Journal of
PERIODONTAL RESEARCH

J Periodont Res 2010; 45: 381–388

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2009.01249.x

Porphyromonas gingivalis stimulates the release of nitric oxide by inducing expression of inducible nitric oxide synthases and inhibiting endothelial nitric oxide synthases

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Sun W, Wu J, Lin L, Huang Y, Chen Q, Ji Y. Porphyromonas gingivalis *stimulates the release of nitric oxide by inducing expression of inducible nitric oxide synthases and inhibiting endothelial nitric oxide synthases. J Periodont Res 2010; 45: 381–388. © 2010 The Authors. Journal compilation © 2010 Blackwell Munksgaard*

Background and Objective: The purpose of this study was to examine the ability of *Porphyromonas gingivalis* to invade human umbilical vein endothelial cells (HUVECs) and to study the effects of *P. gingivalis* ATCC 33277 on the production of nitric oxide (NO) and on the expression of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) in HUVECs. We attempted to throw light on the pathway of damage to endothelial function induced by *P. gingivalis* ATCC 33277.

Material and Methods: P. gingivalis ATCC 33277 was cultured anaerobically, and HUVECs were treated with *P. gingivalis* ATCC 33277 at multiplicities of infection of 1:10 or 1:100 for 4, 8, 12 and 24 h. HUVECs were observed using an inverted microscope and transmission electron microscopy. NO production was assayed through measuring the accumulation of nitrite in culture supernatants. Expression of both iNOS and eNOS proteins was investigated through western blotting.

Results: It was found that *P. gingivalis* ATCC 33277 can adhere to HUVECs by fimbriae, invade into HUVECs and exist in the cytoplasm and vacuoles. *P. gingivalis* ATCC 33277 can induce iNOS and inhibit eNOS expression, and stimulate the release of NO without any additional stimulant.

Conclusion: Our study provides evidence that *P. gingivalis* ATCC 33277 can invade HUVECs, and the ability of *P. gingivalis* ATCC 33277 to promote the production of NO may be important in endothelial dysfunction, suggesting that *P. gingivalis* ATCC 33277may be one of the pathogens responsible for atherosclerosis.

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Key words: *Porphyromonas gingivalis;* nitric oxide; inducible nitric oxide synthase; endothelial nitric oxide synthase; human umbilical vein endothelial cells

Accepted for publication July 24, 2009

Recently, increasing evidence from epidemiological, clinical and animal models, and from *in vitro* studies, attribute a role for periodontal infections in the pathogenesis of atherosclerosis and related events (1). Haraszthy *et al.* (2) demonstrated, by PCR, the existence of genetic material of gingival pocket bacteria in 44% of

atheromatous plaques. The most frequently identified microorganisms were *Bacteroides forsythus* (30%) and *Porphyromonas gingivalis* (26%). In 1998, Deshpande *et al*. (3) first published a paper on the invasion of bovine aortic and cardiac endothelium by *P. gingivalis*, as well as the replication of genetic material, which was observed under a scanning electron microscope. According to these studies, *P. gingivalis* seems to play an important role in atherosclerosis and related diseases and it is thought to be the main infective agent involved in atherosclerosis. However, the relationship between this periodontopathogen and atherosclerosis remains unclear.

The vascular endothelium produces a variety of vasoactive substances that have an important effect on vascular tone, haemostasis, leukocyte and platelet adhesion, vascular permeability and atherogenesis (4). One of the most important of these mediators is nitric oxide (NO). NO is a short-lived bioactive molecule produced by mammalian cells, including endothelial cells, which serves as a messenger molecule for various physiological and pathological procedures (5). NO is synthesized from L-arginine by NO synthase (NOS), and induces vasodilatation via the activation of soluble guanylyl cyclase to generate cyclic guanosine monophosphate (6). Under physiological conditions, two 'constitutive' forms of NOS play a role in NO production, predominantly endothelial NOS (eNOS) and, to a lesser extent, neuronal NOS. The third isoform, inducible NOS (iNOS), is expressed in a number of inflammatory cell types and has an essential role in vascular inflammation. The constitutive enzymes produce low-level NO, important for maintaining vascular homeostasis, whereas iNOS activity results in 'high-output' NO production, which is thought to underlie its activity in inflammation (5).

Expression of iNOS is induced by inflammatory stimulants such as bacterial lipopolysaccharide (LPS) and proinflammatory cytokines, including tumor necrosis factor-α, interleukin-1b and interferon-c, which are present in a variety of cell types following bacterial infections (7). Binion *et al*. (8) indicated that iNOS was not expressed within human umbilical vein endothelial cells (HUVECs) stimulated with bacterial LPS and tumor necrosis factor-α, and the expression of eNOS was also not affected by these stimulatory agents. A great number of reports have shown that *P. gingivalis* is able to induce iNOS-associated NO production in both immune system cells and non-immune system cells, such as macrophages, keratinocytes and spleen cells, as well as in gingival fibroblasts (9).

Several reports have focussed on endothelial cells, showing the expression of both eNOS and iNOS (10,11), which are known to be critical elements in the relationship between periodontal pathogenesis and initial dysfunction of the vessels. The purpose of this study was to investigate the effects of *P. gingivalis* ATCC 33277 on the production of NO and to analyze the expression of iNOS and eNOS within HUVECs.

Material and methods

Bacteria and cell culture conditions

Strain ATCC 33277 of *P. gingivalis* was used throughout this present study. It was grown anaerobically on the surface of enriched Columbia agar (Oxoid Ltd, Cambridge, UK) containing 5% (v/v) sheep blood, or in trypticase soy broth (Oxoid), supplemented with 1 mg/mL of yeast extract (Oxoid), hemin (0.05 mg/mL) and menadione (0.01 mg/mL) in an anaerobic jar (BD, Franklin Lakes, NJ, USA) at 37°C. Plate-grown cultures were routinely incubated for 5 d and used as the inoculums for liquid growth. Bacterial suspensions were prepared in phosphate-buffered saline (PBS), without Mg^{2+}/Ca^{2+}, with reference to established growth curves and using spectrophotometric analysis. Culture purity was assessed by gram staining and plating on solid medium.

HUVECs (ATCC, Manassas, VA, USA) were cultured on 100-mL glass flasks in Dulbecco's modified Eagle's minimal essential medium (Gibco, Los Angeles, CA, USA), containing 10% heat-inactivated bovine calf serum (Gibco), penicillin (100 μg/mL), streptomycin (100 μg/mL) and Hepes (10 mM) at 37°C in an atmosphere of 5% CO_2. Confluent cells were dissociated by the addition of 0.25% trypsinase (Sigma Chemical Co., St Louis, MO, USA) and 0.01% EDTA (Sigma Chemical Co.). HUVECs were used usually between 5 and 10 passages in all experiments. Fresh medium without antibiotics was added to HUVECs before they were treated with *P. gingivalis* ATCC 33277.

Treatment of HUVECs with *P. gingivalis* ATCC 33277

HUVECs were dissociated from the glass flask, washed three times with PBS and viable cells were counted. HUVECs were then seeded into six-well plates (Corning, Acton, MA, USA), at a density of 4×10^5 cells per well, and incubated for at least 6 h to allow them to adhere to the plates. Then, HUVECs were either stimulated with *P. gingivalis* at multiplicities of infection (MOIs) of 1:10 or 1:100, or remained uninfected, for 4, 8, 12, or 24 h, after which culture supernatants were collected and assayed for NO production.

Assessment of HUVECs morphology

HUVECs cultured on six-well plates were co-incubated with *P. gingivalis* ATCC 33277 at MOIs of 1:10 or 1:100, or remained uninfected. Twenty-four hours later, phase-contrast micrographs were taken on a Fisher Micromaster digital inverted microscope (Fisher Scientific, Pittsburgh, PA, USA) using MICRON 2.0 imaging software (Westover Scientific, Mill Creek, WA, USA).

Electron microscopy

Transmission electron microscopy was performed on designated samples to confirm adherence to HUVECs and invasion within the cells by *P. gingivalis* ATCC 33277. HUVECs were cultured in six-well plates and treated as described above, and monolayers were then washed three times with the addition of 0.5 mL of 0.25% trypsin-

ase and 0.01% EDTA. The cell slurry was immediately centrifuged for 4 min at 15,000 *g*. Cell pellets were washed twice with PBS and fixed with 2.5% gluteraldehyde in 0.1 M sodium cacodylate, then postfixed in 1% OsO_4 in 0.1 M sodium cacodylate for 1 h. In principle, the anion liberated by the enzyme is captured by a heavy metal cation, and thus a practically insoluble and electron optically dense precipitation is formed at the site of the enzyme. The ultrathin sections were contrasted with lead citrate and uranyl acetate before examination using the 1200EX electron microscope (JEOL, Tokyo, Japan).

Measurement of NO production

NO production was assayed by measuring the accumulation of the stable oxidative metabolite, nitrite, in culture supernatants. Briefly, HUVECs were stimulated with *P. gingivalis* ATCC 33277 at MOIs of 1:10 or 1:100, or remained uninfected, in six-well plates for the indicated periods of time, cell supernatants were collected and preserved at −70°C and then 100 μL of Griess reagent (1% sulphanilamide, 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 plate (Corning) and left at room temperature for 10 min. The absorbance of these supernatants was read at 540 nm using a Spectra Max 250 ELISA Reader (Bio-TEK, Winooski, VT, USA), and the nitrite concentrations were calculated from a standard curve established with serial dilutions of $NaNO_2$ (Sigma) in culture medium.

Western blot analysis of iNOS and eNOS proteins

HUVECs were seeded in 100-mm tissue-culture dishes (Greiner Bio-one, Frickenhausen, Germany), at a density of 1×10^7 cells per dish, and either treated with *P. gingivalis* ATCC 33277 at MOIs of 1:10 or 1:100, or were uninfected, for the indicated periods of time. After incubation, the HUVECs were washed three times with ice-cold PBS and lysed by incubation for 30 min on ice with 200 μL of lysis buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.002% sodium amide, 0.1% sodium dodecyl sulphate, 1% Nonidet P-40] containing protease inhibitors (1 mM phenylmethanesulphonyl fluoride, 5 mg/mL of aprotinin, 5 mg/mL of pepstatin A and 5 mg/mL of leupeptin). Cell lysates were centrifuged at 10,000 *g* for 10 min to remove insoluble material and protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis on a minigel apparatus (Bio-Rad) and transferred onto poly(vinylidene difluoride) membranes (Bio-Rad). These membranes were blocked by incubation, for 1 h at room temperature, in Tris-buffered saline containing 3% bovine serum albumin and 0.1% Tween-20 , then incubated, at 4°C overnight, with either polyclonal antibody against iNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or polyclonal antibody against eNOS (BD Transduction Laboratories, Lexington, KY, USA) and monoclonal antibody β-actin (BD Transduction Laboratories) diluted in Tris-buffered saline containing 1% bovine serum albumin. Membranes were washed three times in Tris-buffered saline (containing 0.1% Tween-20) and then incubated with the secondary antibodies for 1 h at room temperature. Bands were visualized using the enhanced chemiluminescence detection system (Amersham Biosciences, Bucks, UK) and images were acquired using a ChemiDOCTM XRS instrument (Bio-Rad). Quantification of images was performed using Quantity One 1D analysis software (Bio-Rad).

Statistical analysis

All experiments were performed in duplicate wells for each condition and repeated at least three times. All data were expressed as means ± standard error of the mean, and SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The significance of variability among NO production and the expression of iNOS and eNOS proteins from each group and the corresponding control were determined using analysis of variance and post-hoc tests. A p-value of < 0.05 was considered to be statistically significant.

Results

Morphology of HUVECs infected with *P. gingivalis* ATCC 33277

To explore the capacity of *P. gingivalis* ATCC 33277 to induce the morphological alteration of HUVECs, a series of phase-contrast micrographs was first assessed (Fig. 1). Compared with uninfected controls (Fig. 1A), HUVECs co-incubated with *P. gingivalis* ATCC 33277 at MOIs of 1:10 or 1:100 (Fig. 1B,C) , respectively were present as an intact monolayer, the majority of cells exhibited no morphologic shrinking, rounding and detachment, and the bacteria localized on the surface of HUVECs in 24 h.

Invasion of HUVECs by *P. gingivalis* ATCC 33277

The nature of the interaction of *P. gingivalis* ATCC 33277 and HUVECs was explored by transmission electron microscopy (Fig. 2). *P. gingivalis* ATCC 33277 adhered to HUVECs via fimbria, microvilli were also protruding from HUVECs and surrounded the attached bacteria (Fig. 2B). *P. gingivalis* ATCC 33277 was localized in the cytoplasm of HUVECs after co-incubation for 8 h (Fig. 2C). *P. gingivalis* ATCC 33277 was also detected within vacuoles in HUVECs (Fig. 2D).

NO induction by *P. gingivalis* ATCC 33277

The concentration of nitrite, an indicator of NO production, was measured after the addition of varying concentrations of *P. gingivalis* ATCC 33277 to HUVECs. Within 24 h, *P. gingivalis* ATCC 33277 at MOIs of 1:10 and 1:100 stimulated the release of NO in cultured HUVECs. A statistically significant difference was found between these groups for the 12 and 24 h peri-

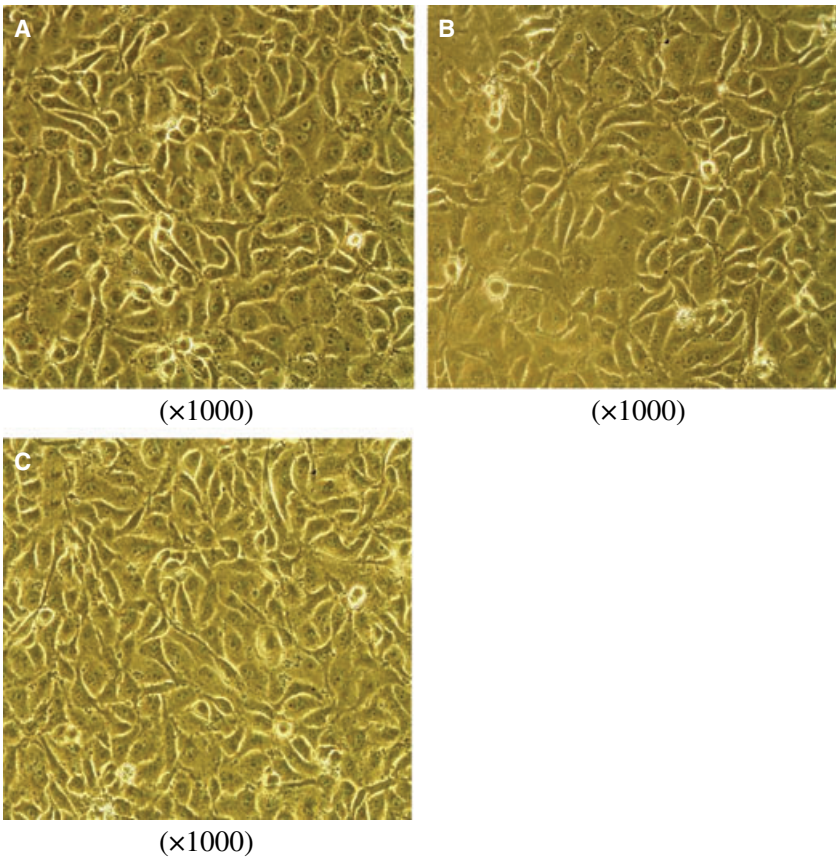

Fig. 1. Infection with *Porphyromonas gingivalis* ATCC 33277 at multiplicities of infection (MOIs) of 1:10 and 1:100 failed to induce morphological changes in human umbilical vein endothelial cells (HUVECs). (A) Uninfected control. (B, C) Infection with *P. gingivalis* ATCC 33277 at MOIs of 1:10 or 1:100, repectivelt, demonstrated normal morphology of an intact monolayer 24 h after infection.

ods of incubation, compared with the vehicle control (co-incubation for 12 h, *p*-values = 0.012, 0.003; co-incubation for 24 h, *p*-values = 0.001, 0.0009 respectively) and maximum NO production (about 96.861 μM) was achieved in the *P. gingivalis* ATCC 33277 MOI 1:100 group when incubated for 24 h. At an MOI of 1:100, the effects of *P. gingivalis* ATCC 33277 were much higher at incubation times of 12 and 24 h, compared with 4 h of incubation (*p*-values = 0.018, 0.002 respectively) (Fig. 3).

Expression of iNOS and eNOS proteins

Expression of both iNOS and eNOS was measured within HUVECs in order to determine which form was involved in stimulating the production of NO following incubation with *P. gingivalis* ATCC 33277 at MOIs of 1:10 and 1:100. We demonstrated an increase in iNOS protein and a decrease in eNOS protein within HUVECs after co-incubation for 12 and 24 h (Fig. 4). Co-incubation for 12 h, compared with vehicle, gave the following results: *p*-values = 0.0009, 0.0009 for eNOS protein and *p*-values = 0.013, 0.0009 for iNOS protein stimulated with *P. gingivalis* ATCC 33277 at MOIs of 1:10 and 1:100 respectively (Fig. 4A); co-incubation for 24 h gave *p*-values = 0.0009, 0.0009 for eNOS protein and *p*-values = 0.0009, 0.0009 for iNOS protein stimulated with *P. gingivalis* ATCC 33277 at MOIs of 1:10 and 1:100 respectively (Fig. 4B). HUVECs stimulated with *P. gingivalis* ATCC 33277 at MOIs of 1:10 and 1:100 for the periods of time indicated expressed a protein of approximately 130 kDa, recognized by specific antibody to iNOS and a protein of approximately 135 kDa, recognized by specific antibody to eNOS.

Discussion

P. gingivalis, as a major aetiologic agent in the pathogenesis of periodontal disease, is an asaccharolytic, gram-negative anaerobic coccobacillus of the subgingival dental plaque or periodontal pocket. *P. gingivalis* outer membrane components, including pili and membrane bubbles, have been shown to adhere to epithelial cells and to dental plaque; moreover, *P. gingivalis* can produce several toxic compounds, such as LPS, gingipains and proteolytic fragment (12). *P. gingivalis* infection can cause local inflammation leading to ulceration of the gingivae and local vascular changes, which increase the incidence and severity of transient bacteraemias when the gingivae are traumatized. Procedures such as dental extraction, periodontal surgery, tooth scaling and even toothbrushing, can lead to the presence of oral bacteria in circulating blood (13). Therefore, endothelial cells can act as primary target cells during infection with *P. gingivalis* (14).

Roth used *P. gingivalis* 381 to infect human aortic endothelial cells and found that *P. gingivalis*, at MOIs of 1:10 to 1:100, has no effect on the morphology of human aortic endothelial cells. Only at a much higher MOIs (1:500 and 1:1000) that *P. gingivalis* can induce endothelial apoptosis (15). In the present study, we also demonstrated that *P. gingivalis* ATCC 33277 at MOIs of 1:10 to 1:100 does not have a deleterious effect on the morphology of HUVECs 24 h after infection.

P. gingivalis ATCC 33277 is the type strain, classified as a less virulent strain, and invades human gingival epithelial cells and human aortic endothelial cells (16). In the present study, *P. gingivalis* ATCC 33277 was found to adhere to HUVECs via fimbriae, to invade HUVECs and to exist in the cytoplasm or vacuoles of HUVECs. Indeed, several recent studies have demonstrated that *P. gingivalis* is able to invade and activate different cell types in the tissue surrounding the teeth (endothelial and gingival epithe-

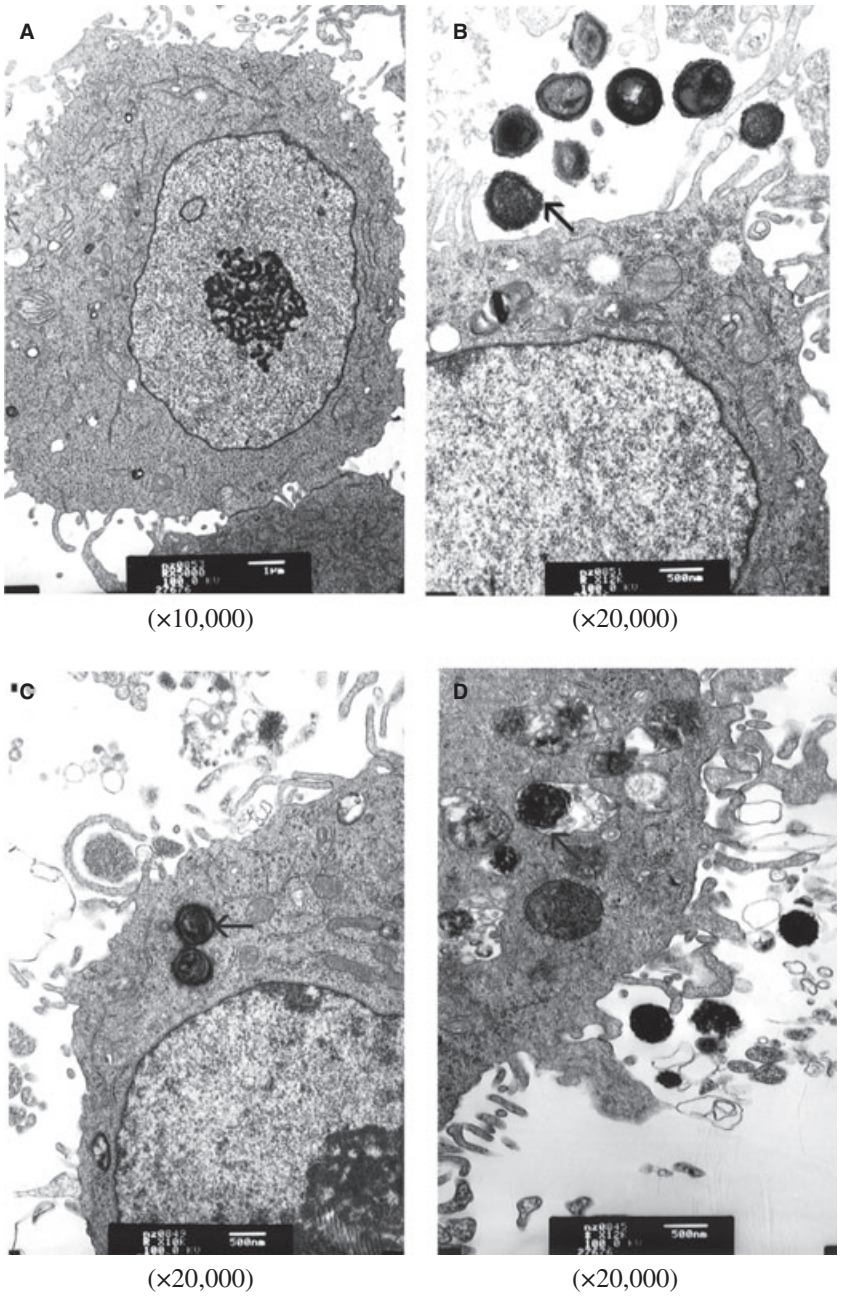

Fig. 2. Transmission electron microscopy demonstrating the invasion of human umbilical vein endothelial cells (HUVECs) by *Porphyromonas gingivalis* ATCC 33277. *P. gingivalis* ATCC 33277 is seen adhering to HUVECs via fimbria (A); this intimate attachment included the presence of microvilli protruding from HUVECs and surrounding the attached bacteria (arrow) (B). *P. gingivalis* ATCC 33277 (arrow) was present in the cytoplasm of HUVECs after co-incubation for 8 h (C). *P. gingivalis* ATCC 33277 (arrow) was detected within vacuoles in HUVECs (D).

lial cells as well as periodontal ligament cells) (17,18). Invasion by *P. gingivalis* has been proposed as a possible mechanism of pathogenesis in periodontal and cardiovascular diseases (3). The initial step of bacterial adherence and invasion to host cells typically requires the bacterial surface component fimbriae (19). The major fimbria of *P. gingivalis* seems to play a major role in adherence and invasion of bovine aortic endothelial cells (BAECs). Because the *fimA* mutant DPG3 cannot adhere to and invade BAEC, invasive strains of *P. gingivalis*, not a noninvasive *fimA* mutant, stimulate the expression of cell adhesion molecules. These include intercellular adhesion molecule 1, vascular cell adhesion molecule 1 and P-/E-selectin on the cell surface of HUVECs (19,20). Within an animal model study, the *P. gingivalis* mutant DPG3 failed to accelerate atherosclerosis in ApoE($^{-/-}$) mice, despite a measurable bacteraemia and localization of the mutant to the aorta (21).

The exact mechanisms by which *P. gingivalis* may enter human endothelial cells are still not fully understood, but the use of lipid rafts as a portal of entry has been suggested (21). The intracellular location of *P. gingivalis* has not been singularly defined. Deshpande *et al.* (3) suggested that *P. gingivalis* resided within uncharacterized vacuoles in BAECs and HUVECs, *P. gingivalis* was reported to be free in the cytoplasm in gingival epithelial cells (22). In this study, we found that *P. gingivalis* ATCC 33277 was free in the cytoplasm and vacuoles within HUVECs. The precise intracellular fate of internalized bacteria, and the exact nature of the processes by which they modulate endothelial function, have also not been completely clarified. *P. gingivalis* ATCC 33277 was capable of replicating within endothelial cells, suggesting that it has the capacity to persist within this host cell and possibly also to alter the integrity of the endothelial cell. This affords them protection from the host immune system and may directly activate signal transduction pathways linked to the proinflammatory responses (3).

NO has recently received considerable attention as a novel type of mediator, which has both pro-atherosclerotic and anti-atherosclerotic effects (23). We used *P. gingivalis* ATCC 33277 at MOIs of 1:10 to 1:100 to infect HUVECs and found that it stimulated HUVECs to release NO through upregulating iNOS and downregulating eNOS in the absence of other stimuli. The gradual increase of NO production in the control group may be caused by cell proliferation within 24 h. NO production was observed in murine macrophages stimulated with LPS or Lipid A-associated

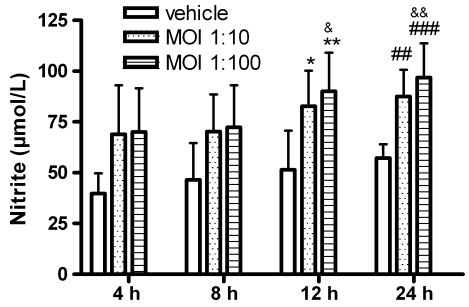

Fig. 3. The concentrations of nitrite in culture supernatants of human umbilical vein endothelial cells (HUVECs) were measured after adding *Porphyromonas gingivalis* ATCC 33277 at multiplicities of infection (MOIs) of 1:10 and 1:100. Within 24 h (compare with vehicle), *P. gingivalis* ATCC 33277 had stimulated the release of NO from HUVECs; statistically significant differences between these groups was found at 12 and 24 h time-points compared with the vehicle: co-incubation for 12 h, *p*-values = 0.012, 0.003 ($^{*}p < 0.05$, $^{**}p < 0.01$); co-incubation for 24 h, *p* values = 0.001, 0.0009 respectively ($^{\#}p < 0.01$, $^{\#\#\#}p < 0.001$), and maximum NO production (about 96.861 μM) was achieved following treatment with *P. gingivalis* ATCC 33277 for 24 h at an MOI of 1:100. At an MOI of 1:100, the effects of *P. gingivalis* ATCC 33277 were much higher at incubation times of 12 and 24 h, compared with 4 h, *p*-values = 0.018, 0.002 respectively ($^{\&}p < 0.05$, $^{\&\&}p < 0.01$).

proteins of *P. gingivalis* (9,24); Hama *et al*. (25) indicated that HUVECs were stimulated with proinflammatory cytokines and LPS extracted from *P. gingivalis*, which induced the expression of iNOS mRNA and protein compared with negative controls. However, the present results disagreed with the study of Binion *et al*. (8), which indicated that iNOS was not expressed by HUVECs stimulated with bacterial LPS plus tumor necrosis factor-α and that eNOS expression was not affected.

eNOS is constitutively expressed in normal endothelial cells and exerts an anti-atherogenic effect through the generation of NO (pM) involved in vasodilation as well as inhibition of leukocyte adhesion, platelet adhesion and smooth muscle cell proliferation (26). Incubation of cultured BAECs with LPS decreased the levels of eNOS mRNA and protein; this decrease is likely to result from an increased degradation rate of its transcript. Lu *et al*. (27) showed that concomitant treatment of cultured bovine coronary endothelial cells with LPS decreased eNOS mRNA levels. Immunoblotting analysis using lysates prepared from HUVECs revealed reduction of eNOS phospholyration at serine 1177 after treatment with *P. gingivalis*–LPS (28). By contrast, iNOS expression can be induced in a variety of cell types (including macrophages, vascular smooth muscle cells and endothelial cells) with suitable agents, such as bacterial LPS, cytokines and other compounds (29). *P. gingivalis* possesses an array of virulence factors and has been shown to induce iNOS expression in inflammatory cells (30). Pathways resulting in the induction of iNOS expression may vary in different cells or in different species. Activation of the transcription factors nuclear factor-κB and signal transducers and activators of transcription 1α, and thereby activation of the iNOS promoter, seems to be an essential step for iNOS induction in most cells (31). *P. gingivalis* strains in-

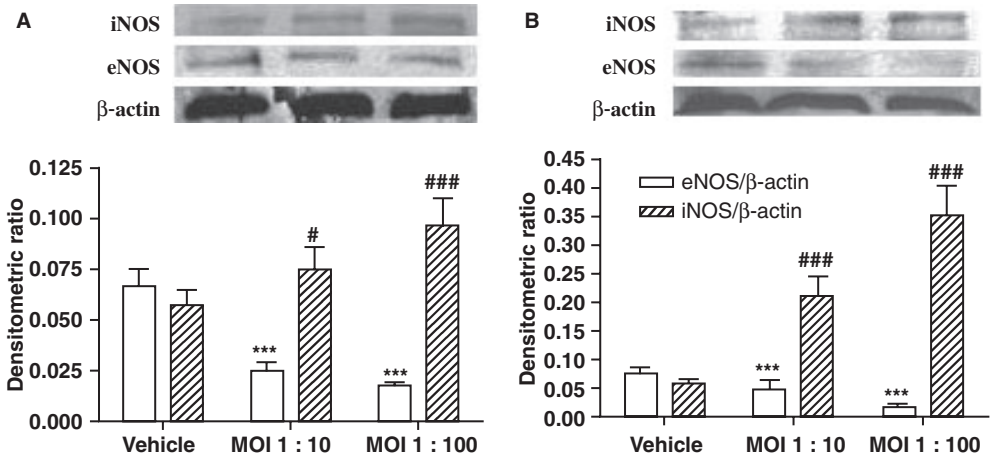

Fig. 4. Western blot analysis to determine the expression of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) proteins by human umbilical vein endothelial cells (HUVECs). The levels of iNOS and eNOS proteins were quantified by densitometry. (A) After incubation with *Porphyromonas gingivalis* ATCC 33277 at multiplicities of infection (MOIs) of 1:10 and 1:100 for 12 h, the iNOS protein level increased and the eNOS protein level decreased in HUVECs, compared with the vehicle: *p*-values = 0.0009, 0.0009 for eNOS protein ($^{***}p < 0.001$) and *p*-values = 0.013, 0.0009 for iNOS protein respectively ($p < 0.05$, $^{\#\#\#}p < 0.001$). (B) After incubation with *P. gingivalis* ATCC 33277 at MOIs of 1:10 and 1:100 for 24 h, the iNOS protein level increased and the eNOS protein level decreased in HUVECs, compared with the vehicle: *p*-values = 0.0009, 0.0009 for eNOS protein ($^{***}p < 0.001$) and *p*-values = 0.0009, 0.0009 for iNOS protein respectively ($^{\#\#\#}p < 0.001$).

duced the phosphorylation of p38 MAPK, the degradation of IκBα (naturally occurring inhibitor of nuclear factor-κB) and the translocation and activation of endothelial cell nuclear factor-κB (14), which translocates to the nucleus where it binds to the promoter regions of various inflammatory genes, including iNOS (32). Binding of nuclear factor-κB to specific nucleotide sequences in the promoter region of the iNOS gene results in rapid and effective transcription of this gene (33). iNOS, once expressed, can generate large amounts of NO (μM) for extended periods of time, and is believed to be involved in cytotoxicity following inflammation (5).

These results indicate that *P. gingivalis* can actively invade HUVECs and that fimbriae are required for this process. It has the capacity to persist within this host cell and affords them protection from the host immune system and possibly to affect endothelial cell function and to directly activate signal transduction pathways linked to the proinflammatory response. *P. gingivalis* ATCC 33277 can stimulate HUVECs to release NO through upregulating iNOS and downregulating eNOS in the absence of other stimulants. The release of NO through stimulation by *P. gingivalis* is a process recognized to occur in a variety of human diseases associated with inflammation. The elevated and sustained levels of this molecule may also lead to tissue breakdown via various mechanisms of cytotoxicity (i.e. oxidation and nitration reactions, enzymatic inhibition, DNA injury and activation of cyclooxygenases and metalloproteinases). Moreover, excess NO and O_2^- can stimulate an increased production of $ONOO^-$, which is known to promote platelet aggregation, thrombogenesis and low-density lipoprotein oxidation (5).

Conclusions

Our data demonstrated that *P. gingivalis* ATCC 33277, a known periodontal pathogen, can actively adhere to and invade endothelial cells. We have shown that *P. gingivalis* ATCC 33277 can stimulate endothelial cells to release NO through the upregulation of iNOS and the downregulation of eNOS. We believe that *P. gingivalis* ATCC 33277 can promote the production of NO, which may be important in endothelial dysfunction and as the pathogen of atherosclerosis.

Acknowledgements

This work was supported by the project of Nanjing Social Developing Fund (no. 200901085, no. ZKX08017) to Weibin Sun, by the National Natural Science Foundation of China (no. 30730044) and the National Basic Research Program (973) (no. 2006CB708509, no. 2006CB503801) to Qi Chen, by the National Natural Science Foundation of China (grant no. 30770891) to Yong Ji and by the QingLan Project.

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