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Clathrin and Lipid Raft-dependent Internalization of *Porphyromonas gingivalis* in Endothelial Cells

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Porphyromonas gingivalis **is one of the most important periodontal pathogens and has been to known to invade various types of cells, including endothelial cells. The present study investigated the mechanisms involved in the internalization of *P. gingivalis* in human umbilical vein endothelial cells (HUVEC). *P. gingivalis* internalization was reduced by clathrin and lipid raft inhibitors, as well as a siRNA knockdown of caveolin-1, a principal molecule of lipid raft-related caveolae. The internalization was also reduced by perturbation of actin rearrangement, while microtubule polymerization was not required. Furthermore, we found that Src kinases are critical for the internalization of *P. gingivalis* into HUVEC, while neither Rho family GTPases nor phosphatidylinositol 3-kinase are required. Taken together, this study indicated that *P. gingivalis* internalization into endothelial cells involves clathrin and lipid rafts and requires actin rearrangement associated with Src kinase activation.**

Key words: *Porphyromonas gingivalis*, endothelial cell, bacterial internalization, clathrin, lipid raft

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Introduction

Periodontal diseases are infectious diseases and are associated with the subgingival growth of periodontopathogens. Of the suspected periodontal pathogens, *Porphyromonas gingivalis* has been most consistently and strongly associated with chronic periodontitis [1]. *P. gingivalis* is a gram-negative black pigmented anaerobe that colonizes in periodontal pockets and spreads into deeper tissues. The host response to *P. gingivalis* is evident in increased serum antibody titers in subjects with periodontitis [2]. It is noteworthy that much of the damage in periodontitis is actually the result of the host response to the bacteria, not the result of the bacteria themselves [3]. Therefore, interactions of periodontopathogens with host cells are clearly of fundamental importance.

Bacterial internalization has been reported to play important roles in various infections. *Mycobacterium avium ssp. paratuberculosis*-induced chemokine production is completely internalization-dependent in intestinal epithelial cells [4]. The traversal of blood brain barrier by *Cryptococcus neoformans* is the crucial step in brain infection [5]. Group B *Streptococcus* can modulate immune functions of dendritic cells through internalization [6]. Furthermore, *P. gingivalis* can stimulate endothelial cells to produce monocyte chemoattractant protein-1, where bacterial internalization was required for the chemokine induction [7].

Phagocytes absorb foreign bodies through phagocytosis,

while non-phagocytic cells can absorb them using other mechanisms of endocytosis pathways. Many pathogenic bacteria use a variety of pathways to be internalized into non-phagocytic cells; there are two major internalization pathways, classified by the basis of dependent molecules, which are clathrin-dependent and lipid raft-dependent pathways. Clathrin is a protein forming coated vesicles for small molecules. However, recent evidence indicates that it also participates in for large molecules such as bacteria [8]. Lipid rafts are cholesterol-rich microdomains of host cell membranes and are also involved in bacterial internalization. *P. gingivalis* can invade many cell types including epithelial, endothelial cells, fibroblasts, and vascular smooth muscle cells [9-12]. There have been several studies reporting characteristics of *P. gingivalis* entry into host cells. Typically, *P. gingivalis* internalization is clathrin-dependent in gingival epithelium [9] and lipid raft-dependent in macrophages [12]. Belanger et al. described *P. gingivalis* internalization in endothelial cells as lipid raft-dependent, based on their unpublished observations [13]. However, clear data on cellular mechanisms of *P. gingivalis* internalization into endothelial cells has not been reported.

Eukaryotic cells contain three main kinds of cytoskeletal filaments, which are actin filaments, intermediate filaments, and microtubules. As these filaments support cell membrane, cytoskeletal rearrangement is a prerequisite for bacterial internalization [14]. There are also signaling molecules mediating uptake of bacteria which are specific for different pathogens and/or in different host cell types. The activation of Src familly kinases, Rho family GTPases, and phosphatidylinositol 3-kinase (PI3K) is notably associated with bacterial internalization [15-17].

As *P. gingivalis* internalization into endothelial cells has been proposed as an important mechanism of pathogenesis in periodontal and cardiovascular diseases [18], a better understanding of the internalization process of *P. gingivalis* into endothelial cells is essential. In this context, we investigated the cellular mechanisms of *P. gingivalis* entry into endothelial cells.

Materials and Methods

Reagents

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Jasplakinolide was purchased from Calbiochem (San Diego, CA, USA). PP2 was from AG Scientific (San Diego, CA, USA). Caveolin-1 siRNA and control siRNA were purchased from Santa Cruz Biotechnology (Dallas, TX, USA)

P. gingivalis culture

P. gingivalis 381 was grown in Trypticase soy broth supplemented with yeast extract (1 mg/ml), hemin (5 μg/ml), and menadione (1 μg/ml), and incubated anaerobically (85% N_2, 10% H_2, and 5% CO_2) at 37℃. Bacteria in logarithmic growth phase were used in all experiments.

Human endothelial cell culture

Primary human umbilical vein endothelial cells (HUVEC) were purchased from Cascade Biologics (Carlsbad, CA, USA) and used between passages 3–7. The cells were cultured in Medium 200 (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco), and 50 μg/ml gentamicin at 37℃ in 5% CO_2.

Antibiotic protection assay

The extent of internalization of *P. gingivalis* was determined by conventional antibiotic protection assay. HUVEC were seeded at a density of 1 X 10^5 cells per well in 12-well plates and incubated overnight. The next day, the cells were treated with inhibitors for 30 min. *P. gingivalis* were added at a multiplicity of infection (MOI) of 100. After 2 h of infection, HUVEC were washed 3 times with Medium 200, and then treated with gentamicin (300 μg/ml) and metronidazole (200 μg/ml) for 1 h to kill the bacteria remained outside HUVEC. The cells were washed 3 times with Medium 200 and finally lysed by 1 ml of distilled water for 30 min and bacterial viable counting was performed. For enumeration of bacteria for invasion plus adhesion, all the procedures of the assay were followed except the antibiotics treatment. The numbers of adherent bacteria were calculated by subtracting invasion from invasion plus adhesion.

siRNA transfection

HUVEC were seeded at a density of 1 X 10^5 cells per well in 12-well plates the day prior to transfection. Treatment of caveolin-1 siRNA and control siRNA was done according to the manufacturer’s protocol. Briefly, 40 nmol siRNA was transfected into HUVEC, using Lipofectamine RNAiMAX transfection reagent from Invitrogen (Carlsbad, CA, USA).

After 6 h, the cultures were replaced with fresh medium and grown for 48 h. Then the cells were washed with fresh medium before the experiments. One set of cell groups was used to determine the caveolin-1 mRNA level by RT-PCR and another set of groups was used for antibiotic protection assay. The sequences of primers were 5'-GGGCAACATCTACAAGCCC AACAA-3', 5'-CTGATGCACTGAATCTCAATCAGGAA-3' for caveolin-1 (372 bp); and 5'-AGCGGGAAATCGTGCGTG-3', 5'-CAGGGTACATGGTGGTGCC-3' for β-actin (300 bp). RT-PCR was done as described previously [19].

Statistical analysis.

All Data are expressed as mean SEM. Statistical analysis of one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons test was performed using GraphPad InStat (GraphPad Software, La Jolla, CA, USA). Statistical significance was marked as *, ** and *** indicating $p<0.05$, $P<0.01$, and $P<0.001$, respectively, relative to control. All experiments were repeated at least three times.

Results

Clathrin is involved in *P. gingivalis* internalization into HUVEC

To determine if clathrin is involved in *P. gingivalis* internalization into endothelial cells, we treated HUVEC with chlorpromazine, a clathrin inhibitor. Chlorpromazine blocks recycling of clathrin and adaptor protein AP2 fromthe endosome. Our results showed that pretreatment of HUVEC with chlorpromazine significantly reduced the invasion of *P. gingivalis* in a dose-dependent manner (Fig. 1A), but the inhibitor did not affect *P. gingivalis* adhesion onto HUVEC (Fig. 1B).

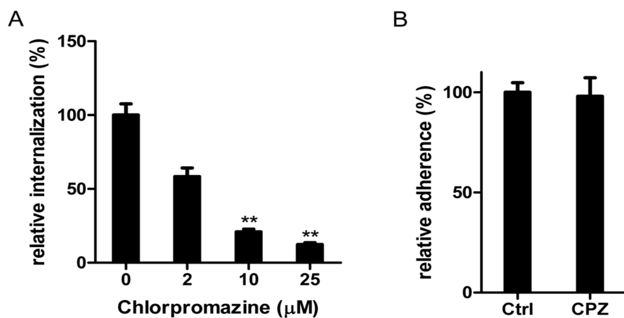


Fig. 1. Clathrin is involved in *P. gingivalis* internalization into HUVEC. (A) Numbers of *P. gingivalis* internalized into HUVEC were determined by antibiotic protection assays in the presence or absence of chlorpromazine (2, 10, and 25 μM). The internalization of *P. gingivalis* into HUVEC in the absence of the inhibitor was set to 100%. (B) Numbers of *P. gingivalis* adhered to HUVEC were also determined in the presence or absence of chlorpromazine (25 μM).

Lipid rafts/caveolin-1 are also involved in *P. gingivalis* internalization

To investigate if *P. gingivalis* uses lipid rafts for its internalization into HUVEC, we treated HUVEC with lipid

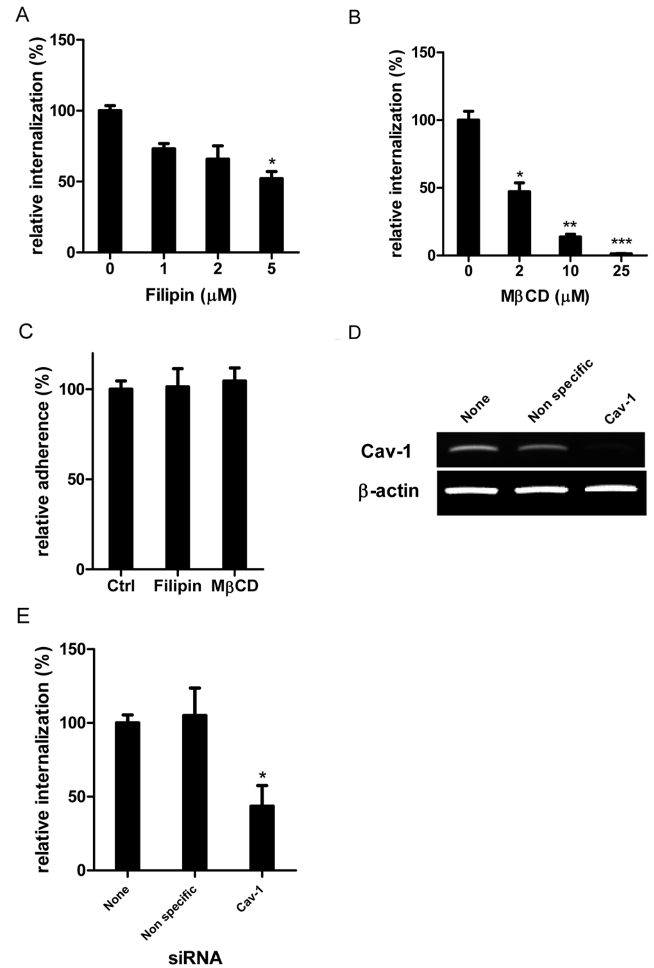
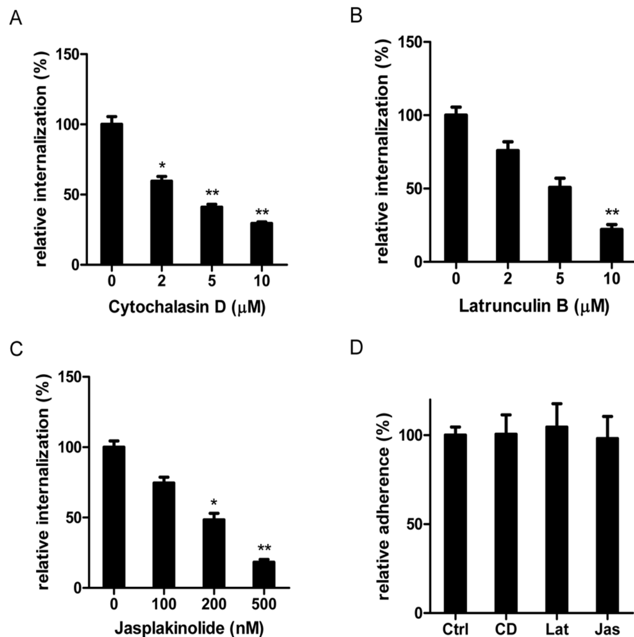


Fig. 2. Lipid rafts/caveolin-1 are involved in *P. gingivalis* internalization into HUVEC. (A-B) Numbers of *P. gingivalis* internalization into HUVEC were determined by antibiotic protection assays in the presence or absence of lipid raft inhibitors, filipin (1, 2, and 5 μM) and MβCD (2, 10, and 25 μM). The internalization of *P. gingivalis* into HUVEC in the absence of the inhibitor was set to 100%. (C) Numbers of *P. gingivalis* adhered to HUVEC were also determined in the presence or absence of lipid raft inhibitors, filipin (5 μM) and MβCD (5 μM). (D) Knockdown of caveolin-1 mRNA by siRNA treatment was confirmed by RT-PCR. (E) Numbers of *P. gingivalis* internalized into HUVEC were also determined after caveolin-1 siRNA treatment.

raft inhibitors, filipin and methyl-β-cyclodextrin (MβCD). The results showed that both MβCD and filipin significantly reduced the internalization of *P. gingivalis* (Fig. 2A and B). The inhibitors did not affect *P. gingivalis* adhesion onto HUVEC (Fig. 2C). Among several types of lipid rafts, caveolae are a special type of lipid rafts and characterized by the principal protein caveolin-1. We compared normal cells and caveolin-1 siRNA transfected cells. Caveolin-1 was successfully knocked down in HUVEC (Fig. 2D), and the transfected cells showed reduced *P. gingivalis* internalization (Fig. 2E).

Actin reorganization is required for *P. gingivalis* internalization into HUVEC

To determine if the internalization of *P. gingivalis* into endothelial cells involves actin rearrangement, we treated HUVEC with three types of actin polymerization inhibitors (cytochalasin D, latrunculin B, and jasplakinolide). The results showed that pretreatment of HUVEC with each inhibitor significantly reduced the internalization of *P. gingivalis* in a dose-dependent manner (Fig. 3A-C). The inhibitors did not affect bacterial adhesion (Fig. 3D). Microtubules might play a role in bacterial internalization. To examine the involvement of microtubules in *P. gingivalis* invasion into HUVEC, we treated HUVEC with two different microtubule inhibitors (nocodazole and colchicine). Neither inhibitors affected *P. gingivalis* invasion (Fig. 3E and F).



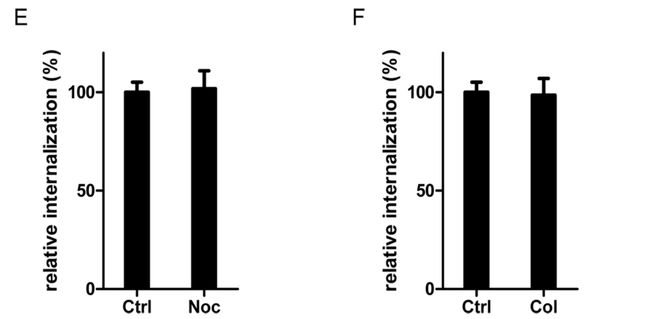


Fig. 3. Actin polymerization is required for *P. gingivalis* internalization into HUVEC. (A-C) Numbers of *P. gingivalis* internalized into HUVEC was determined by antibiotic protection assays in the presence or absence of actin polymerization inhibitors, cytochalasin D (2, 5, and 10 μM), latrunculin B (2, 5, and 10 μM), or jasplakinolide (100, 200, and 500 nM). The internalization of *P. gingivalis* into HUVEC in the absence of the inhibitor was set to 100%. (D) Numbers of *P. gingivalis* adhered to HUVEC were also determined in the presence or absence of actin polymerization inhibitors, cytochalasin D (CD, 10 μM), latrunculin B (Lat, 10 μM), and Jasplakinolide (Jas, 500 nM). (E and F) Numbers of *P. gingivalis* internalized into HUVEC were determined in the presence or absence of microtubule inhibitors, nocodazole (Noc, 10 μM) and colchicine (Col, 10 μM).

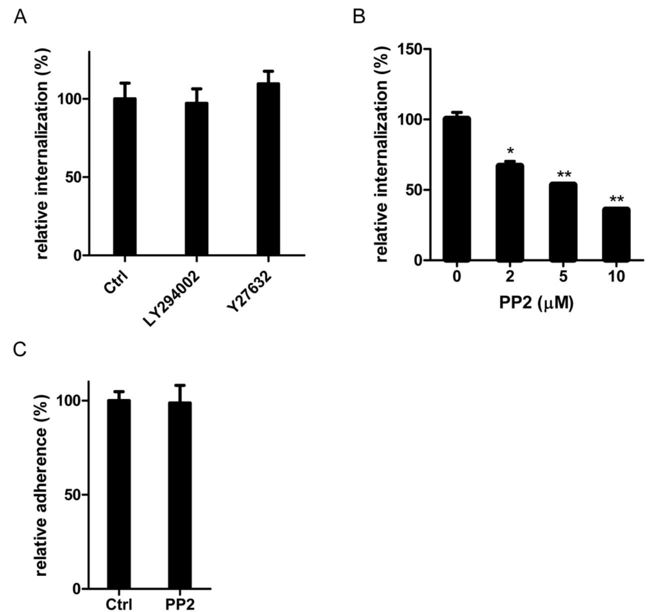


Fig 4. Src family kinase activation is required for *P. gingivalis* internalization into HUVEC. (A) Numbers of *P. gingivalis* internalized into HUVEC were determined by antibiotic protection assays in the presence or absence of Rho GTPases inhibitor, Y27632 (5 μM) and PI3K inhibitor, LY294002 (50 μ M). The internalization of *P. gingivalis* into HUVEC in the absence of the inhibitor was set to 100%. (B) Numbers of *P. gingivalis* internalized into HUVEC were determined in the presence or absence of Src kinase inhibitor PP2 (2, 5, and 10 μ M). (C) Numbers of *P. gingivalis* adhered to HUVEC were also determined in the presence or absence of PP2 (10 μM).

Src family kinase activation is required for *P. gingivalis* internalization

The Rho family of small GTPases, PI3K, and Src kinases have been known to play key roles for internalization of different pathogens. By using specific inhibitors, we investigated whether these enzymes are required for *P. gingivalis* internalization into endothelial cells. The results showed that the Rho GTPases inhibitor, Y27632 or the PI3K inhibitor, LY294002, had no effect on *P. gingivalis* internalization into HUVEC (Fig. 4A). In contrast, the Src kinase inhibitor, PP2, significantly reduced *P. gingivalis* internalization into HUVEC (Fig. 4B). *P. gingivalis* adherence onto HUVEC was not affected by PP2 (Fig. 4C).

Discussion

Investigation of cellular mechanisms governing *P. gingivalis* internalization into host cells is of considerable importance to understand the basis of invasive *P. gingivalis*-associated diseases. Our study demonstrated that *P. gingivalis* exploits both clathrin- and lipid raft-dependent pathways to enter endothelial cells. By using both clathrin-mediated and lipid raft-mediated processes, *P. gingivalis* should effectively invade endothelial cells. Efficient internalization is beneficial for *P. gingivalis* from the aspects of nutrient acquisition and immune evasion. Furthermore, as the two pathways are separate systems, in case one system is not working well, the other system will still operate for *P. gingivalis* internalization. Caveolae are a special type of lipid rafts. We demonstrated that perturbation of caveolin-1 function by siRNA knockdown substantially reduced *P. gingivalis* internalization into endothelial cells. Two studies reported different results regarding the effect of caveolin-1 knockdown on *P. gingivalis* invasion in epithelial cells: One showed significant reduction of *P. gingivalis* invasion and the other showed only a minor effect [9,20].

The involvement of actin cytoskeleton in *P. gingivalis* internalization into endothelial cells was demonstrated in this study. The internalization of *P. gingivalis* was substantially reduced by three different types of inhibitors of actin rearrangement. Cytochalasin D prevents actin polymerization and latrunculin B binds and sequesters actin monomers. Jasplakinolide promotes and stabilizes the assembly of actin filaments, interfering with actin treadmilling [21]. Another component of cytoskeleton is microtubules, but reorganization of which was not required for *P. gingivalis* internalization into HUVEC. Although *P. gingivalis* entry into host cells has been reported to require polymerization of both microfilaments and microtubules in epithelial cells [22,23], involvement of microtubules in *P. gingivalis* entry into endothelial cells has not been known. Our results suggest that *P. gingivalis* can invade endothelial cells even when actions of microtubules are blocked. Further study is needed to clearly explain the role of microtubules in *P. gingivalis* internalization in various cell types.

Previous studies demonstrated the involvement of various signaling molecules in bacterial internalization. Src kinase, PI3K, and Rho family GTPases including cdc42, rhoA are most frequently engaged signals in internalization of a variety of bacterial species. The role of PI3K for bacterial internalization appeared cell type-specific [15-17,24,25]. Our results showed that PI3K is not required for *P. gingivalis* internalization into endothelial cells. This is in accordance with the PI3K-independency of M3 streptococci invasion in HUVEC [16]. An interesting finding in this study is the implication of Src kinase activation in *P. gingivalis* internalization into endothelial cells. Src kinases are critical signal transducers modulating a wide variety of cellular functions. It has been shown that activation of Src kinases is important for bacterial invasion in various settings [16,26]. Nonetheless, no information is currently available concerning the role of Src kinases in *P. gingivalis* internalization into endothelial cells. Here we showed for the first time that Src kinases are key signal transducers involved in *P. gingivalis* internalization into endothelial cells. Our future study will be directed to the downstream molecules of Src kinases such as cortactin, an actin-binding protein [27].

To summarize, the present study indicated that *P. gingivalis* internalization into human endothelial cells is clathrin- and lipid raft/caveolae-dependent, and that in the process of *P. gingivalis* internalization, activation of Src kinases rather than PI3K and Rho family GTPases is required for actin rearrangement.

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Institute.

Conflict of interest

The authors declare that they have no competing interest.

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