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Sphingosine-1-phosphate promotes the proliferation and attenuates apoptosis of Endothelial progenitor cells via S1PR1/S1PR3/PI3K/Akt pathway†

Short running title: S1P affect the proliferation and apoptosis of EPCs

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Abbreviations:
BM, bone marrow; DilAc-LDL，Dil-labeled acetylated low density lipoprotein; DM, diabetes mellitus; EBM-2, endothelial basal medium 2; ECGS, endothelial cell growth supplement; ECs, endothelial cells; ELISA, Enzyme-linked immunosorbent assay; EPCs, Endothelial progenitor cells; FBS, fetal bovine serum; HPFs, high power fields; LSCM，laser scanning confocal microscope； rh, recombinant human; rhVEGF, recombinant human vascular endothelial growth factor; rhIGF-1, recombinant human insulin-like growth factor 1; rhEGF, recombinant human epidermal growth factor; bFGF, basic fibroblast growth factor; S1P，Sphingosine-1-phosphat; S1PR, Sphingosine-1-phosphate receptor; SD, standard deviation; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SPHKs, sphingosine kinases; TUNEL, transferase dUTP nick-end labeling; UEA-1, Ulex europaeus agglutinin-1;

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Abstract

Sphingosine-1-phosphate (S1P) is a bioactive lysophospholipid that involves in numerous pathophysiological processes. Endothelial progenitor cells (EPCs) play a crucial role in endothelial repair and tumor angiogenesis. The aim of study was to determine the effects of S1P on proliferation and anti-apoptosis of EPCs and their signaling pathways. In this study, we showed that S1P, SEW2871 (a selective S1P receptor 1 (S1PR1) agonist), or CYM5541 (a selective S1P receptor 3 (S1PR3) allosteric agonist promotes the proliferation and attenuates apoptosis of bone marrow (BM)-derived EPCs. Futher more, it was showed that S1P could promote EPCs proliferation, which could be significantly inhibited by pretreatment with CAY10444 (an S1PR3 antagonist), VPC23019 (a selective S1PR(1)/S1PR(3) antagonist), or LY294002 (a PI3K inhibitor). Moveover, we discovered that S1P could significantly attenuate H_2O_2-induced apoptosis and activation of caspase-3 in vitro, while W146 (an S1PR1 antagonist), VPC23019, or LY294002 could significantly increase the activation of caspase-3 and subsequent augmented apoptosis. Our results indicated that the protective effect of S1P is mediated by activating the PI3K/Akt pathway. In addition, S1P promotion of EPCs proliferation was observed to be mainly mediated through S1PR3 and attenuation of EPCs apoptosis induced by H_2O_2 was mainly mediated through S1PR1; both of these effects are mediated by activating the PI3K/Akt pathway, which provides potentially useful therapeutic targets for coronary artery disease, diabetes mellitus, and cancer treatment.

1. Introduction

It is well known that endothelial progenitor cells (EPCs) are capable to repair injured blood vessels through proliferation, migration (Hristov et al., 2003), and eventually differentiate into endothelial cells (ECs) (Urbich and Dimmeler, 2004b). EPCs could prevent neointima formation in injured vascular (Rauscher et al., 2003; Urbich and Dimmeler, 2004a) and induce new blood vessels in tumor development (Nolan et al., 2007) and metastasis (Gao et al., 2008). There is accumulating evidence that the number of EPCs in adult peripheral blood, along with their function, is a sensitive and reliable predictive factor for the risks and prognostic endpoints of coronary artery disease (Lambiase et al., 2004; Vasa et al., 2001; Werner et al., 2005), diabetes mellitus (DM) (Fadini et al., 2005), and wound healing processes (Chironi et al., 2007; Ghani et al., 2005). Meanwhile, it was found that the number of EPCs related with Nottingham prognostic index scores o breast cancer patients (Goon et al., 2009)in adult peripheral blood, and it was higher in patients with various types of cancer. It implied that EPCs may contribute to the vascularisation of lung cancer (Nowak et al., 2010), and its quantity was related to the tumor node metastasis stage and histological differentiation of the gastric cancer patients (Ha et al., 2013). Comparing with the patients with resectable HCC or those with liver cirrhosis, the numbers of the EPCs are much higher in the patients with advanced unresectable HCC (Ho et al., 2006). The effective treatment can reduce the number of EPCs in the circulating of patients with gynaecological cancerin (Kim et al., 2013). Therefore, it is important to maintain the quantity and function of EPCs at normal levels in pathological conditions.

Sphingosine-1-phosphate (S1P) is a bioactive lysophospholipid and signaling molecule phosphorylated from sphingosine by sphingosine kinases (SPHKs) in multiple cell types (Ksiazek et al., 2015). It has gained attention in several diseases, such as atherosclerosis, myocardial

infarction (Takuwa et al., 2008), and cancer (Ader et al., 2015; Tornquist, 2013). S1P is a natural constituent of blood, which can be found both in plasma and bold cells (Ksiazek et al., 2015). S1P level in plasma is much higher than in the solid tissues. Both platelets and erythrocytes store large amounts of S1P. Plateles lack of the S1P-degrading enzyme S1P lyase, herein, S1P accumulates intracellularly and can be released after plateles activation (Ulrych et al., 2011). Platelets number in patients with solid tumors is much higher than in normal person and many factors in the tumor microenvironment can activate the platelet (Prokopowicz et al., 2016). It was reported that S1P can directly regulate cellular biological function (Binder et al., 2015; Brocklyn, 2010; Kim et al., 2004; Tabasinezhad et al., 2013) and that S1P can induce the migration and angiogenesis of EPCs (Walter et al., 2007; Wang et al., 2015), protect photoreceptors from oxidative stress-induced apoptosis, and attenuate H_2O_2-induced apoptosis in cultured bovine aortic ECs (Moriue et al., 2008; Rotstein et al., 2010). SEW2871 is the potent and selective S1P receptor 1 (S1PR1) agonist (Lien et al., 2006) and CYM5541 is a novel and selective S1P receptor 3 (S1PR3) allosteric agonist (Jo et al., 2012) However, there is no data to show whether S1P, SEW2871 or CYM5541 can regulate the proliferation and apoptosis of EPCs.

Apoptosis is an essential biological process that can clear up the damaged, superfluous and infected cells through a genetically controlled mechanism. It was reported that apoptosis is the mainly regulatory mechanism for EPC survival (Cui et al., 2015; Kerr et al., 1972). Oxidative stress, which is also known to be excess production of reactive oxygen species (ROS), often leads to EC damage. There is high concentrations of ROS, especially H_2O_2, in the vasculature (Ward, 1991) and H_2O_2 is often involved in oxidative stress-induced apoptosis in EPCs (Fujii et al., 2006; Lee et al., 2013). However, whether S1P could regulate the proliferation and apoptosis of EPCs is still unknow. To address this question, we designed the study to investigate the ability of S1P to promote proliferation and attenuate H_2O_2-induced apoptosis in EPCs, and explored possible mechanisms of action.

Our previous studies have demonstrated that the PI3K/Akt signaling pathway plays an important role in EPCs stimulation by platelet-derived growth factor B (PDGF-BB), and demonstrated that the S1PR3/PDGFR-β/Akt signaling pathway participates in the S1P-induced migration and angiogenesis of EPCs (Wang et al., 2015). However, it was not clear that whether S1P could regulate the proliferation and apoptosis of EPCs through the PI3K/Akt signaling pathway or not. To address these questions, we designed the study to investigate the ability of S1P to promote proliferation and attenuate H_2O_2-induced apoptosis in EPCs, and explored possible mechanisms of action.

2. Materials and Methods

2.1 Isolation and characterization of EPCs

EPCs were isolated and characterized following our established protocol, with some modifications (Yu et al., 2014). Briefly, mononuclear cells (MNCs) were isolated from the bone marrow (BM) of Sprague-Dawley rats (male, 150–180 g; Wuhan, China) by density gradient centrifugation (Lymphoprep 1.077; Axis-Shield PoC AS). After washing for three times, the MNCs were resuspended in EGM-2MV Bullet Kit medium (Lonza), containing endothelial basal medium (EBM-2) and 5% fetal bovine serum, and were supplemented with recombinant human (rh) vascular endothelial growth factor (rhVEGF), basic fibroblast growth factor (rhFGF-B), epidermal growth factor (rhEGF), insulin-like growth factor 1 (rhIGF-1), ascorbic acid, and heparin. Next, the MNCs were seeded on

fibronectin-coated cell culture plates at a density of $2 \times 10^6/cm^2$ and cultured in a 5% CO_2 incubator at 37 °C. EPCs were cultured for 5 to 7 days with medium changed every 3days before being used for the experiments. The cells were incubated with 2.4 μg/ml Dil-labeled acetylated low density lipoprotein (DilAc-LDL; Invitrogen), 10 μg/ml FITC-Ulex europaeus agglutinin-1 (UEA-1; Sigma-Aldrich), and 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Beyotime). Triple-stained cells positive for DilAc-LDL, UEA-1, and DAPI were identified as EPCs. Additionally, before EPC phenotypes were confirmed by flow cytometry (FACS, Beckman couter, Indianapolis, USA), cells (1×10^6) should be incubated with the following monoclonal antibodies: FITC-conjugated anti-rat CD133, CD34, CD45, and VEGFR-2, and their corresponding isotype controls (Bios, Beijing, China).

2.2 EPCs proliferation assay

EPCs proliferation was examined by the colorimetric MTS assay (Cell Titer 96 Aqueous, Promega), as described previously (Wang et al., 2012; Yu et al., 2014). EPCs were seeded in 96-well plates in quintuplicate at a density of 1×10^4/well and cultured in synchronized DMEM/H (supplemented with 0.5% bovine serum albumin [BSA], and lacking fetal bovine serum [FBS] and endothelial cell growth supplement [ECGS]). After culturing for 12 hrs, EPCs were incubated in 20% FBS-supplemented DMEM/H with S1P (0, 200, 800, and 1400 nM). MTS (20 μL/well) was added into the culture medium to incubate for another 8hrs at 37°C. The optical density (OD) at 490 nm was recorded using a 96-well plate reader.

The potent S1PR1 agonist SEW2871 (20 nM; Sigma-Aldrich), S1PR3 agonist CYM5541 (10 nM; Cayman Chemical), S1PR1 antagonist W146 (10 μM; Avanti Polar Lipids), S1PR3 antagonist CAY10444 (10 μM; Cayman Chemical), selective S1PR(1)/S1PR(3) antagonist VPC23019 (10 μM; Avanti Polar Lipids), and PI3K inhibitor LY294002 (10 μM; Cell Signaling Biotechnology) were added into culture medium for 30 min before the addition of S1P. Simultaneously, we detected the effect of S1P treatment on EPC proliferation by cell counts.

Briefly, EPCs were incubated with or without S1P (800 nM). After culturing for7 days, attached cells were stained to detect uptake of DilAc-LDL, UEA-1, and DAPI expression, as described above. Triple-positive cells were considered EPCs and manually quantified in six randomly selected high power fields (HPFs).

2.3 EPCs apoptosis assay

Immunofluorescent apoptosis assays were performed by using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). Adherent cells were incubated in 20% FBS-supplemented DMEM/H and pretreated with S1P (0, 200, 800, and 1400 nM) for 30 min. H_2O_2 (600 μM; Merck, Darmstadt, Germany), as previously described (Wang et al., 2016), it was added to the cells and incubated for 6 hrs. After that, TUNEL staining was performed with the in situ Cell Death Detection Kit (Roche, USA), according to the manufacturer's instructions, and then nuclear staining was performed with DAPI. The numbers of TUNEL-positive and -negative nuclei were counted in five different HPFs per section under a laser scanning confocal microscope (LSCM, Leica, Wetzlar, Germany). Apoptosis activity was expressed in terms of TUNEL labeling index, calculated by dividing the positively labeled cells by the total cell number. SEW2871 (20 nM), CYM5541 (10 nM), W146 (10 μM), CAY10444 (10 μM), VPC23019 (10 μM), and LY294002 (10 μM) were added 30 min before the addition of S1P, where indicated.

2.4 Enzyme-linked immunosorbent assay (ELISA) for secreted VEGF

The concentration of VEGF in the cell supernatants was measured by ELISA Quantikine kit (R&D Systems) according to the manufacturer's protocol. To compare the expression levels of VEGF among the six groups of EPCs, with or without W146, CAY10444, VPC23019, or LY294002 for 30 min each, followed by treatment with or without S1P (800 nM) in DMEM/H. The cell supernatant was collected after culturing for 72 hrs and centrifuged at 6000 × *g* at 4°C for 10 min. The samples were analyzed in triplicate and appropriately diluted to ensure the measured values were in the concentration range of the standard curve.

2.5 Western blot

EPCs were cultured in 6-well plates and allowed to grow to 80% confluence before exposed to 800 nM S1P for different times (10 to 30 min or 24h), or stimulatied for 30 min with W146 (10 μM), CAY10444 (10 μM), VPC23019 (10 μM), or LY294002 (10 μM), and S1P (800 nM) in DMEM/H (supplemented with 1% FBS and without ECGS). The protein concentration of cell lysates was estimated by using the Bradford method. An equal volume of cell lysate was run on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (ThermoFisher) using the iBot Gel Transfer System (Invitrogen). The membranes were blocked with 5% non-fat milk, probed with anti-cleaved caspase-3, anti-VEGF (1:1000; Cell Signaling Biotechnology), anti-Akt, anti-phospho-Akt (1:500; Cell Signaling Biotechnology), or anti-GAPDH (1:1000; Cell Signaling Biotechnology) antibodies, and then stained with horseradish peroxidase-coupled secondary antibodies. The protein bands were visualized via enhanced chemiluminescence (Amersham Pharmacia Biotech) and quantified using Quantity One software (Bio-Rad).

2.6 Statistical analysis

All data, from at least three independent experiments, is expressed as means ± standard deviation (SD). Statistical analyses were performed by using SPSS 18.0 software (IBM). Comparisons in the groups were performed using one-way analysis of variance (ANOVA); $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Characterization of rat BM-derived EPCs.

To confirm the purity and identify phenotype of EPCs from rat BMs, attached cells were characterized by immunofluorescence and FACS analysis after 7 days of cell culture. Most MNCs (92.37 ± 2.19%) were positive for DilAc-LDL, UEA-1 lectin binding, and DAPI (Fig. 1A–D), suggesting EC characteristics. In addition, FACS analysis showed that 92.16 ± 1.08% of these adherent cells expressed CD133 (Fig. 1E), 75.73 ± 4.9% expressed CD34 (Fig. 1F), 88.7 ± 6.63% expressed VEGFR-2 (Fig. 1G), and 2.91 ± 2.73% expressed CD45 (Fig. 1H). These results above confirmed the successful isolation and culture of EPCs from rat BMs.

3.2 S1P enhances EPCs proliferation.

In this study, the effects of S1P, SEW2871, or CYM5541 on EPCs proliferation were explored first. As shown in Fig. 2A and 2B, S1P, SEW2871, or CYM5541 treatment significantly increased EPCs proliferation, compared to untreated cells. The maximum increase in EPC proliferation was

observed with 800 nM S1P treatment; a further increase was not observed with 1400 nM S1P (Fig. 2A). Cultures treated with 800 nM of S1P contained significantly more EPCs after 7 days compared with control cultures (Fig. 2C and 2D). Therefore, S1P (200 nM and 800 nM) significantly enhances EPC proliferation, and 800 nM S1P was used throughout this study.

3.3 The role of the S1PR1/3/Akt pathway in S1P-induced proliferation of EPCs.

We used the MTS assay to detect whether the S1PR1/3/Akt signaling pathway was required for S1P-induced EPCs proliferation. Compared with the S1P-treated group, S1P-induced proliferation was significantly inhibited by pretreatment with W146, CAY10444, VPC23019, or LY294002; the enhanced EPCs proliferation was induced by S1P which completely abolished after treatment with CAY10444, VPC23019, and LY294002. In addition, the proliferation-promoting effect of S1P was observed to be mainly mediated through S1PR3. The results above revealed that the S1PR1/S1PR3/Akt signaling pathway participated in S1P-induced EPCs proliferation (Fig. 2B).

3.4 S1P prevented EPCs apoptosis induced by H_2O_2.

We examined whether S1P, SEW2871, or CYM5541 was able to attenuate apoptotic responses of EPCs. As indicated in Fig.3A and 4A, pretreatment with S1P, SEW2871, or CYM5541 significantly attenuated the TUNEL labeling index of EPCs compared with that of untreated EPCs. The maximum EPCs apoptosis inhibition was observed with 800 nM S1P treatment; a further increase was not observed with 1400 nM S1P (Fig. 3A). At the same time, we found that S1P, SEW2871, or CYM5541 significantly decreased activated cleaved caspase-3 levels ($P < 0.01$; Fig. 3B and 4B). Representative images showed control cultures contained significantly more TUNEL staining compared with cultures pretreated with 800 nM S1P (Fig.3C). Therefore, S1P (200 nM and 800 nM) significantly decreased EPCs apoptosis after H_2O_2 treatment, and 800 nM S1P was used for further experiments.

3.5 The role of the S1PR1/S1PR3/Akt signaling pathway in S1P prevention of EPC apoptosis induced by H_2O_2.

Compared with the S1P treated group, the S1P attenuated TUNEL labeling index of EPCs was significantly increased by pretreatment with W146, CAY10444, VPC23019, or LY294002 (Fig. 4A). Furthermore, pretreatment with W146, CAY10444, VPC23019, or LY294002 significantly increased activated cleaved caspase-3 levels (Fig. 4B). We found that the reduced EPCs apoptosis which induced by S1P was completely abolished after treatment with W146, VPC23019, or LY294002. In addition, it was observed that the anti-apoptotic effect of S1P was mainly mediated through S1PR1. These data revealed that the S1PR1/S1PR3/Akt signaling pathway participated in the S1P-mediated prevention of EPCs apoptosis induced by H_2O_2. (Fig.4C).

3.6 Secretion of VEGF and expression of VEGF by EPCs

Our previous study showed that S1P could induce EPCs to release angiogenic growth factors such as VEGF (Wang et al., 2015). Therefore, we investigated whether W146, CAY10444, VPC23019, or LY294002 can affect the VEGF secretion of EPCs. The results showed that, when cultured with S1P, the concentration of soluble VEGF increased significantly. However, the concentration of soluble VEGF significantly decreased in those groups cultured with S1P + W146, S1P + CAY10444, S1P + VPC23019, and S1P + LY29400 (Fig. 5A).

We also determined whether W146, CAY10444, VPC23019, or LY294002 have activation affects the production of VEGF. Treatment of EPCs with 800 nM S1P led to significantly increase of VEGF protein expression. However, the S1P + W146 group, S1P + CAY10444 group, S1P + VPC23019 group, and S1P + LY294002 group showed significantly decreased VEGF protein expression (Fig. 5B and 5C).

These results revealed an important relationship between S1P and the secretion or expression of VEGF by EPCs and indicated that suppression the S1PR1, S1PR3 and PI3K could reduce S1P-induced secretion and expression of VEGF by EPCs.

3.7 PI3K/Akt activation by S1P is prevented by various inhibitors in EPCs.

Our previous results suggest that Akt phosphorylation is induced by exogenous S1P stimulation in EPCs (Wang et al., 2015). In this study, we confirmed the time-dependent phosphorylation of active Akt (Fig. 6A, 6B). Furthermore, we evaluated whether S1P activates the S1PR1/S1PR3/Akt/signaling pathway in EPCs. As demonstrated in Figure 6C, Akt phosphorylation induced by S1P was significantly blocked (Fig. 6D) by pretreatment with the S1PR1 inhibitor W146, S1PR3 inhibitor CAY10444, selective S1PR(1)/S1PR(3) inhibitor VPC23019, and PI3K inhibitor LY294002. These results suggested that S1P treatment activates Akt through S1PR1 and S1PR3 in EPCs.

4. Discussion

In this study, we demonstrated for the first time that S1P, SEW2871, or CYM5541 could induce the proliferation of EPCs and attenuate the apoptosis induced by H_2O_2. The proliferation-inducing effect of S1P was mainly mediated through S1PR3 and the anti-apoptotic effect was mainly mediated through S1PR1. Furthermore, these effects were both mediated by the PI3K/Akt pathway.

It is known that S1P is a bioactive product of sphingolipid metabolism and has been implicated in numerous pathophysiological processes. It performs pleiotropic actions by binding to and activating specific receptors on S1P cell-surface. These receptors belong to a family of G-protein-coupled receptors that contains five subtypes: S1PR1 through 5. S1PR1, 2, and 3 are widely expressed on the surface of many cell types, while S1PR4 and 5 are expressed only in the nervous system and the immune system. Each receptor subtype could activate diverse downstream signaling pathways, affecting various molecular and cellular events, including cell differentiation, growth, survival, and migration (Spiegel and Milstien, 2003, 2011).

Several PI3K/Akt inhibitors have been developed. LY294002 is one of the first generation of these inhibitors, which are used to elucidate the value of PI3K/Akt as a therapeutic target (Sun et al., 2016). The PI3K/Akt pathway also involves in cell proliferation, survival, motility, and apoptosis (Cantley, 2002; Osaki et al., 2004; Warfel and Kraft, 2015). Several studies showed that activation of the PI3K/Akt pathway might play a major role in VEGF (Gerber et al., 1998) and estrogen (Simoncini et al., 2000) induced alteration of EPCs quantity and their function. This result is consistent with those we obtained using EPCs. Our previous studies have demonstrated that the PI3K/Akt signaling pathway plays an important role following platelet-derived growth factor B (PDGF-BB) stimulation of EPCs (Wang et al., 2012), and demonstrated that the S1PR3/PDGFR-β/Akt signaling pathway participates in the S1P-induced migration and angiogenesis of EPCs (Wang et al., 2015). However, whether S1P could regulate the proliferation

and apoptosis of EPCs was not clear.

It is well known that circulating BM-derived EPCs contribute to the maintenance of endothelial integrity, its normal function, and postnatal neovascularization. The number of EPCs in the circulation varies according to pathologic status. DM can reduce the quantity of EPCs and weaken its function in patients (Fadini et al., 2005), and risk factors for coronary artery disease reduce the number of EPCs in humans (Hristov and Weber, 2004; Pearson, 2009). It was reported that tumors and ischemic tissue has the latent capacity to recruit EPCs from the bone marrow (De Falco et al., 2004; Janic and Arbab, 2010). These pathological states are often accompanied by abnormal elevation of S1P. We proposed that S1P might be an important factor affecting the quantity of EPCs.

In this study, we isolated and identified rat BM-derived EPCs according to protocols established by our group and other investigators (Li et al., 2007; Yu et al., 2014). We found that S1P (200 nM and 800 nM) significantly promoted EPCs proliferation. Furthermore, we showed that S1P receptors are required for these proliferations. In our study, three antagonists and an inhibitor (W146, CAY10444, VPC23019, and LY294002) were used to reveal that inhibiting the activity and phosphorylation of Akt could reduce S1P-induced proliferation of EPCs. These observations indicated that the proliferation effect of S1P was mediated by activating the PI3K/Akt pathway, and this effect was mainly through S1PR3. As EPCs contribute to neovascularization and reendothelialization, augmenting the quantity of EPCs and improving the activity of these cells may be an attractive therapeutic target.

Oxidative stress process which involved in free radicals, oxygen anions, and peroxides often leads to EC damage, which is closely associated with the initiation, development, and progression of vascular diseases. High concentrations of ROS, especially H_2O_2 (Moriue et al., 2008), are known to occur in the vasculature. S1P inhibits apoptosis in various cell types (Moriue et al., 2008; Nakahara et al., 2012), and its role of suppressing oxidative stress is publicly known. Therefore, S1P might have a protective effect against EPC death by stopping oxidative stress. In this study, we administered S1P before H_2O_2 treatment, to evaluate whether it could protect the EPCs against H_2O_2-induced apoptosis. We found that S1P (200 nM and 800 nM) statistically significantly decreased EPCs apoptosis induced by H_2O_2 and that the decrease induced by 800 nM S1P was completely abolished after treatment with W146, VPC23019, and LY294002. TUNEL assay results and caspase-3 levels also showed that the treatment with S1P could inhibit the apoptosis of EPCs. The protective effect of S1P was mediated by activating the PI3K/Akt pathway, and the anti-apoptotic effect was mainly mediated through S1PR1. The protection of EPCs, especially in the presence of oxidative stress, might be of great therapeutic value in the treatment of coronary artery disease.

VEGF plays a key role in endothelial regeneration and reparation of vascular injury. Study showed some agonist could induce the maximal expression and secretion levels of VEGF at the same concentration which induced the maximal proliferation of EPCs (Yu et al., 2014). In the clinic, most of the current anti-angiogenic strategies for cancer therapy are based on blocking VEGF functions (Sonpavde et al., 2012), and anti-VEGF agents have been successfully used for the treatment of certain human cancers (Motzer et al., 2007). The present study provides evidence that S1P significantly increases VEGF secretion from EPCs and the selective inhibition of S1PR1, S1PR3, or S1PR1/S1PR3, and PI3K can reduce VEGF secretion levels.

In conclusion, our results show that S1PR1 and S1PR3 might be essential receptors for

S1P-modulation of EPCs. The S1PR1/S1PR3/PI3K/Akt signaling pathway plays an important role following S1P stimulation on EPCs, which provides potentially useful therapeutic targets for coronary artery disease, DM, and cancer treatment.

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Legends

Fig. 1. Isolation and characterization of endothelial progenitor cells (EPCs).

(A) Bone marrow (BM) derived mononuclear cells (MNCs) showed uptake of DiI-labeled acetylated low density lipoprotein (red: DilAc-LDL) and Ulex europaeus agglutinin-1 (green: UEA-1) (B), and nuclei stained with 4', 6-diamidino-2-phenylindole (blue: DAPI) (C) after 7 days in culture. Most MNCs were triple-stained positive for DilAc-LDL, UEA-1, and DAPI (D). Scale bar = 75 μm. Adherent MNCs were analyzed for expression of CD133 (E), CD34 (F), VEGFR-2 (G), and CD45 (H) by flow cytometry. Dotted histograms represent isotype controls. N = 3. Scale bar = 50 μm.

Fig. 2. Sphingosine-1-phosphate (S1P), SEW2871, or CYM5541 promotes endothelial progenitor cells (EPCs) proliferation. S1P induces EPCs proliferation via the S1P receptor 1/3 (S1PR1/3)/Akt signaling pathway.

(A) Proliferation of EPCs stimulated with different concentrations of S1P for 8 h was determined by MTS assay. (B) Proliferation of EPCs pretreated with or without W146, CAY10444, VPC23019, and LY294002. Incubation was for 30 min, followed by treatment with 800 nM S1P, 20nM SEW2871 or 10 nM CYM5541. EPCs proliferation was examined by MTS assay. (C) Representative images showing overlay of Dil-labeled acetylated low density lipoprotein (DilAc-LDL；red), Ulex europaeus agglutinin-1 (UEA-1；green), and 4’,6-diamidino-2-phenylindole (DAPI；blue) from control cultures and S1P-treated (800 nM) cultures after 7 days. (D) Quantification of the cultures from panel B reveals that S1P (800 nM) treatment of EPCs significantly increases proliferation. *P < 0.05 vs. control group; **P < 0.01 vs. control group; $^{\#}$P < 0.05 vs. S1P group; $^{\#\#}$P < 0.01 vs. S1P (200 nM) group or $^{\#\#}$P < 0.01 vs. S1P group. N =5- 6. Scale bar = 75 μm.

Fig. 3. Sphingosine-1-phosphate (S1P) attenuates H_2O_2-induced apoptosis and activation of caspase-3 in cultured endothelial progenitor cells (EPCs).
(A) Anti-apoptosis of EPCs pretreated with different concentrations of S1P for 30 min was carried out by immunofluorescent terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). Apoptosis activity was expressed in terms of TUNEL labeling index. (B) Representative western blot images are using antibodies and recognizing cleaved caspase-3. Protein level of cleaved caspase-3, as assessed by densitometric analysis. (C) Representative images showed TUNEL staining of EPCs pretreated with S1P (800 nM, 30 min). Green: TUNEL signal. Blue: nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI). Overlay of TUNEL and DAPI indicated DNA breaks were mainly located in nuclei. **P < 0.01 vs. control group; #P < 0.05 vs. S1P (200 nM) group; ##P < 0.01 vs. S1P (200 nM) group. N = 3-6. Scale bar = 75 μm.

Fig. 4. Sphingosine-1-phosphate (S1P) prevented endothelial progenitor cells (EPCs) apoptosis via the S1P receptor 1/3 (S1PR1/3)/Akt pathway. (A) Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) index of EPCs was significantly decreased by S1P, SEW2871 or CYM5541, and significantly increased by pretreatment with W146, CAY10444, VPC23019, or LY294002. (B) Apoptosis level assessment representative western blot images. (C) Protein level of cleaved caspase-3, as assessed by densitometric analysis. *P < 0.05 vs. control group; **P < 0.01 vs. control group; $^{\#}$P < 0.05 vs. S1P (800 nM) group; $^{\#\#}$P < 0.01 vs. S1P (800 nM) group. N = 3-5.

Fig. 5. Secretion of vascular endothelial growth factor(VEGF)and expression of VEGF by endothelial progenitor cells (EPCs)
Pretreatment with various inhibitors attenuated sphingosine-1-phosphate (S1P) stimulated activation upregulates the expression and secretion of vascular endothelial growth factor (VEGF)in endothelial progenitor cells (EPCs). (A) Enzyme-linked immunosorbent assay (ELISA) of VEGF secreted by EPCs after pretreatment with or without W146, CAY10444, VPC23019, and LY294002. Incubation was for 30 min, followed by treatment with 800 nM S1P as indicated for 72hrs. (B and C) Western blot analysis of VEGF expression in EPCs after pretreatment with or without W146, CAY10444, VPC23019, and LY294002. Incubation was for 30 min, followed by treatment with 800 nM S1P as indicated for 24hrs. *P < 0.05 vs. control group; **P < 0.01 vs. control group; #P < 0.05 vs. S1P group. N = 3.

Fig. 6. Pretreatment with various inhibitors attenuated sphingosine-1-phosphate (S1P)-stimulated phosphorylation of Akt. (A and C) Representative western blot images. The values for S1P-stimulated phosphorylation of Akt were normalized to those of total Akt (B). The values for inhibitor pretreatment effects on S1P-stimulated phosphorylation of Akt are normalized to those of total Akt (D). * $P < 0.05$ vs. control group; ** $P < 0.01$ vs. control group; $^{\#\#}P < 0.01$ vs. S1P group. N = 3.

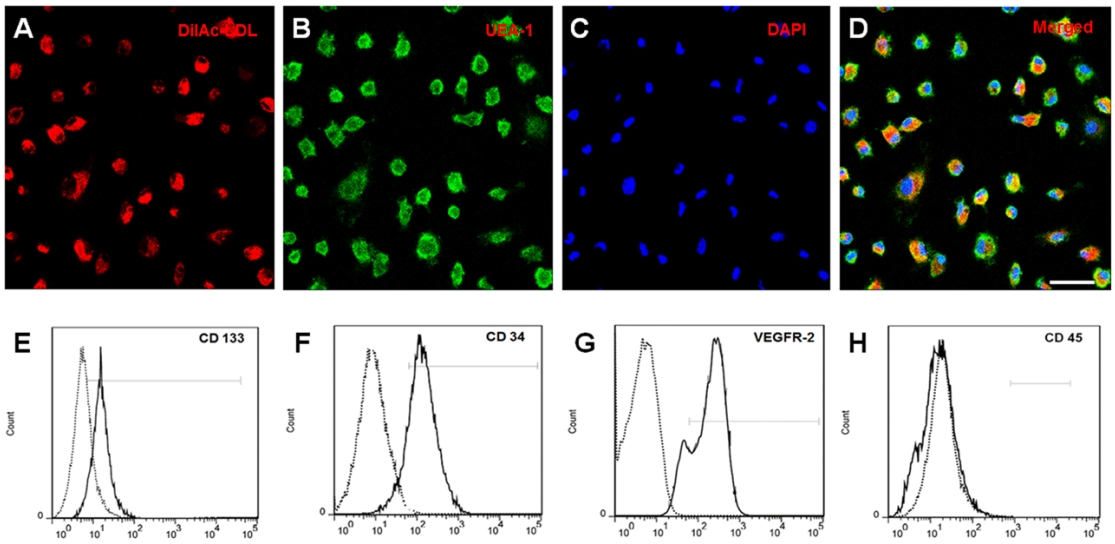

Figure 1

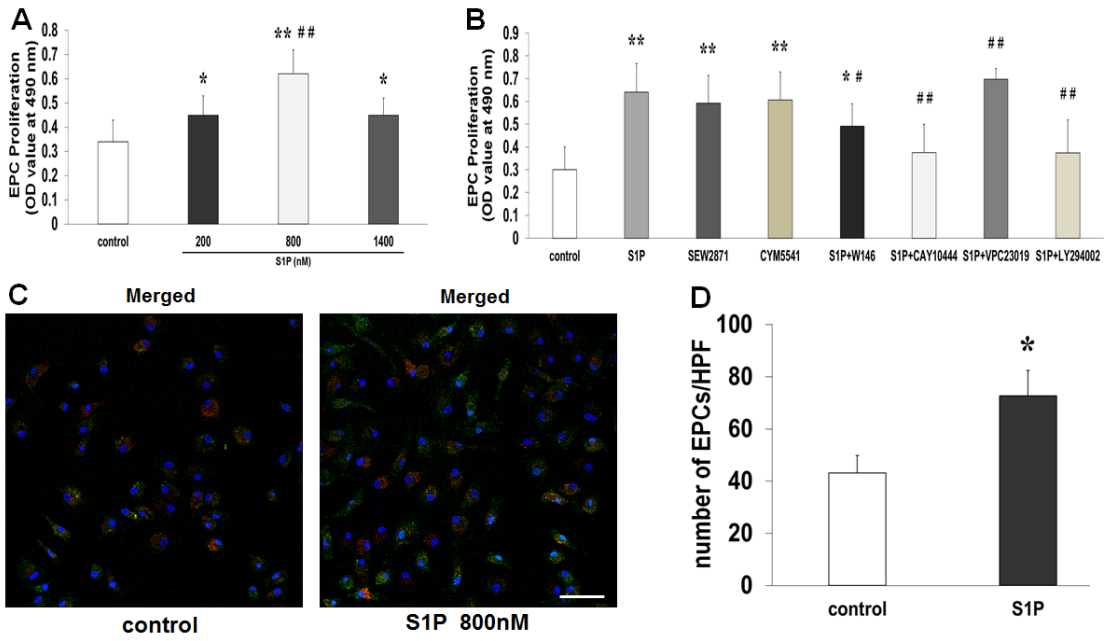

Figure 2

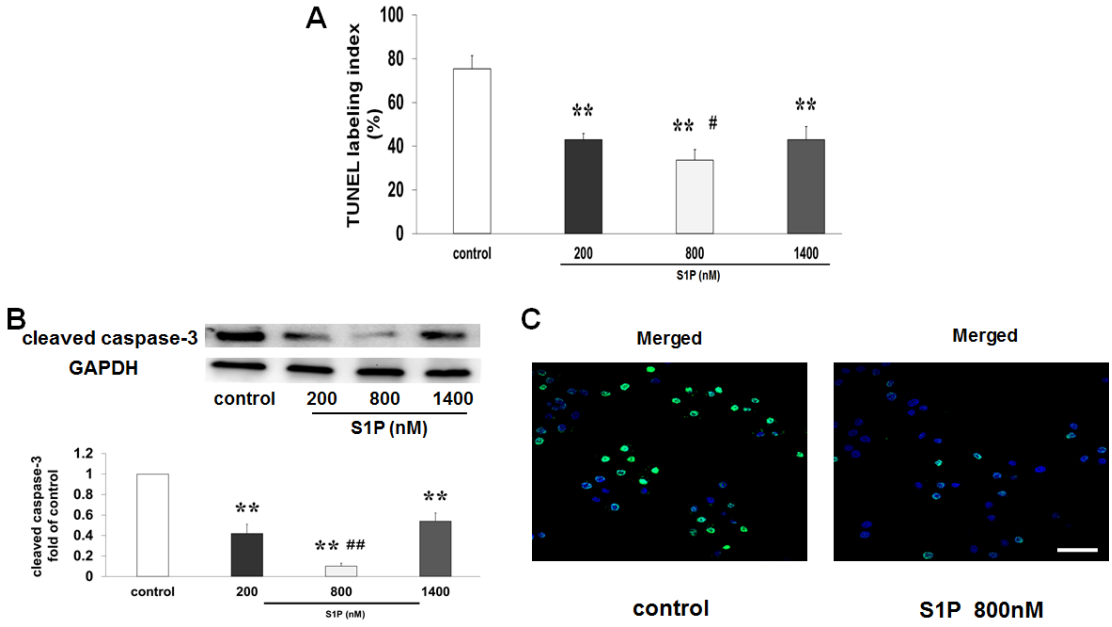

Figure 3

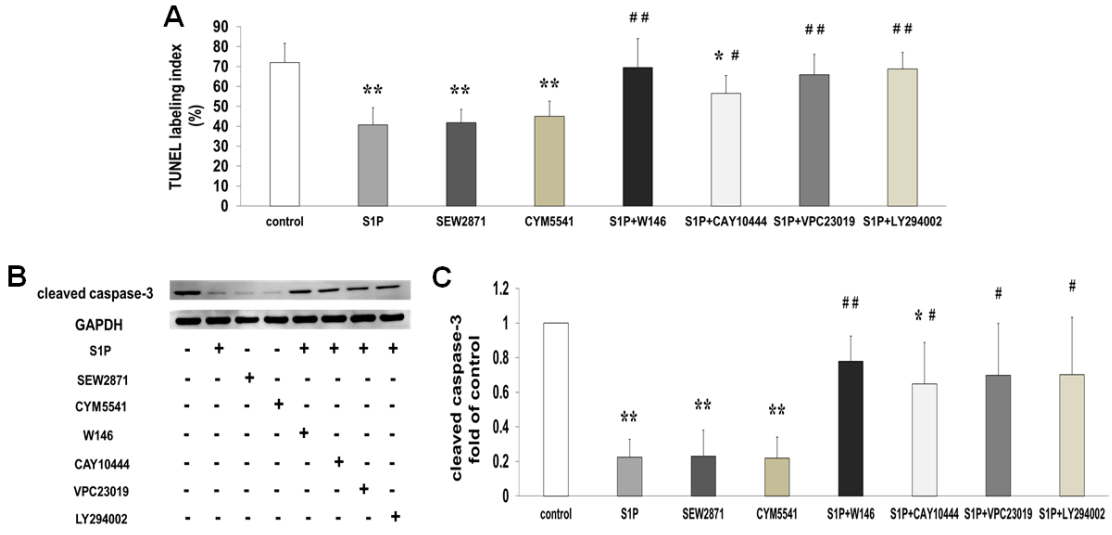

Figure 4

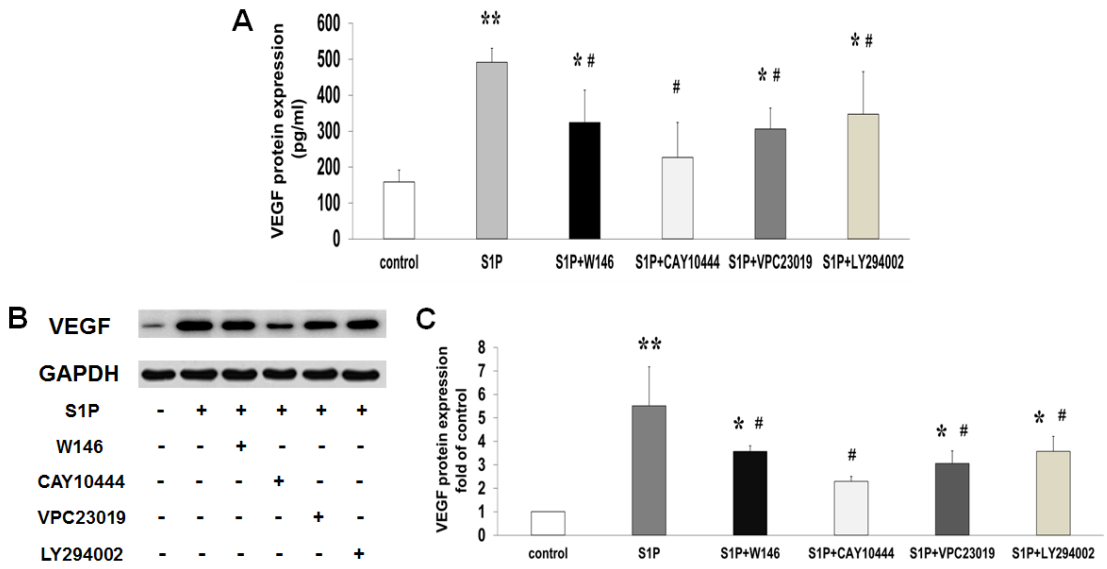

Figure 5

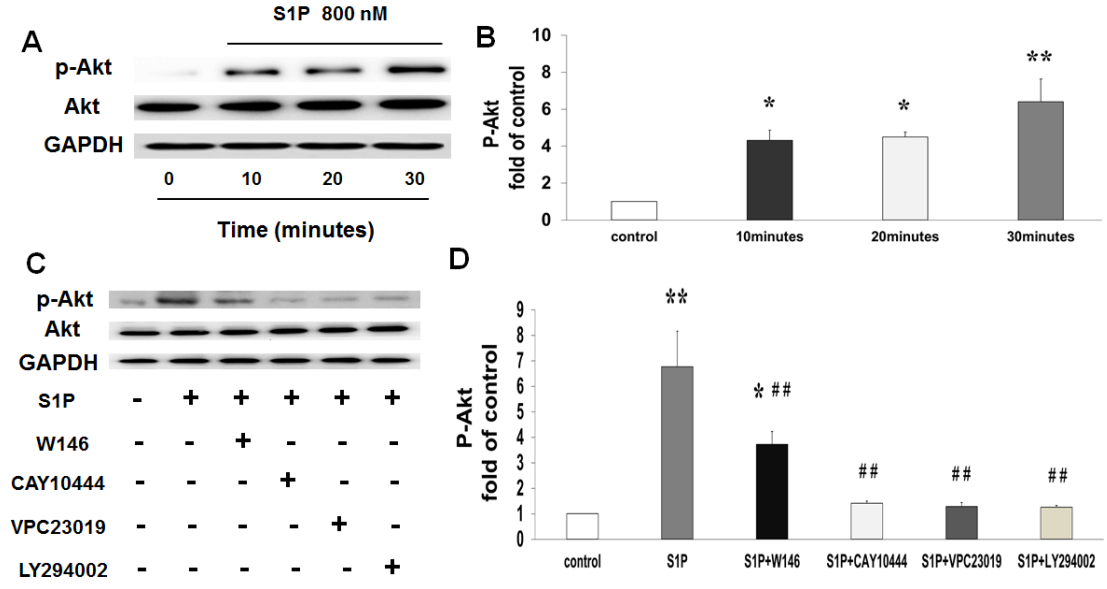

Figure 6