

# Nitric oxide production and inducible nitric oxide synthase expression induced by *Porphyromonas gingivalis* lipopolysaccharide

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## I . Introduction

Nitric oxide (NO) is a short-lived bio-active molecule produced by immunocompetent cells such as macrophages that serves as a messenger molecule for various physiological and pathological processes<sup>1</sup>. It is synthesized from L-arginine by nitric oxide synthase (NOS) present in various tissues<sup>2</sup>. Three distinct isoforms of NOS, neural (nNOS), endothelial (eNOS) and inducible (iNOS), have been reported in mammalian tissues<sup>2</sup>. 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- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and interferon- $\gamma$  (IFN- $\gamma$ ), in a variety of cell types including macrophages, following bacterial infection<sup>3,4</sup>. iNOS, once expressed, can generate large amounts of NO for extended times, and is believed to be involved in cytotoxic effects following inflammation<sup>1</sup>.

Periodontal disease is a chronic inflammatory process accompanied by destruction of surrounding connective tissue and alveolar bone, and sometimes loss of teeth<sup>5</sup>. The

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primary causative agents of periodontal disease are particular gram-negative anaerobic bacteria that accumulate in the gingival sulcus. *Porphyromonas gingivalis* is a major periodontal pathogen that is dominant in the periodontal pockets of patients with adult periodontitis<sup>6,7</sup>.

LPS is a major component of the outer membrane of gram-negative bacteria, including *P. gingivalis*. 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- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8<sup>8</sup>). These cytokines have been implicated in the pathogenesis of inflammatory periodontal disease<sup>9,10</sup>. In addition to these cytokines, NO has recently received considerable attention as a novel type of mediator<sup>11</sup>; inhibition of NOS activity and NO production frequently limits the progression and severity of experimental inflammatory diseases such as osteoarthritis, glomerulonephritis, and colitis<sup>11,12</sup>.

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<sup>13</sup>, and gingival tissues from patients with chronic periodontitis have higher levels of iNOS protein and mRNA than healthy tissue<sup>14-17</sup>. Macrophages, polymorphonuclear cells and fibroblasts are the sources of iNOS in periodontal tissues, with endothelial cells also contributing<sup>14-17</sup>. Moreover, LPS from *Actinobacillus actinomycetemcomitans*, a major pathogen of early-onset periodontitis,

induced significant production of NO in macrophages<sup>18,19</sup>, and LPSs from *Prevotella intermedia* and *Prevotella nigrescens*, the causative agents of inflammatory periodontal disease, fully induced iNOS expression and NO production in the murine macrophage cell line RAW264.7 cells in the absence of other stimuli<sup>20,21</sup>.

The LPS 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 NO. The purpose of this study was to investigate the effects of *P. gingivalis* 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.

## II. Materials and methods

### 1. 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 GAM broth (Nissui, Tokyo, Japan) supplemented with 1  $\mu$ g/ml menadione and 5  $\mu$ g/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  $\times$  g for 20 min at 4 $^{\circ}$ 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. Preparation of LPS

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* using the butanol extraction method of Morrison and Leive<sup>22</sup>. Briefly, cells were suspended in 0.15 M NaCl at 4°C and an equal volume of butanol 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 two times 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 lyophilised. This crude preparation, which contains both lipid A-associated proteins and LPS, was re-suspended in water and ultracentrifuged for at 105,000 × *g* for 3 h. This procedure was repeated once more and the endotoxin lyophilised. LPS was prepared from lyophilized endotoxin by the standard hot phenol-water method<sup>23</sup>. Briefly, 90% phenol was added to the endotoxin 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 35,000 × *g* for 15 min and the pooled aqueous extract was dialysed extensively against distilled water at 4°C. The dialysed extract was centrifuged at 105,000 × *g* for 3 h and lyophilized to yield LPS. *Salmonella typhimurium* LPS (phenol extract) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

## 3. Cell cultures

The murine macrophage cell line RAW 264.7 (American Type Culture Collection, Rockville, MD, USA) 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% NaHCO<sub>3</sub>, 1 mM sodium pyruvate, and 10% [v/v] heat-inactivated FBS in a humidified chamber with 5% CO<sub>2</sub>/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<sup>5</sup> 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.

#### 4. 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<sup>24</sup>. Cells were pretreated with the indicated concentrations of each inhibitor before 24 h incubation with *P. gingivalis* LPS (10µg/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<sub>2</sub> 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, Palo Alto, CA, USA). Cell viability is expressed as a percentage of the control value.

#### 5. Measurement of NO production

NO production was assayed by measuring the accumulation of the stable oxidative metabolite, nitrite (NO<sub>2</sub><sup>-</sup>), in culture supernatants<sup>25</sup>. Briefly, 5×10<sup>5</sup> 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 Chemical, St. Louis, MO, USA) 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<sub>2</sub> (Sigma) in culture medium.

#### 6. Western blot analysis of iNOS

Cells were plated in 60 mm tissue culture dishes at a density of 2×10<sup>6</sup> cells/dish and treated with various concentrations of *P. gingivalis* 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 10,000 x g for 10 min to remove insoluble material, and their protein concentrations determined with the bicinchoninic acid (BCA) 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 SDS-polyacrylamide gel electrophoresis (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, USA) 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 1:2,000 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.

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

Cells were plated in 100 mm tissue culture dishes at a density of  $2 \times 10^7$  cells/dish and treated with various concentrations of *P. gingivalis* 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, 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, Korea) and thermal cycler (GeneAmp PCR system 2400; PE Applied Biosystems, Foster City, CA, USA)<sup>26</sup>.  $\beta$ -actin served as internal control. The number of cycles that ensured nonsaturating PCR conditions was established in preliminary exper-

iments. 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;  $\beta$ -actin, 5'-TCCTTCGTTGCCGGTCCACA-3' (sense) and 5'-C-GTCTCCGGAGTCCATCACA-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 LPS stimulation) and the density of PCR products from iNOS and  $\beta$ -actin mRNA (data not shown).

## 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  $\pm$  S.D. of four independent experiments.

### III. Results

#### 1. NO induction by *P. gingivalis* LPS

Concentrations of nitrite, an indicator of NO production, were measured 24 h after adding various concentrations of isolated *P. gingivalis* 381 LPS to RAW264.7 cells. *P. gingivalis* LPS induced NO release from the RAW264.7 cells over the range 1 ng/ml to 100  $\mu\text{g/ml}$  (Figure 1). Basal nitrite release was about 1  $\mu\text{M}$ . It was effective at a concentration as low as 1  $\mu\text{g/ml}$ , and maximum NO production (about 53  $\mu\text{M}$ ) was achieved at a concentration of 100  $\mu\text{g/ml}$ . *S. typhimurium* LPS, as a control, also stimulated NO

production to a maximum of 62  $\mu\text{M}$ . Its activity was significantly higher than that of *P. gingivalis* LPS with respect of both minimum stimulatory dose and maximum NO produced.

RAW264.7 cells were challenged with 10  $\mu\text{g/ml}$  *P. gingivalis* LPS, and production of NO in the culture supernatant was measured at various times thereafter. After an initial lag of 8 h, NO secretion increased linearly from 8 h to 24 h and plateaued thereafter. Nitrite accumulation reached 29  $\mu\text{M}$  (Figure 2). *S. typhimurium* LPS also caused a marked elevation in NO secretion that leveled off after 24 h.

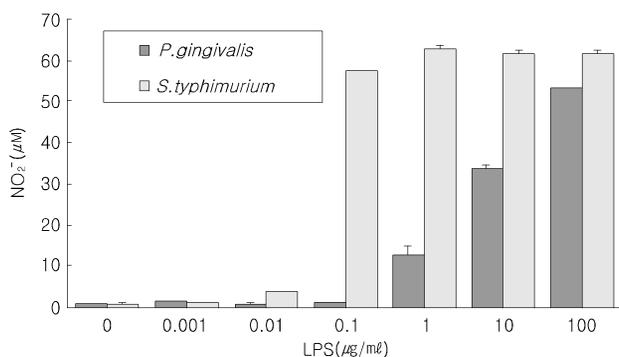


Figure 1. Dose response of NO production by RAW264.7 cells stimulated with *P. gingivalis* LPS. Cells were incubated with increasing concentrations of LPS and supernatants were removed after 24 h and assayed for NO. The results are means  $\pm$  standard deviation of four experiments.

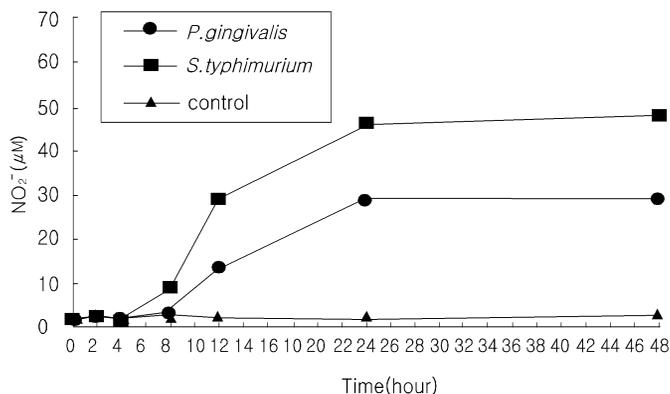


Figure 2. Time course of NO production by RAW264.7 cells stimulated with *P. gingivalis* LPS. Cells were incubated with 10  $\mu\text{g/ml}$  of LPS. Other details as in Figure 1.

## 2. Expression of iNOS protein and mRNA

To determine whether the elevated NO production was due to upregulation of the iNOS protein, we examined the effect of *P. gingivalis* LPS on the expression of iNOS protein and demonstrated an increase in iNOS protein by immunoblotting. Cells stimulated with *P. gingivalis* LPS expressed a protein of approximately 130 kDa, recognized by specific antibody to iNOS (Figure 3). When RAW264.7 cells were exposed to increasing concentrations of *P. gingivalis*

LPS, there was a concentration-dependent accumulation of iNOS (Figure 3A). iNOS protein was detectable with a concentration of *P. gingivalis* LPS as low as 0.1  $\mu\text{g}/\text{ml}$  and reached a maximum at a concentration of 100  $\mu\text{g}/\text{ml}$ . Control cells did not produce detectable iNOS band, as seen in Figure 3A. Figure 3B shows the time course of changes in iNOS protein expression induced by 10  $\mu\text{g}/\text{ml}$  of *P. gingivalis* LPS. iNOS protein showed detectable signal at 4h, and maximum expression was achieved at 12 h.

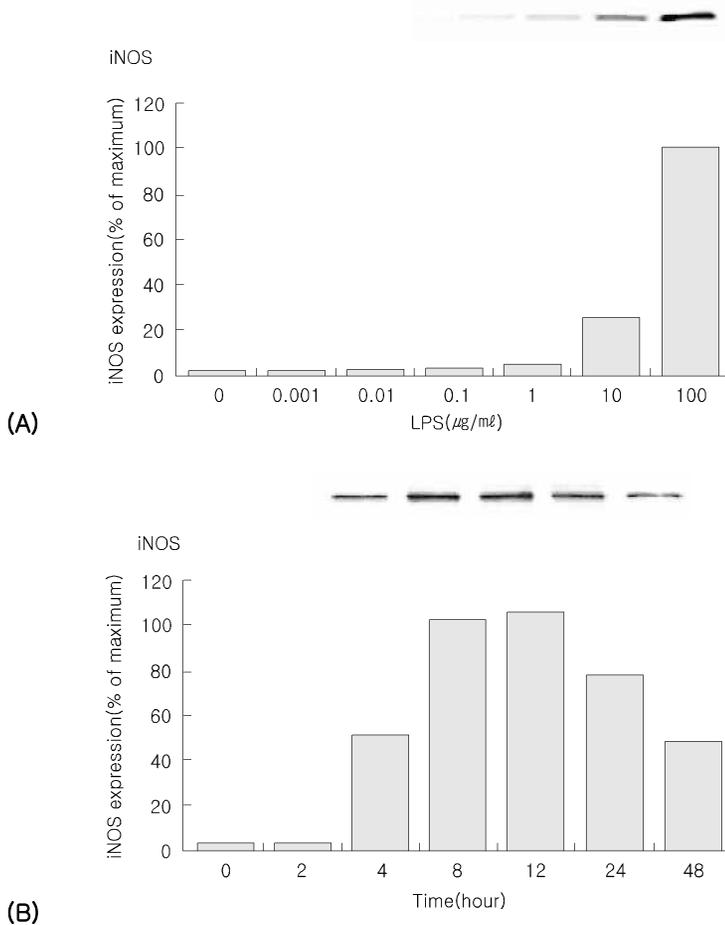


Figure 3. Dose response (A) and time course (B) of iNOS protein expression in RAW264.7 cells stimulated with *P. gingivalis* LPS. iNOS protein synthesis was measured by immunoblot analysis of cell lysates using iNOS-specific antibody and iNOS protein levels were quantitated by densitometry. (A) Cells were incubated with different concentrations of *P. gingivalis* LPS for 24 h. (B) Cells were incubated in the presence of 10  $\mu\text{g}/\text{ml}$  of *P. gingivalis* LPS for different times.

The effect of *P. gingivalis* LPS on iNOS transcription and accumulation of iNOS mRNA was confirmed by RT-PCR. Exposure of cells to *P. gingivalis* LPS enhanced iNOS mRNA expression (Figure 4). When RAW 264.7 cells were exposed to increasing concentrations of *P. gingivalis* LPS, there was a concentration-dependent accumulation of iNOS mRNA (Figure 4A). iNOS mRNA was detectable with a concentration of *P. gingivalis* LPS as low as 0.1  $\mu\text{g}/\text{ml}$ , reached a

maximum at a concentration of 100  $\mu\text{g}/\text{ml}$ . Control cells did not produce detectable signal, as seen in Figure 4A. Figure 4B shows the time course of changes in iNOS mRNA expression induced by 10  $\mu\text{g}/\text{ml}$  of *P. gingivalis* LPS. iNOS mRNA showed detectable signal at 2 h, and maximum expression was achieved at 4 h. Unstimulated RAW264.7 cells did not contain detectable amounts of iNOS mRNA.

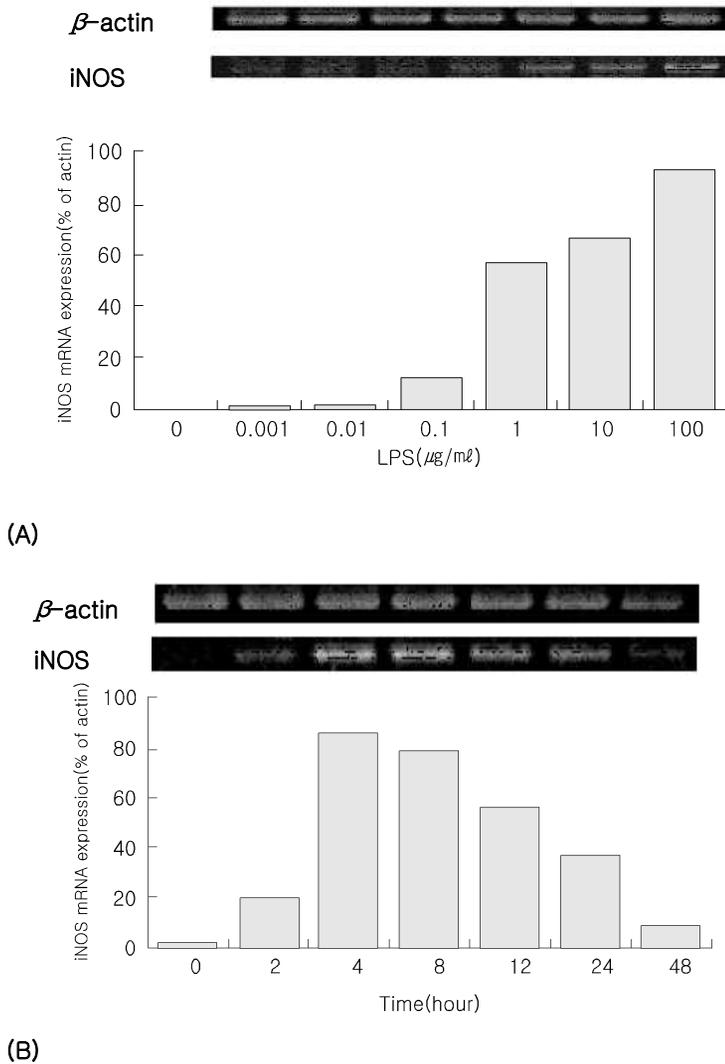
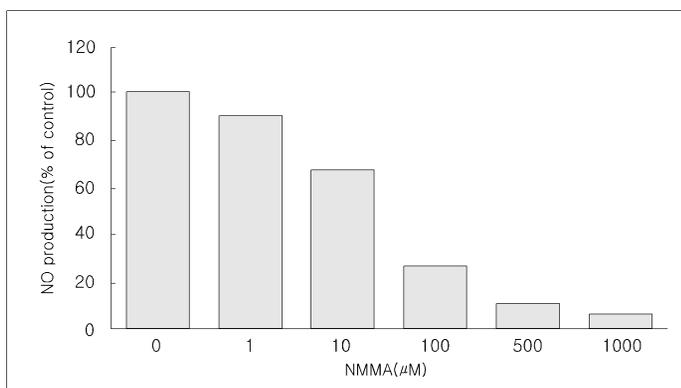


Figure 4. Dose response (A) and time course (B) of iNOS mRNA expression in RAW264.7 cells stimulated with *P. gingivalis* LPS. See Materials and methods for further details. (A) Cells were incubated with different concentrations of *P. gingivalis* LPS for 24 h. (B) Cells were incubated in the presence of 10  $\mu\text{g}/\text{ml}$  of *P. gingivalis* LPS for different times.



**Figure 5.** Influence of NG-monomethyl-L-arginine (NMMA), an L-arginine analogue, on the NO-inducing activity of *P. gingivalis* LPS. RAW264.7 cells were pretreated with the indicated concentrations of NMMA for 1 h before 24 h incubation with *P. gingivalis* LPS (10 μg/ml). Supernatants were removed after 24 h and assayed for NO. The results are means ± standard deviation of four experiments.

### 3. Effects of various inhibitors on NO production

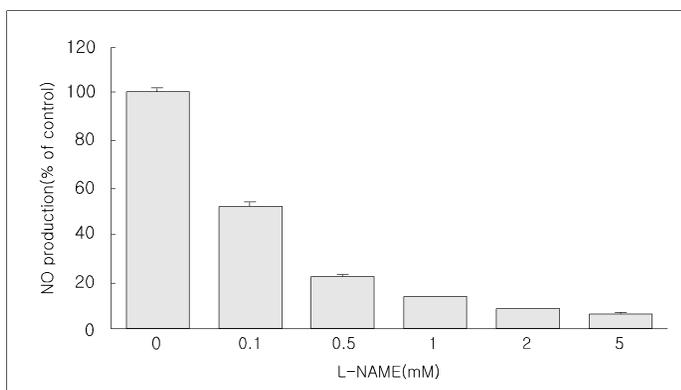
N<sup>G</sup>-monomethyl-L-arginine (NMMA), an L-arginine analogue, is a specific inhibitor of NO production in the L-arginine-dependent pathway<sup>27)</sup>. To determine if the signaling mechanism of *P. gingivalis* LPS-induced NO production involves this pathway, RAW264.7 cells were pretreated with the indicated concentrations of NMMA for 1 h before incubation with *P. gingivalis* LPS. Addition of NMMA inhibited NO production in a concentration-dependent manner (Figure 5).

The effect of NOS inhibitor on *P. gingivalis* 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. gingivalis* LPS. L-NAME suppressed *P. gingivalis* LPS-induced NO production by RAW264.7

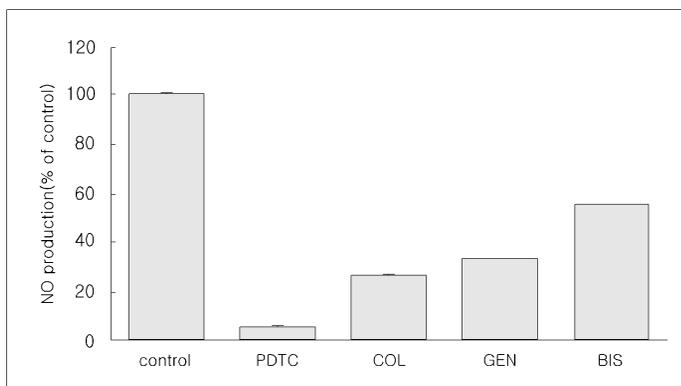
cells (Figure 6).

To elucidate the role of nuclear factor-κB (NF-κB) in *P. gingivalis* LPS-induced NO production, RAW264.7 cells were pretreated with 50 μM pyrrolidine dithiocarbamate (PDTC), an antioxidant that acts as a specific inhibitor of NF-κB activation, for 1 h before incubation with *P. gingivalis* LPS. Pre-treatment with 50 μM PDTC produced about 94% inhibition of NO production (Figure 7).

We also assessed whether production of NO by *P. gingivalis* LPS stimulation depends on microtubule polymerization. RAW264.7 cells were pretreated with 500 μM of colchicine, a mitosis inhibitor, for 30 min before incubation with *P. gingivalis* LPS. *P. gingivalis* LPS-induced NO production was found to be suppressed by colchicine pretreatment (Figure 7). Colchicine showed about 72% inhibition of NO production at the test concentration of 500 μM without affecting cell



**Figure 6.** Influence of nitro-L-arginine methyl ester (L-NAME), a nonspecific NOS inhibitor, on NO-inducing activity of *P. gingivalis* LPS. RAW264.7 cells were pretreated with the indicated concentrations of L-NAME for 1 h before 24 h incubation with *P. gingivalis* LPS (10 g/ml).



**Figure 7.** Effects of pyrrolidine dithiocarbamate (PDTTC, 50  $\mu$ M), colchicine (COL, 500  $\mu$ M), genistein (GEN, 10  $\mu$ M) and bisindolylmaleimide (BIS, 10  $\mu$ M) on the NO-inducing activity of *P. gingivalis* LPS. RAW264.7 cells were pretreated with indicated concentrations of specific inhibitors for 1 h before 24 h incubation with *P. gingivalis* LPS (10  $\mu$ g/ml).

viability.

To investigate the possible involvement of signaling kinases, e.g., protein tyrosine kinase (PTK) and protein kinase C (PKC), in signaling NO induction, cells were pretreated with inhibitors of these kinases for 30 min before incubation with *P. gingivalis*

LPS. As shown in Figure 7, the specific PTK inhibitor, genistein, showed about 67.1 % inhibition of NO production at the concentration of 10  $\mu$ M. The PKC inhibitor bisindolylmaleimide also reduced *P. gingivalis* LPS-induced NO production by about 43.3% at the concentration of 10  $\mu$ M (Figure 7).

## IV. Discussion

Because production of NO has been recognized as a marker in a variety of human diseases associated with inflammation<sup>28,29</sup>, we studied the effects of the LPS of *P. gingivalis*, 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<sup>17</sup>.

*P. gingivalis* LPS stimulated the release of NO from the RAW264.7 cells. Since iNOS is the catalytic enzyme of NO production<sup>30</sup>, we examined the effect of *P. gingivalis* LPS 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. gingivalis* LPS induces iNOS expression predominantly at the transcriptional level.

It is of interest to note that there was some delay between iNOS mRNA expression and NO production. iNOS mRNA showed detectable signals at 2 h, while NO production increased above the control level only at 12 h. Moreover, maximum iNOS mRNA expression was achieved at 4 h. Evidently, in the RAW264.7 cells, iNOS mRNA is produced at high levels, but decrease before NO reaches its maximum, and high levels of NO are found long after expression of iNOS mRNA had started to decline.

We demonstrated an absolute requirement for endogenous L-arginine in NO production. L-NAME, a non-specific NOS inhibitor, at-

tenuated *P. gingivalis* LPS-induced NO production by RAW264.7 cells, indicating that the presence of NOS is a prerequisite for NO production. We also tested PDTC, an antioxidant that acts as a specific inhibitor of NF- $\kappa$ B activation, would affect the induction of NO by *P. gingivalis* LPS. NF- $\kappa$ B, a ubiquitous transcription factor, is known to regulate the transcription of a variety of genes involved in the inflammatory process<sup>31</sup>, and an increasing body of evidence has suggested that the expression of iNOS is dependent on the activation of NF- $\kappa$ B<sup>4,32,33</sup>. Binding of NF- $\kappa$ B to the specific binding nucleotide sequences in the promoter region of the iNOS gene results in rapid and effective transcription of this gene<sup>33</sup>. In the present study, the addition of NF- $\kappa$ B inhibitor, PDTC, markedly suppressed NO synthesis indicating that NF- $\kappa$ B mediates *P. gingivalis* LPS-induced NO production in RAW264.7 cells.

We demonstrated that *P. gingivalis* LPS-induced NO production by RAW264.7 cells was inhibited by the microtubule-disrupting agent colchicine. Our findings suggest that microtubules are involved in NO production by macrophages activated with *P. gingivalis* LPS. We also examined the effect of signaling kinases. Activation of PTK and PKC appears to be necessary for *P. gingivalis* LPS-induced NO production in murine macrophages.

Taken together, these results indicate that multiple signaling pathways such as NF- $\kappa$ B, PTK, and PKC are involved in *P. gingivalis* LPS-stimulated NO production. It would be interesting to find out how these

pathways coordinate for NO production. 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. gingivalis* LPS-induced NO production. As a result, PDTC showed potent inhibition of NO production without affecting cell viability (about 94% inhibition at the test concentration of 50  $\mu$ M). 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.

NO synthesis is increased in periodontal disease, as a result of macrophage infiltration in the periodontal tissues<sup>13,34</sup>. The present study clearly shows that the LPS of *P. gingivalis*, a major periodontal pathogen, fully induced iNOS expression and NO production in the murine macrophage cell line RAW264.7 in the absence of other stimuli.

There are strain-dependent variations in the ability of *P. gingivalis* LPS to induce NO production. LPS from *P. gingivalis* ATCC 33277 failed to induce NO production by RAW264.7 cells similar to that of W50<sup>35,36</sup>. In contrast, NO could be produced by murine macrophages when stimulated with LPS preparation from *P. gingivalis* A7436; however, this LPS was far less potent than LPS from enteric bacteria<sup>36</sup>.

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<sup>37</sup>. The ability of *P. gingivalis* LPS to promote the production of NO

may be important in the establishment of the chronic lesion observed in inflammatory periodontal disease. The precise mechanism by which *P. gingivalis* LPS induces NO production remains to be elucidated.

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# *Porphyromonas gingivalis*의 세균내독소가 RAW264.7세포에서의 nitric oxide의 생성과 inducible nitric oxide synthase의 발현에 미치는 영향 및 기전

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본 연구는 치주질환 주요 병인균주 중의 하나인 *Porphyromonas gingivalis*의 세균내독소가 마우스 대식 세포주인 RAW264.7 세포에서의 nitric oxide의 생성과 iNOS의 발현에 미치는 영향을 분석하고 그 기전을 규명하기 위해 수행되었다. Butanol추출법과 phenol-water법에 의해 *P. gingivalis* 381로부터 세균내독소를 추출하였으며, NO의 생성은 배양 상층액 내의 nitrite 농도를 측정하여 결정하였다. 또한, iNOS의 western blot 분석과 reverse transcription (RT)-PCR 산물의 분석을 수행하였다. *P. gingivalis*의 세균내독소는 부가적인 자극이 없는 상태에서도 iNOS의 발현과 NO 생성을 유발하였으며, NF- $\kappa$ B, microtubule polymerization, protein tyrosine kinase, 그리고 protein kinase C 등이 *P. gingivalis* 세균내독소에 의한 NO 생성에 간여하는 것으로 여겨진다. 또한, *P. gingivalis* 세균내독소에 의한 NO 생성에는 L-arginine이 요구되었다. *P. gingivalis* 세균내독소에 의한 NO 생성은 염증성 치주질환의 발병과 진행에 있어 중요한 역할을 하는 것으로 여겨진다.

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주요어 : *Porphyromonas gingivalis*; Lipopolysaccharide; Nitric oxide; iNOS; RAW264.7 cells