Rho kinase mediates *Porphyromonas gingivalis* outer membrane vesicle-induced suppression of endothelial nitric oxide synthase through ERK1/2 and p38 MAPK

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ARTICLE INFO

ABSTRACT

Objective: To investigate the effect of Rho kinase (ROCK) on *Porphyromonas gingivalis* outer membrane vesicles (OMVs)-induced suppression of endothelial nitric oxide synthase (eNOS) and explore the potential mechanism.

Design: Firstly, we investigated the effect of OMVs on total eNOS expression and eNOS activity in Human Umbilical Vein Endothelial Cells (HUVECs) and if ROCK activation is involved. Furthermore, we estimated the effect of ROCK in regulating eNOS expression and the possible underlying mechanism in vitro. At last we confirmed the results by immunohisochemisty for eNOS expression in mouse aorta endothelium exposed to OMVs and inhibitors.

Results: We found that OMVs suppressed eNOS expression both at RNA and protein levels in a time- and dose-dependent manner. ROCK activity was observed in this process by detecting phosphorylation of myosin light chain (MLC) and myosin-associated phosphatase type 1 (MYPT-1), which lead to reduced eNOS expression. The suppression of eNOS was significantly reversed by ROCK inhibitor Y-27632. Moreover, Y-27632 pretreatment obviously inhibited the activation of ERK1/2 and p38 MAPKs induced by OMVs, whereas that of JNK was not affected. In addition, blocking ERK1/2 or p38 MAPK by PD98059 and SB203580, respectively attenuated the OMVs-induced eNOS phosphorylation. Ex vivo study shows that OMVs reduced eNOS expression in mouse aorta endothelium. Co-treatment with OMVs and inhibitors could significantly reverse the eNOS suppression. Taken together, these results demonstrate that ROCK mediated OMVs-induced eNOS suppression through ERK1/2 and p38 MAPK.

Conclusions: These data suggest that ROCK may mediate OMVs-induced eNOS expression through ERK1/2 and p38 MAPK.

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1. Introduction

Porphyromonas gingivalis (*P. gingivalis*) induces a local chronic host inflammatory response that results in inflammatory bone destruction, which is manifested as periodontal disease[1]. However, mounting evidence has accumulated supporting a role for *P. gingivalis*-mediated periodontal disease as a risk factor for systemic diseases including cardiovascular disease, where several periodontal pathogens have been detected in heart valve lesions and atheromatous plaque[2–5].

The outer membrane vesicles (OMVs), which released by *P. gingivalis* as extracellularly membrane vesicles, retain main virulence constituents including proteins, lipopolysaccharide (LPS), muramic acid, capsule, and fimbriae[6]. OMVs can be internalized into host cells via a lipid-raft-dependent endocytic pathway and subsequently lead to induction of inflammatory pathways. OMVs as well can be found in the blood and cerebrospinal fluid during clinically severe bacterial infections. sera from periodontitis patients had significantly stronger reactivity against an OMVs-producing wild type strain than the isogenic OMVs-depleted strain[6]. It has been proposed that OMVs may contribute to the mortality in clinical diseases[7]. To our knowledge, there are no published reports focused on the effect of OMVs on eNOS expression, which relative to oxidative damage and metabolic dysfunction in endothelial cells.

ROCK is a pivotal downstream effector of RhoA in regulating the actin cytoskeleton by phosphorylation and inhibition of MLCP, which increases MLC phosphorylation and cellular contraction. MYPT-1 is a major downstream effector of ROCK and a regulator of MLC activation for the contraction in cells[8,9]. The levels of MLC and MYPT-1 phosphorylation directly correlate with the level of intracellular ROCK kinase activity[10]. ROCK is involved in mediating diverse cellular functions such as smooth muscle contraction, cell migration and proliferation. Increased ROCK activity is associated with endothelial dysfunction. Its contributory role to vascular inflammation and the atherosclerosis is well studied. It was reported that LPS-induced ROCK activation may directly promote myometrial, thus contributing to preterm labor-mediated preterm birth[11]. However, little is known about how ROCK got involved in the process.

It has been proved that LPS and TNF-α suppress eNOS expression by decreasing the half-life of the eNOS mRNA and lead to reduction of NO level in endothelial cells[12,13]. Although the cross-talk signalling is complex, LPS-induced TNF-α production is known to play a pivotal role in the suppression of eNOS expression via mitogen-activated protein kinases (MAPKs)[14–16]. Since LPS is one of virulence factors in OMVs, whether OMVs share the same pathway as LPS needs to be further investigated.

Our findings indicate that ROCK in HUVECs plays an important role in OMVs-mediated eNOS expression. OMVs-induced ROCK activation is through ERK1/2- and p38 MAPK-dependent mechanism, which is different compared to LPS. This study is instructive for further research on proteomics and functional analysis of *P. gingivalis*-derived OMVs on dysfunction of endothelial cells, which is a main factor for CVD.

2. Materials and methods

2.1. Human umbilical vein endothelial cells isolation and culture

HUVECs were isolated from human umbilical cord veins by collagenase treatment, as described by our previous studies and others[17–19]. Umbilical cords were obtained from placenta donors without health problems. The umbilical vein was cannulated with sterile blunt needles and fixed by clamping the cord over the needles with a sterile string. Then, the vein was filled with 0.1% collagenase (Gibco). The cord was placed in a bottle containing phosphate buffered saline (PBS) and preincubated at 378 C in the water bath. The bottle was incubated for about 15 min at 378 C under 5% CO_2. The collagenase solution containing endothelial cells was flushed from the cord by perfusion with 10 mL of PBS, and was collected in a sterile 10 mL tube. The suspension was centrifuged at 1000 rpm for 8 min. Supernatant was discarded, and endothelial cells were resuspended in 5 mL of high-glucose Dulbecco's modified Eagle's medium culture medium (Gibco) supplemented with 15% fetal calf serum (Gibco) and 100 units/mL of penicillin/streptomycin. The cell suspension was incubated in 25 cm^2 plastic flask (Costar) at 378 C under 5% CO_2. The culture medium was half replaced at 24 h, and then every 2–3 days until cell confluence. Cells used for all experiments were from passages 3 to 5.

2.2. Preparation of P. gingivalis OMVs

Preparation of OMVs was performed from *P. gingivalis* ATCC 33277 as described by others[20,21] with some modification. In brief, the supernatant of a two-day culture of strain 33277 was collected by centrifugation at 4000 × g for 20 min at 4 °C, then filtered through a 0.22 μm PVDF filter and ultra-centrifuged at 100,000 × g for 3 h at 4 °C in a 41 Ti rotor (Beckman Instruments, Inc., USA). The resulting OMVs pellet was re-suspended in 20 mM Tris-Cl (pH 8.0) and the protein concentration was measured by Bradford assay[22] using bovine serum albumin as a standard. Transmission electron microscopy (Philips CM200 EFG, FEI Company, Netherlands) was applied to identify OMVs.

2.3. Polymerase chain reaction (PCR) analysis

Total RNAs were isolated from HUVECs treated with different concentration of OMVs (1, 10, 100, 1000 ng/mL) for different times (1, 4, 8, 12 and 24 h). cDNAs were obtained from 1 μg of total RNAs using a miScript II RT kit. Quantitative real-time PCR (qRT-PCR) was performed with miScript SYBR Green PCR Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. ROCK and eNOS mRNA levels were determined by using iTaqTM SYBR Green Supermix with ROX (BioRad, Hercules, CA) with ABI PRISM 7000 Sequence Detection System (Applied Biosystem) using target-specific primers. The primers for eNOS and β-actin were used as described[23]. PCR reaction conditions of eNOS were as follows: 35 cycles (each cycle consisted of 45 s at 94 °C, 45 s at 60 °C, and 60 s at 72 °C). The fold changes for target gene mRNAs were