Macrophage differentiation induced by PMA is mediated by activation of RhoA/ROCK signaling

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ABSTRACT — In order to investigate the effects of RhoA/ROCK signaling in macrophage differentiation, we used 100 ng/mL PMA to induce macrophage differentiation from U937 cells in vitro. The observation of cell morphology and the expression of CD68 and SR-A were performed to confirm the differentiation induced by PMA. Western blot analysis showed that the expression of ROCK1 and ROCK2 and the phosphorylation of MYPT1 were significantly increased after PMA treatment. Pulldown assay showed that the activation of RhoA was obviously enhanced when U937 cells were treated with PMA. In order to further demonstrate whether RhoA/ROCK signaling could mediate the macrophage differentiation induced by PMA, we successfully suppressed the expression of RhoA, ROCK1 and ROCK2 by performing siRNA technology in U937 cells, respectively. The macrophage differentiation and the expression of CD68 and SR-A were significantly inhibited by the suppression of RhoA, ROCK1 or ROCK2 in PMA-induced U937 cells, indicating that the macrophage differentiation induced by PMA is associated with RhoA/ROCK signaling pathway. In addition, we pretreated U937 cells with Y27632 (ROCK inhibitor, 20 μM) for 30 min and then observed the macrophage differentiation induced by PMA. The result illustrated that Y27632 pretreatment obviously inhibited PMA-induced differentiation and the expression of CD68 and SR-A. In conclusion, the activation of RhoA/ROCK signaling is responsible for the macrophage differentiation induced by PMA.

Key words: PMA, Macrophage, RhoA/ROCK signaling pathway, ROCK inhibitor

INTRODUCTION

Atherosclerosis (AS) is a chronic inflammatory disease of the vessel wall and the first cause of death in the world (van Dijk et al., 2010). Macrophages are the most abundant immune cells in the lesions of AS and play an indispensable role in the process of disease formation, ranging from lesion to plaque rupture (Stary et al., 1994). Monocytes in the blood circulation adhere to the active endothelial cells in the vascular lesions and then transmigrate through the endothelium stimulated by chemical chemokines to differentiate into macrophages (Mangge et al., 2004).

Small Rho GTPases of Rho family plays a role of molecular switching in a series of intracellular activities (Heasman and Ridley, 2008). Rho family members, mainly including RhoA, Rac1 and Cdc42, are the key regulators of actin skeleton and cell adhesive junction (Heasman and Ridley, 2008). Recently, RhoA has been reported to have a certain effect on the maintenance of cell adhesion (Herzog et al., 2011). The suppression of RhoA will lead to the disruption of cell adhesion junction and abnormal cell morphology (Katayama et al., 2011). Rho-associated protein kinase (ROCK), including two subtypes ROCK1 and ROCK2, belongs to the serine/threonine protein kinase and is the effector protein of small Rho GTPases (Bishop and Hall, 2000). ROCK exerts important roles in regulating many intracellular activities, such as cell contraction, cell migration, axon elongation, cell adhesion and differentiation, and is considered as one of the most potential therapeutic targets (Amano et al., 2010). Fasudil is the unique inhibitor for ROCK applied to clinical and has made significant contributions to the treatment of cardiovascular, neurological and cancer diseases (Kishi et al., 2005). It has been reported that RhoA/ROCK signaling pathway regulated actin cytoskeleton and promoted actin myosin contraction, and subsequently regulated cell translocation and shrinkage (Ishizaki et al., 1996; Leung et al., 1996; Nobes and Hall, 1995; Ridley and Hall, 1992). In addition, the regulation of...
RhoA/ROCK signaling on actin cytoskeleton could also promote smooth muscle contraction, stress fiber formation, cell adhesion and differentiation (Chu et al., 2012; Jordan and Canman, 2012; Kishi et al., 2005; Lee et al., 2012; Maddox et al., 2012; Ruiz-Loredo et al., 2011).

However, the specific role of RhoA/ROCK signaling pathway in the differentiation of macrophages is not clear. In this study, we used PMA to induce the macrophage differentiation model in vitro and then investigated the effects of RhoA/ROCK signaling on the differentiation induced by PMA. The expression of CD68 and SR-A was used as molecular indicators of the macrophage differentiation.

MATERIALS AND METHODS

Materials

Dimethylsulfoxide (DMSO), NP-40, SDS-PAGE gel preparation kit, BCA protein assay kit (enhanced) and PMA (phorbol-12-myristate-13-acetate) were purchased from Beyotime Biotechnology (Haimen, Jiangsu, China). Fetal bovine serum (FBS) was obtained from GibcoBRL (Grand Island, NY, USA). RhoA GTPase activity assay kit was purchased from Cytoskeleton (Denver, CO, USA). Antibodies for ROCK1, ROCK2, MYPT1 and phospho-MYPT1 (Thr-853) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies for RhoA, CD68 and SR-A were purchased from Boster Company (Wuhan, China). Antibody for GAPDH and peroxidase-conjugated anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). siRNAs for RhoA, ROCK1 and ROCK2 were synthesized from Sangon Gene Company (Shanghai, China). The scrambled control-siRNA was as follows: TTCCAG-TATGACCTCCAGC. The siRNAs were dissolved in DEPC water at a concentration of 20 μM. The siRNAs and the transfection reagent Lipofectamine 3000 were diluted with RPMI1640 (HyClone, Logan, UT, USA) and then mixed for 20 min at room temperature. The mixture was added to the cells and incubated for 48 hr.

RT-PCR analysis

Total RNA was extracted using the RNA Total Extraction Kit (Centrifugal Column type). The samples were washed with RNase-free water and the concentration of extracted RNA was then determined. One microgram of total RNA was used for reverse transcription with a reverse transcript synthesis kit (TianGen Biotech, Beijing, China). The cDNA was then used as template for PCR with gene specific primers. The primer sequences were as follows: RhoA, (F)5’-CCATCATCCTGGTTGGGAAT-3’ and (R)5’-CCATGTACCCAAAAGCGC-3’; ROCK1, (F)5’-CAAGAGGAGGTGAAGCATCTCA-3’ and (R)5’-TGCCACAGACTTTGCCTCTT-3’; ROCK2, (F)5’-GAAGAGCAGCAGAAGTGGGT-3’ and (R)5’-GGCAGTTAGCTAGGTTTGTTGG-3’; GAPDH, (F)5’-ACAACCTCTCAAGATTGTCAGCAA-3’ and (R)5’-ACTTTGTGAAGCTCATTTCCTGG-3’. The PCR products were analyzed by agarose gel electrophoresis and scanning densitometry.

Pulldown assay

The protein lysates of 500 μg were added to the RBD-binding beads which specifically binding to the active RhoA-GTase (Aspenström, 1999; Ren et al., 1999). The beads and protein lysates were incubated at 4°C for 1 hr and then centrifuged at 4°C for 1 min. The supernatant was removed (do not destroy the RBD-binding beads, if the beads are destroyed, then centrifuge again) and the binding beads were washed using 500 μL of wash buffer each time (make the binding beads completely sus-
pend when wash. This step should be completed within 1 min). The beads were then centrifuged at 4°C for 3 min. Subsequently, the supernatant was removed and 10 μL of 5 × sample protein buffer was added to the Ep tube. Finally, the samples were boiled for 2 min and cooled down in order to subject to Western blot electrophoresis.

Western blot analysis
To extract the total proteins, cells were washed twice with cold PBS and lysed in lysis buffer after PMA treatment (Xu et al., 2015). Lysates were centrifuged at 4°C for 15 min at 12,000 g. Protein concentration was determined by a Bradford assay (Meng et al., 2015; Xu et al., 2015). Afterwards, the proteins were separated by 10% SDS-polyacrylamide gelelectrophoresis (PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk in TBST (pH 7.4) with 0.1% Tween-20 for 2 hr at room temperature and then incubated overnight at 4°C with different primary antibodies. The signals were detected by HRP-conjugated secondary antibodies for 2 hr at room temperature on an ECL detection system (Amersham Biosciences, Little Chalfont, UK). The relative intensities of the signals were quantified by densitometry and Imaging software (Labworks, Upland, CA, USA).

Statistical analysis
Data were expressed as mean ± S.D. All experiments were repeated for at least 3 times. One-way ANOVA followed by the Bonferroni test for multiple-group comparisons was used for the statistical analysis. A p-value of < 0.05 was considered to be statistically significant.

RESULTS
The activation of RhoA/ROCK signaling is involved in the macrophage differentiation induced by PMA
Normal cultured human monocyte cell line (U937) was treated with PMA (100 ng/mL) for 48 hr for the macrophage differentiation in vitro and then directly observed under inverted microscopy. As shown in Fig. 1A, U937

![Fig. 1](image)

**Fig. 1.** The construction of the macrophage differentiation model in vitro. (A) Cultivated human monocyte U937 cells observed by inverted microscopy (at a magnification of 200 ×). Normal U937 cells were round and translucent. However, cell morphology was obviously changed after treatment with PMA for 48 hr, with cells gathering and adhering generously. (B) The expression of CD68 and SR-A were determined by Western blot analysis. Cells were treated with 100 ng/mL PMA for 48 hr. The protein levels were quantified by densitometric analysis, normalized to GAPDH. Cells without treatment belong to the control group. Data are expressed as means ± S.D. (n = 3). * P < 0.05 vs. the control group.
cells without treatment were round and translucent, which was obviously changed after treatment with PMA, with cells gathering and adhering generously. To further confirm the macrophage differentiation induced by PMA, CD68 and SR-A were analyzed by Western blot (Mantovani et al., 2002; Moore and Tabas, 2011; Yu et al., 2009). As shown in Fig. 1B, the expression of both CD68 and SR-A were obviously increased after incubation with PMA, which indicating that the macrophage differentiation model induced by PMA was successfully constructed.

To clarify whether RhoA/ROCK signaling mediated the macrophage differentiation induced by PMA, we investigated the expression of RhoA, ROCK1 and ROCK2 through Western blot analysis. As shown in Fig. 2, there was no obvious increase of total RhoA after PMA treatment, while the expression of both ROCK1 and ROCK2 were obviously increased when treated with PMA. In addition, we analyzed the activation of RhoA and ROCK by performing pulldown assay and Western blot. As shown in Fig. 2A, the active style of RhoA (RhoA-GTP-bound) was significantly increased after PMA treatment. The activation of ROCK was detected by the phosphorylation of MYPT1 (myosin phosphatase target subunit 1) (Arita et al., 2009). As shown in Fig. 2C, there was no obvious change in the expression of the total MYPT1, while the phospho-MYPT1 was significantly enhanced after PMA treatment. The results indicated that PMA treatment stimulates the expression of ROCK and the activation of both RhoA and ROCK.

In brief, the activation of RhoA/ROCK signaling was strongly enhanced after inducement of PMA, which might be responsible for the macrophage differentiation induced by PMA.
RhoA suppression obviously inhibited the macrophage differentiation induced by PMA

To further demonstrate whether the activation of RhoA is involved in the macrophage differentiation, we suppressed the expression of RhoA and investigated the differentiation induced by PMA. The successful knockdown RhoA siRNA was screened by RT-PCR analysis and the RhoA-siRNA1 could successfully inhibit the expression of RhoA (Fig. 3A). The cells suppressing RhoA were treated with PMA (100 ng/mL) for 48 hr, and then observed under inverted microscopy. As shown in Fig. 3B, RhoA suppression could remarkably inhibit the macrophage differentiation induced by PMA. Furthermore, we detected the expression of CD68 and SR-A induced by PMA after RhoA suppression. As shown in Fig. 3C, both the expression of CD68 and SR-A induced by PMA were significantly decreased when RhoA was suppressed. Therefore, we confirmed that the activation of RhoA is involved in the PMA-induced macrophage differentiation.

ROCK suppression obviously inhibited the macrophage differentiation induced by PMA

To further assess the involvement of ROCK expression in the macrophage differentiation induced by PMA, we suppressed the expression of ROCK1 and ROCK2 in U937 cells, respectively. The successful knockdown ROCK-siRNAs were screened by RT-PCR analysis. The ROCK1-siRNA3 and ROCK2-siRNA1 could successfully inhibit the expression of ROCK1 and ROCK2, respectively (Figs. 4A and 5A). Subsequently, the cells suppressing ROCK1 and ROCK2 were treated with PMA for 48 hr, and then observed under inverted microscopy. As shown in Figs. 4B and 5B, the suppression of ROCK1 and ROCK2 could remarkably inhibit the macrophage differentiation induced by PMA. Furthermore, the expression of CD68 and SR-A induced by PMA were significantly inhibited by ROCK1 or ROCK2 suppression (Figs. 4C and 5C). Therefore, the enhancement of the expression of ROCK1 or ROCK2 could mediate the macrophage differentiation after PMA treatment.

Y27632 pretreatment inhibited ROCK activity and the PMA-induced macrophage differentiation

To examine whether the activation of ROCK is involved in the macrophage differentiation, we pretreated the cells with Y27632 (inhibit ROCK1 and ROCK2 with equal potency) for 30 min and then treated with PMA for 48 hr. As shown in Fig. 6A, the macrophage differenti-
Fig. 4. Macrophage differentiation induced by PMA was inhibited by ROCK1 suppression. (A) Detection of ROCK1 suppression via RT-PCR analysis. ROCK1-siRNA: U937 cells were transfected with ROCK1 siRNAs. 1: siRNA1; 2: siRNA2; 3: siRNA3; 4: siRNA4. Control siRNA: U937 cells were transfected with control siRNA. (B) Cells were observed by inverted microscopy (200 ×). U937 cells transfected with ROCK1-siRNA3 were treated with PMA (100 ng/mL) for 48 hr. (C) Western blot analysis of the expression of CD68 and SR-A. The protein levels were quantified by densitometric analysis, normalized to GAPDH. Cells without treatment belong to the control group. Data are expressed as means ± S.D. (n = 3). * P < 0.05 vs. the control group. # P < 0.05 vs. the group treated with PMA.

Fig. 5. Macrophage differentiation induced by PMA was inhibited by ROCK2 suppression. (A) Detection of ROCK2 suppression via RT-PCR analysis. ROCK2-siRNA: U937 cells were transfected with ROCK2 siRNAs. 1: siRNA1; 2: siRNA2; 3: siRNA3; 4: siRNA4. Control siRNA: U937 cells were transfected with control siRNA. (B) Cells were observed by inverted microscopy (200 ×). U937 cells transfected with ROCK2-siRNA1 were treated with PMA (100 ng/mL) for 48 hr. (C) Western blot analysis of the expression of CD68 and SR-A. The protein levels were quantified by densitometric analysis, normalized to GAPDH. Cells without treatment belong to the control group. Data are expressed as means ± S.D. (n = 3). * P < 0.05 vs. the control group. # P < 0.05 vs. the group treated with PMA.
Adaptation was effectively induced by PMA treatment, which could be significantly inhibited by Y27632 pretreatment. Furthermore, the expression of CD68 and SR-A induced by PMA were obviously inhibited by Y27632 pretreatment (Fig. 6B). The results indicated that the activation of ROCK is involved in the macrophage differentiation induced by PMA.

**DISCUSSION**

The investigation of monocytes growth and differentiation is important for understanding the mechanisms of atherosclerosis (AS). U937 cells are not yet fully differentiated but still retain differentiation potential, which can differentiate into macrophage-like blood cells by various stimulations (Geissler *et al*., 1989). Phorbol ester (PMA) and γ-interferon are two important factors that can stimulate the differentiation of monocytes. In this study, we used PMA as a stimulus to induce the differentiation of U937 cells.

Normal U937 cells were round and translucent, which was obviously changed after treatment with PMA, with cells gathering and adhering generously. It has been reported that macrophages in the lesion of AS can highly express CD68, SR-A and other scavenger receptors (SRs) (Aspenström, 1999). Scavenger receptor CD68 is a type I hyperglycosylated transmembrane protein that belongs to the glycoprotein-lysosomal-related membrane protein family and a specific antigen on the surface of monocytes/macrophages (Ren *et al*., 1999). The CD68 receptor has been identified as a macrophage specific molecular marker. The SR-A receptor in macrophages has been reported to be involved in lipids accumulation in the blood vessels, which promotes the formation of foam cells and starts the process of AS. Therefore, the expression of CD68 and SR-A were analyzed as two important molecular markers for the macrophage differentiation in this study. The results showed that the expression of CD68 and SR-A were significantly enhanced after PMA treatment, indicating that we successfully constructed the macrophage differentiation model by PMA in vitro.

Rho GTPases play important regulatory roles in many intracellular activities including cell proliferation, differentiation, and apoptosis and carry out the molecular switching effects through transforming the styles between GTP-bound and GDP-bound (Boureux *et al*., 2007; Zhang *et al*., 2005). The Rho GTPases family is demonstrated to be the key regulators of actin cytoskeleton and cell junction (Heasman and Ridley, 2008). Recent studies have reported that RhoA is essential for maintaining cell adhesion in the rat neuroepithelium (Herzog *et al*., 2011; Katayama *et al*., 2011), indicating that RhoA plays an

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**Fig. 6.** Macrophage differentiation induced by PMA was inhibited by Y27632 pretreatment. U937 cells were pretreated with Y27632 (20 μM) for 30 min and then treated with PMA (100 ng/mL) for 48 hr. (A) Cells were observed by inverted microscopy (200 ×). (B) Western blot analysis of the expression of CD68 and SR-A. The protein levels were quantified by densitometric analysis, normalized to GAPDH. Cells without treatment belong to the control group. Data are expressed as means ± S.D. (n = 3). * P < 0.05 vs. the control group. # P < 0.05 vs. the group treated with PMA.
important role in cell adhesion. Therefore, we speculated whether RhoA is involved in the PMA-induced macrophage differentiation. In this study, we examined the effects of PMA treatment on the expression and activation of RhoA. The results showed that PMA treatment could significantly stimulate the activation of RhoA, but had no effect on the expression of total RhoA. To further confirm the RhoA activity mediated the PMA-induced macrophage differentiation, we successfully suppressed RhoA and observed the effects of RhoA suppression on macrophage differentiation. The results showed that RhoA suppression significantly inhibited PMA-induced U937 cell aggregation and both the expression of CD68 and SR-A, indicating that PMA promoted CD68 and SR-A expression through RhoA activation, which may induce macrophage differentiation and lipids accumulation.

Rho-associated protein kinase (including ROCK1 and ROCK2) is known as the Rho GTPases effector protein and plays important roles in many cell functions (Amano et al., 2010; Bishop and Hall, 2000). ROCK is the most important downstream targets of RhoA. To investigate whether ROCK is involved in the PMA-induced macrophage differentiation, we first examined the expression and activation of ROCK. PMA induction obviously promoted the expression of ROCK1 and ROCK2 and the activation of ROCK. Subsequently, we suppressed ROCK1 and ROCK2, respectively, in U937 cells and observed the differentiation induced by PMA. The results showed that ROCK1 suppression could significantly inhibit the PMA-induced U937 gathering and both the expression of CD68 and SR-A, indicating that ROCK1 is involved in the PMA-induced CD68 and SR-A expression and then in the macrophage differentiation and lipids accumulation. However, ROCK2 suppression completely inhibited the PMA-induced U937 gathering and the expression of CD68, while partially inhibited SR-A expression, suggesting that ROCK2 is mainly involved in the PMA-induced CD68 expression and macrophage differentiation, and may have less impact on SR-A expression and lipids accumulation. In the future, we will further verify the effects of ROCK2 suppression on PMA-induced lipids accumulation. In addition, pretreatment with ROCK inhibitor Y27632 significantly inhibited the PMA-induced cell gathering and both the expression of CD68 and SR-A, indicating that ROCK activation is related to CD68 and SR-A expression induced by PMA, and then involved in the macrophage differentiation and lipids accumulation.

Therefore, we believed that PMA promotes macrophage differentiation through the activation of RhoA and ROCK. RhoA/ROCK pathway may accelerate the progressive process of AS by mediating the differentiation of monocytes into macrophages. Our results also supported that ROCK1 is required for both macrophage differentiation and lipids accumulation induced by PMA through regulating both CD68 and SR-A expression, whereas ROCK2 mainly participates in macrophage differentiation through regulating CD68 expression and may less affect lipids accumulation because of partial inhibition of SR-A expression.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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