Sphingosine 1-phosphate (S1P) inhibits monocyte–endothelial cell interaction by regulating of RhoA activity

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Abstract Recent studies suggest that sphingosine 1-phosphate (S1P) protects against atherosclerosis. We assessed the effects of S1P on monocyte–endothelial interaction in the presence of inflammatory mediators. Pretreatment of THP-1 cells with S1P abolished Phorbol 12 myristate 13-acetate (PMA)-induced THP-1 cell adhesion to human umbilical vein endothelial cells (HUVECs). S1P inhibited PMA-induced activation of RhoA, but not PKCs. S1P activated p190Rho GTPase activation protein (GAP) only in the presence of PMA, suggesting an inhibitory effect of S1P and PMA to suppress RhoA. In conclusion, S1P inhibited monocyte–endothelial interactions by inhibiting RhoA activity which may explain its anti-atherogenic effects.
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1. Introduction

Sphingosine 1-phosphate (S1P), a bioactive lipid mediator, has a variety of actions in several types of cells. In plasma, S1P is mainly present in high-density lipoprotein (HDL) and low-density lipoprotein (LDL), to a lesser extent [1]. Platelets also contain high concentrations of S1P, and a fraction (20–40%) of S1P is released into the circulation upon their activation [2].

Recent studies reported the cytoprotective actions of S1P on vascular endothelial cells (ECs). S1P stimulates the proliferation [3,4], survival [1,5,6], and migration [3,7–9] of ECs. It also induces nitric oxide (NO) synthesis in ECs [10]. Thus, S1P has been proposed as an anti-atherogenic mediator.

Human peripheral monocytes express S1P receptors, the members of G-protein-coupled receptors (GPCRs) [11,12]. However, the direct effects of S1P on peripheral monocytes have not been fully elucidated. The adhesion of peripheral monocytes to vascular endothelium importantly contributes to atherogenesis [13]. The present study tested the effects of S1P on monocyte–endothelial cell interactions in the presence of inflammatory mediators, and the underlying mechanism(s) for this process.

2. Materials and methods

2.1. Cell cultures and reagents

THP-1 cell line (ATCC) was cultured in RPMI-1640 containing 10% FCS. Human umbilical vein endothelial cells (HUVECs) were isolated from normal-term umbilical veins and cultured in 0.1% gelatin-coated tissue culture dishes, then plated on 22-mm fibronectin-coated glass cover slips after 2 or 3 passages for use in a flow chamber apparatus, as previously described [14]. S1P was obtained from Sigma–Aldrich Japan. Go6976 and phorbol-12-myristate-13-acetate (PMA) were obtained from Calbiochem. Rottlerin was obtained from BIOMOL Research Laboratories. Pertussis toxin (PTX) was obtained from List Biological Laboratories. Recombinant human IL-1β was obtained from Genzyme. The antibodies used in the present study were as follows: rabbit anti-protein kinase C (PKC)α and δ polyclonal antibodies (Santa Cruz Biotechnology), rabbit anti-RhoA polyclonal antibody (Upstate), mouse anti-hemagglutinin epitope (HA) antibody (Boehringer Mannheim), rabbit anti-actin polyclonal antibody (Sigma), mouse anti-p190RhoGAP antibody (Upstate), anti-phosphotyrosine-RC20:HRPO(BD Bioscience) and HRP-conjugated goat anti-mouse IgG. To examine cell viability, THP-1 cells were stained with a 0.25% trypan blue solution after incubation with S1P or PMA.

2.2. Monocyte adhesion assays

The protocols of the adhesion assays under flow conditions have been previously described in detail [15]. Briefly, HUVEC monolayers were stimulated with 10 U/mL of IL-1β for 4 h on coverslips and then positioned in a flow chamber mounted on an inverted microscope (IX70, Olympus, Japan). The monolayers were perfused for 5 min with perfusion medium, after which THP-1 cells (1×10^6/mL) were drawn through the chamber with a syringe pump (PHD2000, Harvard Apparatus) for 10 min at a controlled flow rate to generate a shear stress of 1.0 dyne/cm². The entire period of perfusion was recorded on videotape, and then transferred to a personal computer for image analysis to determine the number of rolling and adherent THP-1 cells on HUVEC monolayers in 10 randomly selected 20× microscope fields. In some experiments, THP-1 cells were preincubated with appropriate concentrations of PKC inhibitors (Go6976, 2.6 μmol/L; Rottlerin, 5 μmol/L) prior to S1P or PMA treatment. Though it is difficult to completely control variation of baseline adhesive interaction of HUVEC prepared from donor to donor, we obtain consistent response after IL-1β stimulation as we previously demonstrated [15–17].

2.3. Translocation of PKC in THP-1 cells

To examine the translocation of and PKC from the cytosol to the membrane, an indicator of activation, membrane and total cell lysates of THP-1 cells (1×10^6/mL) were prepared as described previously [17].

2.4. RhoA pull-down assay

RhoA pull-down assay was performed using Rho activation Kit (Upstate) following the manufacturer's protocol [18].

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2.5. Overexpression of RhoA

Wild-type (pEF-BOS-HA-WT-RhoA) and dominant active (pEF-BOS-HA-DA-RhoA) mutant forms of RhoA cDNA constructs were kindly provided by Dr. Shinya Kuroda (University of Tokyo, Tokyo, Japan), and transfected into THP-1 cells by lipofection as previously described [15]. We previously observed that 40% of cells were transfected with plasmid cDNA by this method [19]. The transfected THP-1 cells were harvested 24 h after transfection and RhoA expression was determined by Western blotting analysis with a monoclonal antibody against the HA epitope.

2.6. Immunoprecipitations

Immunoprecipitations were performed as described previously [20] and probed with monoclonal antibodies against p190RhoGAP or phosphotyrosine (RC20). The relative amount of phosphorylated p190RhoGAP was determined by measuring the amount of phosphorylated p190RhoGAP immunoprecipitated relative to the total amount of p190RhoGAP.

2.7. Monocyte isolation

Monocytes were freshly isolated from a buffy coat, obtained from healthy volunteers, using a MACS monocyte isolation kit (Miltenyi Biotec). Isolated monocytes were cultured in media containing 20% human serum (The Interstate Companies), 10 ng/ml of M-CSF (Genzyme Techne), 100 IU/ml penicillin, 100 μg/ml and streptomycin in RPMI 1640 (Sigma).

2.8. Statistical analysis

Results are presented as the means ± S.E.M. Data were analyzed using analysis of variance (ANOVA), with a value of $P < 0.05$ considered significant.

3. Results

3.1. S1P inhibits PMA-induced THP-1 cell adhesion to HUVECs

We assessed the effect of S1P on THP-1 cell adhesion in the presence of PMA. PMA, a potential activator of PKC families, induces inflammatory process in many types of cells. Indeed, PMA alone remarkably enhanced THP-1 cell adhesion to activated HUVECs. THP-1 cells were preincubated with various concentrations of S1P for 18 h before the addition of PMA. PMA-induced THP-1 cell adhesion to HUVECs was significantly decreased in a S1P concentration-dependent manner and reached a plateau after 5 μmol/L S1P (Fig. 1A). With this concentration, PMA-induced THP-1 cell adhesion was completely inhibited after 18 h of preincubation (Fig. 1B). Thus, we chose to incubate THP-1 cells with 5 μmol/L S1P for 18 hours in the following experiments.

S1P also inhibited PMA-induced adhesion of human peripheral blood monocytes to HUVECs (Fig. 1C). We then examined whether S1P inhibits THP-1 cell adhesion induced by SDF-1α, a chemokine that induces monocyte adhesion. S1P also inhibited SDF-1α-induced THP-1 cell adhesion to HUVECs (Fig. 2).

3.2. PTX reverses S1P inhibition of THP-1 cell adhesion

THP-1 cells as well as human peripheral monocytes express S1P receptors, the members of G-protein-coupled receptors (GPCRs) [11]. We examined whether inhibitory effect of S1P is mediated by S1P receptor. When THP-1 cells were pretreated with pertussis toxin (PTX), a specific Gi protein inhibitor, S1P failed to inhibit PMA-induced THP-1 cell adhesion (Fig. 3).
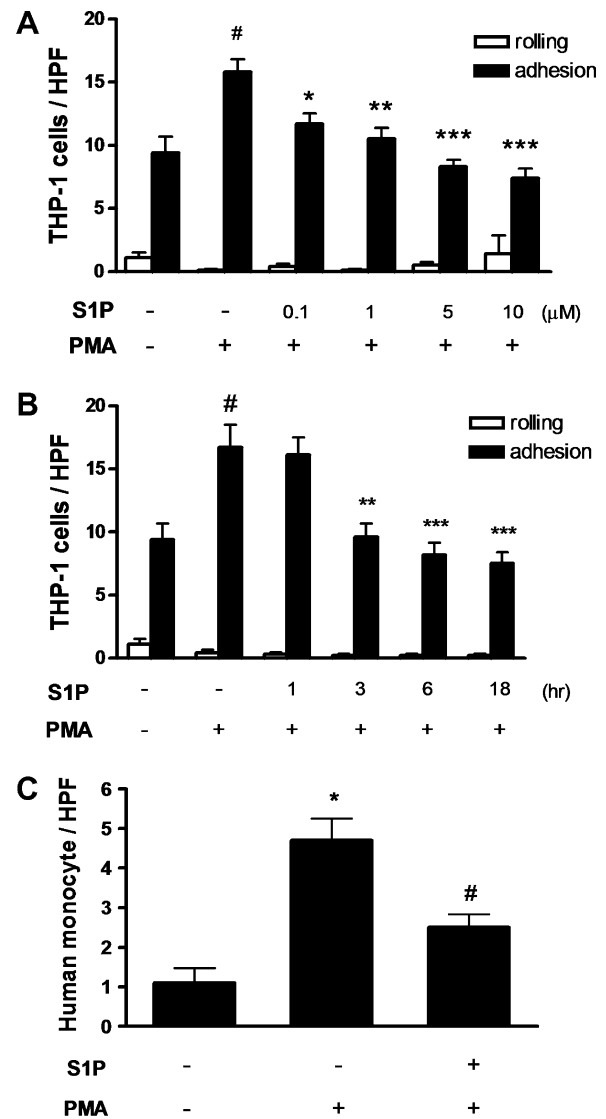

Fig. 1. S1P inhibits PMA-induced THP-1 cell adhesion to HUVECs. (A) THP-1 cells (1×10^6/mL) were treated with 250 nM of PMA for 10 min and various concentrations of S1P for 18 h before incubation with 250 nmol/L of PMA for 10 min. The cells were perfused over activated (IL-1β 10 U/mL, 4 h) HUVEC monolayers at a flow rate of 1.0 dyne/cm² as described in materials and methods. Adhered and rolling cells were counted as described in materials and methods. Data are representative of the results of three separate experiments. $^\#P < 0.001$ vs. S1P (−)/PMA (−). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. S1P (−)/PMA (+). (B) THP-1 cells were treated with 250 ng/mL of PMA for 10 min, or 5 μmol/L of S1P for indicated hours before incubation with 250 ng/mL of PMA and a flow assay was performed as described in A. Data are representative of the results of three separate experiments. $^\#P < 0.001$ vs. S1P (−)/PMA (−). $^{***}P < 0.001$ vs. S1P (−)/PMA (+). (C) Human monocytes (1×10^6/mL) were treated with S1P or PMA and a flow assay was performed as described in A. Data are representative of the results of three separate experiments. $^*P < 0.001$ vs. S1P (−)/PMA (−). $^\#P < 0.05$ vs. S1P (−)/PMA (+).

3.3. Effects of S1P and PMA on PKC activation in THP-1 cells

The PKC family plays an important role in several mechanisms that promote atherosclerosis [21], and increase monocyte–endothelial interactions by activating RhoA and modulating the expression and activation of integrins [17,22]. Thus, we examined whether S1P affect PKCα and PKCδ activ-
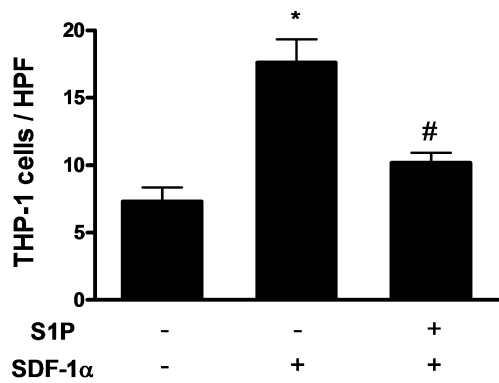
Fig. 2. S1P inhibits SDF-1α- induced THP-1 cell adhesion to HUVECs. THP-1 cells were treated with 100 ng/mL of SDF-1α for 10 min, or 5 μmol/L of S1P for 18 hours before incubation with 100 ng/mL of SDF-1α and a flow assay was performed as described in Fig. 1A. Data are representative of the results of three separate experiments. *$P < 0.001$ vs. S1P (−)/SDF-1α (−). #$P < 0.001$ vs. S1P (−)/SDF-1α (+).
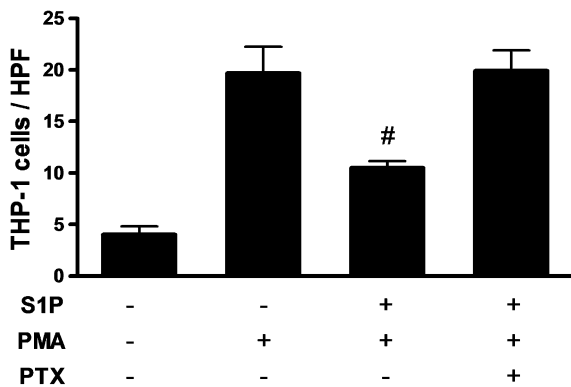

Fig. 3. PTX reverses inhibition of THP-1 cell adhesion by S1P. THP-1 cells were incubated in the absence or presence of 100 ng/mL of pertussis toxin (PTX) for 24 h, then incubated with PMA and a flow assay was performed as described in Fig. 1A. Data are representative of the results of three separate experiments. #$P < 0.01$ vs. S1P (−)/PMA (+)/PTX (−).

ity in the presence of PMA. PMA alone induced PKCα and δ activation in THP-1 cells. Contrary to the result of adhesion assays, S1P further augmented PMA-induced PKC activation (Fig. 4A). Go6976 (a PKCα inhibitor) or rottlerin (a PKCδ inhibitor) inhibited PMA-induced THP-1 cell adhesion. However, inhibitory effect of S1P on THP-1 cell adhesion was not affected by Go6976 or rottlerin (Fig. 4B).

3.4. Effects of S1P and PMA on RhoA activation in THP-1 cells

We then examined the effect of S1P on RhoA activity in the presence of PMA, PMA activated RhoA in THP-1 cells (Fig. 5A), which was inhibited by Go6976 or rottlerin (data not shown). S1P inhibited PMA-induced RhoA activation (Fig. 5A). To investigate the potential role of RhoA in THP-1 cell adhesion, THP-1 cells were transfected with cDNA carrying wild-type (WT) or dominant active (DA) RhoA. We confirmed the expression of exogenous RhoA in transfected THP-1 cells (Fig. 5B). Compared to WT-RhoA transfected THP-1 cells, DA-RhoA transfected THP-1 cells showed higher adhesiveness to HUVECs, which was not affected by PMA or
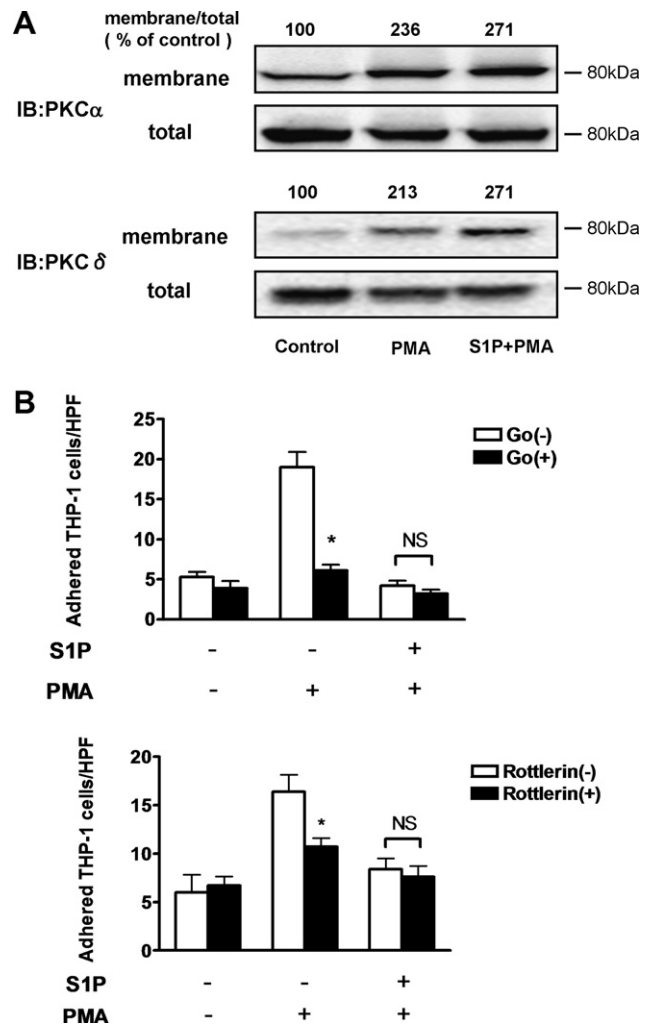

Fig. 4. Effects of S1P and PMA on activation of PKCs in THP-1 cells. (A) THP-1 cells were treated with S1P and PMA as described in Fig. 1A. PKCα and δ activation was detected in the membrane and cytosol lysates of THP-1 cells (1×10^6/mL) under each condition by Western blotting. Blots are representative of three separate experiments. (B) THP-1 cells were incubated in the absence or presence of 5 μmol/L of rottlerin or 2.6 μmol/L of Go6976 for 30 min, then incubated with S1P and PMA and a flow assay was performed as described in Fig. 1A. Data are representative of the results of three separate experiments. *$P < 0.05$ vs. Go6976 (−) or rottlerin (−).

S1P treatment (Fig. 5C). Taken together, these results indicate that RhoA plays a dominant role in THP-1 cell adhesion. To elucidate this mechanism, we measured the activity of GTPase activation protein (GAP) that inactivates RhoA. PMA alone did not affect tyrosine phosphorylation of p190RhoGAP. Interestingly, in the presence of PMA, S1P induced tyrosine phosphorylation of p190RhoGAP, suggesting its activation (Fig. 5D). S1P alone did not affect p190RhoGAP activity (data not shown). These results suggest that S1P, in conjunction with PMA, activates p190RhoGAP, causing RhoA inactivation in THP-1 cells.

4. Discussion

The present study demonstrated that S1P inhibited PMA-induced THP-1 cell adhesion by inhibiting RhoA activity.
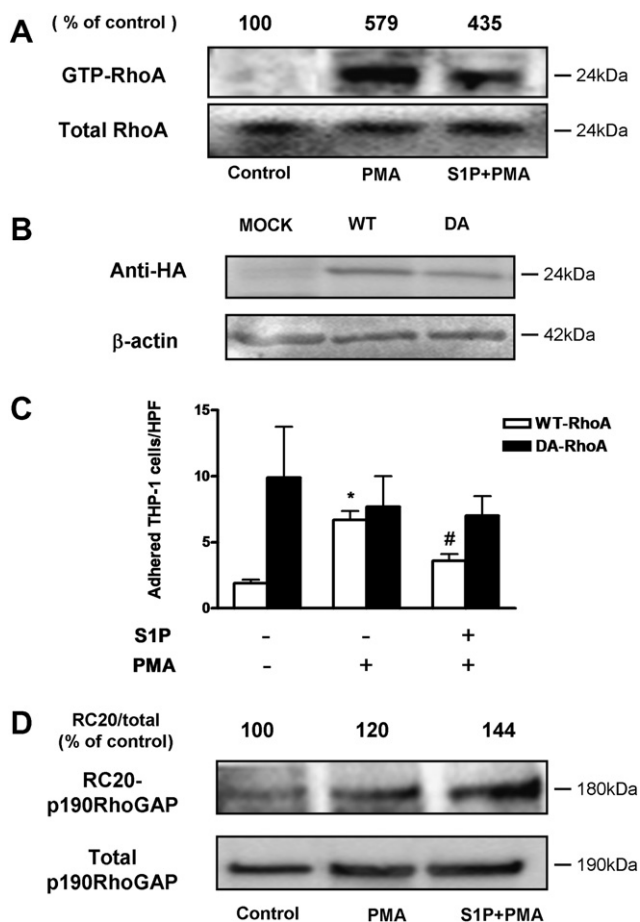
Fig. 5. Effects of S1P and PMA on activation of RhoA in THP-1 cells. (A) THP-1 cells were incubated with S1P and PMA as described in Fig. 1A, before being subjected to RhoA pull down assay. Blots are representative of three separate experiments. (B) The expression levels of transfected WT-RhoA and DA-RhoA in THP-1 cells were evaluated by Western blotting with an anti-HA tag monoclonal antibody (anti-HA) at 24 h after transfection. (C) THP-1 cells were transfected with WT-RhoA and DA-RhoA, then treated as described in B, after which flow assays with activated (IL-1β 10 U/mL, 4 h) HUVECs were carried out. $^{*}P < 0.001$ vs. S1P (−)/PMA (−), $^{\#}P < 0.01$ vs. S1P (−)/PMA (+). (D) Phosphorylation of p190Rho-GAP was determined by immunoprecipitation, analysis of immune complexes by SDS–PAGE, and immunoblotting with anti-phospho-tyrosine antibody. Blots are representative of three separate experiments.

S1P also inhibited SDF-1α-induced THP-1 cell adhesion. These results suggest that S1P inhibits monocyte–endothelial interaction under the conditions that cause inflammatory processes, supporting its anti-atherogenic property.

It has been reported that S1P prevents monocyte–endothelial interactions [23–25]. S1P prevents monocyte–endothelial interactions through endothelial S1P1 or S1P3 receptor. Although S1P receptors are also found on monocytes, the effect of S1P on monocytes in not clear. We provide the first evidence that S1P protects the vasculature against inflammatory signal-induced monocyte–endothelial interactions. S1P's effects were inhibited by PTX, indicating that they are mediated by S1P receptors in G$_i$-protein dependent manner [26]. Kimura et al. reported that PTX-sensitive S1P receptors are responsible for inhibitory pathways for adhesion molecule expression in ECs [24].

We previously showed that PKCα and PKCδ play important roles in adhesion of THP-1 cells by activating RhoA [17]. PMA is one of the potential activators of PKC families [27], and induces monocyte adhesion to ECs. However, preincubation of THP-1 cells with S1P inhibited PMA-induced THP-1 cell adhesion in spite that PKC was further activated. This urged us to focus on the role of RhoA, downstream of PKCs as a modulator of cell motility and cell adhesion. RhoA is an important molecule involved in the regulation of the actin cytoskeleton, integrins, and monocyte–endothelial interaction [15]. Some papers reported that S1P activate Rho GTPase in other cell types [28–30]. We observed that S1P alone slightly activate RhoA activation in THP-1 cells (data not shown). However, induction of THP-1 cell adhesion by S1P is much smaller that induced by PMA. Experiments using THP-1 cells transfected with DA RhoA showed that RhoA could regulate cell adhesion. Indeed, S1P inhibited PMA-induced RhoA activation, although PKCs were activated.

We also examined the possible involvement of other Rho family proteins such as Rac1 and Cdc42. S1P attenuated PMA-induced Cdc42 activation but not affect Rac1 activation (data not shown), although S1P induces Rac1 activation in endothelial cells [31,32]. These results suggest the distinct role of Rho family proteins in regulation of cell adhesive interactions in THP-1 cells.

The activities of the Rho family including RhoA are determined by the dynamic balance between the activated form (GTP) and inactive form (GDP). Two distinct family of proteins; Rho guanine nucleotide exchange factor (GEF) (GDP to GTP) and RhoGAP (GTP to GDP) regulate these two forms of RhoA [33]. We found that S1P activates p190Rho-GAP in the presence of PMA. These results suggest that S1P inactivates RhoA by activating p190RhoGAP. Mechanism by which PMA and S1P activate p190RhoGAP remains unknown. Recently, several phospholipids such as phosphatidic acid (PA) [34] and phosphatidylserine (PS) [35] are reported to increase GAP activity in synergism with PMA. Moreover, co-incubation with PKC, diacylglycerol and phosphatidylserine induced the phospholylation of p190RhoGAP in fibroblasts and the translocation of p190RhoGAP was completely blocked by pretreatment of cells with PKC inhibitors [36]. These results suggest p190RhoGAP activation could be influenced by PKCs. Our findings suggest that PKC activity augmented by PMA and S1P not only directly affect Rho GTPase but also induce p190RhoGAP activation. The precise mechanism for RhoA regulation in synergism with PMA will require further investigations.

The concentration of S1P is from 0.2 μM to 0.9 μM in plasma and serum [37]. Previous studies have reported that high concentrations of S1P (1–20 μmol/L) had atherogenic effects, while anti-atherogenic effects have been shown at low concentrations [25,38]. However, our results showed that S1P has inhibitory effects on monocyte–endothelial interaction at concentrations as high as 5 μmol/L. This is the first to show that the high concentration of S1P exerts inhibitory effects depending on the underlying conditions.

In conclusion, S1P inhibited PMA-induced THP-1 adhesion to ECs by inhibiting RhoA activity via p190RhoGAP. Atherogenesis involves multiple inflammatory processes. Thus, under atherosclerosis-prone conditions, S1P may exert an anti-atherogenic effect by inhibiting monocyte adhesion to ECs.

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