PPARγ affects nitric oxide in human umbilical vein endothelial cells exposed to Porphyromonas gingivalis

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Objective: Porphyromonas gingivalis induces nitric oxide (NO) synthesis in human umbilical vein endothelial cells (HUVECs). Peroxisome proliferator-activated receptor (PPARγ) has an anti-inflammatory function, and its involvement in this NO induction process requires elucidation. Here, we focused on PPARγ expression in HUVECs exposed to P. gingivalis, and investigated its effects on NO synthesis.

Materials and methods: HUVECs were time-dependently stimulated by P. gingivalis W83 for 0–24 h. PPARγ expression was assessed at the mRNA and protein levels, and PPARγ activation was measured using dual-luciferase reporter assays. NO synthesis and NO synthase (NOS) expression in response to P. gingivalis were examined in HUVECs pretreated with representative PPARγ agonist (15-deoxy-D12,14-prostaglandin J2, 10 μM) or antagonist (GW9662, 10 μM). In addition, NO synthesis and NOS expression in the P. gingivalis-infected and control groups were detected.

Results: The PPARγ mRNA level in HUVECs increased after exposure to P. gingivalis for 1 h and its protein level increased at 2 h. Luciferase-induced PPARγ increased in P. gingivalis-exposed HUVECs. NO synthesis in the infected group at 4 h, and in the PPARγ-activated group at 8 h, was higher than that in controls. Inducible NOS increased in the infected and PPARγ-activated groups at 4 and 8 h. The total endothelial NOS (eNOS) and phospho-eNOS levels were lower in the infected group than controls, but did not change in the PPARγ-activated group.

Conclusions: Activated PPARγ induces NO generation through the NOS pathway in HUVECs exposed to P. gingivalis.

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1. Introduction

Porphyromonas gingivalis, a common periodontal pathogen, is a Gram-negative anaerobic bacterium (Socransky, Haffajee, Cugini, & Smith, 1998). Not only do periodontal pathogens cause periodontal tissue inflammation, but they also enter the circulation and invade vascular endothelial cells (Deshpande, Khan, & Genco, 1998; Nakano et al., 2008). P. gingivalis activates endothelial cells, enhances the expression of inflammatory cytokines (Kim et al., 2013; Rodrigues et al., 2012), and induces molecules that interfere with endothelial integration (Rodrigues et al., 2012; Wan, Liu, & Ouyang, 2015; Assinger et al., 2011).

In the inflammation process, the transcription factor peroxisome proliferator-activated receptor-gamma (PPARγ) is known to inhibit inflammatory expression (Blanquart, Barbier, Fruchart, Staels, & Glineur, 2003; Jiang, Ting, & Seed, 1998; Ricote, Li, Willson, Kelly, & Glass, 1998). PPARγ is a nuclear hormone receptor that binds to the peroxisome proliferator response element (PPRE) on target DNA as a heterodimer with the retinoid X receptor and is trans-activated by its ligands. PPARγ is expressed in vascular endothelial cells and activated by the natural endogenous ligand 15-deoxy-D12,14-prostaglandin J2 (15dPGJ2), and by insulin-sensitizing thiazolidinediones (Kliewer, Xu, Lambert, & Willson, 2001). Activated PPARγ inhibits the expression of cytokines, matrix metalloproteases, and acute phase proteins (Blanquart et al., 2003; Jiang et al., 1998; Ricote et al., 1998). It also modulates the oxidative stress-induced nuclear factor-κB (NF-κB) pathway (Delerive et al., 1999), but its involvement in human umbilical vein endothelial cells (HUVECs) in response to P. gingivalis requires elucidation.

Nitric oxide (NO) is induced in the endothelium when stimulated by P. gingivalis (Sun et al., 2010). Not only does it have antimicrobial functions (Nathan & Shiloh, 2000) but it also modulates the production and function of cytokines (Bogdan.
In endothelial cells, there are two isoforms of NO synthase (NOS): endothelial NOS (eNOS) and inducible NOS (iNOS). eNOS is expressed constitutively by endothelial cells and is transcriptionally regulated by cytokines (Fürstermann, Boisiel, & Kleiner, 1998). iNOS is expressed in response to inflammatory stimuli such as bacterial proteins and cytokines (Alderton, Cooper, & Knowles, 2001). The iNOS promoter is activated by transcription factors such as NF-kB (Ganster, Taylor, Shao, & Geller, 2001). Both eNOS and iNOS are regulated by PPARγ (Sung, Park, Yu, & Chung, 2006; Li et al., 2014). Several investigators have shown that activated PPARγ increases the production of NO in endothelial cells (Wang et al., 2006; Zhao et al., 2011). However, the effect of PPARγ on NO production in HUVECs stimulated by P. gingivalis remains unclear.

We hypothesized that PPARγ plays a role in regulating NO production in HUVECs infected by P. gingivalis. This study investigated the expression and activity of PPARγ and its role in NO production by HUVECs exposed to P. gingivalis.

2. Materials and methods

2.1. Cell culture conditions

HUVEA.h9296 cells (CRL2922™), purchased from the ATCC (LOT number: 61034681), were established by fusing primary human umbilical vein cells with a thioquinaine-resistant clone of A549. EA.h9296 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Paisley, Scotland, UK) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), in 10-cm dishes under 5% CO₂ at 37 °C. Cells were seeded into 6-well plates (Corning, Acton, MA, USA) at 10⁴ cells/well, grown overnight, and then used in experiments.

2.2. Bacterial strain and culture conditions

P. gingivalis W83 was kindly provided by the Department of Microbiology of Peking University School of Stomatology. The bacteria were grown on brain heart infusion (BHI; Bacto, Sparks, MD, USA) broth agar plates supplemented with 5% (v/v) defibrinated sheep blood, 5 μg/mL hemin, and 0.4 μg/mL menadione in an anaerobic system (5% CO₂, 10% H₂, and 85% N₂) at 37 °C for 5–7 days. The bacteria were inoculated into fresh BHI broth supplemented with 5 μg/mL hemin and 0.4 μg/mL menadione and grown for more than 24 h until the optical density at 600 nm reached 1.0 (Deshpande et al., 1998).

2.3. Exposure of HUVECs to P. gingivalis

P. gingivalis were centrifuged, washed with PBS (pH 7.2), and re-suspended in DMEM with 10% FBS at a final concentration of 10⁵ cells/mL (Deshpande et al., 1998). Bacterial suspensions were added to the HUVEC cultures at a multiplicity of infection (MOI) of 10:1 for the indicated times (0, 0.5, 1, 1.5, 2, 4, 8, 12, and 24 h) at 37 °C under 5% CO₂. Control cultures were incubated with medium alone. Both cells and culture supernatants were collected individually and used in subsequent experiments. The viability of HUVECs was assessed using the 0.2% trypan blue exclusion test, which gave values >80%. All assays were performed in triplicate.

2.4. RNA isolation and quantitative real-time polymerase chain reaction (qPCR) analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions and the RNA concentration was measured spectrophotometrically. Total RNA (2 μg) was then reverse-transcribed into single-stranded cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and oligo (dT)₁₅ primers according to the manufacturer’s protocol. qPCR was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Warrington, UK) using SYBR Green reagent (Roche, Indianapolis, IN, USA). β-actin was used as the endogenous control gene. The primers for the PPARγ gene (product size 100 bp) were: sense 5′-ACAAAGTCAATCAAAGTGA-3′ and antisense 5′-TAGGGGAGTTGAAGGCTCT-3′; and the primers for β-actin (product size 146 bp) were: sense 5′-TTGCTTACCCCTTCTT-3′ and antisense 5′-CCATTGCTGTCAGTTT-3′. The standard PCR conditions were 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. All reactions were performed in triplicate. Data are presented as the relative amount of mRNA in one sample versus a control (fold change) using the formula 2⁻ΔΔC(T) (i.e., we used the difference between the CT of a gene of interest and that of β-actin for one sample (ΔCT) and then compared this value to that of the calibrator (control) sample (ΔΔCT)).

2.5. Western blot assay

After incubation, the HUVECs were washed three times with ice-cold PBS and lysed in RIPA buffer (Applygen, Beijing, China) containing protease inhibitors and phosphatase inhibitors. Cell lysates were centrifuged at 10,000 g for 10 min to remove insoluble material, and protein concentrations were determined using a BCA kit (CWBio, Beijing, China). Equal amounts (50 ng) of protein samples were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes by wet blotting. The membranes were blocked in 10% nonfat dry milk for 1 h and probed with antibodies against PPARγ (1:200; Cell Signaling Technology, Danvers, MA, USA) and β-actin (1:1000; ZSGB-BIO, Beijing, China) at 4 °C overnight. After incubation with peroxidase-linked secondary antibodies (1:1000; ZSGB-BIO, Beijing, China), a chemiluminescence detection system (Thermo, Rockford, IL, USA) was used to visualize the immunoreactive proteins. The density of each protein was normalized against each density of β-actin. All the data are from three independent experiments.

2.6. Dual-luciferase reporter assay system

A DNA fragment containing three copies of PPARGRE consisting of the nucleotide sequence 5′-GTC GAC AGG GGA CCA GGA CAA AGG TCA GTG TCC GGA GTG GAC-3′ repeated three times in tandem (consensus PPARGRE underlined) was purchased from Addgene (pGL3–3xPPRE). HUVECs grown in 48-well plates were transfected with 40 ng pGL3-3xPPRE and 4 ng pRL-TK, a plasmid-expressing Renilla luciferase (Promega). The culture medium was changed after 12 h, and P. gingivalis was added. After another 12 h, the dual-luciferase reporter assay was performed as reported (Gijbers et al., 2011).

2.7. Detection of NO and NOS

The role of PPARγ in NO production was investigated in four groups: control, P. gingivalis-infected, PPARγ-activated, and PPARγ-blocked groups. In the control group, HUVECs were cultured with DMEM only. In the infected group, HUVECs were cultured with P. gingivalis (MOI = 10:1). In the PPARγ-activated group, 30 min before stimulation, the cells were incubated with the PPARγ ligand 15d-PCJ₂ (Sigma-Aldrich, St. Louis, MO, USA) at 10 μM (Liu et al., 2009) and then cultured with P. gingivalis. While in the PPARγ-blocked group, 30 min before stimulation, the cells were incubated with the PPARγ antagonist 2-chloro-5-nitro-N-phenylbenzamide (GW9662, Sigma-Aldrich) at 10 μM (Kotlowski et al., 2014), and then cultured with P. gingivalis. At 0, 0.5, 1, 1.5, 2, 4, 8, 12, and 24 h, culture media was collected and preserved at
−80 °C. Because the volumes of the samples were equal, we analyzed the concentration of NO produced. NO concentration was measured as the accumulation of the stable oxidative metabolite, nitrite, in culture supernatants using Griess reagent. Briefly, 50 μL of Griess R1 reagent (Applygen) was added to equal volumes of culture supernatants in a 96-well plate (Corning) and left at room temperature for 5 min. Then, Griess R2 reagent was added, incubated at room temperature, and protected from light for 5 min. The absorbance was read at 540 nm using a Bio-Rad Reader (Bio-Rad, Hercules, CA, USA), and the NO concentrations were calculated from a standard curve established with serial dilutions of NaNO₂ in culture medium. At 1.5, 4, 8 and 12 h in these four groups, proteins were extracted and Western blot assays were performed according to the steps described above; antibodies against iNOS, eNOS, and phospho-eNOS (p-eNOS) (1:1000; Cell Signaling Technology) were applied.

2.8. Statistical analysis

Data were expressed as means ± standard deviation (SD). One-way ANOVA was used to compare 2^{ΔΔCt} values at different time points followed by the least significant difference (LSD) post hoc test. The transcriptional activity of luciferase was compared between two groups using the independent sample t-test. One-way ANOVA was performed to evaluate the differences in NO production or NOS among groups at each time point, followed by the LSD post hoc test using SPSS for Windows software (ver. 10.0; SPSS, Inc., Chicago, IL, USA). The alpha value of P was set as <0.05.

3. Results

3.1. PPARγ mRNA and protein expression in HUVECs exposed to P. gingivalis

The expression of PPARγ mRNA extracted from HUVECs exposed to P. gingivalis for 1 h increased by 3.15 ± 1.73-fold (P<0.01) relative to baseline. When HUVECs were exposed to P. gingivalis for 1.5 h, the expression of PPARγ mRNA recovered (Fig. 1). Western blots (Fig. 2A) showed that PPARγ expression at 2 h increased by 6.39 ± 5.04-fold compared to baseline (Fig. 2B). When HUVECs were exposed to P. gingivalis for 4 h, the PPARγ protein expression recovered (Fig. 2B). These data showed that expression of PPARγ increased significantly at both the mRNA and protein levels when HUVECs were infected with P. gingivalis.

3.2. PPARγ activity in HUVECs exposed to P. gingivalis

The dual-luciferase reporter assay system was used to assess the transcriptional activity of PPARγ in HUVECs exposed to P. gingivalis. The luciferase activity induced in HUVECs exposed to P. gingivalis for 12 h was significantly higher than that in the control group (HUVECs not exposed to P. gingivalis) (1.57 ± 0.17 vs. 1.06 ± 0.04; P<0.05; Fig. 3).

3.3. NO production at each time-point in various groups

This experiment was designed to determine whether activated PPARγ played a role in NO expression in HUVECs exposed to P. gingivalis. Data collected from 0.5 to 24 h showed higher NO production in the infected group (53.25 ± 12.91 μM), PPARγ-activated group (55.44 ± 16.21 μM), and PPARγ-blocked group (52.36 ± 10.99 μM) compared to the control group (45.97 ± 12.11 μM; P<0.05). Furthermore, the P. gingivalis-infected group, PPARγ-activated group, and PPARγ-blocked group did not differ from one another. NO production in the infected group at 4 h was slightly less than that at the corresponding point in the control group (59.14 ± 18.67 μM vs. 42.56 ± 13.74 μM; P<0.05). NO production in the PPARγ-activated group at 8 h was higher than that at the corresponding point in the control group (68.34 ± 31.68 μM vs. 42.63 ± 11.14 μM; P<0.05). NO production in HUVECs exposed to P. gingivalis peaked at 8 h in the PPARγ-activated group (Fig. 4).

3.4. NOS expression at each time point in various groups

Western blots of the expression levels of iNOS, eNOS, and p-eNOS in the four groups (Fig. 5A,B) showed that iNOS expression at 1.5 and 4 h was higher in the infected and PPARγ-activated groups than in controls (P<0.05; Fig. 5C). NOS expression at 4 h in the PPARγ-blocked group was higher than in controls (P<0.05; Fig. 5C). The p-eNOS levels were lower in the infected and PPARγ-blocked groups from 4 to 12 h than in controls (P<0.05); the level was slightly but not significantly higher in the PPARγ-activated group from 1.5 to 12 h than in controls; and it was higher in the PPARγ-activated group than in the PPARγ-blocked and infected groups from 1.5 to 12 h (Fig. 5D). The total eNOS level was lower in the infected group from 4 to 12 h than in controls (P<0.05; Fig. 5E).

![Fig. 1](image-url)  
**Fig. 1.** The expression of PPARγ mRNA in HUVECs exposed to P. gingivalis increased. After HUVECs exposed to P. gingivalis for 1 h, relative quantification real-time PCR assessed that the expression level of PPARγ mRNA was significantly up-regulated compared to the baseline and was recovered from 1.5 h *, P<0.05 as compared with the baseline (n=6).
In the present study, we showed that *P. gingivalis* induced the upregulation of PPARγ expression at both the mRNA and protein levels in HUVECs. To the best of our knowledge, the present study is the first to document the involvement of PPARγ in the process of endothelial cell stimulation by *P. gingivalis*. These results are somewhat consistent with those from previous studies. Huang et al. reported that PPARγ expression was upregulated in endothelial cells in mice infected with *Chlamydia pneumonia* (Huang, Dong, Mai, & Li, 2005). Cantini et al. showed that PPARγ overexpression resulted in a reduction of inflammatory secretions during the process of interfering with tumor necrosis factor alpha and interferon gamma inflammatory activity in human endothelial cells (Cantini et al., 2010). Decreased PPARγ protein expression and activity showed association with pathogenic conditions (Wolf et al., 2014). Our findings from the luciferase assay indicated that PPARγ was activated by its natural ligand in HUVECs exposed to *P. gingivalis*. Activated PPARγ might be self-protective in endothelial cells exposed to *P. gingivalis*.

We showed that NO production was significantly higher in the *P. gingivalis*-infected group at 4 h than in the control group (Fig. 4), and iNOS expression increased at 1.5 and 4 h while eNOS expression significantly decreased from 4 to 12 h (Fig. 5A, C and E). Similarly, Sun et al. showed that when HUVECs were stimulated by *P. gingivalis* (ATCC 33277 at an MOI of 10:1), the release of NO from HUVECs showed a significant increase at 12 and 24 h compared to the vehicle (Sun et al., 2010). In addition, they demonstrated that the increased NO production was due to induced expression of iNOS and inhibited expression of eNOS (Sun et al., 2010). However, in this study, we further demonstrated that p-eNOS expression (Fig. 5D) was significantly inhibited. One possible explanation for the higher NO production in the *P. gingivalis*-infected group than in the control group is a self-amplifying signal mechanism. Umansky et al. (1998) showed that when NF-κB was pre-stimulated, adding NO enhanced the activation of NF-κB, which upregulated iNOS, thus suggesting a self-amplifying mechanism in the inflammatory response. Zhang et al. (2011) proved that NF-κB was activated when HUVECs were exposed to *P. gingivalis*. Together, these facts support our finding that NO production from HUVECs exposed to *P. gingivalis* for 4 h was significantly higher than in controls. However, in Sun's study,
the NO production and iNOS expression peak appeared at 12 and 24 h. This discrepancy may be attributed to the different strains of *P. gingivalis* used, as recent studies have shown that *P. gingivalis* W83 is a pathogenic strain and more virulent than *P. gingivalis* ATCC 33277 (Chen et al., 2004; Lin, Pan, & Li, 2006). Rodrigues et al. (2012) showed *P. gingivalis* W83 remained viable up to 48 h in human coronary artery endothelial cells, whereas ATCC 33277 was cleared by 24 h; the virulence mechanisms among different *P. gingivalis* strains varied.

We showed that NO production in the PPARγ-activated group at 8 h was significantly higher than in the control group (Fig. 4), and iNOS expression increased at 1.5 and 4 h, while p-eNOS expression showed no significant decrease from 1.5 to 12 h compared with the controls (Fig. 5C and D). These results indicated that NO synthesis...
in the PPARγ-activated group occurred by increasing iNOS and maintaining p-eNOS expression. Previous studies showed that activation of PPARγ increased NO production through activation of the phosphatidylinositol 3 kinase/Akt/eNOS pathway (Li et al., 2014) or increased heat shock protein 90-eNOS interaction and eNOS ser1177 phosphorylation in endothelial cells (Polikandriotis, Mazella, Rupnow, & Hart, 2005). ENOS, and p-eNOS in particular, play essential roles in modulating vascular tone and upholding endothelial integrity (Alderton et al., 2001). High levels of NO produced by iNOS were directly toxic and used for killing bacteria (Lundberg & Govoni, 2004). While eNOS generates NO levels in the picomolar range, iNOS generates larger quantities of NO in the nanomolar range (Alderton et al., 2001), which may explain why the peak of NO production in the PPARγ-activated group was not significantly higher than that in the P. gingivalis-infected group. One possible explanation for the delayed peak in the PPARγ-activated group is that pre-activation of PPARγ attenuated the induction of inflammatory cytokines (Cantini et al., 2010) and the self-amplifying signal mechanism (Umansky et al., 1998). Using *P. gingivalis* lipopolysaccharide (LPS)-treated mucous acinar cells from sublingual salivary gland of Sprague-Dawley rats, Slomiany et al. reported that activation of PPARγ by cigitazine resulted in a greater decrease of iNOS activity and NO generation than LPS alone (Slomiany & Slomiany, 2003). Slomiany et al. employed mucous acinar cells but not the human endothelial cells in their study. The cells in their study were pretreated with the indicative concentrations of cigitazine from 5 to 30 μM, then exposed to *P. gingivalis* LPS at 500 ng/mL and incubated for 16 h. PPARγ agonists inhibited the iNOS pathway in murine macrophages (Colville-Nash, Qureshi, Willis, & Willoughby, 1998) and rat primary microglial cultures (Bernardo, Levi, & Minghetti, 2000), and in a rat model with renal ischemia following reperfusion (Betz et al., 2012). In contrast, we found no difference in iNOS expression (1.5–4 h) and NO levels (0–24 h) between the *P. gingivalis*-infected group and PPARγ-activated group. The inconsistent conclusions between our study and Slomiany’s work might be attributed to the different cell types employed, the different PPARγ agonist, *P. gingivalis* or LPS concentrations used and the different stimulation periods. In *P. gingivalis*-stimulated cells, NO production in the PPARγ-blocked group did not differ from the controls at any time point (Fig. 4), indicating that PPARγ played a role in partial modulation of NO production in HUVECs exposed to *P. gingivalis*. iNOS expression was significantly lower in the PPARγ-blocked group than that in the *P. gingivalis*-infected group and PPARγ-activated group after 1.5 h of stimulation, but did not differ from the control group, indicating that blocking PPARγ partially inhibited the expression of iNOS when HUVECs were exposed to *P. gingivalis*. While iNOS expression was significantly higher in the PPARγ-blocked group than that in the control group after 4 h of stimulation, there were no differences among the PPARγ–activated, PPARγ-blocked and *P. gingivalis*-infected groups (Fig. 5C), indicating that the iNOS pathway was partially modulated by PPARγ at the beginning stage of *P. gingivalis* exposure to HUVECs.

While NO production by NOS is well-described, an alternative pathway for NO generation has been observed in recent years. In this alternate pathway, known as the nitrate–nitrite–NO pathway, the inorganic anions nitrate and nitrite are reduced to NO and other reactive nitrogen intermediates (Hezel & Weitzberg, 2015). Our study suggested that PPARγ might be involved in the regulation of NO production through the NOS pathway in HUVECs exposed to *P. gingivalis*. This finding is important because PPARγ might be considered a new potential target in treating endothelial cells induced by *P. gingivalis*. As PPARγ agonists are available clinically, such therapeutics should be investigated for improving endothelial function in patients with periodontitis.

### 5. Conclusions

PPARγ was activated in HUVECs exposed to *P. gingivalis* W83, and activated PPARγ induced NO generation through the NOS pathway. However, the pathogenetic mechanisms require further investigation.

### Conflicts of interest

The authors declare no conflicts of interest with any of the products listed in this manuscript.

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### Ethical approval

No.

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### References


