**Chlamydia pneumoniae** infection induces vascular smooth muscle cell migration via Rac1 activation

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**INTRODUCTION**

*Chlamydia pneumoniae* is a Gram-negative, obligate intracellular bacterium that mainly causes respiratory tract infections. There is increasing evidence indicating that chronic or recurrent *C. pneumoniae* infection is strongly associated with the development of atherosclerosis and coronary artery diseases (Liu et al., 2005; Chen et al., 2010b; Luque et al., 2012). *C. pneumoniae* can infect several types of cells, such as circulating macrophages, vascular smooth muscle cells (VSMCs) and endothelial cells (Dechend et al., 1999; Gaydos et al., 1996). Our previous study demonstrated that *C. pneumoniae* can infect VSMCs and directly promote VSMC migration (Zhang et al., 2012), which is considered as a key event in the atherogenic process. Secretion of some adhesion molecules and inflammatory cytokines stimulated by *C. pneumoniae* infection has been shown to enhance the migration of atherosclerosis-related cells (Uriarte et al., 2004; MacIntyre et al., 2003). However, whether *C. pneumoniae* infection directly regulates cytoskeletal dynamics to induce VSMC migration is not fully understood. Therefore, elucidation of the alterations in the activity of the intracellular cytoskeletal regulatory proteins may reveal a novel mechanism for *C. pneumoniae* infection-induced VSMC migration.

To initiate motility, cells rearrange their actin cytoskeleton to form a thin protruding sheet (the lamellipod) with filaments oriented towards the membrane (Verkhovsky et al., 2003), thereby impinging on and pushing the leading edge forward. Ras-related C3 botulinum toxin substrate 1 (Rac1), a member of the Rho family of small GTPases, is a key regulator of the actin cytoskeletal remodelling, especially lamellipodia formation, and plays a pivotal role in a variety of processes, such as morphogenesis, neuronal development, cell adhesion and movement (Migotte et al., 2011; Chen et al., 2010a; Maddal a et al., 2011). It has recently been demonstrated that leucine-rich repeat and fibronectin type III domain-containing protein 4 (LRNF4) functions in monocyte cell elongation via Rac1-mediated actin cytoskeleton reorganization (Konakahara et al., 2012). Kumerz et al. (2011) also found that resveratrol can inhibit Rac1 activation and lamellipodia formation in response to epidermal growth factor (EGF). Reports also showed that...
Rac1 activation was deregulated in several cancer cells, which was associated with their migration and invasion (Akunuru et al., 2011; Makrodouli et al., 2011). Eitel et al. (2012) reported that Rac1 is activated in *C. pneumoniae*-infected mononuclear cells. However, the role of Rac1 activation in *C. pneumoniae* infection-induced VSMC migration is not well understood.

Rac1, cycling between a GDP-bound state (inactive form) and a GTP-bound state (active form), acts as a molecular switch in multiple signal transduction pathways to translate signals into series of intracellular events that ultimately regulate the activities of cytoskeletal proteins and actin assembly (Raftopoulou & Hall, 2004). However, how signals are transmitted to Rac1 and how Rac1 receives stimuli to generate changes are not yet well defined. Phosphatidylinositol 3-kinase (PI3K), another important intracellular enzyme, is widely involved in controlling cell polarity and migration (Welf et al., 2012). It has been reported that PI3K signalling may participate in activation of the Rho family GTPases Rac1 and Cdc42, leading to the initiation of actin polymerization (Dawes & Edelstein-Keshet, 2007). Furthermore, PI3K inhibitors can block Rac1 activation, subsequently inhibiting hyaluronan-induced VSMC migration and ephrin-1-stimulated endothelial cell migration (Goue¨ffic et al., 2006; Brantley-Sieders et al., 2004). Our previous work has shown that activation of PI3K plays an important role in regulating the migration of rat primary VSMCs (rVSMCs) stimulated by *C. pneumoniae* infection (Wang et al., 2013). Therefore, it would be interesting to determine whether PI3K is involved in *C. pneumoniae* infection-mediated activation of Rac1 signalling pathway in rVSMCs.

In this study, we focused on the role of Rac1 activation in the migration of rVSMCs infected with *C. pneumoniae*, and established a link between PI3K and Rac1 in this process. We demonstrate that *C. pneumoniae* infection markedly increased GTP–Rac1 expression in rVSMCs, which was downregulated after the addition of inhibitors of Rac1 or PI3K, thereby inhibiting *C. pneumoniae* infection-induced rVSMC migration. Our data suggest that PI3K-dependent Rac1 activation plays a vital role in *C. pneumoniae* infection-induced rVSMC migration.

### Methods

**Reagents and antibodies.** FBS and Dulbecco’s modified Eagle’s medium (DMEM) were from Gibco. Vancomycin and gentamicin were obtained from Amresco. Glutathione–Sepharose 4B was obtained from GE Healthcare Biosciences. IPTG and the PI3K inhibitor LY294002 were purchased from Promega. Cell lysis buffer and the BCA Protein Assay Reagent kit were from the Beyotime Institute of Biotechnology. BSA and glutathione–Sepharose 4B were obtained from Amresco. The glutathione S-transferase–p21-activated kinase–p21 binding domain (GST–PAK–PBD) recombinant plasmid was obtained from Dr Margaret J. Wheelock, University of Nebraska Medical Center, NE, USA. The Rac1 inhibitor NSC23766 was from Merck. The following antibodies were commercially obtained: mouse anti-Rac1 mAb (BD Biosciences), mouse anti-β-actin mAb (Beijing Zhongshan Goldenbridge Biotechnology) and horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories).

**Cell culture.** rVSMCs were prepared from aortas of Sprague–Dawley rats as described previously (Zhang et al., 2012). In brief, the cells were cultured in DMEM supplemented with 10 µg gentamicin ml⁻¹, 25 µg vancomycin ml⁻¹ and 10% FBS and incubated in a humidified incubator at 37 °C with 5% CO₂. The medium was changed every fourth day until the cells became confluent. For all experiments, rVSMCs were used between the third and eighth passage. In some experiments, the cells were pre-incubated for 1 h with 50 µM Rac1 inhibitor NSC23766 or 25 µM PI3K inhibitor LY294002 prior to *C. pneumoniae* infection. In the migration assays, rVSMCs were pretreated with 0.8 mM hydroxy carbamide for 12 h before exposure to *C. pneumoniae* to inhibit cell proliferation.

**C. pneumoniae propagation and infection of rVSMCs.** *C. pneumoniae* AR-39 strain (ATCC 53592) was purchased from the American Type Culture Collection. *C. pneumoniae* was propagated and purified in HEp-2 cells as described previously by Zhang et al. (2011). The purified bacteria were suspended in chlamydial sucrose/phosphate/glutamate medium and stored at −70 °C until use. The titre of *C. pneumoniae* was determined in HEp-2 cells and concentrations were expressed as inclusion-forming units (i.f.u.).

Cultured rVSMCs were inoculated with *C. pneumoniae* as described previously (Zhang et al., 2012). Briefly, rVSMCs were seeded into six-well plates at a density of 3 × 10⁵ cells per well. Once confluent, the cells were infected with *C. pneumoniae* at an infectious dose of 5 × 10⁶ i.f.u., centrifuged at 1700 g for 50 min at room temperature and subsequently incubated in a humidified incubator at 37 °C with 5% CO₂.

**Pull-down assay for Rac1 activity.** Rac1 activation was measured using a GST–PAK–PBD fusion protein, which binds to activated Rac1. The activity of Rac1 was assessed by the detection of its GTP-loaded form (GTP–Rac1). rVSMCs were infected with *C. pneumoniae* at an infectious dose of 5 × 10⁵ i.f.u. rVSMCs were stimulated with *C. pneumoniae* at different time points and then washed with cold PBS and lysed in lysis buffer (50 mM Tris/HCl pH 7.2, 500 mM NaCl, 10 mM MgCl₂, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF). After centrifugation at 3000 g at 4 °C for 10 min, the supernatants were collected and the protein concentrations were determined using a BCA Protein Assay kit. The proteins were incubated with glutathione–Sepharose 4B beads coupled with the GST–PAK–PBD fusion protein at 4 °C for 6–8 h. The protein–bead complexes were then recovered by centrifugation and washed with PBS. Following the last wash, the protein–bead complexes were resuspended in SDS sample buffer and resolved by 12% SDS-PAGE. The precipitated GTP–Rac1 and total Rac1 were then incubated with glutathione–Sepharose 4B beads coupled with the GST–PAK–PBD fusion protein, which binds to active Rac1. The activity of Rac1 was assessed by the detection of its GTP–Rac1 expression in rVSMCs, which was downregulated after the addition of inhibitors of Rac1 or PI3K, thereby inhibiting *C. pneumoniae* infection-induced rVSMC migration. Our data suggest that PI3K-dependent Rac1 activation plays a vital role in *C. pneumoniae* infection-induced rVSMC migration.

**Wound-healing assay.** A wound-healing assay was performed to assess cell migration ability. rVSMCs were seeded into six-well plates and incubated until 90% confluent and serum-free medium with 0.8 mM hydroxy carbamide was then added. A sterile pipette tip was used to make a wound by scratching across the monolayer, and detached cells were washed away. Cells were pre-treated with the Rac1 inhibitor NSC23766 (50 µM) for 1 h and then infected with *C. pneumoniae*. Photographs of the wound were taken at 0 and 24 h post-wounding with or without exposure to *C. pneumoniae* under an inverted phase-contrast microscope (×100 magnification). The area of cell recovery were determined with image analysis software. The ratio of cell recovery area to the original wound area was calculated to evaluate migration.

**Transwell assay.** To further assess *C. pneumoniae* infection-induced rVSMC migration, we performed a Transwell assay as described.
previously (Zhang et al., 2012). rVSMCs were incubated with C. pneumoniae at an infectious dose of $5 \times 10^8$ i.f.u. for 14 h and then seeded into the upper chamber of a Transwell system to allow cell migration through the membrane for 8 h. The number of cells that migrated to the lower side of the membranes from six independent, randomly chosen visual fields was counted under an inverted phase-contrast microscope ($\times 200$ magnification) to quantify cell migration.

**Statistical analysis.** All experiments were performed with triplicate samples and repeated three times. Data were expressed as means $\pm$ SEM. The difference of means between multiple groups was evaluated by ANOVA. Statistical significance was established using Student–Newman–Keuls analysis. Statistical differences were considered significant for $P<0.05$.

### RESULTS

**C. pneumoniae** infection induces activation of Rac1 in rVSMCs

Our previous work showed that C. pneumoniae infection significantly promotes rVSMC migration (Zhang et al., 2012). Rac1 is required at the front of the cells to regulate actin polymerization and membrane protrusion, which is thought to contribute to cell movement (Maddala et al., 2011; Konakahara et al., 2012). We therefore investigated whether C. pneumoniae infection could induce Rac1 activation in rVSMCs by a GST pull-down assay. As shown in Fig. 1, a low level of GTP–Rac1 was observed in quiescent rVSMCs. C. pneumoniae infection activated Rac1 in rVSMCs from 30 min to 8 h after infection with a peak at 3 h post-infection ($P<0.05$) and then returned to the basal level. These results implied that C. pneumoniae infection-induced rVSMC migration might be associated with activation of Rac1.

**Rac1 activation is essential for rVSMC migration stimulated by C. pneumoniae infection**

To determine whether C. pneumoniae infection-induced rVSMC migration depends on Rac1 activity, we analysed the effects of inhibition of Rac1 activity using a selective Rac1 inhibitor, NSC23766 (50 μM), on rVSMC migration using a wound-healing assay and a Transwell assay. The GST pull-down assay demonstrated that C. pneumoniae infection markedly stimulated Rac1 activation compared with that of the control group ($P<0.05$); NSC23766 (50 μM) significantly reduced GTP–Rac1 expression without changing the expression levels of total Rac1 in C. pneumonia-infected rVSMCs (1.12 $\pm$ 0.11 vs 0.67 $\pm$ 0.06; $P<0.05$; Fig. 2).

An in vitro wound-healing model of the rVSMC monolayer was used to assess the effects of Rac1 on C. pneumoniae infection-induced rVSMC migration. As shown in Fig. 3(a), C. pneumoniae infection accelerated wound closure compared with the control group 24 h after the cell monolayer was scratched with a pipette tip (0.35 $\pm$ 0.05 vs 0.24 $\pm$ 0.06; $P<0.05$). Compared with C. pneumoniae-infected rVSMCs, wound closure was significantly suppressed after the use of NSC23766 (50 μM) (0.23 $\pm$ 0.04 vs 0.35 $\pm$ 0.05; $P<0.05$). In addition, the Transwell migration assay showed that the number of rVSMCs infected with C. pneumoniae migrating through the membrane was higher than in the control group (88.56 $\pm$ 7.06 vs 54.67 $\pm$ 4.87; $P<0.05$; Fig. 3b). NSC23766 (50 μM) markedly suppressed C. pneumoniae infection-induced rVSMC migration compared with C. pneumoniae infection group (61.78 $\pm$ 5.78 vs 88.56 $\pm$ 7.06; $P<0.05$; Fig. 3b). These results suggested that Rac1 plays an indispensable role in C. pneumoniae infection-mediated rVSMC migration.

**C. pneumoniae infection-induced Rac1 activation in rVSMCs is PI3K dependent**

PI3K has been widely implicated in controlling cell migration and polarity and has been reported as an upstream signalling molecule for Rac1 activation (Welf et al., 2012; Dawes & Edelstein–Keshet, 2007). Therefore, in this study, the PI3K inhibitor LY294002 was added to the medium 1 h before C. pneumoniae infection; activation of Rac1 was detected at 3 h

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**Fig. 1.** C. pneumoniae infection induces activation of Rac1 in rVSMCs. Cells were infected with C. pneumoniae at $5 \times 10^8$ i.f.u. and the activity of Rac1 was assessed by the detection of its GTP-loaded form (GTP–Rac1) using GST pull-down assays at the indicated time points and analysed by Western blotting. Results are shown as means $\pm$ SEM for three independent experiments. *$P<0.05$ compared with 0 h after C. pneumoniae infection.
post-infection. LY294002 not only decreased the basal level of Rac1 activity but also markedly suppressed *C. pneumoniae* infection-induced Rac1 activation (*P*<0.05; Fig. 4).

**DISCUSSION**

Recent studies have highlighted the role of *C. pneumoniae* infection in the process of cell migration (Uriarte et al., 2004; Schmidt et al., 2006; Högdahl et al., 2008). Some chemokines and adhesion molecules secreted by *C. pneumoniae*-infected human endothelial cells can promote transendothelial migration of neutrophils and monocytes (Uriarte et al., 2004; MacIntyre et al., 2003). Our previous studies have shown that *C. pneumoniae* infection can directly stimulate the adhesion and migration of HEp-2 cells and VSMCs (Zhang et al., 2011, 2012). However, whether *C. pneumoniae* infection induces VSMC migration by reorganizing the actin cytoskeleton is unknown.

Rac1 is an important regulator of the actin cytoskeletal rearrangement and plays a pivotal role in cell migration (Maddala et al., 2011; Konakahara et al., 2012). Accordingly, in this study, we first investigated whether *C. pneumoniae* infection induced Rac1 activation in rVSMCs using a GST pull-down assay. The results showed that *C. pneumoniae* infection induced a sustaining activation of Rac1 in rVSMCs from 30 min to 8 h after infection, which is consistent with previous reports (Eitel et al., 2012; Lin et al., 2011) showing that Rac1 is activated in *C. pneumoniae*-infected monocytes and *C. pneumoniae* heat-shock protein 60-treated endothelial cells. Lin et al. (2011) demonstrated that treatment with GroEL1 (a heat-shock protein from *C. pneumoniae*) rapidly induced Rac1 activation in a dose-dependent manner, which also supports our results. Uniquely, our report focused on the time-dependent changes of Rac1 activation induced by *C. pneumoniae* infection. Subtil et al. (2004) also found that Rac1 activation is involved in *Chlamydia caviae* entry.

![Fig. 2. Effects of NSC23766 on Rac1 activation in *C. pneumoniae*-infected rVSMCs. rVSMCs were infected with *C. pneumoniae* at 5×10⁵ i.f.u. for 3 h, and the activity of Rac1 was assessed by the detection of its GTP-loaded form (GTP–Rac1) using GST pull-down assays in the presence or absence of NSC23766 (50 μM) and analysed by Western blotting. Results are shown as means ± SEM for three independent experiments. *P*<0.05 compared with the control group; *#P*<0.05 compared with the *C. pneumoniae* infection group.](image)

![Fig. 3. Effects of Rac1 on *C. pneumoniae* infection-induced rVSMC migration. Cells were pre-treated with NSC23766 (50 μM) for 1 h before *C. pneumoniae* infection. (a) Cell migration in the wound-healing assay was evaluated by the migration index (percentage of cellular recovery area relative to the original wound area). (b) Cell migration in the Transwell assay was quantified by counting the number of migrated cells from six independent, randomly chosen visual fields. Results are shown as means ± SEM for three independent experiments. *P*<0.05 compared with the control group; *#P*<0.05 compared with *C. pneumoniae* infection group.](image)
into the host cell, which to some degree supports our view that Rac1 activation is associated with *C. pneumoniae* infection.

Several groups have reported that Rac1 activation is involved in insulin-like growth factor-I-induced VSMC migration and EGF-stimulated human hepatoma HepG2 cells migration (Meng et al., 2008; Hu et al., 2012). To further determine the roles of Rac1 activation in the process of *C. pneumoniae* infection-induced rVSMC migration, NSC23766 was applied to inhibit intracellular Rac1 activity, and cell migration assays were then performed to assess the migration ability of rVSMCs. Our data showed that NSC23766 inhibited Rac1 activation and rVSMC migration stimulated by *C. pneumoniae* infection. Recent studies have shown that knockout of Rac1 also impairs the migration of keratinocytes and results in delayed re-epithelialization (Tscharntke et al., 2007). Faroudi et al. (2010) also demonstrated that Rac1-deficient T-cells have multiple defects in cell migration and home very inefficiently to lymph nodes (Faroudi et al., 2010). These studies suggest that Rac1 activation may be involved in *C. pneumoniae* infection-induced VSMC migration.

PI3K has also been involved in controlling cell migration (Welf et al., 2012; De Laurentiis et al., 2011; Choi et al., 2010). Activated PI3K–Akt signalling has been shown to be able to cause F-actin rearrangement to promote the migration of VSMCs (Brachmann et al., 2005). Rac1 and PI3K, the two important intracellular enzymes, regulate cellular function through various, often overlapping, signalling pathways. Huang et al. (2005) reported that PI3K–Cdc42/Rac1 plays an important role in EGF-induced MDA-MB-231 cell migration. In the process of EGF-stimulated colonic epithelial cell migration, Rac1 activation seems to depend largely on the activation of PI3K and Src family kinases (Dise et al., 2008). In addition, the PI3K pharmacological inhibitor LY294002 or dominant-negative PI3K constructs could inhibit Ephrin-A1-stimulated endothelial cell migration partly by reducing Rac1 activity (Brantley-Sieders et al., 2004). Our present study shows that LY294002 significantly suppresses Rac1 activation induced by *C. pneumoniae* infection, which is consistent with the current view that Rac1 acts as a downstream effector of PI3K-controlled signalling pathways as demonstrated in VSMCs (Goueffic et al., 2006) and endothelial cells (Lai et al., 2011). Our results indicate that PI3K-dependent Rac1 activation may be associated with VSMC migration induced by *C. pneumoniae* infection.

Once activated, Rac1 interacts with its downstream targets to generate a variety of intracellular responses (Bishop & Hall, 2000). IQ domain GTPase-activating protein 1 (IQGAP1), a key regulator of actin polymerization (Brown et al., 2008; Le Clainche et al., 2007), plays an important role in cell migration by interacting with activated Rac1 (Fukata et al., 2002; Tang et al., 2011). Our previous study demonstrated that *C. pneumoniae* infection can promote VSMC migration possibly by upregulating IQGAP1 expression (Zhang et al., 2012). However, it remains to be determined whether *C. pneumoniae* infection promotes IQGAP1 binding to activated Rac1 to induce VSMC migration.

In conclusion, in the present study, we demonstrated that Rac1 is activated in a PI3K-dependent manner in rVSMCs upon exposure to *C. pneumoniae*, and inhibition of Rac1 activation suppresses rVSMC migration induced by *C. pneumoniae* infection, indicating that Rac1 plays an important role in *C. pneumoniae* infection-induced rVSMC migration. Our results reveal a new mechanism whereby *C. pneumoniae* infection plays an important role in the development of atherosclerosis by inducing VSMC migration through activating Rac1.
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