathological processes [1]. It is synthesized from L-arginine by nitric oxide synthase (NOS) present in various tissues [2]. Three distinct isoforms of NOS, neural (nNOS), endothelial (eNOS) and inducible (iNOS), have been reported in mammalian tissues [2]. Expression of iNOS, also commonly called NOS-2, is induced by inflammatory stimuli such as bacterial lipopolysaccharide (LPS), and proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ), in a variety of cell types including macrophages, following bacterial infection [3,4]. iNOS, once expressed, can generate large amounts of NO for extended times, and is believed to be involved in cytotoxic effects following inflammation [1].

Periodontal disease is a chronic inflammatory process accompanied by destruction of connective tissue and alveolar bone, and sometimes loss of teeth [5]. The primary causative agents of periodontal disease are gram-negative anaerobic bacteria that accumulate in the gingival sulcus. *Prevotella nigrescens* is a putative periodontal pathogen that is frequently recovered from subgingival flora in patients with gingivitis [6] and adult periodontitis [7,8]. This bacterium has also been reported to be associated with endodontic infections [9,10] and peri-implantitis [11].

LPS is a major component of the outer membrane of gram-negative bacteria, including *P. nigrescens*. It has
the ability to trigger a number of host cells, especially mononuclear phagocytes, to produce and release a wide variety of immunologically active mediators, including TNF-α, IL-1β, IL-6, and IL-8 [12]. These cytokines have been implicated in the pathogenesis of inflammatory periodontal disease [13,14]. In addition to these cytokines, NO has recently received considerable attention as a novel type of mediator [1]; inhibition of NOS activity and NO production frequently limits the progression and severity of experimental inflammatory diseases such as osteoarthritis, glomerulonephritis, and colitis [15,16].

NO is thought to have a role in the pathogenesis of inflammatory periodontal disease as it does in other inflammatory diseases. Enhanced production of NO has been demonstrated in periodontal disease [17,18], and LPS from Actinobacillus actinomycetemcomitans-induced significant production of NO in macrophages [19,20]. Moreover, gingival tissues from patients with periodontitis have higher levels of iNOS protein and mRNA than healthy tissue [21–24]. Macrophages, polymorphonuclear cells and fibroblasts are the sources of iNOS in periodontal tissues, with endothelial cells also contributing [21–24].

The LPS of P. nigrescens may play a role as a virulence factor in the development and progression of inflammatory periodontal disease and endodontic infections, stimulating the host cells to produce and release proinflammatory mediators. At present, however, no reports exist concerning the ability of P. nigrescens LPS to induce the release of proinflammatory mediators. The purpose of this study was to investigate the effects of purified P. nigrescens LPS on the production of NO and the expression of iNOS 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 NO production.

2. Materials and methods

2.1. Bacteria and culture conditions

Prevotella nigrescens ATCC 33563 was grown anaerobically on the surface of enriched Trypticase soy agar containing 5% (v/v) sheep blood, or in GAM broth (Nissui, Tokyo, Japan) supplemented with 1 μg/ml menadione and 5 μg/ml hemin. Plate grown cultures were routinely incubated for 4 days and used as the inoculum for liquid growth. Liquid-grown cells were incubated for approximately 24 h, to late exponential growth phase. They were collected by centrifugation at 12,000g for 20 min at 4 °C, washed three times with phosphate-buffered saline (PBS, pH 7.2) and lyophilized. Culture purity was assessed by gram staining and plating on solid medium.

2.2. LPS isolation

LPS was prepared from lyophilized P. nigrescens ATCC 33563 cells by the standard hot phenol–water method [25]. Briefly, 90% phenol was added to bacteria suspended in pyrogen-free distilled water and the mixture was extracted twice at 68 °C for 20 min. After cooling, the aqueous phase was separated by centrifugation at 7000g for 15 min and the pooled aqueous extract was dialyzed extensively against distilled water at 4 °C. The dialyzed extract was centrifuged at 105,000g for 3 h and lyophilized to yield crude extract. This was treated with DNase (25 μg/ml; Sigma Chemical, St. Louis, MO) and RNase (25 μg/ml; Sigma) in 0.1 M Tris (pH 8.0) at 37 °C overnight to remove nucleic acids. Any contaminating protein was then hydrolyzed with protease K (50 μg/ml; Sigma), followed by heating at 60 °C for 1 h and incubating overnight at 37 °C. The protein content of the purified LPS, determined by the method of Markwell et al. [26], was less than 0.1%. Coomassie blue staining of overloaded sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) did not reveal any visible protein bands in the purified LPS, confirming the purity of the preparation (data not shown). Salmonella typhimurium LPS (phenol extract) was purchased from Sigma Chemical Co. (St. Louis, MO). The protein content of S. typhimurium LPS (Sigma), used as a control, was about 3%.

2.3. Cell cultures

The murine macrophage cell line RAW264.7 (American Type Culture Collection, Rockville, MD) was grown in Nunc flasks in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 U/ml of penicillin, 100 μg/ml streptomycin, 10 mM Hepes, 2 mM L-glutamine, 0.2% NaHCO3, 1 mM sodium pyruvate, and 10% (v/v) heat-inactivated FBS in a humidified chamber with 5% CO2/95% 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 1 × 106 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 LPS were added and the cells were cultured for the indicated times, after which culture supernatants were collected and assayed for NO.

2.4. Cytotoxicity assay

The cellular toxicity of several inhibitors was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-
lium bromide (MTT) assay, which is based on the conversion of MTT to formazan by mitochondrial dehydrogenases [27]. Cells were incubated with each inhibitor for the indicated time, and MTT was added to the cultures to a final concentration of 0.5 mg/ml. After incubation at 37 °C in 5% CO₂ for 2 h, the supernatant was removed and the cells were solubilized in dimethyl sulfoxide (DMSO). 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, USA). Cell viability was expressed as a percentage of the control value.

2.5. Measurement of NO production

NO production was assayed by measuring the accumulation of the stable oxidative metabolite, nitrite (NO₂⁻), in culture supernatants [28]. Briefly, 1 × 10⁶ cells/well were stimulated in 24-well tissue culture plates for the indicated times, 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, USA), and nitrite concentrations were calculated from a standard curve established with serial dilutions of NaNO₂ (Sigma) in culture medium.

2.6. Western blot analysis of iNOS

Cells were plated in T-25 culture plates at 5 × 10⁶ cells/plate and treated with various concentrations of P. nigrescens LPS for the indicated times. After incubation, they were washed three times with ice-cold PBS 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% SDS, 1% Nonidet P-40] containing protease inhibitors (1 mM PMSF, 5 mg/ml aprotinin, 5 mg/ml pepstatin A, and 5 mg/ml leupeptin). The cell lysates were centrifuged at 10,000g for 10 min to remove insoluble material, and their protein concentrations determined with the bicinchoninic acid (BCA) protein assay reagents (Pierce, USA) according to the manufacturer’s instructions. The same amount of protein (50 μg) from each supernatant was then subjected to SDS–PAGE 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 PBST (PBS with 0.1% Tween-20) containing 3% nonfat dry milk, followed by incubation with polyclonal antibody against iNOS (Santa Cruz Biotechnology, Santa Cruz, CA) diluted (1:50) in PBS containing 1.5% goat serum for 1 h at room temperature. They were then washed three times for 10 min each with PBST, incubated with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) at room temperature for 1 h and visualized by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, USA) as recommended. The intensity of each protein-specific band was quantified by densitometer with densitometric software.

2.7. Reverse transcription-polymerase chain reaction and analysis of PCR products

Cells were plated in 100 mm tissue culture dishes at a density of 2 × 10⁷ cells/dish and treated with 1 μg/ml of P. nigrescens LPS for the indicated times. Following incubation, they were washed twice with PBS and collected by centrifugation. Total RNA was isolated with an RNeasy Mini Kit (Qiagen, Valencia, CA), 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, Korea) and thermal cycler (GeneAmp PCR system 2400; PE Applied Biosystems, USA) [29]. β-actin served as internal control. PCR amplification of the cDNA with primers specific for iNOS and β-actin was carried out in the same tube. The number of cycles that ensured nonsaturating PCR conditions was established in preliminary experiments. PCR amplification of iNOS 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: iNOS, 5’-TCACTGGGACAGCAACAAT-3’ (sense) and 5’-TGGTGCTGCAAGATGTGCTGA-3’ (antisense) (corresponding to positions 348–367 and 857–838, respectively, of the published mouse iNOS mRNA sequence), yielding a 510-bp product; β-actin, 5’-TCTTTGTTG-CGGTCCACA-3’ (sense) and 5’-CGTCTCGGAG-TCCATCACA-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 UV light. The intensities of the PCR bands on gel photographs were quantified by densitometry, and expression of iNOS mRNA was calculated as the ratio of the densities of the iNOS and actin bands.

2.8. Statistical analysis

Statistical analysis was performed using Student’s paired t test with p < 0.05 considered statistically significant. Data are expressed as means ± SD of four independent experiments.
3. Results and discussion

Because production of NO has been recognized as a marker in a variety of human diseases associated with inflammation [30,31], we studied the effects of the LPS of *P. nigrescens*, the causative agent of inflammatory periodontal disease and endodontic infections, on the production of NO and expression of iNOS in the murine macrophage cell line RAW264.7. Macrophages are known to be the main source of iNOS in periodontal tissues [24].

Concentrations of nitrite, an indicator of NO production, were measured 24 h after adding various concentrations of purified *P. nigrescens* ATCC 33563 LPS to RAW264.7 cells. *P. nigrescens* LPS-induced NO release from the RAW264.7 cells over the range from 0.1 ng/ml to 10 μg/ml (Fig. 1). Basal nitrite release was about 2.6 μM. It was effective at a concentration as low as 10 ng/ml, and maximum NO production (about 65 μM) was achieved at the concentrations between 0.1 and 10 μg/ml. *S. typhimurium* LPS, as a control, also stimulated NO production to a maximum of 63 μM. Its activity was similar to that of *P. nigrescens* LPS with respect of both minimum stimulatory dose and maximum NO produced.

RAW264.7 cells were challenged with 1 μg/ml *P. nigrescens* LPS, and production of NO in the culture supernatant was measured at various times thereafter. After an initial lag of 4 h, NO secretion increased linearly from 8 to 24 h and plateaued thereafter. Nitrite accumulation reached 58 μM (Fig. 2). *S. typhimurium* LPS also caused a marked elevation in NO secretion that leveled off after 24 h.

Since iNOS is the catalytic enzyme of NO production [32], we examined the effect of *P. nigrescens* LPS on the expression of iNOS protein and demonstrated an increase in iNOS protein by immunoblotting. Cells stimulated with *P. nigrescens* LPS expressed a protein of...
approximately 130 kDa, recognized by specific antibody to iNOS (Fig. 3). When RAW264.7 cells were exposed to increasing concentrations of P. nigrescens LPS, there was a concentration-dependent accumulation of iNOS (Fig. 3(a)). iNOS protein was detectable with a concentration of P. nigrescens LPS as low as 1 ng/ml and reached a maximum at a concentration of 10 μg/ml. Control cells produced either no detectable iNOS band, or only a very weak signal. Fig. 3(b) shows the time course of changes in iNOS protein expression induced by 1 μg/ml of P. nigrescens LPS. iNOS protein showed detectable signal at 2 h, and maximum expression was achieved at 8–12 h of stimulation. The level of iNOS protein started to decline after 24 h of incubation.

iNOS is controlled mainly at the transcriptional level and we confirmed that P. nigrescens LPS induces iNOS expression predominantly at the transcriptional level. The effect of P. nigrescens LPS on iNOS transcription and accumulation of iNOS mRNA was confirmed by RT-PCR. Preliminary experiments established that there was a linear relationship between total RNA levels in cell extracts (after 24 h LPS stimulation) and the density of PCR products from iNOS and β-actin mRNA (data not shown). Exposure of cells to P. nigrescens LPS enhanced iNOS mRNA expression (Fig. 4). iNOS 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 iNOS mRNA.

It is of interest to note that there was some delay between iNOS expression and NO production. Both iNOS protein and mRNA showed detectable signals at 2 h, while NO production increased above the control level only at 8 h. Evidently, in the RAW264.7 cells, iNOS protein and mRNA are produced at high levels, but decrease before NO reaches its maximum, and high levels of NO are found long after expression of iNOS protein and mRNA had started to decline.

We demonstrated an absolute requirement for endogenous L-arginine in NO production. ΔG-monomethyl-L-arginine (NMMA), an L-arginine analogue, is a specific inhibitor of NO production in the L-arginine-dependent pathway [33]. To determine if the signaling mechanism of P. nigrescens LPS-induced NO production involves this pathway, RAW264.7 cells were pretreated with the indicated concentrations of NMMA for 1 h before incubation with P. nigrescens LPS. Addition of NMMA inhibited NO production (Fig. 5(a)).

The effect of NOS inhibitor on P. nigrescens LPS-induced NO synthesis was tested. Cells were pretreated with the indicated concentrations of nitro-L-arginine methyl ester (L-NAME), a nonspecific NOS inhibitor, for 1 h before incubation with P. nigrescens LPS. L-NAME attenuated P. nigrescens LPS-induced NO production in a concentration-dependent manner, indicating that the presence of NOS is a prerequisite for NO production (Fig. 5(b)).

We also tested whether pyrrolidine dithiocarbamate (PDTC), an antioxidant that acts as a specific inhibitor of the nuclear factor-κB (NF-κB) activation, would affect the induction of NO by P. nigrescens LPS. NF-κB, a ubiquitous transcription factor, is known to regulate the transcription of a variety of genes involved in the inflammatory process [34], and an increasing body of evidence has suggested that the expression of iNOS is dependent on the activation of NF-κB [4,35,36]. Binding of NF-κB to the specific binding nucleotide sequences in the promoter region of the iNOS gene results in rapid and effective transcription of this gene [36]. LPS-induced activation of NF-κB leads to its dissociation and enables it to translocate into the nucleus and bind to the κB site in the promoter region of the iNOS gene, thus activating transcription [1]. RAW264.7 cells were pretreated with PDTC for 1 h before incubation with P. nigrescens LPS. In the present study, the addition of NF-κB inhibitor, PDTC, markedly suppressed NO synthesis indicating that NF-κB mediates P. nigrescens LPS-induced NO production in RAW264.7 cells (Fig. 5(c)).

We also assessed whether production of NO by P. nigrescens LPS stimulation depends on microtubule polymerization. Microtubules have some regulatory function in LPS-induced NO production by macrophages [37]. RAW264.7 cells were pretreated with various concentrations of colchicine for 30 min before incubation with P. nigrescens LPS. Colchicine is a mitosis inhibitor which binds directly to the beta subunit of tubulin [38]. We demonstrated that P. nigrescens LPS-induced NO production by RAW264.7 cells was
suppressed by the microtubule-disrupting agent colchicine (Fig. 5(d)). Our findings suggest that microtubules are in some way involved in NO production by macrophages activated with *P. nigrescens* LPS. These results are in accordance with previous studies showing that NO levels were partially suppressed when this microtubule-disrupting agent was added to murine peritoneal macrophages stimulated with LPS or taxol [37]. This previous study also indicated that iNOS protein was the target of colchicine [37].

To investigate the possible involvement of signaling kinases, e.g., protein tyrosine kinase (PTK) and protein kinase C (PKC), in signaling *P. nigrescens* LPS-induced NO production, cells were pretreated with inhibitors of these kinases for 30 min before incubation with *P. nigrescens* LPS. As shown in Fig. 5(e), the specific PTK inhibitor, genistein, reduced LPS-induced nitrite production. Activation of tyrosine kinase therefore appears to be necessary for *P. nigrescens* LPS-induced NO production. However, the depressant effect of genistein on NO production was partly due to its cytotoxic action, as demonstrated by MTT assay (Table 1). Genistein reduced cell viability by 27% at 50 μM. We also examined the effect of the PKC inhibitor bisindolylmaleimide. Bisindolylmaleimide-reduced NO production by about 90% at a concentration of 50 μM (Fig. 5(f)). However, the depressant effect of bisindolylmaleimide on NO production by *P. intermedius* LPS-stimulated RAW264.7 cells was essentially due to its cytotoxic action (Table 1). Bisindolylmaleimide reduced cell viability by 96% at 50 μM.

The inhibitors of NO production have been considered as potential anti-inflammatory agents. In this study, we evaluated the effects of various inhibitors on *P. nigrescens* LPS-induced NO production (Fig. 5). As a result, PDTC showed potent inhibition of NO production without affecting cell viability (about 85% inhibition at the test concentration of 100 μM). The inhibition of NO production by PDTC may be useful in the therapy of inflammatory diseases such as periodontitis. This hypothesis, however, remains to be tested.

Fig. 5. Effects of various inhibitors on *P. nigrescens* LPS-induced NO production. RAW264.7 cells were pretreated with the indicated concentrations of each inhibitor for 1 h ((a)–(c)) or 30 min ((d)–(f)) before 24 h incubation with *P. nigrescens* LPS (1 μg/ml). Supernatants were removed after 24 h and assayed for NO. The results are means ± SD of four experiments.
The periodontium is consistently in contact with LPS produced by gram-negative periodontopathogenic bacteria. LPS from *Actinobacillus actinomycetemcomitans*, a major periodontal pathogen, has been shown in vitro to induce NO production in murine macrophages [19,20]. However, NO production is not a general phenomenon of LPS from all periodontal pathogenic bacteria. LPS isolated from the major periodontal pathogen *Porphyromonas gingivalis* failed to induce NO production by RAW264.7 cells [39]. Exogenous L-arginine was a prerequisite in *P. gingivalis* LPS-induced NO production. There are no previous reports of the ability of *P. nigrescens* LPS to stimulate the release of NO by inducing iNOS expression, and the present study clearly shows, for the first time, that *P. nigrescens* LPS alone, without the addition of IFN-γ, fully induced iNOS expression and NO production in RAW264.7 cells. The ability of *P. nigrescens* LPS to promote the production of NO may be important in the establishment of the chronic lesion accompanied by osseous tissue destruction observed in inflammatory periodontal disease and endodontic infections. The precise mechanism by which *P. nigrescens* LPS induces NO production remains to be elucidated.

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### References


