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Original Article

Activation of peroxisome proliferator-activated receptor γ improves endothelial dysfunction induced by *Porphyromonas gingivalis* through the PI3K/Akt, ERK and NFκB pathways

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Abstract: Objectives: The aim of this study was to investigate the mechanisms related to functional improvement of human umbilical vein endothelial cells (HUVECs) after exposure to *Porphyromonas gingivalis* through the activation of peroxisome proliferator-activated receptor (PPARγ). Methods: HUVECs were treated with PPARγ agonist (15-deoxy-Δ12,14-prostaglandin J_2, 10 μM) or antagonist (GW9662 10 μM) and assessed for the levels of nitric oxide (NO), endothelial NO synthase (eNOS), inducible NOS (iNOS), protein kinase B (Akt), p-Akt, extracellular signal-regulated protein kinase (ERK) and p-ERK using Western blots after exposing the cells to *P. gingivalis*. The nuclear translocation of nuclear factor-kappa B (NF-κB) p65 was examined using immunofluorescence microscopy. Results: The levels of NO were significantly higher in HUVECs exposed to *P. gingivalis* or activated for PPARγ than those in controls. The expression of p-eNOS, p-Akt and p-ERK was significantly down-regulated after *P. gingivalis* exposure but remained unchanged after PPARγ activation. The iNOS expression and activation of NFκB were significantly higher after *P. gingivalis* infection and PPARγ activation as compared with control. Conclusions: Activated PPARγ improves endothelial dysfunction induced by *P. gingivalis* through the regulation of the PI3K/Akt and ERK and NFκB pathways.

Keywords: Peroxisome proliferator-activated receptor, nitric oxide, *Porphyromonas gingivalis*, endothelial dysfunction

Introduction

Porphyromonas gingivalis (*P. gingivalis*) is one of the most common periodontal etiological bacteria. It is Gram-negative and anaerobic [1]. Periodontal pathogens can not only cause periodontal tissue inflammation, but also enter the blood circulation and invade vascular endothelial cells [2, 3]. *P. gingivalis* is shown to decrease the expression of endothelial nitric oxide synthase (eNOS) in human umbilical vein endothelial cell (HUVECs) [4], resulting in endothelial dysfunction. Cohort and case-control studies also show that periodontitis are often associated with endothelial dysfunction [5, 6]. After debridement, endothelial function of patients with periodontal disease can be improved [7].

The ligand-activated transcription factor peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear hormone receptor that binds to peroxisome proliferators response element (PPRE) on target DNA as a heterodimer with retinoid X receptor and is trans-activated by its ligands. PPARγ regulates metabolism, cell proliferation and inflammation. Activated PPARγ is shown to increase the expression of eNOS in endothelial cells [8, 9]. In our early study, we found that once activated by endogenous ligand (15-deoxy-Δ12,14-prostaglandin J_2 (15dPGJ_2)) after exposed to *P. gingivalis*, PPARγ in HUVECs can increase the expression of eNOS [10]. However, the mechanisms related to PPARγ effect on eNOS expression in HUVECs are still unclear.

Membrane-associated second messenger protein, phosphatidylinositol 3-kinase (PI3K) and its downstream kinase, protein kinase B (Akt), are shown to improve endothelial dysfunction via upregulating eNOS expression [11]. Akt has been implicated in the eNOS activation in several types of endothelial cells [12]. Previous

study shows that treatment with agonist increases vascular PPARγ expression accompanied by the restoration of PI3K/Akt/eNOS signaling activation, leading to improved endothelial function in spontaneously hypertensive rats [13]. Another important signaling pathway involved in eNOS expression is extracellular signal-regulated protein kinase (ERK1/2) which plays an important role in the regulation of eNOS in a agonist-dependent manner [14]. The expression of activated of ERK1/2 is markedly down-regulated after bovine aortic endothelial cells were treated with protein extracts from *P. gingivalis* [15]. Cale et al showed that eNOS activity is dependent on the mitogen-activated protein kinase (MAPK) /ERK1/2 signaling pathway [14]. However, neither PI3K/Akt nor ERK1/2 pathway has been investigated in PPARγ-mediated attenuation of endothelial dysfunction induced by *P. gingivalis*.

Inducible nitric oxide synthase (iNOS) is an isoform of nitric oxide synthase (NOS) and its expression is regulated by inflammatory stimuli such as bacterial proteins and cytokines [16], and is responsive to transcription factors such as NF-κB [17]. There are evidences from animal and in vitro studies that PPARγ agonist may have suppressive effects on NF-κB action [18, 19]. However whether PPARγ regulates iNOS expression through NF-κB has not been documented.

Therefore, it is very likely that the Akt, ERK and NFκB pathways are associated with *P. gingivalis*-induced endothelial dysfunction in HUVECs. We hypothesized that PPARγ plays a role in regulating NOS expression through the PI3K/Akt, ERK1/2 and NFκB pathways. The aim of this study was to investigate how the activated PPARγ improve NOS expression in HUVECs exposed to *P. gingivalis*. The finding may provide insights into the molecular mechanisms underlying activated PPARγ-mediated attenuation of endothelial dysfunction and new strategies for treatment of periodontal tissue inflammation.

Materials and methods

Cell line and culture conditions

HUVEC line EA.hy926 (CRL2922™) was purchased from American Type Collection Center (ATCC, Cat. no 61034681). It was established by fusing primary human umbilical vein cells with a thioguanine-resistant clone of A549. EA.hy926 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Paisley, Scotland, UK) containing 10% fetal bovine serum (FBS, HyClone, Logan, UT), in 10-cm dishes under 5% CO_2 at 37°C. Cells were seeded into 6-well plates (Corning, Acton, MA, USA) at 10^6 cells/ml/well, grown overnight, and then used in experiments.

Bacterial strain and culture conditions

P. gingivalis W83 was a gift of the Department of Microbiology of Peking University School of Stomatology. The bacteria were grown in 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% CO_2, 10% H_2, and 85% N_2) at 37°C for 5-7 days. The cultures were inoculated into fresh BHI broth supplemented with 5 μg/ml hemin and 0.4 μg/ml menadione and grown for 24 h or until the optical density at 600 nm reached 1.0.

Exposure of HUVECs to P. gingivalis

P. gingivalis was centrifuged, washed with PBS (pH 7.2), and re-suspended in DMEM with 10% FBS at a final concentration of 10^8 cells/ml [3]. The bacterial suspensions were added to the HUVEC cultures at a multiplicity of infection (MOI) of 10:1 for the indicated times (1.5, 4, 8, 12 h) at 37°C under 5% CO_2. Control cultures were incubated with medium alone. Both cells and culture supernatants were collected separately for subsequent experiments. The viability of HUVECs was assessed using the 0.2% trypan blue exclusion test. All assays were performed in triplicate.

To investigate the effects of PPARγ on NOS, HUVECs were either cultured in DMEM (control), exposed to *P. gingivalis* (*P. gingivalis* infection), activated for PPARγ by adding PPARγ agonist 15d-PGJ_2 (Sigma-Aldrich, St. Louis, MO, USA) at 10 μM [20] or blocked for PPARγ by adding PPARγ antagonist 2-chloro-5-nitro-N-phenylbenzamide (GW9662, Sigma-Aldrich, St. Louis, MO, USA) at 10 μM [21] 30 min before being exposed to *P. gingivalis*. At 1.5, 4, 8, 12 h, cells and culture supernatants were collected, proteins were extracted for Western blot analysis. To investigate the pathways in which PPARγ modulates NOS, HUVECs were treated with PI3K inhibitor wortmannin (100 nM) or

eNOS inhibitor cavtratin (10 nM) for 30 min before the bacterial exposure.

Western blot analysis

HUVECs were washed three times with ice-cold PBS and lysed in RIPA buffer (Applygen, Beijing, China) containing proteinase inhibitors and phosphatase inhibitors. Cell lysates were centrifuged at 10000 g for 10 min to remove insoluble materials, and protein concentrations were determined using a BCA kit (CWBIO, Beijing, China). 50 μg proteins were separated on 12% sodium dodecyl sulfate-polyacrylamide gel by electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked in 10% non-fat milk for 1 h and probed with antibodies against eNOS, phospho-eNOS (p-eNOS) and iNOS (1:1000; Cell Signaling Technology, Danvers, MA, USA), Akt, p-Akt, ERK1/2, p-ERK1/2 (1:500; Cell Signaling Technology, Danvers, MA, USA) and β-actin (1:1000; ZSGB-BIO, Beijing, China) as internal reference, separately, 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 was normalized against β-actin. All the data were from three independent experiments.

Immunofluorescence analysis

Immunofluorescence assays were carried out according to the previously described protocol [22]. Briefly, HUVECs were plated on culture slides. Then, the cells were fixed, permeabilized and incubated with NF-κB p65 antibody (1:300; Cell Signaling Technology, Danvers, MA, USA). Fluorescein isothiocyanate-conjugated immunoglobulin G (1:500, ZSGB-BIO, Beijing, China) was added as the secondary antibody. NF-κB antigen was identified in the cytoplasm or nuclei (green). The nuclei were stained by DAPI (blue). Cells were visualized under a fluorescence microscope equipped with a digital camera (OLYMPUS BX51, OLYMPUS, Tokyo, Japan). The nuclear translocation of NF-κB p65 in the *P. gingivalis*-exposed HUVECs was observed. The percentages of translocation cells over the total number of cells were calculated.

NO detection

Culture medium was collected and NO concentration was measured as the total contents of stable oxidative metabolite, nitrite using the Griess reagent [23]. Briefly, 50 μl of Griess R1 reagent (Applygen, Beijing, China) was added to equal volumes of culture supernatant in a 96-well plate (Corning, Acton, MA, USA) and left at room temperature for 5 min. Then, Griess R2 reagent was added, left at room temperature in the darkness for 5 min. The absorbance was then read at 540 nm using a Bio-Rad plate reader (Bio-Rad, Hercules, CA, USA), and the NO concentrations were calculated from a standard curve established with serial dilutions of $NaNO_2$ in culture medium.

Statistical analysis

All experiments were performed in triplicate wells for each condition and repeated at least three times. Data were expressed as the mean ± standard deviation (SD). One way ANOVA was performed to evaluate the differences in NO production or protein levels or cell counts among groups at each time point followed by LSD post hoc using SPSS 10.0 software (SPSS, Inc., Chicago, IL, USA). A P value of < 0.05 was considered statistically significant.

Results

NO content and NOS expression

The data showed that NO contents in HUVECs 1.5 h to 12 h after *P. gingivalis* exposure or PPARγ activation were significantly higher than that in the control (54.14 ± 15.76 μM and 54.64 ± 18.93 μM vs 44.61 ± 11.28 μM, $P < 0.05$), while the cells treated with the PPARγ antagonist did not change the NO level (**Figure 1A**). Western blot analysis showed that during the period, p-eNOS levels were lowered in the *P. gingivalis*-exposed and PPARγ-antagonist treated cells than those in controls (**Figure 1B**, **1C**) $P < 0.05$) while the level was slightly but not significantly higher in the PPARγ-activated group than in controls ($P < 0.05$). p-eNOS level was higher in the PPARγ-activated group than in the PPARγ-blocked and infected groups. The total eNOS level was lower in the infected group than in control ($P < 0.05$; **Figure 1C**).

P. gingivalis attenuated and PPARγ agonist augmented PI3K/Akt/eNOS expression

We then analyzed the expression of proteins in the PI3K/Akt/eNOS pathways. The results showed that total Akt, p-Akt, total eNOS and

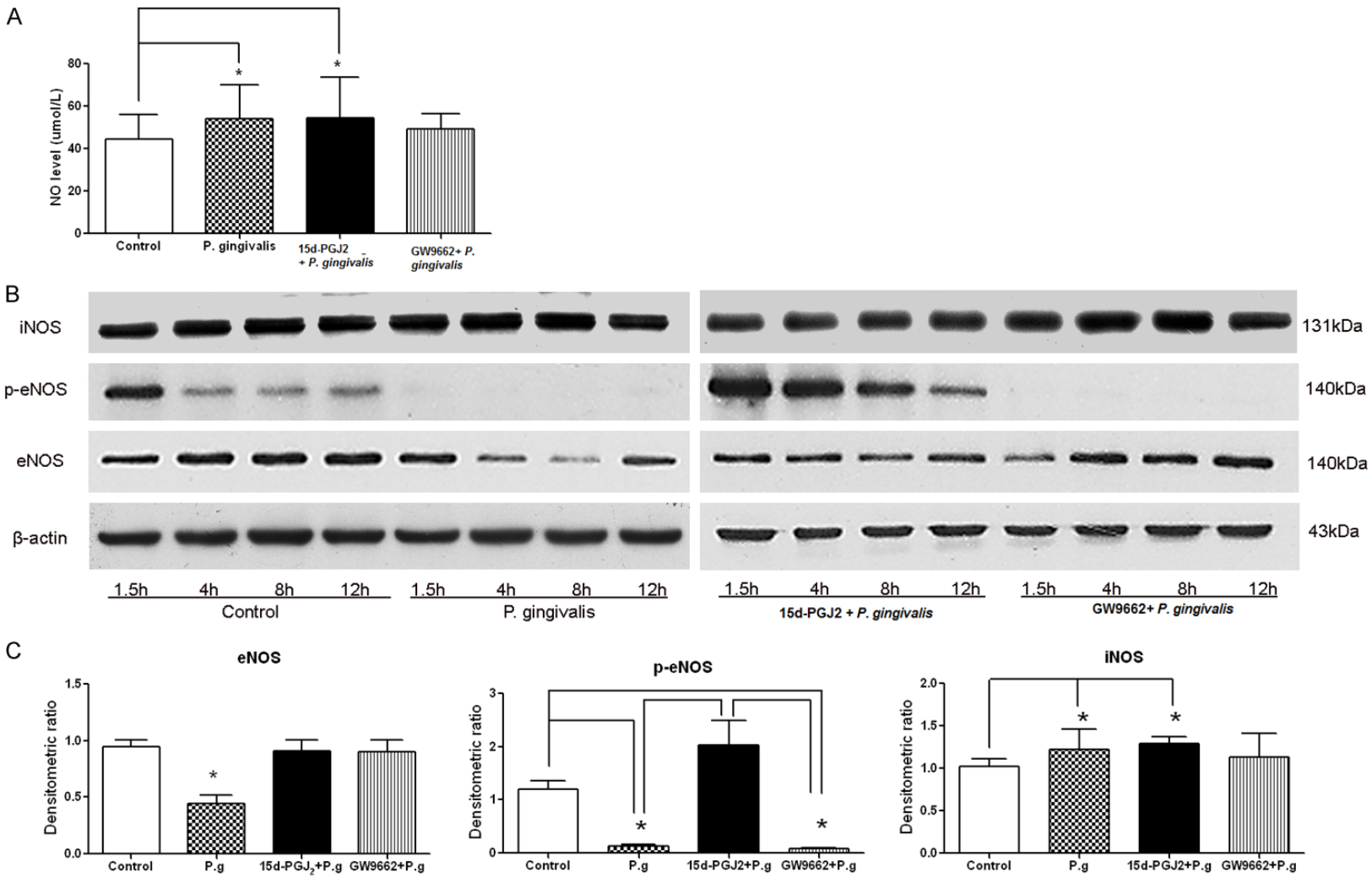
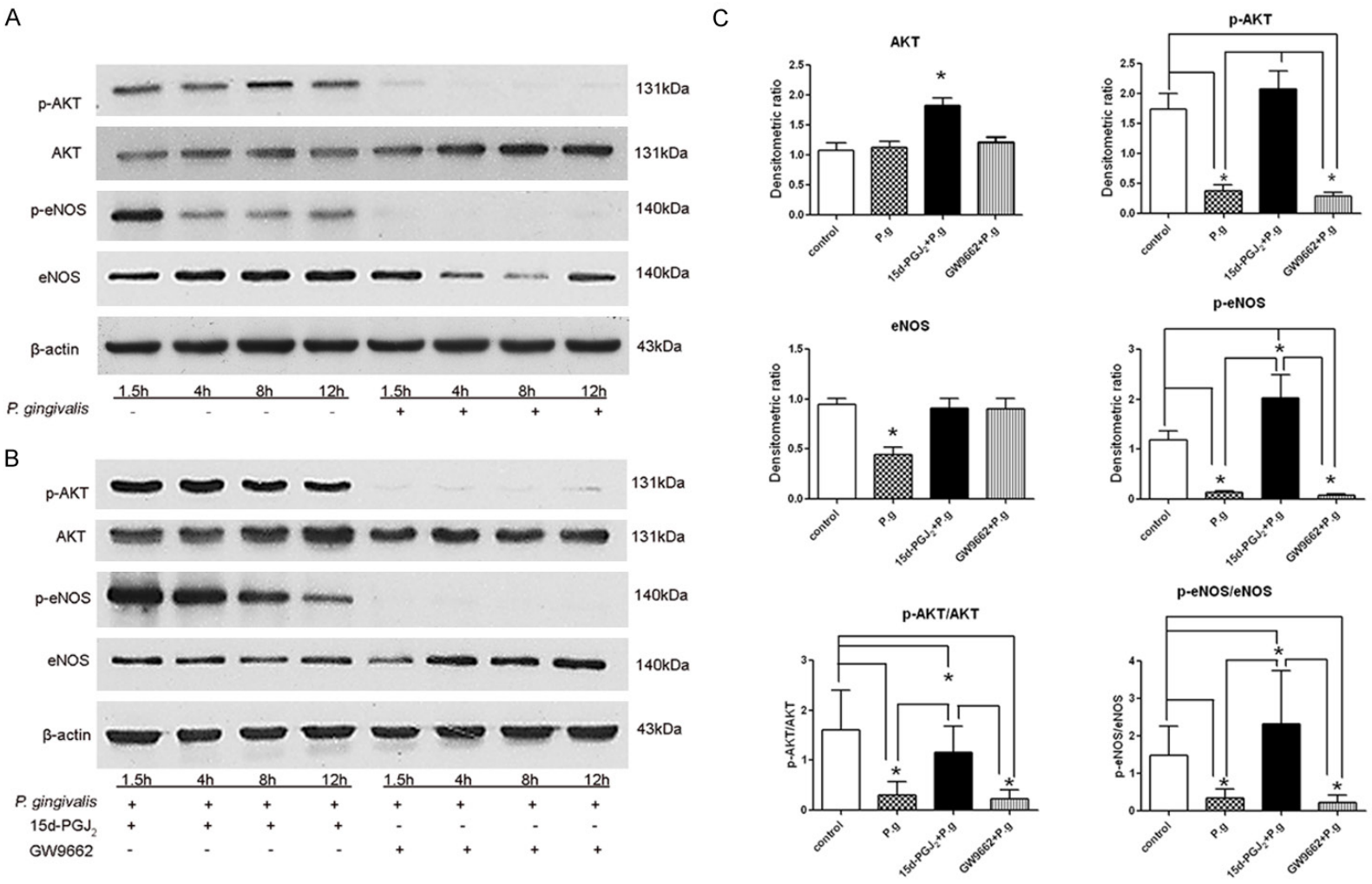


Figure 1. Effect of *P. gingivalis*, PPARγ agonist 15d-PGJ_2 and antagonist GW9662 on NO content and NOS expression in HUVECs. A. Averaged NO content 1.5 to 12 h after the treatment; B. Representative Western blots of iNOS, eNOS, p-eNOS 1.5 to 12 h after the treatment; C. Averaged levels of iNOS, eNOS, p-eNOS 1.5 to 12 h after the treatment. *denotes $P < 0.05$ compared between two experimental groups (n = 24).


A
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15d-PGJ2+P.g
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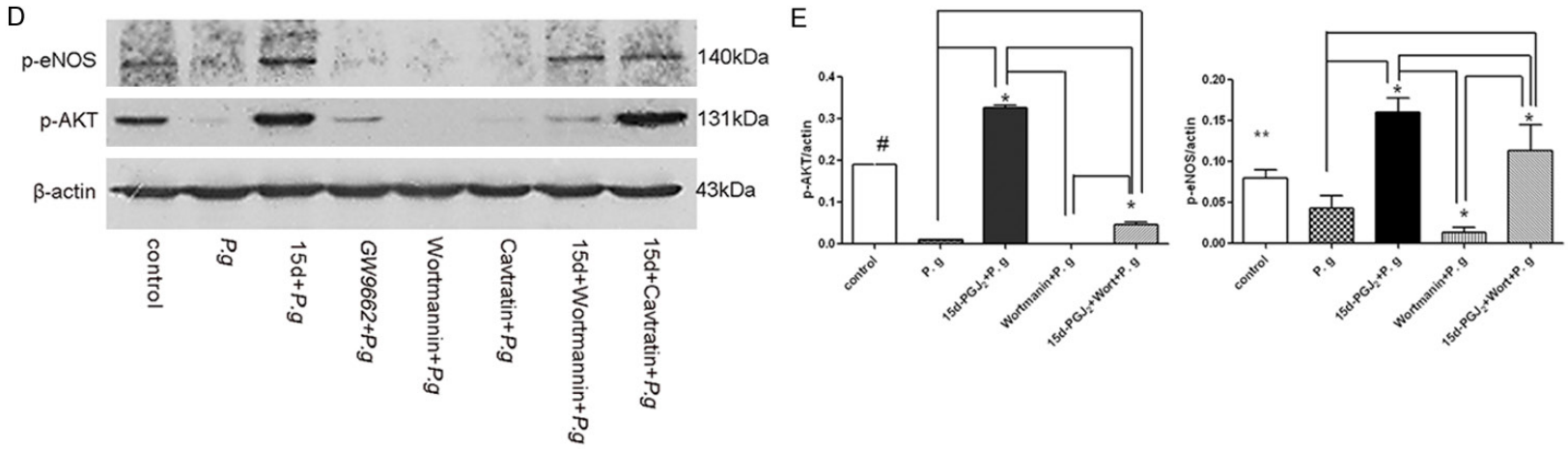

Figure 2. Effect of *P. gingivalis*, PPARγ agonist 15d-PGJ_2, antagonist GW9662, PI3K inhibitor wortmannin and eNOS cavtratin on gene expression in PI3K/Akt/eNOS pathways in HUVECs. A and B. Representative Western blots showing the expression of gene expression in the PI3K/Akt/eNOS pathways; C. The averaged levels of Akt, p-Akt, eNOS and p-eNOS in the PI3K/Akt/eNOS pathways 1.5 to 12 h after the treatments; D. Representative Western blots showing the expression of gene expression in the PI3K/Akt/eNOS pathway affected by wortmannin (100 nM) and cavtratin (10 nM); E. The levels of p-Akt and p-eNOS affected by wortmannin (100 nM) and cavtratin (10 nM). *denotes $P < 0.05$ compared between two experimental groups, **denotes $P < 0.05$ compared with *P. g*, 15d-PGJ_2 + *P. g* and wortmannin + *P. g* group, and #denotes $P < 0.05$ compared with other four groups.

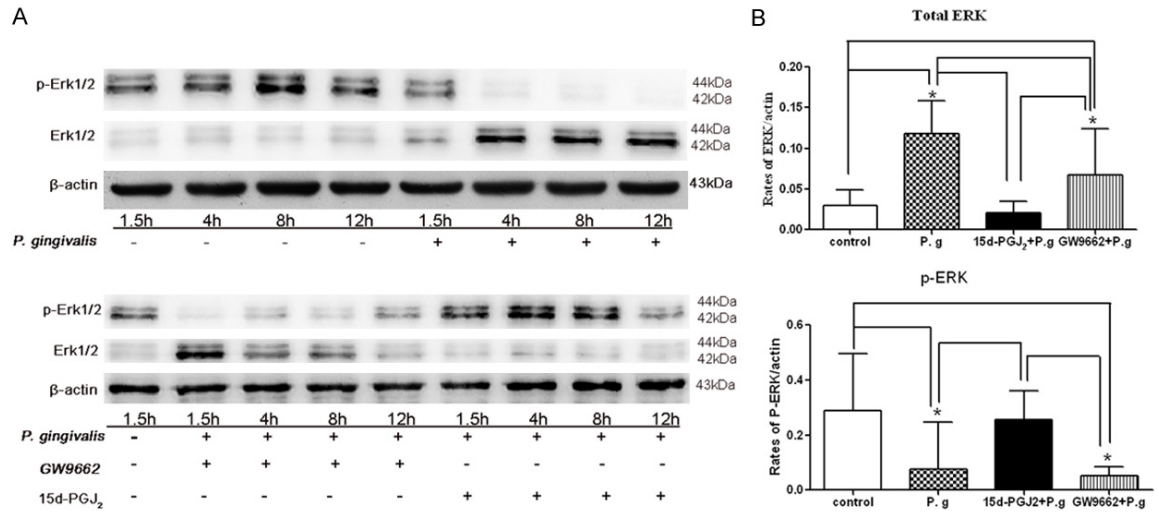

Figure 3. Effect of *P. gingivalis*, PPARγ agonist 15d-PGJ_2, antagonist GW9662, PI3K inhibitor wortmannin on ERK expression and phosphorylation in HUVECs. A. Representative Western blots showing the expression of ERK and phospho-ERK; B. The averaged levels of total ERK and p-ERK 1.5 to 12 h after the treatments. *denotes $P < 0.05$ compared between two experimental groups.

p-eNOS were up-regulated in HUVECs following agonist treatment, while *P. gingivalis* attenuated the expression (**Figure 2A-D**). Compared with control, p-Akt, p-eNOS and total eNOS levels in *P. gingivalis* cells were significantly lower ($P < 0.05$; **Figure 2C**), while the total Akt levels were similar between these two groups (**Figure 2C**). These data show that expression of the Akt/eNOS pathways was inhibited significantly when HUVECs were exposed to *P. gingivalis*.

As shown in **Figure 2B** and **2C**, the expression of total Akt and p-eNOS in the PPARγ-activated group was significantly up-regulated compared to other three groups, Analysis shows that the level of p-Akt in the PPARγ-activated group was significantly higher than that in the *P. gingivalis*- and PPARγ antagonist-treated groups, but similar to that of control (**Figure 2C**). These data indicate that PPARγ activation could increase eNOS expression through the Akt/eNOS pathway.

We further investigated if PI3K inhibitor Wortmannin and eNOS inhibitor Cavtratin would inhibit the Akt/eNOS pathways. The results show that they inhibited the expression of p-eNOS and p-Akt in HUVEC exposed to *P. gingivalis,* while the p-Akt expression was significantly higher following treating the cells with PPARγ agonist 15d-PGJ_2 with or without Wortmannin or Cavtratin after *P. gingivalis* exposure (**Figure 2D**, **2E**). These data indicate that 15d-PGJ_2-activated PPARγ could increase the eNOS expression through the PI3K/Akt pathway.

P. gingivalis attenuated and PPARγ agonist augmented ERK1/2 expression

We then investigated the expression of ERK1/2 following the *P. gingivalis* exposure and PPARγ activation. Western blot analyses showed the p-ERK expression and the rates of p-ERK/ERK after 1.5 h to 12 h treatment were lower in HUVECs after the cells were exposure to *P. gingivalis* or treated with the PPARγ antagonist than the controls. The activation of PPARγ did not alter the expression or ratio as compared with the control (**Figure 3A**, **3B**) but significantly increased these as compared with *P. gingivalis* exposure (**Figure 3A**, **3B**).

PPARγ modulated iNOS expression through NFκB pathway

The nuclear translocation of NFκB in HUVECs following *P. gingivalis* infection and PPARγ activation was then examined using immunofluorescence microscopy. The results showed that both *P. gingivalis* and PPARγ agonist 15d-PGJ_2 + *P. gingivalis* significantly increased the translocation as compared with the control, while the antagonist GW9662 + *P. gingivalis* signifi-

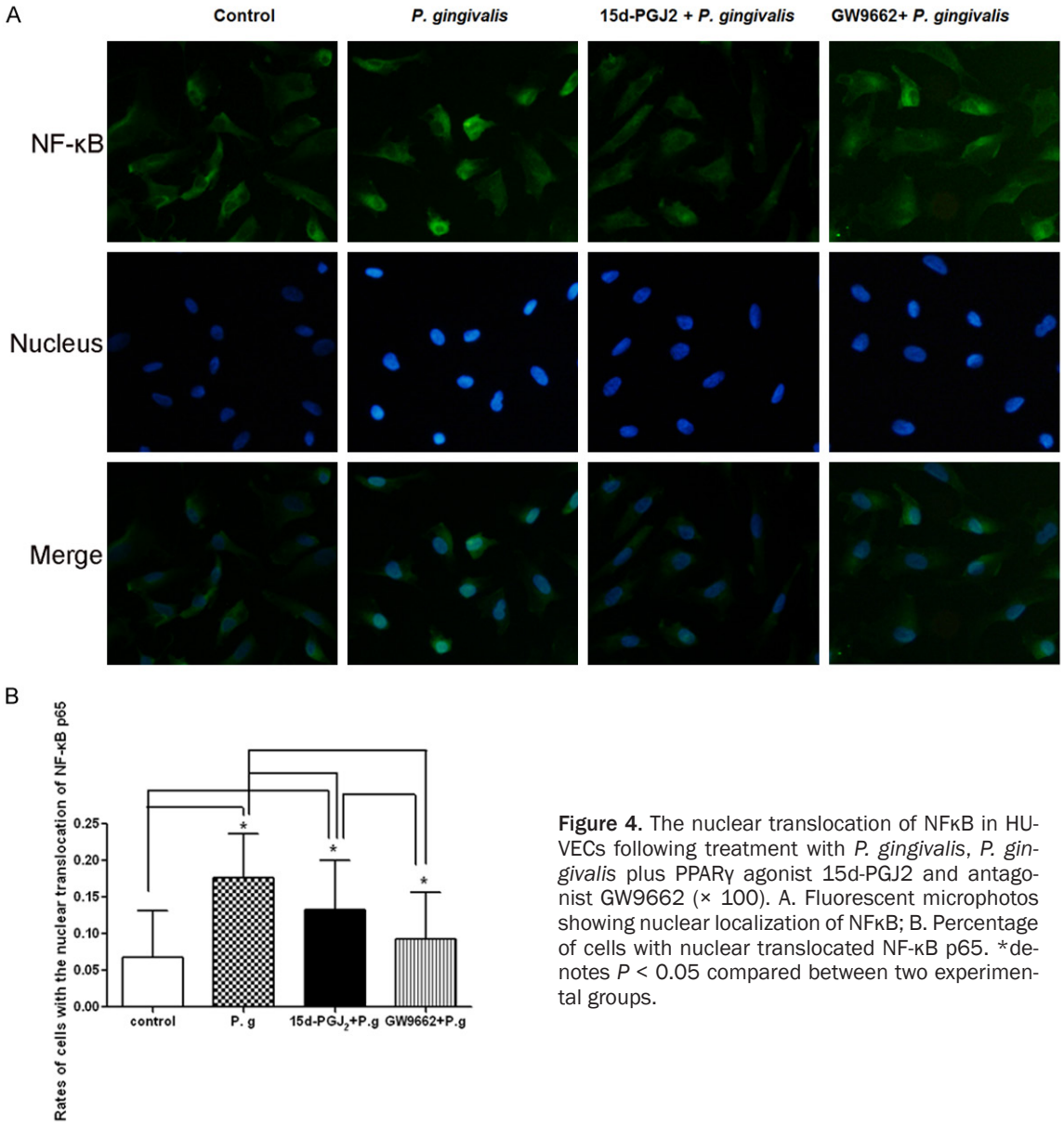

Figure 4. The nuclear translocation of NFκB in HUVECs following treatment with *P. gingivalis*, *P. gingivalis* plus PPARγ agonist 15d-PGJ2 and antagonist GW9662 (× 100). A. Fluorescent microphotos showing nuclear localization of NFκB; B. Percentage of cells with nuclear translocated NF-κB p65. *denotes $P < 0.05$ compared between two experimental groups.

cantly reduced the increased translocation as compared with the infection group and PPARγ activated group ($P < 0.05$, **Figure 4A**, **4B**). These data suggest that *P. gingivalis* activated the NF-κB pathway while PPARγ activation might partially inhibit the activation.

Discussion

Due to increasing prevalence of periodontal disease, it is important to have a better understanding of the mechanism by which PPARγ agonists prevent and treat vascular diseases caused by *P. gingivalis*. The major new finding of the present study is that the activation of PPARγ improves *P. gingivalis*-induced endothelial dysfunction through increasing eNOS expression in the PI3K/Akt and ERK pathways and decreasing iNOS expression in the NFκB signaling pathway.

A hallmark of arterial endothelial dysfunction is impaired endothelium-dependent dilation, which is predictive of future cardiovascular disease events [24, 25]. NO, a key endothelium-derived relaxing factor, plays an important role in the maintenance of vascular tension and activity. In endothelial cells, there are two iso-

forms of NOS, eNOS and iNOS. eNOS is expressed constitutively in endothelial cells [26]. Endothelial dysfunctions are often characterized by decreased generation of NO by eNOS. iNOS is expressed in response to inflammatory stimuli such as bacterial proteins and cytokines, and over-production of NO induced by iNOS is toxic [16]. Previous studies, including ours, have indicated that in endothelial cells exposed to *P. gingivalis*, increased NO production is due to increased expression of iNOS and decreased expression of eNOS [4]. Phosphoinositide 3-kinase/Akt and ERKs are two important signaling pathways that regulate eNOS activity. Previous studies have demonstrated that Akt can regulate eNOS activation in vascular endothelial cells [27, 28], and represents a novel Ca^{2+}-independent regulatory mechanism for the activation of eNOS [27]. Our data show that *P. gingivalis* weakened the phosphorylation of Akt and eNOS in HUVECs, while 15d-PGJ_2 enhanced the phosphorylation of Akt and phosphorylated eNOS expression in HUVECs exposed to *P. gingivalis*. This is consistent with the previous studies [27, 28]. *P. gingivalis* is shown to attenuate the PI3K/Akt signaling pathway via the proteolysis of gingipains, resulting in the destruction of epithelial barriers in gingival epithelial cells [29]. In present study, wortmannin and cavtratin are found to block the phosphorylation of PI3K and eNOS, respectively, while even pre-incubation of wortmannin together with 15d-PGJ_2, increased the eNOS expression in HUVECs exposed to *P. gingivalis* (**Figure 2D**). Li et al also showed that 15d-PGJ_2 increases eNOS expression through the PI3K/Akt pathways in pulmonary artery endothelial cells [11].

ERK1/2 plays an important role in the regulation of cellular processes such as proliferation, differentiation and survival [30, 31]. For example, the ERK pathway is shown to involve in modulating eNOS activity through influent delivery of arginine to eNOS by cationic amino acid transporter-1 [32]. The molecular mechanisms of ERK1/2 modulating the eNOS activity include changing intracellular Ca^{2+} concentration, phosphorylation and, possibly, intracellular trafficking [14]. A key observation in this study is the decreased expression of p-ERK following *P. gingivalis* exposure, which is also observed in earlier studies. For example, p-ERK expression was reduced when periodontal ligament cells was exposed to *P. gingivalis* [33]. Using 30 min 15d-PGJ_2 incubation before *P. gingivalis*, the ratio of p-ERK/total ERK increased to the control level (**Figure 3A**), suggesting that p-ERK in the HUVECs is attenuated due to *P. gingivalis* infection, while the activation of PPARγ can increase the p-ERK expression in HUVECs infected by *P. gingivalis*.

NFκB as an important transcription factor is responsible for regulating expression of genes that control cell adhesion, proliferation, inflammation, redox status, and tissue specific enzymes. *P. gingivalis* triggers intracellular signaling pathways [34] leading to an activation of a kinase mediated-phosphorylation and degradation of the inhibitors of NFκB (IκB) [35]. This results in translocation of the NFκB heterodimer to the nucleus where it binds to the promoters of gene targets [35]. Pierce et al showed that inhibition of endothelial cell NFκB nuclear translocation improves endothelium-dependent dilation [36]. Inhibiting NFκB signaling might also limit the vicious cycles of inflammation and oxidative stress. In mice models injected with bacterial lipopolysaccharide, NFκB kinase inhibitor significantly attenuated iNOS expression [37], while Ricote et al showed PPARγ agonist may have suppressive effects on NF-κB action in monocytes [18]. Zhang et al showed that in HUVECS stimulated by *P. gingivalis* for 30 to 90 minutes, IκB expression was significantly higher than the controls [18]. In present study, translocation of the NFκB heterodimer was higher after *P. gingivalis* infection (**Figure 4B**). This is consistent with the early result [35]. In present study we also found that the translocation of the NFκB heterodimer was fewer following PPARγ activation as compared with *P. gingivalis* exposure, suggesting that the PPARγ ligand 15d-PGJ_2 may partially inhibit NFκB activity, which is similar to Ricote's conclusion [18]. It's worth noting that Maggi et al have recently shown that 15d-PGJ_2 has an inhibitory effect on iNOS expression in monocyte-macrophage cell lines [38]. Also, agonist of PPAR is shown to suppress NFκB and stimulated IκB expression in human body [19].

In conclusion, activation of peroxisome proliferator-activated receptor γ improves endothelial dysfunction induced by *P. gingivalis* through the PI3K/Akt and ERK and NFκB pathways.

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Disclosure of conflict of interest

None.

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