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Involvement of clathrin and β-arrestins in *Aggregatibacter actinomycetemcomitans* endocytosis of human vascular endothelial cells

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Phosphorylcholine (PC) has been reported to help PC-bearing *Aggregatibacter actinomycetemcomitans* to gain access to the general circulation via interaction with the platelet-activating factor receptor (PAFR) on endothelial cells. However, the underlying mechanism remains unclear. Here, we found that inhibition of clathrin and β-arrestins blocked bacterial PC binding to PAFR which significantly attenuated *A. actinomycetemcomitans* invasion of human vascular endothelial cells (HUVEC). These results demonstrated the involvement of clathrin and β-arrestins in PC-bearing *A. actinomycetemcomitans* invasion of HUVEC.

Key words: *Aggregatibacter actinomycetemcomitans,* clathrin, β-arrestins, human vascular endothelial cells.

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a significant periodontal pathogen implicated in aggressive periodontitis and adult periodontitis (Asikainen et al., 1997; Meyer and Fives-Taylor, 1997; Zambon, 1985). This Gram-negative bacterium has been shown to adhere and penetrate epithelial and endothelial cells (Meyer et al., 1996; Schenkein etal., 2000).

Phosphorylcholine (PC) has been reported as a virulence factor in a number of pathogenic prokaryotes, including Gram-positive bacteria *Streptococcus pneumoniae* and the Gram-negative species *Haemophilus influenzae* (Harnett and Harnett, 1999). PC not only plays an important role in *S. pneumoniae* adherence and invasion of host cells (Cundell et al., 1995), but also enhances *H. influenzae* biofilm maturation (Hong et al., 2007). Recent studies have identified *A. actinomycetemcomitans* as

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Abbreviations: PC, Phosphorylcholine; **PAFR,** platelet-activating factor receptor; **HUVEC,** human vascular endothelial cells; **GPCRs,** G proteins coupled receptors; **MDC,** monodansylcadaverine; **CPZ,** chlorpromazine; **MOI,** multiplicity of infection.

Table 1. Plasmids of β-arrestin-1/-2 siRNA.

Plasmid	Sequence
ARRB1-3	5'- GGATCCTGAGTATCTCAAAGATTCAAGAGATCTTTGAGATACTCAGGATCCTT -3'
ARRB2-1	5'- GGACCAGGGTCTTCAAGAAGTTTCAAGAGAACTTCTTGAAGACCCTGGTCCTT -3'
ARRB-NC	5'- GTTCTCCGAACGTGTCACGT *CAAGAGATT* ACGTGACACGTTCGGAGAA TT -3'

Table 2. Primers used in real time PCR.

Primer	Sequence	Amplified region (bp)
ARRB1-F	5'-ACACGGGTGTATTACATTCGG-3'	88
ARRB1-R	5'-CCAGTGAGGTGGGAAGAGC-3'	
ARRB2-F	5'-CCCTCAATGTAAATGTCCACG-3'	86
ARRB2-R	5'-GATGTCGGCGTACTGTCTCAC-3'	
β-actin-F	5'-CCCTGGCACCCAGCAC-3'	70
β-actin-R	5'-GCCGATCCACACGGAGTAC-3'	

variable in reaction with PC specific monoclonal antibodies, TEPC-15 (Schenkein et al., 2000; Purkall et al., 2002). PC helps PC-bearing *A. actinomycetemcomitans* to invade human vascular endothelial cells via the platelet-activating factor receptor (PAFR) as well as *S. pneumoniae* and *H. influenza* (Schenkein etal., 2000; Cundell et al., 1995; Swords et al., 2000).

As typical of G proteins coupled receptors (GPCRs), PAFR activation by PC could be regulated by β-arrestins (McLaughlin et al., 2006; Eckels et al., 2009). β-Arrestins could bind to activated GPCRs and clathrin to promote the clathrin-mediated endocytosis and transcytosis (Shenoy and Lefkowitz, 2011; Gyombolai et al., 2013). The role of β-arrestins in inflammation process has been well known by regulation of chemokine responsiveness (Vroon et al., 2006; Cattaruzza et al., 2013; Porter et al., 2010). It has been reported that clathrin and β-arrestin-1 could mediate *S. pneumonia* and *Acinetobacter baumannii* invasion of epithelial cells (Radin et al., 2005; Smani et al., 2012). However, there are no studies about clathrin and β-arrestins in *A. actinomycetemcomitans* internalization by epithelial or endothelial cells. In this work, we aimed to study whether clathrins and β-arrestins participated in endocytosis of *A. actinomycetemcomitans*.

MATERIALS AND METHODS

Bacterial strains and media

A. actinomycetemcomitans strains HK1651 and ATCC29523 were purchased from ATCC Bioresource Center (USA). HK1651 was identified as PC-positive strain and ATCC29523 was PC-negative strain before. The brain heart infusion medium was used as the liquid medium and Tryptic soy agar (TSA) was used as the solid medium (Huankai Microbial Sci. &Tech. Co. Let, Guangdong, China). The *A. actinomycetemcomitans* strains were grown in humidified 5% CO_2 atmosphere at 37°C.

Cell culture

The human umbilical vein endothelial cells (HUVEC) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). They were cultured in DMEM medium, supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA). Cells were incubated at 37°C, 5% CO_2, 95% air humidified atmosphere.

Plasmids and transfection

The double-stranded siRNAs for β -arrestin-1 and β -arrestin-2 were chemically synthesized and sequenced by Shanghai GenePharma Co., Ltd. The sequences of β -arrestin-1 and β -arrestin-2 siRNAs used in this study Are listed in Table 1. HUVEC was transfected with the plasmids using Lipofectamine™ LTX and PLUS™ Reagent (Invitrogen, USA) according to the manufacture's instruction. Briefly, 0.5 μg of DNA in 100 μl of Opti-MEM® I Reduced Serum Medium (Hyclone, USA) without serum were mixed with 1.25 μl of Lipofectamine™ LTX and 0.5 μl PLUS™ Reagent. The mixture was then added to the wells containing HUVEC at 37°C for 24 h. After the transfection, the cells were observed under an inverted fluorescence microscope and the real-time reverse-transcription PCR was used to examine the β -arrestin-1 and β -arrestin-2 expression.

Real-time reverse-transcription PCR

The total cellular RNA was extracted with TRIzol® (TaKaRa Biotechnology Co., Ltd.) and reverse transcribed into cDNA using the ExScript RT Reagent Kit (TaKaRa Biotechnology Co., Ltd.). Real-time PCR was performed by employing the SYBR® Premix Ex Taq™ Kit (TaKaRa Biotechnology Co., Ltd.) and was run on the ABI 7500 Real-Time PCR System (Applied Biosystems, CA, USA). The β-actin was used as an internal control. Primers used in this study are listed in Table 2. The thermal cycling was initiated with a first denaturation step of 30 s at 95°C, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s and 72°C for 90 s. Melting curve analysis was performed and concentration values were measured. The detected cycle threshold (Ct) values were interpolated into the standard curves of the plasmid constructs, and the mRNA expression in the samples was calculated by $2^{-\triangle\triangle Ct}$.

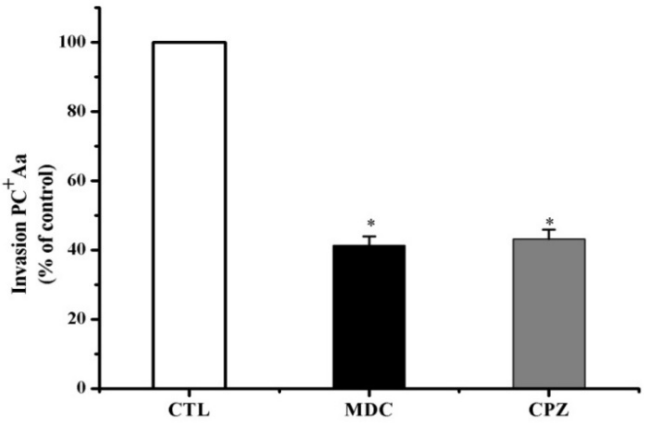

Figure 1. Clathrin inhibitors block PC positive *A. actinomycetemcomitans* invasion of HUVEC. Confluent HUVEC monolayers on the 24-well plate were treated with the indicated concentrations of MDC and CPZ for 30 min before addition of the bacteria. Results are presented as relative invasion of PC positive *A. actinomycetemcomitans* (%). Data are means ± standard deviation of five independent experiments done in triplicate (*P<0.05).

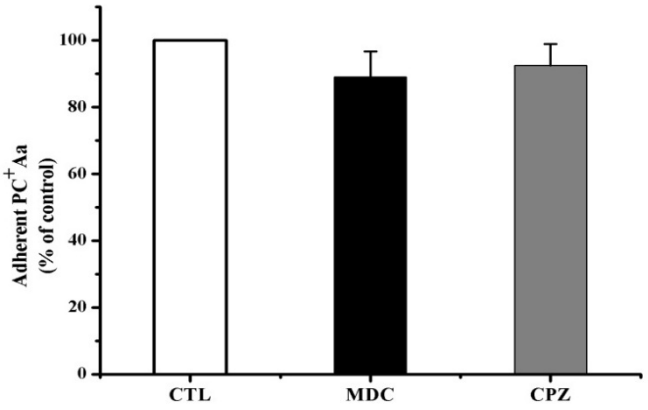

Figure 2. Relative adhesion of PC positive *A. actinomycetemcomitans* (%) after clathrin inhibitors were blocked. The adhesion examination was carried out as described earlier. Data are means ± standard deviation (P<0.05).The adhesion showed no significant difference between pretreated and unpretreated groups.

Bacterial invasion and adhesion assays

To determine the role of clathrin in *A. actinomycetemcomitans* internalization by HUVEC, the cells were pretreated with monodansylcadaverine (MDC) or chlorpromazine (CPZ) for 30 min prior to infection with PC positive and negative *A. actinomycetemcomitans* strains (OD650=0.9) at a multiplicity of infection (MOI) of 100. After 4 h incubation at 37°C, the invasion assay was performed as described by Schenkein et al. (2000).

Briefly, the monolayers were incubated with gentamicin (50 mg/ml) for 2 h to kill bacteria external to the HUVEC. Then the monolayers were lysed and bacteria were plated to enumerate total cell-associated bacteria. For adhesion assay, the number of total cell-associated bacteria was determined as above without the gentamicin step.

To study the role of β-arrestins in *A. actinomycetemcomitans* internalization by HUVEC, PC positive and negative *A. actinomycetemcomitans* strains were added to the confluent monolayers of HUVEC transfected or not with siRNA of β-arrestin-1, β-arrestin-2 and control. The adhesion and internalization assays were done as described above.

Statistical analysis

The group data are presented as mean ± S.D. A student *t*-test was used to determine the differences between means. The difference was considered significant at $P < 0.05$. The SPSS (version 13.0) statistical package was used (SPSS Inc., Chicago, IL).

RESULTS

Clathrin role in *A. actinomycetemcomitans* internalization by HUVEC

To investigate whether the clathrin was involved in the *A. actinomycetemcomitans* endocytosis process during *A. actinomycetemcomitans* invading HUVEC, the impact of clathrin inhibitors, monodansylcadaverine (MDC) and chlorpromazine (CPZ) were studied in *A. actinomycetemcomitans* adhesion and invasion of HUVEC process. It was found that MDC and CPZ effectively inhibited the PC positive *A. actinomycetemcomitans* invasion of HUVEC to 41.33 ± 2.59% and 43.22 ± 2.67% of the control (Figure 1), respectively.

However, there was no significant difference between the MDC or CPZ pretreated and nonpretreated groups on adherence (Figure 2), which showed that the MDC and CPZ inhibition of clathrin could not stop *A. actinomycetemcomitans* adhesion to HUVEC.

β-Arrestins role in *A. actinomycetemcomitans* internalization by HUVEC

To study the role of β-arrestins in the process of *A. actinomycetemcomitans* adhesion and invasion of HUVEC, double-stranded siRNAs for β-arrestin-1, β-arrestin-2 and control were chemically synthesized and transfected in HUVEC, and then the effects on *A. actinomycetemcomitans* adhesion and invasion of HUVEC were investigated. First, it was detected that intracellular level of β-arrestins was targetedly reduced after transfection when compared with untransfected group and negative control group (Figures 3 and 4). Then the invasion of PC positive *A. actinomycetemcomitans* into HUVEC was tested and it was 53.12 ± 2.81 and 56.00 ± 2.37% of the control (Figure 5), respectively. The invasion was effectively blocked by β-arrestins siRNA.

However, there was no significant difference in the number of bacteria adhered to the cells (Figure 6), which indicated that β-arrestins siRNA could not inhibit the PC binding to PAFR on the cell surface.

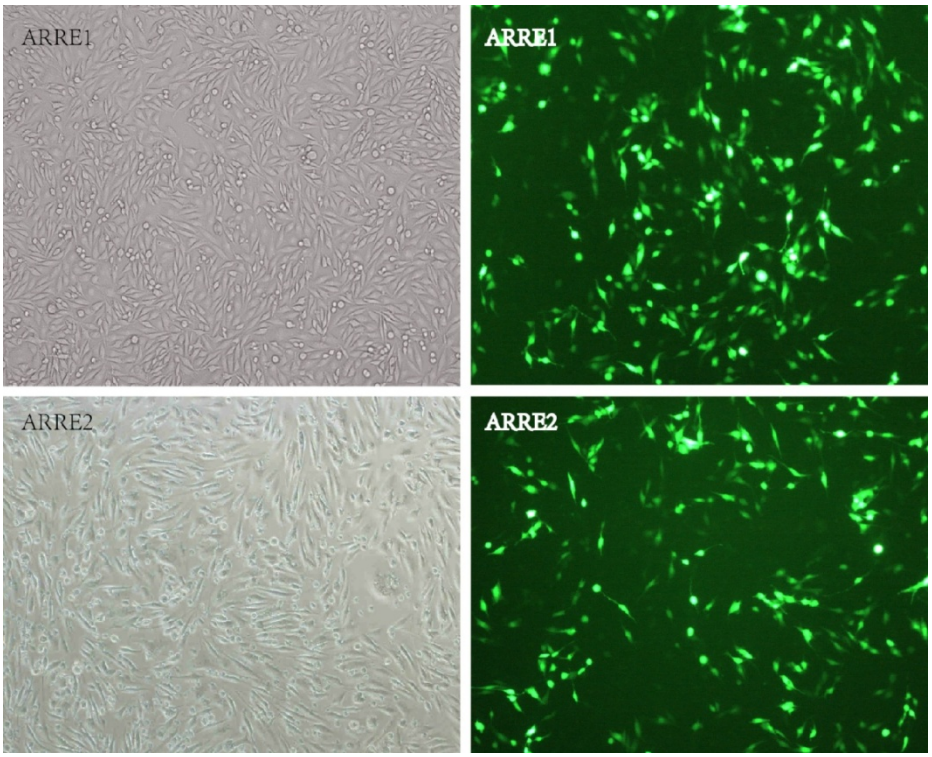

Figure 3. Cell transfection of β-arrestin-1/-2 siRNA observed under a fluorescence microscope (40×). Transfected cells showed a green fluorescence.

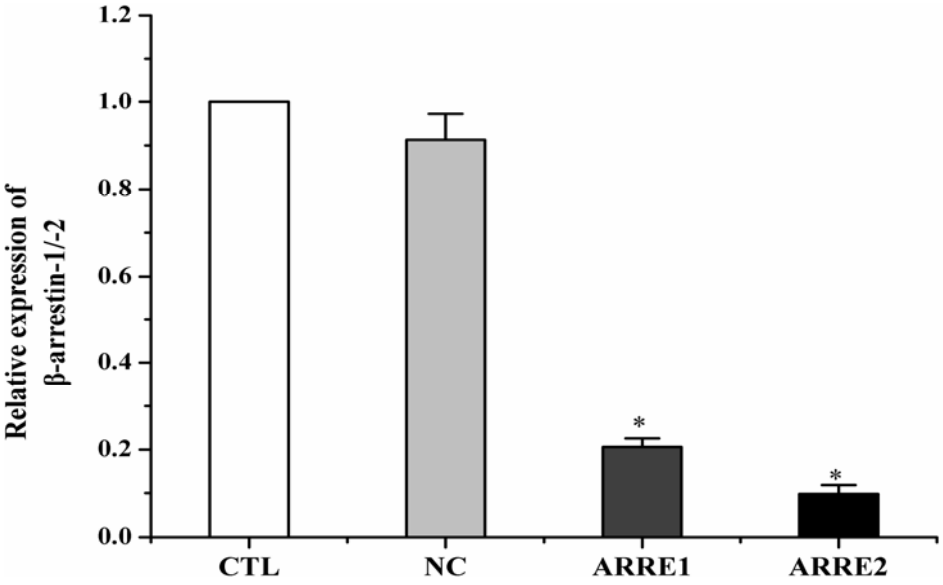

Figure 4. Real-time reverse-transcription PCR was used to detect β-arrestin-1 mRNA expression in HUVEC as described in the 'materials and methods' section. Values shown are expressed as percent of level of each siRNA of β-arrestin in control-transfected HUVEC. Error bars indicate standard deviations (*P<0.05).

Besides, we also examined the effect of clathrin and β-arrestins in PC negative *A. actinomycetemcomitans* groups, there was no significant difference in the adhesion and invasion of HUVEC between PC negative

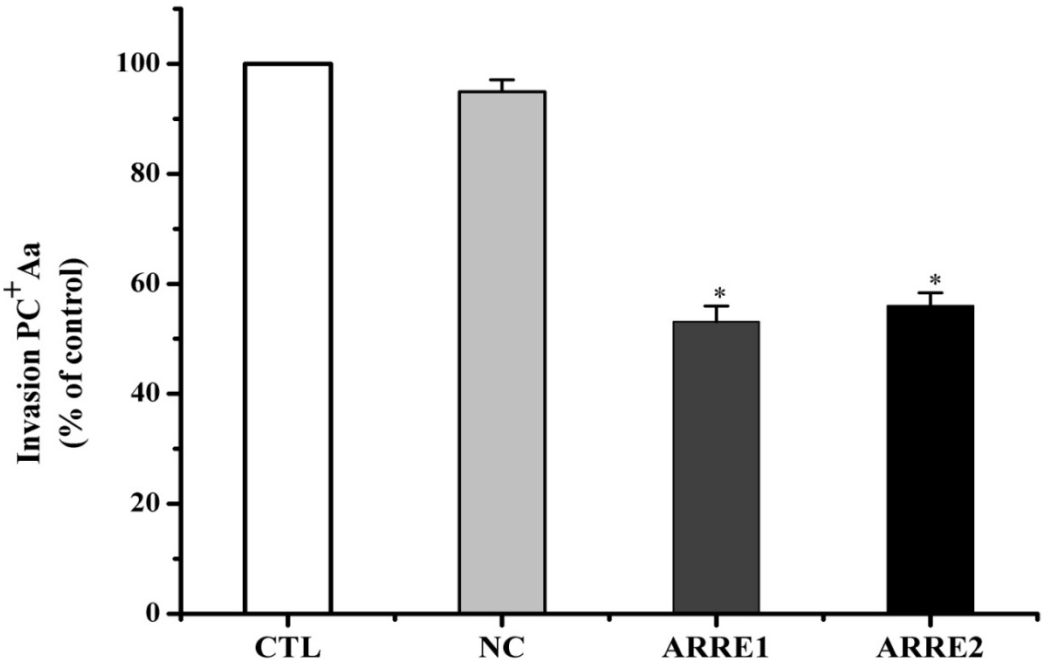

Figure 5. β-Arrestin-1/-2 siRNA block PC positive *A. actinomycetemcomitans* invasion of HUVEC. The relative invasion of PC positive *A. actinomycetemcomitans* (%) was examined as described earlier. Data are means ± standard deviation (*P<0.05). CTL: Group without cell transfection; NC: Negative control group.

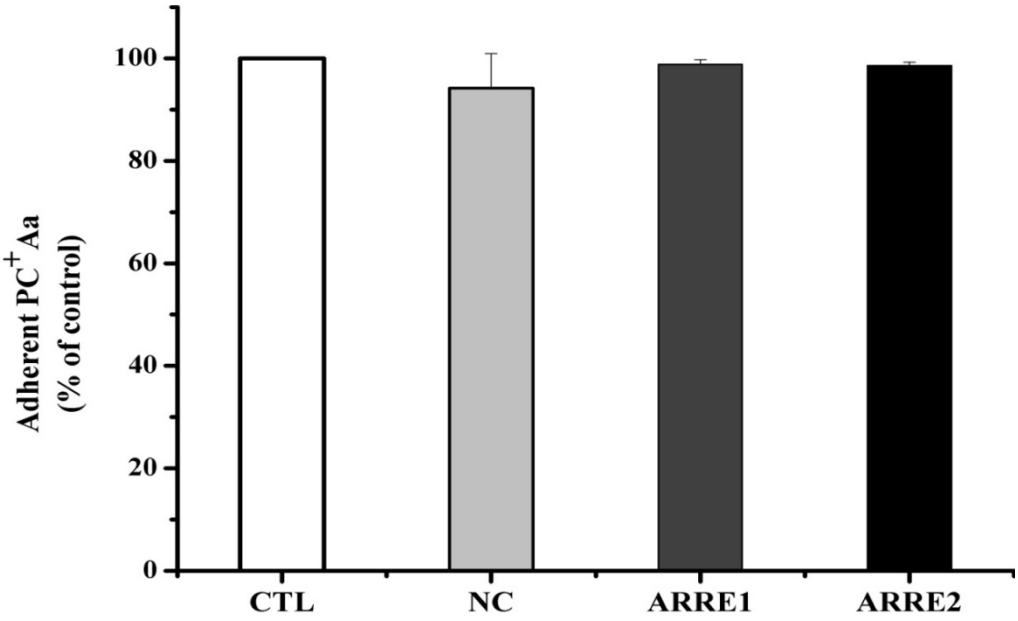

Figure 6. Relative adhesion of PC positive *A. actinomycetemcomitans* (%) after β-arrestin-1/-2 siRNA trasfected into HUVEC. The adhesion examination was carried out as described before (P<0.05). The adhesion showed no significant difference between transfected and untransfected groups.

groups (data not shown).

DISCUSSION

In the present study, we showed new evidences of the mechanism involved in adherence and invasion in human vascular endothelial cells by *A. actinomycetemcomitans* bearing PC. We demonstrated here that clathrin and β-arrestins participated in *A. actinomycetemcomitans* invasion of host cells.

Previous data have extensively indicated that *A. actinomycetemcomitans* interacts with host cells via a mechanism of specific receptors (Meyer et al., 1997). Schenken and coworkers had reported that PAFR had participated in the invading HUVEC by *A. actinomycetemcomitans* bearing PC (Schenkein et al., 2000; Purkall et al., 2002). Clathrin and β-arrestins have been shown to play important roles in regulating PAFR during bacterial endocytosis. *S. pneumoniae* required the scaffold function of the β-arrestins to recruit clathrin in PAFR-mediated endocytosis by endothelial cells (Radin et al., 2005). Besides, Smani et al. (2012) reported that clathrin and β-arrestin-1 and 2 are involved in *A. baumannii* invasion of lung epithelial cells. In this study, we found that clathrin inhibitors MDC and CPZ significantly inhibited *A. actinomycetemcomitans* bearing PC invasion of HUVEC. Additionally, β-arrestin-1 and - 2 siRNA prevented *A. actinomycetemcomitans* internalization of HUVEC. These findings are the first report for clathrin and β-arrestin-1 and - 2 that participated in *A. actinomycetemcomitans* invasion of endothelial cells.

Conclusion

In summary, we proposed that PC-positive *A. actinomycetemcomitans* may invade endothelial cells via a mechanism dependent upon the involvement of clathrin and β-arrestins in mediating the PAFR activation by bacterial PC. However, β-arrestins-mediated receptor internalization can also regulate signal transduction by activating signaling proteins such as ERK1/2, p38 MAPK and JNK (Vroon et al., 2006), which also take part in *A. actinomycetemcomitans* invasion of host cells. Therefore, further studies are needed to demonstrate these other mechanisms involved in β-arrestins-mediated infection by *A. actinomycetemcomitans* bearing PC.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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