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Nitric oxide production by a human osteoblast cell line stimulated with *Aggregatibacter actinomycetemcomitans* lipopolysaccharide

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Background/aim: Human osteoblasts induced by inflammatory stimuli express an inducible nitric oxide synthase (iNOS). The aim of the present study was to test the hypothesis that *Aggregatibacter actinomycetemcomitans* lipopolysaccharide stimulates the production of nitric oxide (NO) by a human osteoblast-like cell line (HOS cells).
Methods: Cells were stimulated directly with *A. actinomycetemcomitans* lipopolysaccharide or pretreated with the following L-NIL (an iNOS inhibitor), anti-CD14, Toll-like receptor 2 (TLR2), or TLR4 antibody before stimulation with *A. actinomycetemcomitans* lipopolysaccharide. The role of the cyclic nucleotides was assessed by pretreating the cells with the following; ODQ (a guanylyl cyclase inhibitor); SQ22536 (an adenylyl cyclase inhibitor); db-cAMP (a cyclic adenosine monophosphate analog); br-cGMP (a cyclic guanosine monophosphate analog); forskolin (an adenylyl cyclase activator), IBMX [a non-specific phosphodiesterase (PDE) inhibitor], or KT5720 [a protein kinase A (PKA) inhibitor]. The cells were also preincubated with genistein [a protein tyrosine kinase (PTK) inhibitor], bisindolylmaleimide [a protein kinase C (PKC) inhibitor], BPB [a phospholipase A2 (PLA2) inhibitor], and NDGA (a lipoxygenase inhibitor). The iNOS activity and nitrite production in the cell cultures were determined spectrophotometrically.
Results: The results showed that *A. actinomycetemcomitans* lipopolysaccharide stimulated both iNOS activity and nitrite production by HOS cells; this was reduced by L-NIL, anti-CD14, or anti-TLR4 antibody, SQ22536, KT5720, genistein, bisindolylmaleimde, BPB, and NDGA, but was enhanced by db-cAMP, IBMX, and forskolin.
Conclusion: These results therefore suggest that *A. actinomycetemcomitans* lipopolysaccharide may induce the production of NO by HOS cells via a CD14–TLR4 molecule complex, a cAMP–PKA pathway, as well as by a PTK, PKC, PLA2, and lipoxygenase-dependent mechanism.

Key words: *Aggregatibacter actinomycetemcomitans;* cyclic adenosine monophosphate; human osteoblast cell line; inducible nitric oxide synthase; lipopolysaccharide; lipoxygenase; nitric oxide; phospholipase A2; protein kinases

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Nitric oxide (NO) is a gaseous molecule synthesized by the metabolism of L-arginine catalyzed by nitric oxide synthase (NOS). It plays a crucial role in many human biological systems (27). Three isoforms of NOS, neural NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2), and endothelial NOS (eNOS or NOS-3), have been recognized (29). Human osteoblasts have been

shown to express constitutively eNOS, but the expression of iNOS molecules is induced following stimulation with a combination of either interleukin-1β, tumor necrosis factor-α, or interferon-γ with lipopolysaccharide (13, 24, 35, 36). However, *Prevotella intermedia* and *Escherichia coli* lipopolysaccharide alone was able to induce the production of NO by rodent osteoblasts (33, 34). The exact mechanism by which NO may play a role in bone remodeling remains to be elucidated. It seems plausible that the rapid production of osteoblast-derived NO induced by inflammatory stimuli may play a pivotal role in bone resorption (33, 34), whereas the slow release of osteoblast-derived NO induced by physiological stimuli such as estradiol (31), estrogen (38), and fluid shear stress (17) may stimulate bone formation.

Aggregatibacter (Actinobacillus) actinomycetemcomitans, a gram-negative oral bacterium, has been associated with the destructive stage of periodontal diseases, involving rapid alveolar bone destruction (25, 47). Indeed, lipopolysaccharide from this bacterium has been shown to stimulate bone resorption *in vivo* and *in vitro* in an animal model (14, 16, 30, 37). Furthermore, we and others have demonstrated that lipopolysaccharide from *A. actinomycetemcomitans* induces the production of NO by murine macrophages and splenocytes (3, 39, 40). The induction of the immune response to *A. actinomycetemcomitans* lipopolysaccharide in mice, both *in vivo* and *in vitro*, appears to be regulated by NO (42, 43).

A human osteoblast-like cell line (HOS cells), originated from human osteosarcoma, has been shown to form nodules, produce both alkaline phosphatase and hydroxyapatite crystals, and express receptors such as bone morphogenetic protein and progesterone receptors (9, 15, 23, 44). Furthermore, this cell line is responsive to growth factors such as transforming growth factor-β (12), estrogen (20), and cytokines such as interleukin-1 (28) and as such seems to be similar to human primary osteoblasts and to function as osteogenic cells. Bacterial lipopolysaccharide alone stimulates the production of NO by rodent osteoblasts (13, 33) so the aim of the present study was to determine if lipopolysaccharide from *A. actinomycetemcomitans* induces the production of NO by HOS cells.

Materials and methods

Preparation of bacterial lipopolysaccharide

A. actinomycetemcomitans Y4 (serotype b) was grown in Todd–Hewitt broth supplemented with 1% (weight/volume) yeast extract (Difco Laboratories, Detroit, MI) anaerobically as described elsewhere (40). Lipopolysaccharide from this periodontopathic bacterium was prepared using hot phenol–water, treated with nuclease (Sigma, St Louis, MO), and then lyophilized as previously described (40). Trace amounts of protein, assessed using a BCA Protein Assay (Pierce, Rockford, IL), in the extract were not detected. The extract also showed the typical lipopolysaccharide ladder-like pattern with polyacrylamide gel electrophoresis and silver staining (Bio-Rad Laboratories Inc., Hercules, CA). The extract was subsequently dissolved at 1 mg/ml in culture medium (see below).

Cell cultures

A human osteoblast-like cell line (HOS cells) (ATCC No: CRL-1543) was purchased from American Type Culture Collection (ATCC; Rockville, MD) and grown in Dulbecco's modified Eagle's minimal essential medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Sigma) and 1% penicillin–streptomycin (Sigma) until confluence was reached. After harvesting and washing, a single cell suspension (1×10^6 cells/ml) was prepared in the above medium. Two hundred microliters of cell suspension containing 2×10^5 cells/well with or without the presence of 1, 5, or 10 μg/ml of *A. actinomycetemcomitans* lipopolysaccharide was plated in 96-well plates (Nunc, Roskilde, Denmark) and incubated for 3 days at 37°C in a humidified atmosphere and 5% CO_2. Medium only was used as a negative control. The HOS cells were pretreated with L-N^6-(1-iminoethyl)-lysine (L-NIL; Sigma). One million HOS cells in 1 ml of culture medium in the presence of 10 μM or 100 μM L-NIL were incubated for 1 h at room temperature. After washing, the cells were resuspended, stimulated with 10 μg/ml *A. actinomycetemcomitans* lipopolysaccharide and incubated for 3 days at 37°C in a humidified atmosphere and 5% CO_2. All cultures were in triplicate.

The role of CD14, Toll-like receptor 2 (TLR2), and TLR4

One million HOS cells in 1 ml culture medium were incubated with various concentrations of anti-human CD14, Toll-like receptor 2 (TLR2) or TLR4 antibody (Santa Cruz Biotech, Santa Cruz, CA) for 1 h at room temperature. After washing, the cells were resuspended, stimulated with 10 μg/ml *A. actinomycetemcomitans* lipopolysaccharide and incubated for 3 days at 37°C in a humidified atmosphere and 5% CO_2.

The role of cyclic nucleotide pathway

9-(Tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536, an adenylyl cyclase inhibitor) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, a guanylyl cyclase inhibitor) were dissolved in distilled water and ethanol, respectively. Dibutyryl cyclic adenosine monophosphate db-cAMP (a cAMP analog) and 8-bromo cyclic guanosine monophosphate (br-cGMP, a cGMP analog) were dissolved in distilled water, whereas forskolin (an adenylyl cyclase activator), isobutyl-1-methylxanthine [IBMX, a non-specific phosphodiesterase (PDE) inhibitor], and KT5720 [a protein kinase A (PKA) inhibitor] were dissolved in dimethyl sulfoxide. All inhibitor-containing solutions were adjusted to obtain 1 mM stock solution and filter sterilized. One million cells were incubated in 1 ml of the culture medium containing various concentrations of SQ22536 or ODQ for 30 min at room temperature. The same number of cells was also incubated in culture medium containing various concentration of forskolin, KT5720, or IBMX for 1–2 h at room temperature. After washing, the HOS cells were cultured in the presence of 10 μg/ml *A. actinomycetemcomitans* lipopolysaccharide as described above. In other experiments, various concentrations of db-cAMP or br-cGMP were added to the culture medium and the cells were subsequently cultured in the presence of *A. actinomycetemcomitans* lipopolysaccharide as above. The morphology and growth of cells in cultures containing 0.001% dimethyl sulfoxide alone were not affected (data not shown). All inhibitors were purchased from Sigma.

The effect of various inhibitors

Genistein [a protein tyrosine kinase (PTK) inhibitor], bisindolylmaleimide [a protein kinase C (PKC) inhibitor], bromophenacyl bromide [BPB; a phospholipase A2 (PLA2) inhibitor] and nordihydroguaiaretic acid (NDGA; a lipoxygenase inhibitor) were dissolved in the culture medium to obtain 1 mM stock solution and filter sterilized. One million cells in 1 ml of the culture medium were incubated with various concentrations of the respective inhibitors for 20 min at room temperature.

After washing, the HOS cells were cultured in the presence of 10 μg/ml *A. actinomycetemcomitans* lipopolysaccharide as described above. All inhibitors were purchased from Sigma.

Nitric oxide assay

The levels of nitrite, a stable end-product of NO oxidation, were assessed in the culture supernatants by the Griess reaction as previously described (40). All cultures were in triplicate and each assessment was repeated three times. Briefly, 100 μl culture supernatant was mixed with an equal volume of the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) and read in an automated reader μQuant spectrophotometer (Biotek-Instrument Inc., Winooski, VT) at 540 nm. The nitrite concentration was calculated from a standard curve prepared with known concentrations of sodium nitrite. All reagents for the NO assay were purchased from Sigma.

iNOS activity assay

After the culture supernatants had been discarded carefully, the cells were washed three times in RPMI-1640 medium, lysed by incubating with 100 μl of 0.1% Triton X-100, and shaken for 30 min. The protein concentration of the cell lysates was measured using the Bradford protein assay (Bio-Rad, Hercules, CA). The iNOS activity was assessed as previously described (42). Briefly, 50 μg cell lysate was incubated for 120 min at 37°C in 100 μl 20 mM Tris–HCl (pH 7.9) containing 4 μM cofactor tetrahydobiopterin (BH_4), 4 μM flavin adenine dinucleotide (FAD), 3 mM dithiothreitol, 2 mM NADPH and 2 mM L-arginine. The reaction was then stopped by adding lactate dehydrogenase (20 U/ml) and the levels of nitrite were measured by the Griess reagent as described above. Unless otherwise stated, all materials were purchased from Sigma.

Statistical analysis

The results were analyzed using a repeat measurement test for the doses and time–course of the iNOS activity and nitrite production by *A. actinomycetemcomitans* lipopolysaccharide-stimulated HOS cells. A one-way analysis of variance followed by Fischer's least-square differences was used to analyze the data from the remaining experiments. The data were analyzed using the statistical software package SPSS (SPSS co., Chicago, IL).

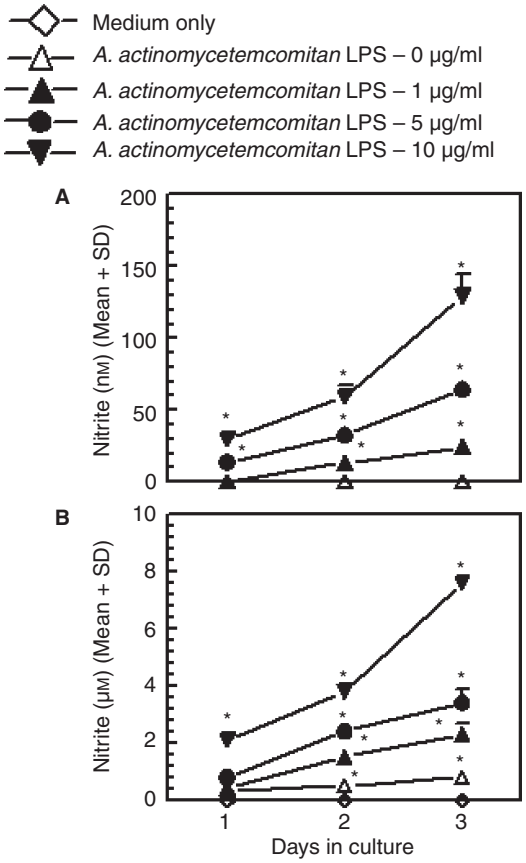

Fig. 1. Dose- and time–course-dependent inducible nitric oxide synthase (iNOS) activity (A) and nitrite production (B) by *Aggregatibacter actinomycetemcomitans* lipopolysaccharide-stimulated human osteoblast cell line (HOS) cells. The cells were stimulated with various concentration of *A. actinomycetemcomitans* lipopolysaccharide and cultured for 3 days. The cells without the presence of *A. actinomycetemcomitans* lipopolysaccharide (*A. actinomycetemcomitans* LPS – 0 μg/ml) were used as a control to assess the levels of both iNOS and NO, whereas the plates containing culture medium only were also used as a control to assess the levels of NO $*P < 0.05$.

Results

iNOS activity and NO production

As depicted in Fig. 1, basal nitrite released by HOS cells alone could be detected at day 1 to day 3 compared with cultures containing medium only ($P < 0.05$). Both iNOS activity and nitrite production by HOS cells stimulated with various concentrations of *A. actinomycetemcomitans* lipopolysaccharide increased steadily up to day 3 ($P < 0.05$). Increased iNOS activity and nitrite production by HOS cells were seen with increased concentrations of *A. actinomycetemcomitans* lipopolysaccharide, particularly on days 2 and 3 ($P < 0.05$) (Fig. 1). There was no iNOS activity in the HOS cell cultures incubated in the absence of *A. actinomycetemcomitans* lipopolysaccharide (Fig. 1). Furthermore, the iNOS activity and nitrite production by *A. actinomycetemcomitans* lipopolysaccharide-stimulated HOS cells were decreased in the presence of L-NIL ($P < 0.05$) (Fig. 2).

The role of CD14 and TLR molecules

The present study showed that both iNOS activity and nitrite production by *A. actinomycetemcomitans* lipopolysaccharide-stimulated HOS cells were reduced when the cells were pretreated with anti-CD14 and anti-TLR4, antibody ($P < 0.05$) (Fig. 3). Pretreatment with anti-TLR2 antibody failed to alter the iNOS activity and nitrite production ($P > 0.05$) (Fig. 3).

The role of the cyclic nucleotide pathway

Pretreatment with SQ22536 or KT5720 reduced iNOS activity and nitrite production while db-cAMP, IBMX, or forskolin enhanced iNOS activity and nitrite production by *A. actinomycetemcomitans* lipopolysaccharide-stimulated HOS cells

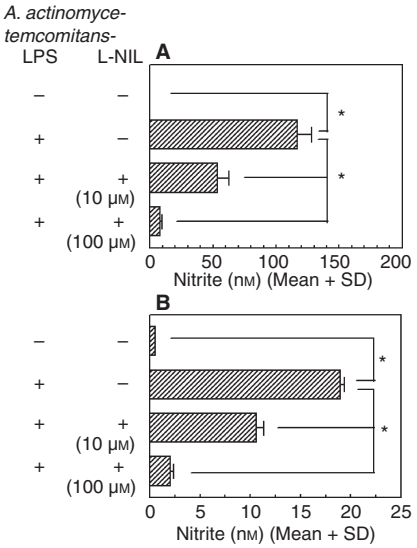

Fig. 2. The effect of L-NIL [an inducible nitric oxide synthase (iNOS) inhibitor] on the iNOS activity (A) and nitrite production (B) by *Aggregatibacter actinomycetemcomitans* lipopolysaccharide-stimulated human osteoblast cell line (HOS) cells. HOS cells were pretreated with L-NIL, washed and cultured with 10 μg/ml *A. actinomycetemcomitans* lipopolysaccharide for 3 days. The cultures of cells with or without the presence of lipopolysaccharide were used as the controls. $*P < 0.05$.

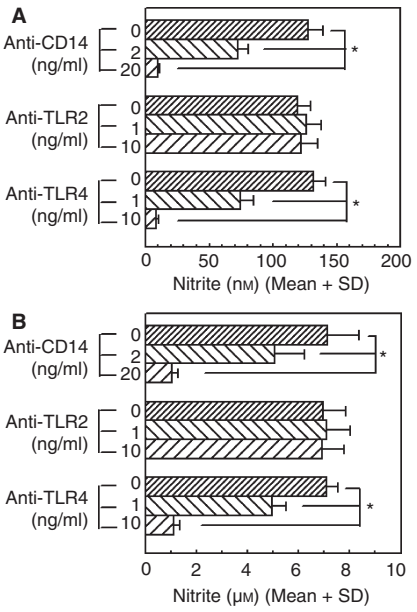

Fig. 3. The role of CD14, Toll-like receptor 2 (TLR2) and TLR4 on the inducible nitric oxide synthase (iNOS) activity (A) and nitrite production (B) by *Aggregatibacter actinomycetemcomitans* lipopolysaccharide-stimulated human osteoblast cell line (HOS) cells. HOS cells were precoated with anti-human CD14, TLR2 or TLR4 antibody, washed and cultured with the presence of 10 μg/ml *A. actinomycetemcomitans* lipopolysaccharide for 3 days. The cultures of uncoated cells in the presence of lipopolysaccharide were used as the controls. $*P < 0.05$.

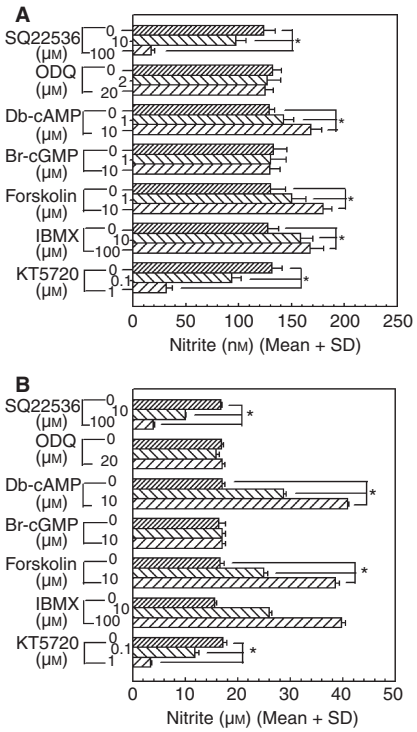

Fig. 4. The role of cyclic nucleotide on the inducible nitric oxide synthase (iNOS) activity (A) and nitrite production (B) by *Aggregatibacter actinomycetemcomitans* lipopolysaccharide-stimulated human osteoblast cell line (HOS) cells. HOS cells were pretreated with SQ22536 (an adenylyl cyclase inhibitor), ODQ (a guanylyl cyclase inhibitor), forskolin (an adenylyl cyclase activator), IBMX (a non-specific phosphodiesterase inhibitor), or KT5720 (a protein kinase A inhibitor), washed and cultured in the presence of 10 μg/ml *A. actinomycetemcomitans* lipopolysaccharide for 3 days. db-cAMP (a cAMP analog) or br-cGMP (a cGMP analog) were added to *A. actinomycetemcomitans* lipopolysaccharide-stimulated cell cultures. The cultures of untreated cells in the presence of lipopolysaccharide were used as the controls. $*P < 0.05$.

in a dose-dependent fashion ($P < 0.05$) (Fig. 4). The presence of ODQ or br-cGMP failed to alter iNOS activity and nitrite production by *A. actinomycetemcomitans* lipopolysaccharide-stimulated HOS cells ($P > 0.05$) (Fig. 4).

The effect of various inhibitors

To elucidate the role of protein kinases on NO production, HOS cells were pretreated with various concentrations of genistein or bisindolylmaleimide before stimulation with *A. actinomycetemcomitans* lipopolysaccharide. The results showed that pretreatment with these inhibitors significantly reduced both iNOS activity and nitrite production by *A. actinomycetemcomitans* lipopolysaccharide-stimulated HOS cells ($P < 0.05$) (Fig. 5). Furthermore, pretreatment with BPB or NDGA before stimulation with *A. actinomycetemcomitans* lipopolysaccharide also suppressed the iNOS activity and nitrite production by HOS cells ($P < 0.05$) (Fig. 5).

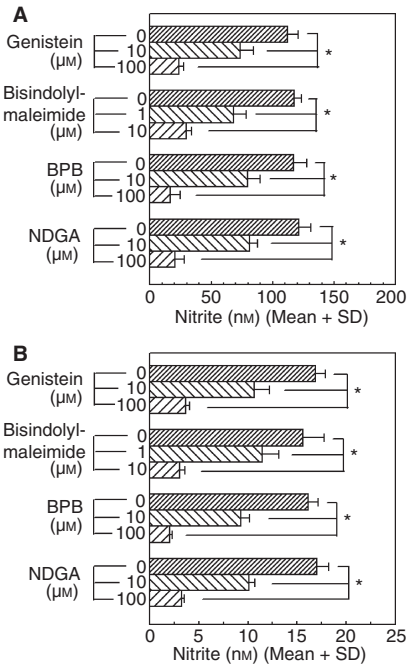

Fig. 5. The effect of various intracellular enzyme inhibitors on the inducible nitric oxide synthase (iNOS) activity (A) and nitrite production (B) by *Aggregatibacter actinomycetemcomitans* lipopolysaccharide-stimulated human osteoblast cell line (HOS) cells. HOS cells were pretreated with genistein (a protein tyrosine kinase inhibitor), bisindolylmaleimide (a protein kinase C inhibitor), bromophenacyl bromide (BPB; a phospholipase A2 inhibitor) and nordihydroguaiaretic acid (NDGA, a lipoxygenase inhibitor), washed and cultured in the presence of 10 μg/ml *A. actinomycetemcomitans* lipopolysaccharide for 3 days. The cultures of untreated cells in the presence of lipopolysaccharide were used as the controls. $*P < 0.05$.

Discussion

The present study has shown that *A. actinomycetemcomitans* lipopolysaccharide stimulates iNOS activity and nitrite production by HOS cells in a dose- and time-dependent manner. These results were further confirmed by the fact that pretreatment with L-NIL suppressed both iNOS activity and nitrite production by *A. actinomycetemcomitans* lipopolysaccharide-stimulated HOS cells. These results are in accordance with those from previous reports that bacterial lipopolysaccharide

induces iNOS activity by rodent osteoblasts (13, 34, 35). Detectable basal nitrite released in the unstimulated HOS cell cultures seen in the present study might be the result of the activity of constitutive NOS (8, 13, 24, 35), because the iNOS activity in these cultures was absent.

Previous studies indicated that *A. actinomycetemcomitans* lipopolysaccharide binds to a CD14–TLR4 molecular complex on the surface of human gingival fibroblasts (10), murine dendritic cells (19), and murine macrophages (41). Indeed, the present results also showed that both iNOS activity and nitrite production by *A. actinomycetemcomitans* lipopolysaccharide-stimulated HOS cells were dependent on the CD14–TLR4 molecular complex, suggesting that the binding between *A. actinomycetemcomitans* lipopolysaccharide and the CD14–TLR4 molecular complex on the surface of HOS cells may initiate the signal transduction leading to the production of NO.

The role of the cyclic nucleotide pathway in the production of NO by lipopolysaccharide-stimulated human osteoblasts is still far from clear. Altered iNOS activity and nitrite production by SQ22536, db-cAMP, forskolin, IBMX, or KT5720, but not by ODQ or br-cGMP, seen in the present study suggests that the cAMP-PKA pathway may play a crucial role in the production of NO by *A. actinomycetemcomitans* lipopolysaccharide-stimulated HOS cells. This is similar to that previously reported for the bacterial lipopolysaccharide-stimulated murine macrophage cell line RAW264.7 (4, 32). In sharp contrast, cytokine-stimulated NO production by human osteoblasts has previously been shown to be the result of a cGMP-dependent mechanism (35). The discrepancy between the present and previous (35) results may be the result of the use of antigen. Furthermore, the regulatory role of phosphodiesterase in the production of NO by *A. actinomycetemcomitans* lipopolysaccharide-stimulated HOS cells was not unexpected because this enzyme plays a key role in determining the levels of intracellular cAMP and, hence, the cAMP-PKA pathway (7).

The exact signal transduction in lipopolysaccharide-induced NO production by osteoblasts remains to be elucidated. The present study revealed some insights into the complexity of this signal transduction involving PTK, PKC, PLA2, and lipoxygenase. This complexity is also seen in lipopolysaccharide-stimulated rodent macrophages (5, 46, 49), rodent osteoblasts (45), and human glial cells (48). It is possible, therefore, that the production of NO by *A. actinomycetemcomitans* lipopolysaccharide-stimulated HOS cells may involve not only the cAMP–PKA pathway but also other cellular signaling system(s). Yet, this hypothesis remains to be determined.

The extrapolation of the present study to the immunopathogenesis of periodontal disease is speculative. Although an increased iNOS expression in gingival inflammatory cells, endothelial cells, and keratinocytes has been documented in patients with periodontal disease (2, 18, 21), the expression of this enzyme in alveolar bone cells is unknown. Failure to develop experimental bone loss in iNOS-deficient mice (11) and iNOS inhibitor-treated mice (6, 22) suggested that NO may play a role in the bone loss in these animal models. Indeed, rapid healing of bone defects *in vivo* observed in iNOS inhibitor-treated rats (1) and the involvement of NO produced by *Prevotella intermedia* lipopolysaccharide-stimulated murine osteoblasts in bone resorption *in vitro* (33) further support this assumption. It is unknown if bacterial lipopolysaccharide directly stimulates alveolar osteoblasts during the course of periodontal disease in humans, such that the possibility that NO produced by periodontopathogen-stimulated osteoblasts may play a crucial role in the induction of alveolar bone loss during the course of periodontal diseases cannot be ignored and clearly requires further investigation. The ability of bacterial lipopolysaccharide topically applied on the gingival tissues of rat to induce the expression of cyclooxygenases 1 and 2 on alveolar osteoblasts (26) highlights the above contention.

In conclusion, *A. actinomycetemcomitans* lipopolysaccharide stimulated iNOS activity and nitrite production by HOS cells. Both iNOS activity and nitrite production by *A. actinomycetemcomitans* lipopolysaccharide-stimulated HOS cells were reduced by pretreatment with L-NIL, anti-CD14 or anti-TLR4 antibody, SQ22536, KT5720, genistein, bisindolylmaleimide, BPB, or NDGA, but enhanced by db-cAMP, IBMX, or forskolin. Therefore, the results of the present study suggest that the binding between *A. actinomycetemcomitans* lipopolysaccharide and the HOS cell surface involves a CD14–TLR4 molecular complex which in turn, may stimulate the production of NO in a cAMP–PKA pathway-dependent and PTK-, PKC-, PLA2-, and lipoxygenase activation-dependent fashion.

Acknowledgments

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