

Heat-shock protein 60 of *Porphyromonas gingivalis* may induce dysfunction of human umbilical endothelial cells via regulation of endothelial-nitric oxide synthase and vascular endothelial-cadherin

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Abstract. Accumulating evidence has established that periodontitis was an independent risk factor for coronary heart disease (CAD). *Porphyromonas gingivalis* (*P. gingivalis*), a major periodontal pathogen, has already been shown to have a significant role in the inflammatory response of CAD *in vivo*. The aim of the present study was to identify whether *P. gingivalis* heat-shock protein 60 (HSP60) induced the dysfunction of human umbilical vein endothelial cells (HUVECs) *in vitro*. HUVECs were stimulated with a range of *P. gingivalis* HSP60 concentrations (1, 10 and 100 ng/l) at different time-points. The levels of vascular endothelial (VE)-cadherin, endothelial nitric oxide synthase (eNOS) and cysteinyl aspartate-specific protease-3 (caspase-3) were measured using western blot analysis. The apoptotic rate of HUVECs was detected using flow cytometry. *P. gingivalis* HSP60 at a concentration of 10 ng/l significantly decreased the expression levels of VE-cadherin and eNOS protein at 24 h stimulation, whereas no difference in these proteins was identified following a low dose of *P. gingivalis* HSP60 (1 ng/l). *P. gingivalis* HSP60 at 100 ng/l significantly downregulated the expression levels of VE-cadherin and eNOS protein at 12 h in HUVECs. However, the cleavage of caspase-3 showed an opposing change at different concentrations. Consistently, *P. gingivalis* HSP60 induced apoptosis of HUVECs in a concentration-dependent

manner. These results indicated that *P. gingivalis* HSP60 may induce dysfunction and apoptosis in HUVECs via downregulating the expression levels of VE-cadherin and eNOS, and promoting the cleavage of caspase-3.

Introduction

Previous studies indicated that periodontitis may be associated with a higher risk of coronary heart disease (CAD) (1-4), independent of established cardiovascular risk factors. Periodontitis is a chronic tissue-destruction inflammatory state that is predominantly induced by *Porphyromonas gingivalis* (*P. gingivalis*) in the gingival pockets of certain individuals with advanced and severe periodontal disease.

P. gingivalis may promote transient bacteremia during tooth brushing, chewing or dental procedures (5-7). Certain studies have identified that *P. gingivalis* was detected frequently in atheromatous plaques of the aorta and coronary artery, and it was reported to perpetuate systemic inflammation (8-10). Additionally, *P. gingivalis* induces macrophage foam cell formation (11) and stimulates oxidation of low-density lipoprotein (12). Certain studies show that *P. gingivalis* lipopolysaccharide (LPS) could induce the expression of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 in human umbilical vein endothelial cells (HUVECs) (13,14), which significantly enhances trans-endothelial migration of inflammatory cells.

Furthermore, atherosclerosis can be triggered and aggravated by the pathogen-driven antigenic peptide from *P. gingivalis* heat-shock protein 60 (HSP60) (15-17). An overall 55% homology exists between human and bacterial HSP60 that can even reach 72% at certain domains of the 573-amino-acid-long molecule (18). *P. gingivalis* HSP60 is reported to accelerate the development of experimental atherosclerosis by cross-reactivity of the immune response to bacterial HSPs (19). However, Jeong *et al* (20) found that *P. gingivalis* HSP60 peptides have distinct roles in the development of atherosclerosis;

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peptide 14 or 19 from *P. gingivalis* HSP60 may have either an anti- or pro-atherogenic role, respectively, in the ApoE(-/-) mouse model of infection-triggered atherosclerosis through distinct mechanisms operating in the polarization of T cells. Additionally, in a clinical study, a strong positive correlation was found between high levels of soluble HSP60 and the risk of CAD (21). Soluble HSP60 levels directly correlate with the presence of classic risk factors of atherosclerosis, such as elevated low-density lipid cholesterol levels, and with particular proinflammatory markers, such as tumor necrosis factor- α (22).

However, the potential pathways linking periodontitis and cardiovascular disease remain to be elucidated (23-25) and the underlying molecular mechanisms from *P. gingivalis* HSP60 regarding the association between periodontitis and atherosclerosis require further investigation. In the present study, the aim was to investigate whether *P. gingivalis* HSP60 treatment leads to the dysfunction of HUVECs directly by affecting the protein expression levels of endothelial nitric oxide synthase (eNOS) and vascular endothelial (VE)-cadherin.

Materials and methods

Cell culture. HUVECs were kindly provided as a gift by Dr Yun Mu (Tianjin Medical University, Tianjin, China). Cell culture media and supplements were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Gibco (Thermo Fisher Scientific, Inc.). *P. gingivalis* HSP60 was purchased from Hongling Longcheng Technology Co., Ltd. (Beijing, China). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS at 37°C in a humidified incubator with 5% CO₂. The culture medium was exchanged every 48 h. HUVECs up to passage 6 were used for the experiments.

Cell viability. Cell viability was determined using the MTT assay. HUVECs were seeded in 96-well culture plates at a density of 0.5x10⁴ cells/well and incubated overnight at 37°C. Following treatment with *P. gingivalis* HSP60 at different concentrations (1, 10 and 100 ng/l), cells were incubated with 5 mg/ml MTT for 24 h. Subsequently, the MTT-containing growth medium was replaced with 100 μ l of dimethyl sulfoxide (DMSO) and mixed thoroughly for 10 min. The optical density readings of each well were determined at 570 nm using a microplate reader (ELx808; BioTek Instruments, Inc., Winooski, VT, USA). The effect of *P. gingivalis* HSP60 on cell viabilities was expressed as the percentage of viable cells in the treated groups compared to the DMSO control. Values [mean \pm standard deviation (SD)] are from three independent experiments.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. The cell layer was washed with 3 ml of phosphate-buffered saline (PBS) twice. Following treatment (1, 10 and 100 ng/l *P. gingivalis* HSP60 for 2, 6, 12 or 24 h), the cells were homogenized in an ice bath using sonification (3 times for 15 sec, 50 Hz) with 1 ml of radioimmunoprecipitation assay lysis buffer containing 400 μ l of protease inhibitors of phenylmethylsulfonyl fluoride (Thermo Fisher Scientific, Inc.). The homogenate was

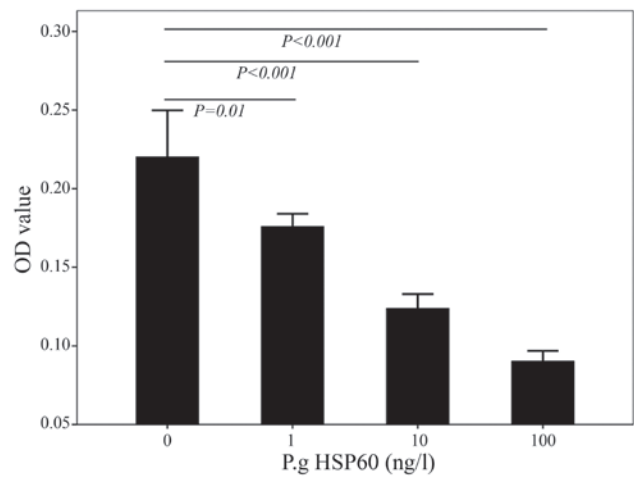


Figure 1. Viabilities of HUVECs following *P. gingivalis* HSP60 treatment at various concentrations for 24 h. Data are expressed as % viability to that of the controls. Values are mean \pm standard deviation, n=3. Compared to 1 ng/l *P. gingivalis* HSP60-treated HUVECs, P=0.004 for 10 ng/l and P<0.001 for 100 ng/l. Compared to 10 ng/l *P. gingivalis* HSP60-treated HUVECs, P=0.035 for 100 ng/l. OD, optical density; P. g, *Porphyromonas gingivalis*; HSP60, heat-shock protein 60; HUVECs, human umbilical endothelial cells.

collected and centrifuged at 12,000 x g for 15 min, and the supernatant was used as a lysate for further determinations. Protein concentration was determined by the BCA™ protein assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Western blot analysis was performed as described previously (26). Equal amounts of cellular protein (30 μ g) underwent electrophoresis on a gradient SDS-PAGE (4-10% gel) and the samples were electrotransferred onto nitrocellulose membranes in a buffer consisting of 25 mM Tris, 192 mM glycine and 20% methanol (pH 8.4) for 2 h at a constant voltage (100 V) with cooling. The following primary antibodies were used for blotting: β -actin (cat. no. M20010; monoclonal mouse anti-human; 1:500; Abmart, Inc., Berkeley Heights, NJ, USA), VE-cadherin (cat. no. SAB1306131; polyclonal rabbit anti-human; 1:500; Sigma-Aldrich, St. Louis, MO, USA), eNOS (cat. no. SAB4502013; polyclonal rabbit anti-human; 1:5,000; Sigma-Aldrich), caspase-3 (cat. no. C9598; polyclonal rabbit anti-human; 1:500; Sigma-Aldrich) and cleaved caspase-3 (cat. no. SAB4503294; polyclonal rabbit anti-human; 1:500; Sigma-Aldrich). The secondary antibodies include IRDye (1:3,000; Abmart, Inc.). Immunocomplexes were detected using the ECL western blotting detection kit (GenMed, Inc., Houston, TX, USA). All the other reagents were purchased from Sigma-Aldrich. The specific proteins were visualized by an Odyssey™ infrared imaging system (LI-COR, Inc., Lincoln, NE, USA).

Flow cytometry analysis for apoptosis quantification. Following the designated treatment (1, 10 and 100 ng/l *P. gingivalis* HSP60), annexin V-fluorescein isothiocyanate-conjugated (FITC)/propidium iodide (PI) apoptosis detection kit (Invitrogen, Thermo Fisher Scientific, Inc.) was used according to the manufacturer's protocol. In brief, the cells were centrifuged at 300 x g for 5 min, washed with cold PBS, and resuspended in 100 μ l of binding buffer. Annexin V-FITC (5 μ l) and PI (5 μ l) were added to each sample, and the mixture was incubated

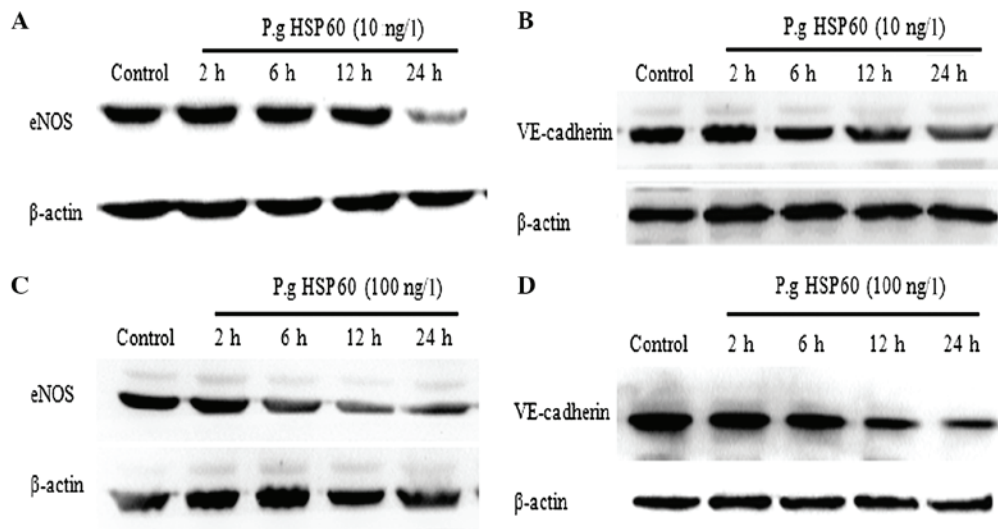


Figure 2. Effects of *P. gingivalis* HSP60 on the protein expression levels of eNOS and VE-cadherin at different time-points. The HUVECs were cultured with *P. gingivalis* HSP60 stimulation (10 and 100 ng/l) at different time-points (2, 6, 12 and 24 h). Effects of *P. gingivalis* HSP60 (10 ng/l) on protein expression levels of (A) eNOS and (B) VE-cadherin at different time-points. Effects of *P. gingivalis* HSP60 (100 ng/l) on protein expression levels of (C) eNOS and (D) VE-cadherin at different time-points. P.g, *Porphyromonas gingivalis*; HSP60, heat-shock protein 60; eNOS, endothelial nitric oxide synthase; VE, vascular endothelial; HUVECs, human umbilical endothelial cells.

at 4°C in the dark for 15 min. The cells were immediately subjected to fluorescence-activated cell sorting analysis (BD Accuri C6; BD Biosciences, San Jose, CA, USA) within 1 h. For cells in the early apoptotic stage, membrane phosphatidylserine was exposed and combined with annexin V. The cells were stained with annexin V with no PI fluorescence and recorded as annexin V (+)/PI (-). The membranes of dead cells and cells in the late apoptotic stage were permeable to PI. These cells were stained with annexin V and PI and were recorded as annexin V (+)/PI (+). Finally, annexin V (+)/PI (-) and annexin V (+)/PI (+) cells were detected under flow cytometry, and the percentages of the total number of cells in each group were compared.

Statistical analysis. Statistical analyses were performed with SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Results are provided as mean \pm SD. One-way or two-way analysis of variance tests were applied to compare the different groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

P. gingivalis HSP60 inhibits the proliferation of HUVECs. To examine the effects of *P. gingivalis* HSP60 on HUVECs, the HUVECs were first treated with different concentrations of *P. gingivalis* HSP60, and the cell viability was detected using the MTT assay. *P. gingivalis* HSP60 at 1, 10 and 100 ng/l significantly altered the viability of HUVECs ($P < 0.05$) (Fig. 1).

P. gingivalis HSP60 downregulates the expression levels of eNOS and V-cadherin in HUVECs. HUVECs were incubated with *P. gingivalis* HSP60 (1, 10 and 100 ng/l) at different time-points (2, 6, 12 and 24 h) and the levels of VE-cadherin and eNOS were detected by western blot analysis. The expression levels of VE-cadherin and eNOS proteins were comparable in HUVECs treated with 1 ng/l of *P. gingivalis* HSP60. The protein expression levels of eNOS at 24 h following treatment

with *P. gingivalis* HSP60 (10 ng/l) were significantly decreased as compared with those at 2, 6 and 12 h (Fig. 2A), and the protein expression level of VE-cadherin had an opposing effect (Fig. 2B). Additionally, HUVECs treated with *P. gingivalis* HSP60 (100 ng/l) exhibited a significantly lower eNOS protein expression level following 12 h of treatment in contrast to the levels at 2 and 6 h (Fig. 2C), and the protein expression of VE-cadherin was significantly decreased after 12 h (Fig. 2D). Taken together, these results provide evidence that *P. gingivalis* HSP60 may lead to endothelial dysfunction by the regulation of VE-cadherin and eNOS protein expression levels.

P. gingivalis HSP60 upregulates the expression of caspase-3 and induces apoptosis of HUVECs in a concentration-dependent manner. Caspase-3 was detected by western blot analysis. The expression of the cleavage of caspase-3 at 24 h was significantly increased as compared with that of 2, 6 and 12 h treatment with *P. gingivalis* HSP60 (10 ng/l) (Fig. 3A), and when HUVECs were treated with *P. gingivalis* HSP60 at 100 ng/l, the expression of the cleavage of caspase-3 at 12 h was significantly increased as compared with that at 2 and 6 h (Fig. 3B). The apoptosis of HUVECs was analyzed by annexin V/FITC staining, as shown in Fig. 3C and D. The percentage of total apoptotic cells was 3.86 ± 0.60 , 5.52 ± 0.82 , 22.99 ± 2.28 and $27.11 \pm 3.87\%$ in cells untreated and cells treated with 1, 10 and 100 ng/l of *P. gingivalis* HSP60, respectively. These results showed that *P. gingivalis* HSP60 (1, 10 and 100 ng/l) stimulation for 24 h significantly increased the apoptotic rate and induced significant apoptosis on HUVECs in a concentration-dependent manner.

Discussion

There is increasing evidence that *P. gingivalis* has a key role in contributing to the progression of atherosclerosis (8-11). According to a recent study, LPS of *P. gingivalis* may induce

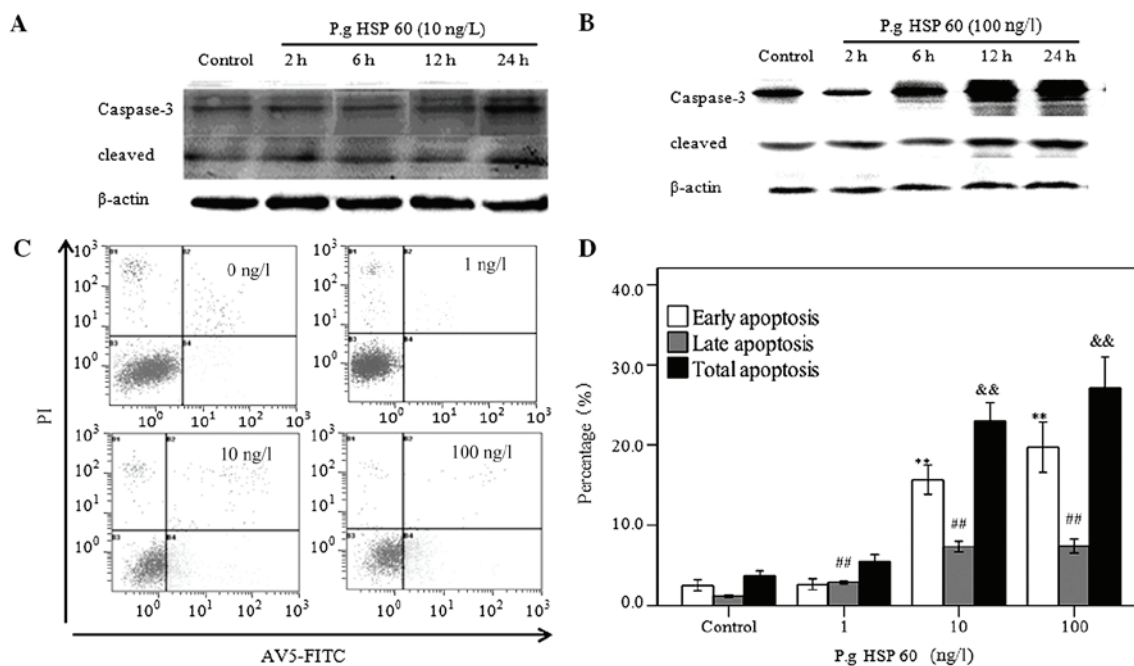


Figure 3. Effects of *P. gingivalis* HSP60 on the protein expression of cleaved caspase-3 protein and apoptosis of HUVECs induced by *P. gingivalis* HSP60. The HUVECs were cultured with *P. gingivalis* HSP60 stimulation (10 and 100 ng/l) at different time-points (2, 6, 12 and 24 h). Effects of *P. gingivalis* HSP60 (10 and 100 ng/l) on protein expression of (A and B) cleaved caspase-3 at different time-points. (C and D) Measurement of apoptotic cells treated with *P. gingivalis* HSP60 (1, 10 and 100 ng/l) for 24 h was performed. Data are shown as mean \pm standard deviation of three independent experiments. * P <0.05 and ** P <0.01 vs. early apoptosis in the control; # P <0.05 and ## P <0.01 vs. late apoptosis in the control; & P <0.05 and && P <0.01 vs. total apoptosis in the control. *P. g.*, *Porphyromonas gingivalis*; HSP60, heat-shock protein 60; HUVECs, human umbilical endothelial cells.

arterial endothelial cell apoptosis and the expression of adhesion molecules in endothelial cells *in vitro*, which may promote atherogenesis (27). Although the *P. gingivalis*-induced dysfunction of HUVECs, including LPS and the immunological mechanism, are well established, little is known regarding the mechanisms involved in *P. gingivalis* HSP60. The association between *P. gingivalis* HSP60 stimulation and HUVEC dysfunction and associated mechanisms are insufficient and require further analysis. The present study assessed the impact of *P. gingivalis* HSP60 on HUVECs. The results proved that co-culture of HUVECs with *P. gingivalis* HSP60 led to decreased viability of HUVECs, as determined by the MTT assay.

As endothelial dysfunction and apoptosis are vital factors in the progression of atherosclerosis, the associated mechanisms of *P. gingivalis* HSP60 on the induction of HUVECs dysfunction and apoptosis were investigated in the present study. The data showed that *P. gingivalis* HSP60 downregulated the expression level of the eNOS protein, which is able to modulate the biologically active gas production of NO. Endothelium-derived NO has an important physiological role in the regulation of vascular tone, and endothelial cell survival and migration (28,29).

Additionally, similar influences of VE-cadherin expression were observed in *P. gingivalis* HSP60-treated HUVECs. VE-cadherin is localized to the adherens junctions, associating with α -catenin, β -catenin, p120-catenin and plakoglobin via its cytoplasmic domains. It has previously been reported that VE-cadherin, as a major regulator of adherens junctions, in particular has an essential role in the regulation of endothelial cell permeability (30-32), migration and assembly of new blood

vessels. Loss of entire VE-cadherin or a lack of its cytoplasmic domain induced endothelial apoptosis and prevented normal vascular development *in vivo* (33). VE-cadherin is an endothelial cell-specific adhesive molecule for the integrity of endothelial cell contacts (31). VE-cadherin has significant functions in the processes of atherosclerosis (33). Thus far, there have been limited studies regarding VE-cadherin expression when HUVECs were treated with *P. gingivalis* HSP60. Information is limited regarding the mechanisms of *P. gingivalis* HSP60-induced atherosclerosis involving the expression of VE-cadherin. The present data identified that VE-cadherin may be one of the factors leading to endothelial dysfunction.

Additionally, the present results showed that *P. gingivalis* HSP60 induced significant apoptosis of HUVECs in a concentration-dependent manner, as shown by the annexin V-FITC/PI assay. Apoptosis is primarily mediated by the activity of caspases. The extrinsic apoptotic pathway involves binding of specific ligands to membrane-bound death receptors, such as Fas/cluster of differentiation 95, which in turn activates caspase-8, facilitating the subsequent activation of terminal effector caspases, such as caspase-3, -6 and -7 (34,35). Thus, caspase-3 is pivotal to the death process. Furthermore, *P. gingivalis* HSP60 was also found to a certain extent to induce HUVECs apoptosis through a mechanism that involved caspase-3 activation. It was proved that apoptosis of VE cells resulted in the loss of endothelial integrity, and was a risk factor of atherosclerosis. Therefore, the present study verified the mechanism of *P. gingivalis* HSP60, which led to atherosclerosis by further accelerating apoptosis in HUVECs.

In conclusion, taken together with the results of other studies, we hypothesize that *P. gingivalis* HSP60 has an essential role in

the dysfunction of HUVECs via the mechanism of regulating eNOS and VE-cadherin expression levels, as well as apoptosis by activating caspase-3 in a unique manner. These findings provide new mechanistic insights into the effect of *P. gingivalis* HSP60 on HUVECs and the associated pathogenesis of cardiovascular disease and periodontitis. Further study is required to determine the pathway of the *P. gingivalis* HSP60-induced decreased expression of VE-cadherin and eNOS.

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