J Periodont Res 2004; 39; 424–431
Printed in the UK. All rights reserved

JOURNAL OF PERIODONTAL RESEARCH
doi: 10.1111/j.1600-0765.2004.00757.x

Prevotella intermedia lipopolysaccharide stimulates release of nitric oxide by inducing expression of inducible nitric oxide synthase

Sung-Jo Kim[1], Mi-Suk Ha[1], Eun-Young Choi[2], Jeom-Il Choi[1], In-Soon Choi[2]

[1]Department of Periodontology, College of Dentistry, Pusan National University and [2]Department of Life Science, College of Natural Science, Silla University, Busan, South Korea

Kim S-J, Ha M-S, Choi E-Y, Choi J-I, Choi I-S. Prevotella intermedia *lipopolysaccharide stimulates release of nitric oxide by inducing expression of inducible nitric oxide synthase. J Periodont Res 2004; 39; 424–431. © Blackwell Munksgaard 2004*

Objectives: The purpose of this study was to examine the effects of lipopolysaccharide from *Prevotella intermedia*, a major cause of inflammatory periodontal disease, 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. intermedia* lipopolysaccharide-induced NO production.

Material and methods: Lipopolysaccharide from *P. intermedia* ATCC 25611 was prepared by the standard hot phenol–water method. NO production was assayed by measuring the accumulation of nitrite in culture supernatants. Western blot analysis of iNOS and analysis of reverse transcription-polymerase chain reaction (RT-PCR) products were carried out.

Results: We found that *P. intermedia* lipopolysaccharide can induce iNOS expression and stimulate the release of NO without additional stimuli and demonstrated an important role of the transcription factor nuclear factor-κB (NF-κB) and microtubule polymerization in NO production. The production of NO required L-arginine but not activation of protein kinase C or protein tyrosine kinase.

Conclusions: The present study clearly shows that *P. intermedia* lipopolysaccharide fully induced iNOS expression and NO production in RAW264.7 cells in the absence of other stimuli. The ability of *P. intermedia* lipopolysaccharide to promote the production of NO may be important in the pathogenesis of inflammatory periodontal disease.

Sung-Jo Kim, DDS, PhD, Department of Periodontology, College of Dentistry, Pusan National University, 1–10 Ami-Dong, Seo-Ku, Busan 602-739, South Korea
Tel: +82 51 240 7461
Fax: +82 51 245 8388
e-mail: sungjokim@pusan.ac.kr

Key words: inducible nitric oxide synthase; nitric oxide; lipopolysaccharide; *Prevotella intermedia*

Accepted for publication May 27, 2004

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, and proinflammatory cytokines, such as tumor necrosis factor-α, interleukin-1β, and interferon-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 surrounding connective tissue and alveolar bone, and sometimes loss of teeth (5). The primary causative agents of periodontal disease are particular gram-negative anaerobic bacteria that accumulate in the gingival sulcus. *Prevotella intermedia* is a major periodontal pathogen (6) that is dominant in the periodontal pockets of patients with adult periodontitis (7, 8). This bacterium has also been frequently recovered from subgingival flora in patients with acute necrotizing ulcerative gingivitis (9) and pregnancy gingivitis (10).

Lipopolysaccharide is a major component of the outer membrane of gram-negative bacteria, including *P. intermedia*. It has the ability to trigger a number of host cells, especially mononuclear phagocytes, to produce and release a wide variety of pharmacologically active mediators, including tumor necrosis factor-α, interleukin-1β, interleukin-6, and interleukin-8 (11). These cytokines have been implicated in the pathogenesis of inflammatory periodontal disease (12, 13). 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 (14, 15).

NO is thought to have an important 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 (16), and lipopolysaccharide from *Actinobacillus actinomycetemcomitans* induced significant production of NO in macrophages (17, 18). Moreover, gingival tissues from patients with chronic periodontitis have higher levels of iNOS protein and mRNA than healthy tissue (19–22). Macrophages, polymorphonuclear cells and fibroblasts are the sources of iNOS in periodontal tissues, with endothelial cells also contributing (19–22).

The lipopolysaccharide of *P. intermedia* 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 proinflammatory mediators. The lipopolysaccharide of this bacterium induced expression of the interleukin-10 receptor gene (23) and of genes encoding proinflammatory cytokines (24, 25), such as interleukin-6 and interleukin-8, in human dental pulp cell cultures. Although *P. intermedia* lipopolysaccharide induced the release of NO from fetal mouse osteoblasts in organ culture (26), no information regarding its effect in other cell types is available. The purpose of this study was to investigate the effects of purified *P. intermedia* lipopolysaccharide 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.

Materials and methods

Bacteria and culture conditions

P. intermedia ATCC 25611 was used throughout. It 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 12,000 *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.

Lipopolysaccharide isolation

Lipopolysaccharide was prepared from lyophilized *P. intermedia* ATCC 25611 cells by the standard hot phenol–water method (27). 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 7000 *g* for 15 min and the pooled aqueous extract was dialyzed extensively against distilled water at 4°C. The dialyzed extract was centrifuged at 105,000 *g* for 3 h and lyophilized to yield crude extract. This was treated with DNase (25 μg/ml; Sigma Chemical, St. Louis, MO, USA) and RNase (25 μg/ml; Sigma) in 0.1 M Tris-HCl (pH 8.0) at 37°C overnight to remove nucleic acids. Any contaminating protein was then hydrolyzed with proteinase K (50 μg/ml; Sigma), followed by heating at 60°C for 1 h and incubating overnight at 37°C. The yield of lipopolysaccharide was approximately 0.26%. The protein content of the purified lipopolysaccharide, determined by the method of Markwell *et al*. (28), was less than 0.1%. Coomassie blue staining of overloaded sodium dodecyl sulfate–polyacrylamide gels did not reveal any visible protein bands in the purified lipopolysaccharide, confirming the purity of the preparation (data not shown). *Salmonella typhimurium* lipopolysaccharide (phenol extract) was purchased from Sigma.

Cell cultures

The murine macrophage cell line RAW264.7 (American Type Culture Collection, Rockville, MD, USA) was grown in Nunc flasks in Dulbecco's modified Eagle's medium supplemented with 100 U/ml of penicillin, 100 μg/ml 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 1×10^6 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 lipopolysaccharide were added and the cells were cultured for the indicated times, after which culture supernatants were collected and assayed for NO.

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 incubated with each inhibitor for 24 h, and MTT was added to the cultures to a final concentration of 0.5 mg/ml. After incubation at 37°C in 5% CO_2 for 2 h, the supernatant was removed and the cells were solubilized in dimethyl sulfoxide. 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 NO production

NO production was assayed by measuring the accumulation of the stable oxidative metabolite, nitrite (NO_2^-), in culture supernatants (30). Briefly, 1×10^6 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 20°C 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 NO synthase

Cells were plated in T-25 culture plates at 5×10^6 cells/plate and treated with various concentrations of *P. intermedia* lipopolysaccharide for the indicated times. After incubation, they were washed three times with ice-cold phosphate-buffered saline and lyzed by incubating for 30 min on ice with 200 μl of lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.002% sodium azide, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mg/ml aprotinin, 5 mg/ml pepstatin A, and 5 mg/ml 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, interleukin, 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 with 0.1% Tween-20 containing 3% non-fat dry milk, followed by incubation with polyclonal antibody against iNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted (1:50) in phosphate-buffered saline containing 1.5% goat serum for 1 h at 20°C. They were then washed three times for 10 min each with phosphate-buffered saline with Tween-20, incubated with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) at 20°C for 1 h and visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, USA) as recommended. The intensity of each protein-specific band was quantified by densitometer with densitometric software.

Reverse transcription-polymerase chain reaction (RT-PCR) and analysis of PCR products

Cells were plated in 100-mm tissue culture dishes at a density of 2×10^7 cells/dish and treated with 1 μg/ml of *P. intermedia* lipopolysaccharide for the indicated times. 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 RT-PCR were carried out with an AccuPower RT/PCR Premix kit (Bioneer, Korea) and thermal cycler (GeneAmp PCR system 2400; PE Applied Biosystems, Foster City, CA, USA) (31). β-Actin served as internal control. The number of cycles that ensured non-saturating 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′-TCACTGGGACAGCACAGAAT-3″ (sense) and 5′-TGTGTCTGCAGATGTGCTGA-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′-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 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. Preliminary experiments established that there was a linear relationship between total RNA levels in cell extracts (after 24 h lipopolysaccharide stimulation) and the density of PCR products from iNOS and β-actin mRNA (data not shown).

Statistical analysis

Statistical analysis was performed using Student's paired *t*-test with $p < 0.05$ considered statistically

significant. Data are expressed as means ± standard deviation of four independent experiments.

Results

NO induction by *P. intermedia* lipopolysaccharide

Concentrations of nitrite, an indicator of NO production, were measured 24 h after adding various concentrations of purified *P. intermedia* ATCC 25611 lipopolysaccharide to RAW264.7 cells. *P. intermedia* lipopolysaccharide induced NO release from the RAW264.7 cells over the range 0.1 ng/ml to 10 μg/ml (Fig. 1). Basal nitrite release was approximately 3 μM. It was effective at a concentration as low as 10 ng/ml, and maximum NO production (approximately 64 μM) was achieved at a concentration of 10 μg/ml. *S. typhimurium* lipopolysaccharide, as a control, also stimulated NO production to a maximum of 63 μM. Its activity was similar to that of *P. intermedia* lipopolysaccharide with respect of both minimum stimulatory dose and maximum NO produced, although, at a concentration of 10 ng/ml, NO induction by *S. typhimurium* lipopolysaccharide was significantly greater than with *P. intermedia* lipopolysaccharide.

RAW264.7 cells were challenged with 1 μg/ml *P. intermedia* lipopolysaccharide, 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 h to 48 h. Nitrite accumulation reached 66 μM (Fig. 2). *S. typhimurium* lipopolysaccharide also caused a marked elevation in NO secretion that leveled off after 24 h.

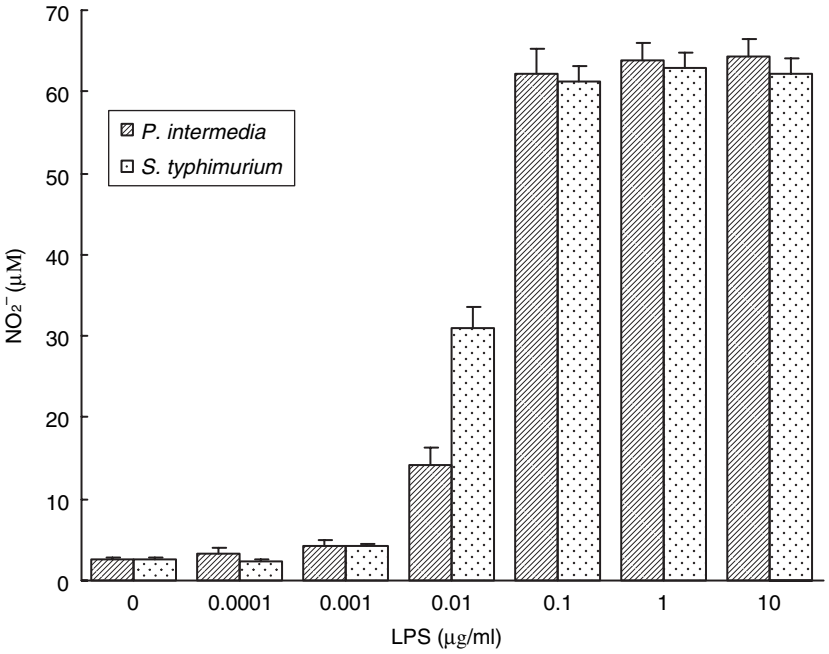

Fig. 1. Dose–response of nitric oxide (NO) production by RAW264.7 cells stimulated with *Prevotella intermedia* lipopolysaccharide (LPS). *Salmonella typhimurium* LPS was used as a control. Cells were incubated with increasing concentrations of LPS and supernatants were removed after 24 h and assayed for NO. The results are means ± SD of four experiments.

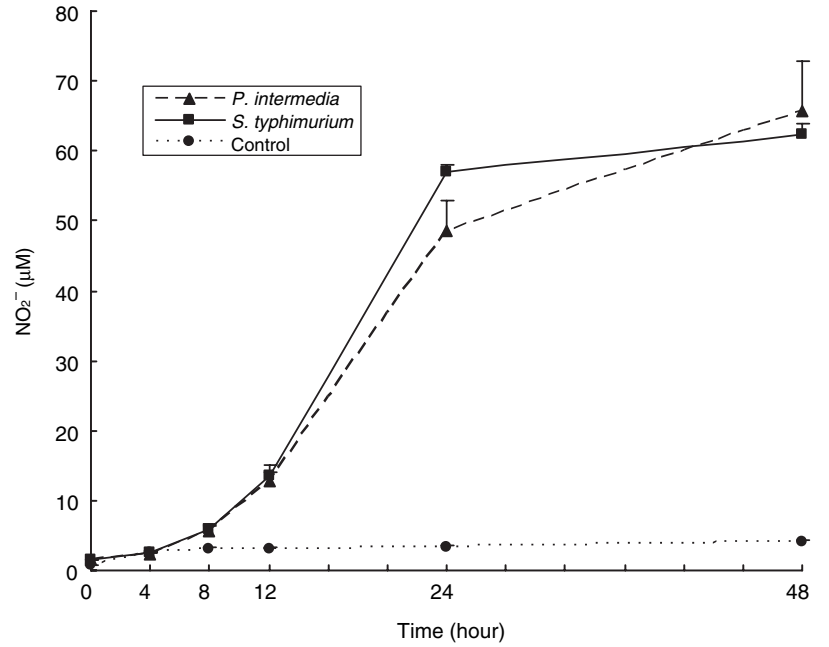

Fig. 2. Time course of nitric oxide (NO) production by RAW264.7 cells stimulated with *Prevotella intermedia* lipopolysaccharide (LPS). Cells were incubated with 1 μg/ml of LPS. Other details as in Fig. 1.

Expression of inducible NO synthase protein and mRNA

To determine whether the elevated NO production was due to up-regulation of the iNOS protein, we evaluated the effects of *P. intermedia* lipopolysaccharide on the expression of iNOS protein. Cells stimulated with *P. intermedia* lipopolysaccharide 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. intermedia* lipopolysaccharide, there was a concentration-dependent accumulation

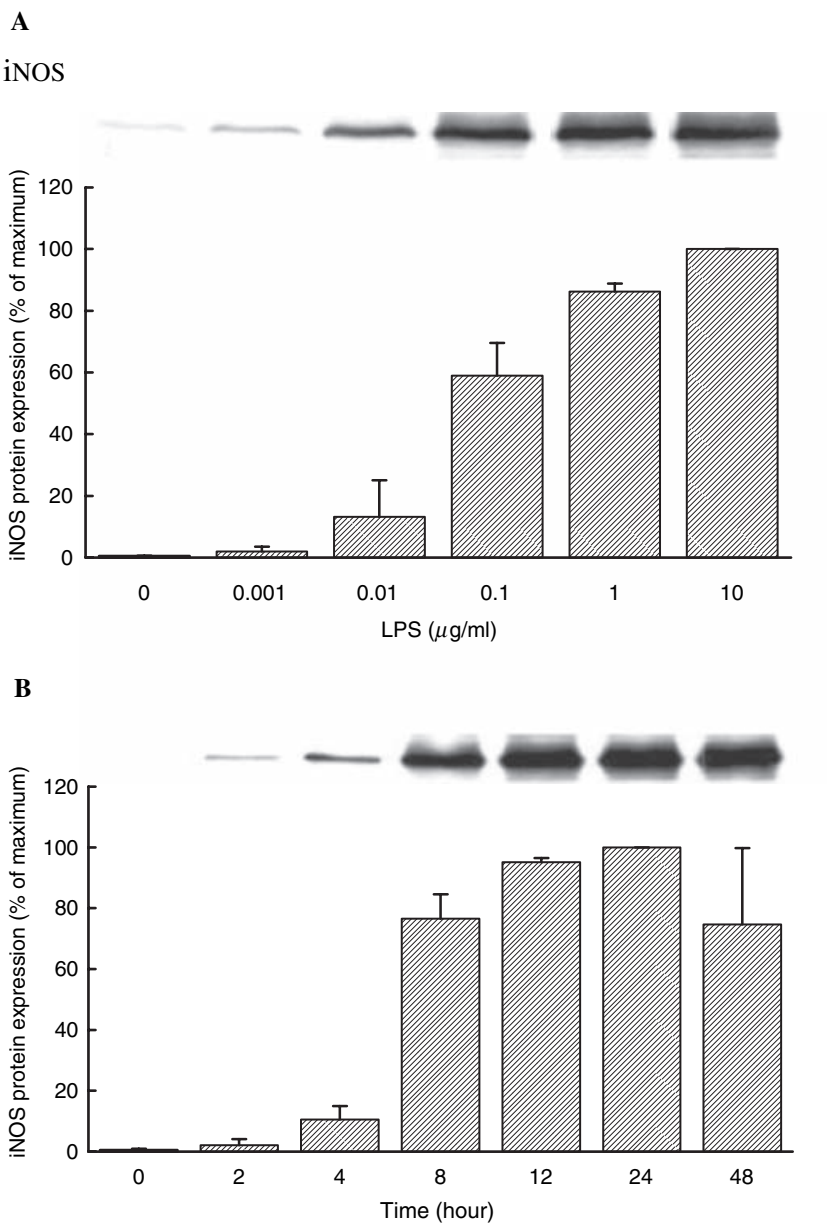

Fig. 3. Dose–response (A) and time course (B) of inducible nitric oxide synthase (iNOS) protein expression in RAW264.7 cells stimulated with *Prevotella intermedia* lipopolysaccharide (LPS). iNOS protein synthesis was measured by immunoblot analysis of cell lysates using iNOS-specific antibody, and iNOS 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. intermedia* LPS for 24 h. (B) Cells were incubated in the presence of 1 μg/ml of *P. intermedia* LPS for different times.

of iNOS (Fig. 3A). iNOS protein was detectable with a concentration of *P. intermedia* lipopolysaccharide 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, as seen in Fig. 3(A). Figure 3(B) shows the time course of changes in iNOS protein expression induced by 1 μg/ml of *P. intermedia* lipopolysaccharide. iNOS protein showed detectable signal at 2 h, and maximum expression was achieved at 24 h.

The effect of *P. intermedia* lipopolysaccharide on iNOS transcription and accumulation of iNOS mRNA was confirmed by RT-PCR. Exposure of cells to *P. intermedia* lipopolysaccharide enhanced iNOS mRNA expression (Fig. 4). iNOS mRNA increased linearly from 2 h to 24 h. Unstimulated RAW264.7 cells did not contain detectable amounts of iNOS mRNA.

Effects of various inhibitors on NO production

N^G-monomethyl-L-arginine (NMMA), an L-arginine analog, is a specific inhibitor of NO production in the L-arginine-dependent pathway (32). To determine if the signaling mechanism of *P. intermedia* lipopolysaccharide-induced NO production involves this pathway, RAW264.7 cells were pretreated with the indicated concentrations of NMMA for 1 h before incubation with *P. intermedia* lipopolysaccharide. Addition of NMMA inhibited NO production (Table 1).

The effect of NOS inhibitor on *P. intermedia* lipopolysaccharide-induced NO synthesis was tested. Cells were pretreated with the indicated concentrations of nitro-L-arginine methyl ester (L-NAME), a non-specific NOS inhibitor, for 1 h before incubation with *P. intermedia* lipopolysaccharide. L-NAME suppressed *P. intermedia* lipopolysaccharide-induced NO production in a concentration-dependent manner (Table 1).

To elucidate the role of nuclear factor-κB (NF-κB) in *P. intermedia* lipopolysaccharide-induced NO production, RAW264.7 cells were pretreated with pyrrolidine dithiocarbamate (PDTC), an antioxidant that acts as a specific inhibitor of NF-κB activation, for 1 h before incubation with *P. intermedia* lipopolysaccharide. Pretreatment with PDTC produced substantial inhibition of NO production (Table 1).

We also assessed whether production of NO by *P. intermedia* lipopolysaccharide 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. intermedia* lipopolysaccharide. *P. intermedia* lipopolysaccharide-induced NO production was found to be suppressed by colchicine pretreatment (Table 1).

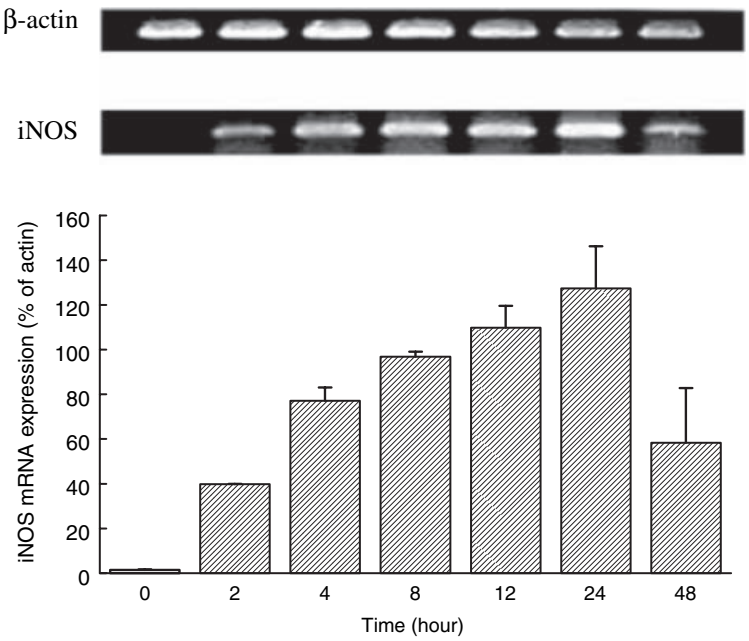

Fig. 4. Time course of inducible nitric oxide synthase (iNOS) mRNA expression in RAW264.7 cells stimulated with *Prevotella intermedia* lipopolysaccharide (LPS). Cells were incubated in the presence of 1 μg/ml of *P. intermedia* LPS for different time periods. See Materials and methods for further details. The polymerase chain reaction bands on a gel photograph in one of two separate experiments yielding similar results are shown.

To investigate the possible involvement of signaling kinases, e.g. protein tyrosine kinase and protein kinase C, in signaling NO induction, cells were pretreated with inhibitors of these kinases for 30 min before incubation with *P. intermedia* lipopolysaccharide. As shown in Table 1, the specific protein tyrosine kinase inhibitor, genistein, reduced lipopolysaccharide-induced nitrite production. However, this inhibitory effect was due to a cytotoxic effect of genistein, as demonstrated by MTT assay (Table 1). The protein kinase C inhibitor bisindolylmaleimide reduced NO production by approximately 90% at a concentration of 50 μM (Table 1). However, bisindolylmaleimide was highly toxic to the cells at this concentration (Table 1).

Cytotoxicity assay

To exclude the possibility that the effect of the various inhibitors on NO production was essentially due to cell death, the effects of each inhibitor on macrophage viability were determined by MTT assay. Treatment of RAW264.7 cells with NMMA, L-NAME, PDTC, and colchicine at the concentrations used did not significantly affect cell viability up to 24 h of incubation, whereas genistein and bisindolylmaleimide reduced cell viability by 27% at 50 μM and 97% at 100 μM, respectively (Table 1).

Discussion

Because production of NO has been recognized as a marker in a variety of human diseases associated with inflammation (33, 34), we studied the effects of the lipopolysaccharide of *P. intermedia*, a major cause of inflammatory periodontal disease, 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 (22). To minimize the effects of contaminating protein, the *P. intermedia* lipopolysaccharide preparation was treated with proteinase K. The protein content of *S. typhimurium* lipopolysaccharide (Sigma), used as a control, was approximately 3%.

The ability of *P. intermedia* lipopolysaccharide to induce NO production in murine macrophages proved to be approximately the same as that of *S. typhimurium* lipopolysaccharide. As iNOS is the catalytic enzyme of NO production (35), we examined the effect of *P. intermedia* lipopolysaccharide on the expression of iNOS protein and demonstrated an increase in iNOS protein by immunoblotting. iNOS is controlled mainly at the transcriptional level and we confirmed in this study that *P. intermedia* lipopolysaccharide induces iNOS expression predominantly at the transcriptional level.

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, whereas NO production increased above the control level only at 8 h. Moreover, both iNOS protein and mRNA were higher at 24 h than at 48 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. L-NAME, a non-specific NOS inhibitor, attenuated *P. intermedia* lipopolysaccharide-induced NO production by RAW264.7 cells in a concentration-dependent manner, indicating that the presence of NOS is a prerequisite for NO production. We also tested whether PDTC, a specific inhibitor of the NF-kB activation, would affect the induction of NO by *P. intermedia* lipopolysaccharide. NF-κB, a ubiquitous transcription factor, is known to regulate the transcription of a variety of genes involved in the inflammatory process (36), and an increasing body of evidence has suggested that the expression of iNOS is dependent on the activation of NF-κB (4, 37, 38). 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 (38). lipopolysaccharide-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,

Table 1. Effects of various inhibitors on *Prevotella intermedia* lipopolysaccharide (LPS)-induced nitric oxide (NO) production

Reagent	Concentration	NO_2^- (μM)	Cell viability (% of control)
NMMA	0 μM	57.5 ± 0.5	100
	1 μM	56.3 ± 0.7	100.5 ± 2.0
	10 μM	48.4 ± 0.8	99.1 ± 1.2
	100 μM	24.7 ± 1.4	99.4 ± 0.8
	500 μM	9.8 ± 0.4	102.2 ± 2.5
	1000 μM	7.8 ± 0.4	96.5 ± 1.9
L-NAME	0.0 mM	57.5 ± 0.5	100
	0.1 mM	42.6 ± 1.2	105.3 ± 1.5
	0.5 mM	21.7 ± 1.3	105.3 ± 1.7
	1.0 mM	13.9 ± 0.5	101.4 ± 1.6
	2.0 mM	9.4 ± 0.8	95.8 ± 1.5
	5.0 mM	5.4 ± 0.7	99.1 ± 2.8
PDTC	0.0 μM	57.5 ± 0.5	100
	0.1 μM	57.0 ± 0.7	101.1 ± 1.3
	1.0 μM	54.3 ± 0.6	96.3 ± 1.9
	10.0 μM	38.9 ± 1.2	98.4 ± 3.2
	50.0 μM	14.1 ± 1.7	97.1 ± 1.0
	100.0 μM	9.6 ± 1.8	96.1 ± 3.5
Colchicine	0 μM	56.6 ± 1.7	100
	10 μM	51.5 ± 1.1	104.2 ± 0.8
	50 μM	51.0 ± 1.9	104.2 ± 1.1
	100 μM	49.0 ± 1.0	99.7 ± 1.2
	500 μM	40.1 ± 0.6	93.1 ± 1.0
	1000 μM	21.3 ± 0.1	97.0 ± 3.0
Genistein	0 μM	52.7 ± 0.6	100
	5 μM	45.9 ± 2.2	92.7 ± 0.7
	10 μM	45.6 ± 2.7	97.4 ± 0.8
	20 μM	40.1 ± 2.1	84.6 ± 4.3
	30 μM	41.0 ± 2.5	81.2 ± 3.4
	50 μM	30.8 ± 1.5	73.1 ± 1.0
Bisindolylmaleimide	0 μM	57.9 ± 0.8	100
	1 μM	56.7 ± 0.8	95.8 ± 0.1
	5 μM	56.5 ± 0.3	94.0 ± 0.2
	10 μM	53.6 ± 0.7	99.4 ± 5.3
	50 μM	8.3 ± 1.5	3.6 ± 0.3
	100 μM	6.0 ± 0.2	3.0 ± 0.3

RAW264.7 cells were pretreated with the indicated concentrations of each inhibitor before 24 h incubation with *P. intermedia* LPS (1 μg/ml). Supernatants were removed after 24 h and assayed for NO. Viability of RAW264.7 cells was assessed by the MTT assay. Results are expressed as means ± SD of four experiments.
NMMA, N^G-monomethyl-L-arginine; L-NAME, nitro-L-arginine methyl ester; PDTC, pyrrolidine dithiocarbamate.

thus activating transcription (1). In the present study, the addition of NF-κB inhibitor, PDTC, markedly suppressed NO synthesis, indicating that NF-κB mediates *P. intermedia* lipopolysaccharide-induced NO production in RAW264.7 cells.

Microtubules have some regulatory function in lipopolysaccharide-induced NO production by macrophages (39) and we demonstrated that *P. intermedia* lipopolysaccharide-induced NO production by RAW264.7 cells was partially inhibited by the microtubule-disrupting agent colchicine. Our findings suggest that microtubules are in some way involved in NO production by macrophages activated with *P. intermedia* lipopolysaccharide. 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 lipopolysaccharide or taxol (39).

The concentrations of each inhibitor used in the present study, with the exception of colchicine, are within the specific inhibitory-concentration ranges reported previously in murine macrophages (18, 40, 41). However, we cannot exclude the possibility that the effect of colchicine at high concentrations (500 and 1000 μM) in the present study was essentially due to non-specific inhibition.

The periodontium is consistently in contact with lipopolysaccharide produced by gram-negative periodontopathogenic bacteria. Lipopolysaccharide from *A. actinomycetemcomitans*, a major pathogen of early onset periodontitis, has been shown *in vitro* to induce NO production in murine macrophages (17, 18). There are no previous reports of the ability of *P. intermedia* lipopolysaccharide to stimulate the release of NO by inducing iNOS expression, and the present study clearly shows, for the first time, that *P. intermedia* lipopolysaccharide alone, without the addition of interferon-γ, fully induced iNOS expression and NO production in the murine macrophage cell line RAW264.7.

NO synthesis is enhanced in periodontal disease, as a result of macrophage infiltration in the periodontal tissues (16, 42). NO 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 (43). The ability of *P. intermedia* lipopolysaccharide 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. The precise mechanism by which *P. intermedia* lipopolysaccharide induces NO production remains to be elucidated.

Acknowledgements

This work was supported by Korea Research Foundation Grant (KRF-2001–041-F00251).

References

1. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide. physiology, pathology, and pharmacology. *Pharmacol Rev* 1991;**43:**109–142.

2. Nathan C, Xie QW. Nitric oxide synthases: roles, tolls and controls. *Cell* 1994;**78:**915–918.
3. Geller DA, Nussler AK, Di Silvio M *et al*. Cytokines, endotoxin, and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. *Proc Natl Acad Sci USA* 1993;**90:** 522–526.
4. Nathan C, Xie QW. Regulation of biosynthesis of nitric oxide. *J Biol Chem* 1994;**269:**13725–13728.
5. Williams RC. Periodontal disease. *N Engl J Med* 1990;**322:**373–381.
6. Socransky SS, Haffajee AD. The bacterial etiology of destructive periodontal disease: current concepts. *J Periodontol* 1992;**63:**322–331.
7. Slots J, Bragd L, Wikstrom M, Dahlen G. The occurrence of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius* in destructive periodontal disease in adults. *J Clin Periodontol* 1986;**13:**570–577.
8. Tanner ACR, Haffer C, Bratthall GT, Visconti RA, Socransky SS. A study of the bacteria associated with advancing periodontitis in man. *J Clin Periodontol* 1979;**6:**278–307.
9. Chung CP, Nisengard RJ, Slots J, Genco RJ. Bacterial IgG and IgM antibody titers in acute necrotizing ulcerative gingivitis. *J Periodontol* 1983;**54:**557–562.
10. Kornman KS, Loesche WJ. The subgingival microbial flora during pregnancy. *J Periodont Res* 1980;**15:**111–122.
11. Morrison DC, Ryan JL. Endotoxins and disease mechanisms. *Annu Rev Med* 1987;**38:**417–432.
12. Honig J, Rordorf-Adam C, Siegmund C, Wiedemann W, Erard F. Increased interleukin-1 beta concentration in gingival tissue from periodontitis patients. *J Periodont Res* 1989;**24:**362–367.
13. Stashenko P, Jandinski JJ, Fujiyioshi P, Rynar J, Socranski SS. Tissue levels of bone resorptive cytokines in periodontal disease. *J Periodontol* 1991;**62:**504–509.
14. Southey A, Tanaka S, Murakami T *et al*. Pathophysiological role of nitric oxide in rat experimental colitis. *Int J Immunopharmacol* 1997;**19:**669–676.
15. Weinberg JB, Granger DL, Pisetsky DS *et al*. The role of nitric oxide in the pathogenesis of spontaneous murine autoimmune disease: increased nitric oxide production and nitric oxide synthase expression in MRL-lpr/lpr mice, and reduction of spontaneous glomerulonephritis and arthritis by orally administered N^G-monomethyl-L-arginine. *J Exp Med* 1994;**179:**651–660.
16. Matejka M, Partyka L, Ulm C, Solar P, Sinzinger H. Nitric oxide synthesis is increased in periodontal disease. *J Periodont Res* 1998;**33:**517–518.
17. Blix IJ, Helgeland K. lipopolysaccharide from *Actinobacillus actinomycetemcomitans* and production of nitric oxide in murine macrophages J774. *Eur J Oral Sci* 1998;**106:**576–581.
18. Sosroseno W, Barid I, Herminajeng E, Susilowati H. Nitric oxide production by a murine macrophage cell line (RAW264.7) stimulated with lipopolysaccharide from *Actinobacillus actinomycetemcomitans*. *Oral Microbiol Immunol* 2002;**17:**72–78.
19. Batista AC, Silva TA, Chun JH, Lara VS. Nitric oxide synthesis and severity of human periodontal disease. *Oral Dis* 2002;**8:**254–260.
20. Hirose M, Ishihara K, Saito A, Nakagawa T, Yamada S, Okuda K. Expression of cytokines and inducible nitric oxide synthase in inflamed gingival tissue. *J Periodontol* 2001;**72:**590–597.
21. Kendall HK, Haase HR, Li H, Xiao Y, Bartold PM. Nitric oxide synthase type-II is synthesized by human gingival tissue and cultured human gingival fibroblasts. *J Periodont Res* 2000;**35:**194–200.
22. Lappin DF, Kjeldsen M, Sander L, Kinane DF. Inducible nitric oxide synthase expression in periodontitis. *J Periodont Res* 2000;**35:**369–373.
23. Tokuda M, Nagaoka S, Torii M. Interleukin-10 receptor expression in human dental pulp cells in response to lipopolysaccharide from *Prevotella intermedia*. *J Endod* 2003;**29:**48–50.
24. Nagaoka S, Tokuda M, Sakuta T *et al*. Interleukin-8 gene expression by human dental pulp fibroblast in cultures stimulated with *Prevotella intermedia* lipopolysaccharide. *J Endod* 1996;**22:**9–12.
25. Tokuda M, Sakuta T, Fushuku A, Torii M, Nagaoka S. Regulation of interleukin-6 expression in human dental pulp cell cultures stimulated with *Prevotella intermedia* lipopolysaccharide. *J Endod* 2001;**27:**273–277.
26. Pelt P, Zimmermann B, Ulbrich N, Bernimoulin JP. Effects of lipopolysaccharide extracted from *Prevotella intermedia* on bone formation and on the release of osteolytic mediators by fetal mouse osteoblasts in vitro. *Arch Oral Biol* 2002;**47:**859–866.
27. Westphal O, Jann K. Bacterial lipopolysaccharides: extraction with phenol–water and further applications of the procedure. In: Whistler RL, ed. *Methods in carbohydrate chemistry*. New York: Academic Press, 1965: 83–91.
28. Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 1978;**87:**206–210.
29. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;**65:**55–63.
30. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem* 1982;**126:**131–138.
31. Rappolee DA, Wang A, Mark D, Werb Z. Novel method for studying mRNA phenotypes in single or small numbers of cells. *J Cell Biochem* 1989;**39:**1–11.
32. Sakuma I, Stuehr DJ, Gross SS, Nathan C, Levi R. Identification of arginine as a precursor of endothelium-derived relaxing factor. *Proc Natl Acad Sci USA* 1988;**85:**8664–8667.
33. Kaur H, Halliwell B. Evidence for nitric oxide-mediated oxidative damage in chronic inflammation. Nitrotyrosine in serum and synovial fluid from rheumatoid patients. *FEBS Lett* 1994;**350:**9–12.
34. Rachmilewitz D, Stamler JS, Bachwich D, Karmeli F, Ackerman Z, Podolsky D. Enhanced colonic nitric oxide generation and nitric oxide synthase activity in ulcerative colitis and Crohn's disease. *Gut* 1995;**6:**718–723.
35. Stuehr DJ, Nathan CF. Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J Exp Med* 1989;**169:**1543–1555.
36. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. *Annu Rev Immunol* 1994;**12:**141–179.
37. Baldwin AS Jr. The NF-κB and I-κB proteins: new discoveries and insights. *Annu Rev Immunol* 1996;**14:**649–683.
38. Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J Biol Chem* 1994;**269:**4705–4708.
39. Kirikae T, Kirikae F, Oghiso Y, Nakano M. Microtubule-disrupting agents inhibit nitric oxide production in murine peritoneal macrophages stimulated with lipopolysaccharide or paclitaxel (taxol). *Infect Immun* 1996;**64:**3379–3384.
40. Gross A, Spiesser S, Terraza A, Rouot B, Caron E, Dornand J. Expression and bactericidal activity of nitric oxide synthase in *Brucella suis*-infected murine macrophages. *Infect Immun* 1998;**66:**1309–1316.
41. Chung HS, Jeong HJ, Hong SH *et al*. Induction of nitric oxide synthase by *Oldenlandia diffusa* in mouse peritoneal macrophages. *Biol Pharm Bull* 2002;**25:**1142–1146.
42. Paquette DW, Williams RC. Modulation of host inflammatory mediators as a treatment strategy for periodontal diseases. *Periodontol 2000* 2000;**24:**239–252.
43. Brennan PA, Thomas GJ, Langdon JD. The role of nitric oxide in oral diseases. *Arch Oral Biol* 2003;**48:**93–100.