

Sphingosine-1-phosphate signaling and biological activities in the cardiovascular system

| | |
|-------|---|
| メタデータ | 言語: eng 出版者: 公開日: 2017-10-03 キーワード (Ja): キーワード (En): 作成者: メールアドレス: 所属: |
| URL | http://hdl.handle.net/2297/11734 |

Sphingosine-1-phosphate signaling and biological activities

in the cardiovascular system

Yoh Takuwa*, Yasuo Okamoto, Kazuaki Yoshioka, Noriko Takuwa

Department of Physiology, Kanazawa University Graduate School of Medicine,

13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan

* All correspondence should be addressed at Department of Physiology, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan. E-mail: ytakuwa@med.kanazawa-u.ac.jp

Key words: Sphingosine-1-phosphate, Sphingosine kinase, S1P Receptor, Signaling, Endothelial cells, Smooth muscle, Cardiomyocyte, Migration, Angiogenesis, Cardiac remodeling

Abstract

The plasma lysophospholipid mediator sphingosine-1-phosphate (S1P) is produced exclusively by sphingosine kinase (SPHK) 1 and SPHK2 *in vivo*, and plays diverse biological and pathophysiological roles by acting largely through three members of the G protein-coupled S1P receptors, S1P₁, S1P₂ and S1P₃. S1P₁ expressed on endothelial cells mediates embryonic vascular maturation and maintains vascular integrity by contributing to eNOS activation, inhibiting vascular permeability and inducing endothelial cell chemotaxis via Gi-coupled mechanisms. By contrast, S1P₂ is expressed in high levels on vascular smooth muscle cells (VSMCs) and certain types of tumor cells, inhibiting Rac and cell migration via a G_{12/13}- and Rho-dependent mechanism. In rat neointimal VSMCs, S1P₁ is upregulated to mediate local production of platelet-derived growth factor, which is a key player in vascular remodeling. S1P₃ expressed on endothelial cells also mediates chemotaxis toward S1P and vasorelaxation via NO production in certain vascular bed, playing protective roles for vascular integrity. S1P₃ expressed on VSMCs and cardiac sinoatrial node cells mediates vasopressor and negative chronotropic effect, respectively. In addition, S1P₃, together with S1P₂ and

SPHK1, is suggested to play a protective role against acute myocardial ischemia. However, our recent work indicates that overexpressed SPHK1 is involved in cardiomyocyte degeneration and fibrosis *in vivo*, in part through S1P activation of the S1P₃ signaling. We also demonstrated that exogenously administered S1P accelerates neovascularization and blood flow recovery in ischemic limbs, suggesting its usefulness for angiogenic therapy. These results provide evidence for S1P receptor subtype-specific pharmacological intervention as a novel therapeutic approach to cardiovascular diseases and cancer.

1. Introduction

Sphingosine-1-phosphate (S1P) is a lysophospholipid mediator circulating in blood, which induces a wide variety of biological responses in a diversity of cell types [1-5]. These include stimulation of cell proliferation, inhibition of apoptosis and regulation of cell shape and cell motility, among others. S1P is present at the order of 10^{-7} M concentration in the plasma, largely in forms bound to plasma proteins including HDL and albumin [1]. S1P is generated by the phosphorylation of sphingosine by sphingosine kinases (SPHKs) 1 and 2, which share a conserved catalytic domain but are expressed in spatiotemporally distinct manners [6]. Deletion of either SPHK1 or SPHK2 is functionally fully compensated by the other isozyme, whereas SPHK1/SPHK2 double knockout mice are embryonic lethal, indicating that S1P is produced exclusively by SPHK's *in vivo*. The major source of plasma S1P is believed to be red blood cells and activated platelets [1, 7], which lack S1P degrading enzyme S1P lyase. In addition, extracellular SPHK1, which is derived from vascular endothelial cells and other types of cells, also likely contributes to production of plasma S1P [8].

Identification of a family of G protein-coupled cell surface receptors specific for S1P

provided strong support for the notion that most, if not all, of the diversity of biological activities of S1P are mediated through either of the S1P receptors [2-5]. However, in lower organisms, which lack S1P receptors, and in certain biological events in mammalian cells S1P and its precursors likely play regulatory roles. Indeed, a recent study demonstrated that SPHK1KO mice show defective decidual formation and decidual angiogenesis in the SPHK2 heterozygous genetic background, without a reduction in S1P levels but with marked elevations in sphingosine levels and intracellular formation of abnormal membranous structures [9]. Intracellular accumulation of sphingosine is suggested to cause inhibition of polyp growth in SPHK1KO mice in murine genetic intestinal polyposis model, in which S1P levels were not different from wild type mice [10]. An intracellular role for S1P is suggested in mast cell activation [11].

Among five S1P receptor subtypes S1P₁₋₅, S1P₁, S1P₂ and S1P₃ are widely expressed in various tissues [2-5], whereas S1P₄ and S1P₅ are expressed mainly in the immune system and the nervous system, respectively. The diversity of S1P actions depends upon their subtype-specific distinct repertoire of heterotrimeric G protein coupling, in

combination with tissue- and cell type-specific expression patterns (Fig. 1).

In this review, we focus on the cardiovascular system and angiogenesis, describing current knowledge on S1P actions and signaling mechanisms in this system, which includes novel actions of S1P that we recently identified. Understanding the cardiovascular actions of S1P provides insights into the pathophysiological roles and therapeutic significance of S1P in the cardiovascular system.

2. S1P receptor activities and signaling in the cardiovascular system revealed from gene knockout studies

S1P₁ is coupled exclusively via G_i to Ras-MAP kinase, phosphoinositide (PI) 3-kinase-Akt pathway and phospholipase C pathway, whereas S1P₂ and S1P₃ are coupled to multiple G proteins, i.e. G_q, G_{12/13} and G_i to activate phospholipase C pathway and Rho pathway as well as the above mentioned G_i-dependent pathways [2-6].

S1P₁ is a major S1P receptor expressed on vascular endothelial cells, and is required for recruitment of mural cells and vascular maturation at the embryonic stage. Either

systemic disruption of S1P₁ gene or endothelial cell-selective knockout of S1P₁ resulted in embryonic lethality with a defect in vascular maturation (see below) [12], a phenotype similar to that in SPHK1/SPHK2 double KO mice [13] which essentially lack S1P, underscoring the importance of S1P₁ in S1P actions during embryonic phase. In addition, both S1P₁KO and SPHK1/SPHK2 double KO mice show defects in development of the nervous system. S1P₁ is also suggested to be involved in the maintenance of vascular integrity after birth, which includes inhibition of vascular permeability and endothelial cell survival [16-18].

S1P₁ receptors expressed on lymphocytes are responsible for their migration out of thymus and lymph nodes into the bloodstream, which is a crucial step in lymphocyte recirculation and thus immunity [14]. The immunosuppressant prodrug FTY720 is specifically phosphorylated by SPHK2 to become an active agonist for S1P₁, however, upon engagement to phosphorylated form of FTY720 S1P₁ undergoes rapid internalization and degradation through a ubiquitin-dependent process and thus strong downregulation of lymphocyte S1P₁ receptor [15]. This FTY720 phosphate activity as a functional S1P₁ antagonist leads to failure of lymphocyte egress from thymus and

peripheral lymphoid organs, and thus functional lymphopenia.

S1P₂ receptor is essential for morphogenesis of the heart in *Zebrafish* [19]. Although it is dispensable for murine cardiac development [20-22], S1P₂ receptor is required for functional integrity of the central nervous system in the weaning age and maintenance of normal structure and function of the inner ear in the adulthood [21, 22]. The latter role of S1P₂ appears to involve functional maintenance of the vasculature in the stria vascularis in the inner ear, in which S1P₂-mediated vasoconstriction controls arterial perfusion appropriately.

S1P₁ and S1P₃ are dominant S1P receptors expressed in vascular endothelial cells, whereas S1P₂ appears to be expressed only in endothelial cells of certain vascular beds [23]. Activation of S1P₂ on endothelial cell inhibits their migration and the formation of tube-like structures on Matrigel, most likely through mechanisms involving Rho-dependent inhibition of Rac and/or stimulation of the 3'-specific phosphoinositide phosphatase PTEN [23, 24]. In murine model of hypoxia-induced retinal angiogenesis, it is reported that S1P₂ expressed on the endothelium in the retinal vessels exerts an inhibitory effect on angiogenesis in neonatal avascular area of the retina, whereas S1P₂

expressed on vessels in vascular tuft invading vitreal body rather promotes pathological neo-angiogenesis, which leads to retinal hemorrhage and thus blindness [25]. The results suggest complex roles of S1P₂ in the regulation of pathological angiogenesis. In addition, activation of S1P₂ induces disruption of adherens junctions and resultantly vascular hyperpermeability [26].

S1P₃ receptor is abundantly expressed on vascular endothelial and medial smooth muscle cells, as well as cardiomyocytes [27]. S1P₃KO mice are phenotypically normal [28], however, S1P₃ deletion abrogates a variety of S1P effects on the cardiovascular system. These include negative chronotropic and hypertensive effects after intravenous administration of S1P *in vivo*, constriction of basilar artery, and endothelium-dependent vasodilation [27, 29]. S1P₃ mediates bradycardia through activation of inward rectifying G protein-activated potassium channels (GIRK1/GIRK4) via G_i in sinoatrial node cells [30], vasoconstriction through G_q-coupled Ca²⁺ mobilization and G_{12/13}-coupled Rho-dependent myosin light chain phosphatase inhibition in vascular smooth muscle cells (VSMCs) of certain vascular bed [27], and activation of eNOS and nitric oxide production in vascular endothelial cells through G_i- and Akt-mediated phosphorylation

of eNOS in concert with a G_q -mediated, Ca^{2+} /calmodulin-dependent activation process [31]. Being a constituent of plasma HDL, S1P is proposed to partially contribute to HDL-induced, NO-dependent vasorelaxation. However, S1P potently reduces myocardial perfusion in a manner dependent exclusively upon $S1P_3$ [32], despite the fact that HDL increases it. S1P is also vasoconstrictive for various vascular beds including kidney, mesentery and brain [33]. These observations, together with hypertensive effects of S1P, suggest that direct constrictive effects of S1P on vascular smooth muscle may dominate its endothelium-dependent dilatory effect in many vascular beds.

3. $S1P_2$ -mediated inhibition of vascular smooth muscle cell migration, a possible therapeutic strategy

Regulation of VSMC migration is a potential target of vascular medicine in the near future. Upon endothelial damage, medial VSMCs are induced to migrate into the intima, which, together with bone marrow-derived vascular progenitor cells recruited to the site of endothelial lesion, undergoes phenotypic conversion to neointimal cells to contribute

to the formation of vascular occlusive lesions, including atheroma and post-angioplasty restenosis. Migration of VSMCs is induced by chemoattractants such as platelet-derived growth factor (PDGF) [34]. Chemoattractants induce chemotaxis, which is a directed cell migration toward an increasing concentration of chemoattractant. In such an environment polarized stimulation of the chemoattractant receptors at the front of the cell that faces increasing concentration of the ligand induces polarized activation of PI 3-kinase and the Rho family small GTPase Rac, resulting in directed cell movement [35]. Growth factor receptors and chemokine receptors thus mediate chemotaxis through activation of PI 3-kinase and Rac, at the site downstream of receptor tyrosine kinases and $\beta\gamma$ subunit of G_i , respectively.

PDGF, which is a potent chemoattractant for VSMCs and released from the intimal VSMCs [34], acts on its cognate receptor tyrosine kinase to induce receptor tyrosyl auto-phosphorylation, followed by activation of PI 3-kinase and Rac, all of which are essential for a chemotactic response [17, 35]. S1P stimulation of $S1P_2$ abolishes PDGF-directed chemotaxis. $S1P_2$ activation also abolishes Rac activation and Rac-dependent actin re-organization, without inhibiting PDGF receptor

autophosphorylation or PI 3-kinase stimulation [2, 17, 36]. Thus, inhibition of Rac activation is very likely a major mechanism for S1P₂-mediated inhibition of cell migration. S1P₂ by itself mediates Rho activation. Since botulinum C3 toxin, which inhibits Rho, abolishes inhibitory regulation of S1P₂ it is likely that Rho activation is involved in S1P inhibition of Rac activity in VSMCs (S. Takashima et al., unpublished observation).

We analyzed in detail the G protein responsible for coupling S1P₂ to inhibition of Rac and cell migration by studying the effects of expression of molecular probes, which include C-terminal mini-peptides of the heterotrimeric G protein α -subunits (G α -CTs), which specifically inhibit receptor-G α protein interaction, as well as β Ark-CT, which acts as a scavenger for $\beta\gamma$ [37]. The results indicate that, among three classes of the heterotrimeric G proteins G_{12/13}, G_q and G_i that S1P₂ activates [38], S1P₂-mediated inhibition of Rac and cell migration requires both G_{12/13} and G_q (S. Takashima et al., unpublished observation). The G_{12/13} class induces Rho activation by direct physical and functional interaction between G α _{12/13} and a group of Rho-guanine nucleotide exchange factors (Rho-GEFs), which includes p115RhoGEF, PDZ-RhoGEF and LARG [39], at

their conserved structural motif known as the regulator of G protein signaling domain (the RGS box). G_q was previously demonstrated to mediate Rho stimulation through mechanisms involving direct association of $G\alpha_q$ with Rho-GEFs in certain non-smooth muscle cell types. In VSMCs AT_1 angiotensin II receptor couples to G_q to induce robust phospholipase C activation, however, AT_1 by itself does not mediate either Rho stimulation or Rac inhibition (S. Takashima et al., unpublished observation). Phospholipase C or its downstream second messengers Ca^{2+} or protein kinase C are not likely to be involved in $S1P_2$ -mediated Rho activation in VSMCs either. The observations indicate that G_q activation by itself is not sufficient for effective Rho activation required for inhibition of Rac and migration in VSMCs. It is unknown whether this is due to lack of the expression in VSMCs of a Rho-GEF type that G_q can associate with, or due to alternative mechanisms. It is likely that G_q exerts a permissive effect on Rho activation in cooperation with $G_{12/13}$.

There are several GPCR agonists that inhibit VSMC motility through G_s -coupled receptors and elevation in cellular cyclic AMP. These include prostaglandin E_2 (PGE_2), dopamine and adrenomedullin [40]. Different from these G_s -coupled receptors, which

suppress VSMC cell migration in the absence of a concentration gradient of the ligand [40], S1P₂ mediates inhibition of cell migration only in the presence of S1P concentration gradient [36]. This property of S1P is a hallmark of a chemorepellant. Certain bioactive factors including the semaphorine and the ephrine family members have recently been defined as chemorepellants for neuronal and vascular cells. These molecules act as ligands for cell surface receptors that are distinct from GPCRs. S1P₂ is the first example of a chemorepellant GPCR with an inhibitory activity for Rac [2].

Sanchez et al. [26] showed that S1P₂ inhibition of cell migration is mediated through stimulation of the 3'-specific PI phosphatase, PTEN, and in a Rho kinase-dependent manner. They suggested that PTEN stimulation by S1P₂ may be responsible for inhibition of cell migration, because 3'-PIs participate in cell migration through multitudes of mechanisms including actin cytoskeletal re-organization, the protein kinase Akt activation, and Rac-GEF activation. However, we observed in VSMCs that knockdown of PTEN expression by RNAi did not affect S1P₂-mediated inhibition of cell migration although PTEN knockdown effectively suppressed Akt activation (Sugimoto N et al., unpublished observations). The observations suggest that S1P₂

mediates stimulation of PTEN in VSMCs as well, but that stimulation of PTEN does not mediate inhibition of VSMC migration. These observations may suggest that $S1P_2$ -mediated inhibition of cell migration depends upon different subcellular mechanisms among cell types.

A number of growth factors, hormones and cytokines have been implicated in the process of vascular restenosis, including PDGF, fibroblast growth factor (FGF), angiotensin II and transforming growth factor- β . Inhibitors of these receptors and downstream signaling pathways have been demonstrated to successfully inhibit stenosis in animal models. Among these, rapamycin, an inhibitor of mTOR which is a protein kinase located downstream of the PI 3-kinase signaling pathway, is proven to be effective in diminishing restenosis in patients when rapamycin-coated stents are implanted at stenotic sites. Selective activation of $S1P_2$ receptor in VSMCs induces inhibition of the intracellular signaling molecules Rac and Akt, leading to inhibition of VSMC migration. Inhibition of Rac and Akt may also lead to anti-proliferation and apoptosis of VSMCs. If a selective $S1P_2$ antagonist is locally administered at stenotic sites as a drug-eluting stent, it could inhibit accumulation of VSMCs into the luminal

surface by uniquely acting as both a chemorepellent and anti-proliferative agent, exerting a protective effect against vascular restenosis.

4. *In vivo* angiogenic activity of S1P and its application for angiogenic therapy in tissue ischemia

The stimulatory activities of S1P in endothelial cell proliferation, migration and capillary-like tube formation *in vitro* are mediated via S1P₁ and S1P₃ and are reported to be as potent as well characterized angiogenic peptide growth factors including vascular endothelial growth factor (VEGF) and FGF-2 [16]. S1P also stimulates angiogenesis *in vivo* via S1P₁ and S1P₃ in the Matrigel implant assay [16]. Disruption of either S1P₁ gene or both SPHK1 and SPHK2 genes in mice demonstrated that S1P and its receptor S1P₁ in vascular endothelial cells are essentially required for the recruitment process of pericytes and smooth muscle cells to the nascent capillaries and thus vascular maturation [12, 13]. A recent investigation [41] demonstrated that RNAi-mediated S1P₁ silencing inhibited tumor angiogenesis and tumor growth *in vivo* in an animal model of subcutaneous tumor implantation, indicating that endogenous S1P is involved in tumor

angiogenesis via S1P₁. It is also reported that anti-S1P antibody potently reduced tumor growth [42]. Indeed, S1P stimulates barrier integrity of endothelial cells *in vitro* in part via S1P₁-mediated upregulation of VE-cadherin and stimulation of intercellular adhesion [18, 44]. *In vivo*, S1P and the S1P receptor agonist FTY720-phosphate acutely suppress pulmonary edema and VEGF-induced vascular hyperpermeability [45]. In contrast, S1P₂ and S1P₃ disrupt endothelial integrity *in vitro* and increase vascular permeability *in vivo* [45, 46]. S1P₂ expressed in endothelial cells also potently inhibits endothelial cell migration and capillary-like tube formation [17, 23]. These observations may suggest that selective S1P₁ activation in combination with S1P₂ activation should be a most promising strategy as an angiogenic treatment.

In the light of high morbidity and mortality due to ischemic cardiovascular diseases in the developed countries, stimulating revascularization in underperfused tissues is an attractive novel therapeutic strategy [47]. To date, however, the results of clinical studies on topical and systemic administration of the angiogenic peptide growth factors, including VEGF, FGF-2 and hepatocyte growth factor, and their expression plasmids, have not been very satisfactory.

The mouse hindlimb ischemic model is one of the well-established animal models for ischemia-induced angiogenesis *in vivo* to evaluate the potential of angiogenic factors as a therapeutic agent. We recently showed for the first time that S1P is useful in stimulating angiogenesis *in vivo* in the mouse hindlimb after ischemia [48]. The ligation and removal of unilateral femoral artery induces a 95 % reduction in the blood flow as compared to the contralateral non-ischemic limb the day after operation (day 1), as evaluated with a laser Doppler blood flow analyzer. The blood flow thereafter gradually recovers over 4 weeks. Daily intramuscular administration of S1P in hindlimb muscle dose-dependently accelerates blood flow recovery, resulting in up to twice as much blood flow at days 7 and 14 as compared to vehicle control. The stimulatory effect on the blood flow by the optimal dose of S1P is similar in the magnitude as that induced by the potent angiogenic peptide growth factor FGF-2, providing evidence that *in vivo* angiogenic strength of S1P is comparable to that of the known angiogenic factors. S1P-induced stimulation of the blood flow recovery is accompanied by an increase in the capillary density in the ischemic hindlimb muscle, suggesting that S1P-induced stimulation of blood flow recovery is due to neovessel formation. We recently generated

SPHK1-transgenic (SPHK1-Tg) mice (see below), which exhibits a marked increase in SPHK activity and a moderate increase in the S1P level in limb muscle as well as in other tissues. In SPHK1-Tg mice, the post-ischemic blood flow recovery and angiogenesis are accelerated as compared to WT littermates. One of the drawbacks in previous trials of angiogenic therapy is local tissue edema due to vascular leakage [47].

Daily S1P injection in wild type mice does not induce an increase in vascular leakage nor does SPHK1-Tg mice exhibit an increased vascular permeability in the ischemic limb, indicating that S1P-induced neo-angiogenesis is not accompanied by vascular hyperpermeability. S1P organizes endothelial tight junctions and adherens junctions [49] and promotes vascular mural cell recruitment at least in part via upregulation of N-cadherin [12, 50]. These effects of S1P likely lead to the protection of the neovessels from hyperpermeability. Daily local administration of S1P exhibits no adverse effect in mice, in terms of general conditions, the blood cell count or the cardiovascular parameters including the heart rate and blood pressure. These data collectively suggest potential usefulness of S1P or S1P₁ agonist for therapeutic angiogenesis in ischemic conditions.

Recent studies showed the contribution of bone marrow-derived circulating endothelial precursor cells to the new blood vessel formation in ischemic tissues. CD34⁺ vascular endothelial progenitors express S1P₃ receptor [51]. Stimulation of progenitor S1P₃ receptor with S1P or synthetic analog FTY720 activates the CXCR4 chemokine receptor which is essential for the effectiveness of progenitor cell therapy for angiogenesis. Therefore, S1P may stimulate angiogenesis not only by directly stimulating pre-existing endothelial cells but also through recruitment of circulating endothelial precursor cells.

5. S1P and cardiac remodeling

Cardiomyocytes express S1P₁, S1P₂ and S1P₃ [27]. S1P induces hypertrophy of cardiomyocytes *in vitro* via S1P₁ [52]. In addition, all of the three major S1P receptor subtypes are also expressed in cardiac fibroblasts which participate in cardiac hypertrophy and fibrosis, i.e. cardiac remodeling [53]. S1P reduces mortality of hypoxic cardiac myocytes *in vitro* [54]. S1P is also playing protective roles for myocardial

ischemia. For example, in an *in vivo* mouse model of myocardial ischemia/reperfusion (IR), exogenous HDL and its lipid component S1P attenuated infarct size dramatically [55]. These effects of HDL and S1P were completely absent in S1P₃KO mice. Deletion of both S1P₂ and S1P₃ receptors, but not either alone, in mice resulted in aggravation of acute myocardial infarction after experimental coronary artery ligation [56]. It is also reported, however, S1P potentially reduces coronary perfusion [32], possibly via coronary artery smooth muscle contraction. SPHK1 has been shown to have protective effects against IR injury in the perfused heart, and is involved in the process of ischemic preconditioning, in which mild ischemic pretreatment alleviates the extent of infarction caused by the subsequent ischemic session [57].

The heart expresses two SPHK isoforms, SPHK1 and SPHK2. Recent studies have demonstrated for various types of cells that stimulation with extracellular signals further stimulates cellular SPHK1 activity over the basal constitutive level. These include PDGF, nerve growth factor, muscarinic acetylcholine agonists, tumor necrosis factor- α , interleukin-1 and crosslinking of cell surface Fc ϵ RI or Fc γ RI, raising the possibility that SPHK1 and S1P are involved in certain pathological conditions. Indeed, Deutschman et

al. have reported that serum S1P level is the most reliable marker that predicts cardiovascular events to occur [58]. Overexpression of SPHK1 in cultures cells stimulates cell proliferation with accerelated G1 to S phase cell cycle progression, and in NIH3T 3 cells it resulted in cell transformation, leading to the notion that wild type SPHK1 gene is an oncogene [59].

We have generated SPHK1a-Tg mice to evaluate the effect of SPHK1a overexpression *in vivo* (N. Takuwa et al., unpublished observations). The TG mice overexpress functional SPHK1a in diverse tissues with more than 10-fold increases in the enzymatic activity. However, the TG mice of both sexes were born without malformation, showed normal development with growth curves identical to those of WT littermates, and were fertile, indicating that SPHK1a overexpression does not compromise regulated cell proliferation, differentiation or apoptosis that are required for development and reproduction. The lifespan of the TG mice are not shorter than the WT littermates. Unexpectedly, we found that the SPHK1a-Tg mice exhibited degenerative changes in cardiomyocytes and myocardial fibrosis, which aggravated with age. The SPHK1a-Tg mouse heart showed a slight but significant increase in the cardiac S1P

content, but normal sphingosine and ceramide levels. Immunohistochemical analysis of SPHK1a in the TG heart tissue revealed SPHK1a overexpression in the myocardium.

The SPHK1a-Tg mice were normotensive and have normal blood cholesterol and glucose levels. Coronary blood vessels in the Tg mice were normal. These observations indicated that cardiac fibrosis in the Tg heart is not secondary to hypertension, ischemic heart disease or other metabolic disorders. Accumulated evidence in recent investigation suggests the involvement of the Rho family GTPases, particularly Rac and Rho, in cardiac remodeling including hypertrophy and fibrosis in mice and human [60]. S1P receptor signaling induces either activation or inhibition of Rac and activation of Rho, depending on the receptor subtype [35]. We observed activation of Rac and Rho in the Tg heart as compared to WT littermates. Long term administration of an HMG CoA reductase inhibitor pitavastatin, which inhibits geranylgeranylation and thus membrane targeting of both Rac and Rho [60], strongly inhibited the development of cardiac fibrosis, consistent with the notion that the Rho family GTPases are involved in the pathogenesis of cardiac fibrosis in SPHK1a-Tg mice. Development of cardiac fibrosis is strongly suppressed in S1P₃KO mice, indicating that S1P₃ is involved in cardiac fibrosis

in the SPHK1a-Tg mice. These observations show that transgenic overexpression of SPHK1a in the mouse heart tissue leads to progressive cardiomyocyte degeneration and cardiac fibrosis, which likely involves activation of the S1P₃-Rho family small G protein signaling pathway as a mechanism. The relevance of these observations in transgenic mice to human cardiac fibrosis of the ischemic, hypertensive and other origins deserves further investigation.

6. Conclusion

Recent investigation has revealed an ever increasing list of previously unrecognized important roles for the S1P signaling system in homeostasis and pathophysiology of the cardiovascular system (Fig. 1). These include embryonic vascular maturation, regulation of angiogenesis and vascular integrity, vascular contraction and NO-mediated relaxation, autoregulation of vascular tone and the heart rate, as well as roles for coronary perfusion, ischemic preconditioning, protection against ischemia and myocardial remodeling. Modulation of the S1P signaling system by targeting S1P receptors and

sphingolipid-metabolizing enzymes, including SPHK1 and possibly other members, are expected to provide new therapeutic modalities for cardiovascular diseases, such as vascular occlusive diseases, tissue ischemia, and cardiac remodeling.

Acknowledgments

The authors are grateful to Drs. Naotoshi Sugimoto, Isao Inoki, Osamu Oyama, Shin-ichiro Takashima, Sei-ichiro Okura, So-ichiro Usui and other members in our laboratory for useful discussion. We thank C. Hirose for excellent secretarial and technical assistances. This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan and the Japan Society for the Promotion of Science, and Practical Application Research Program of JST (Japan Science and Technology Agency) Innovation Plaza Ishikawa.

References

[1] Y. Yatomi, Y. Ozaki, T. Ohmori, Y. Igarashi, Sphingosine 1-phosphate: synthesis and release, *Prostaglandins Other Lipid Mediat.* 64(2001)107-122.

- [2] Y. Takuwa. Subtype-specific differential regulation of Rho family G proteins and cell migration by the Edg family sphingosine-1-phosphate receptors, *Biochim. Biophys. Acta.* 1582(2002)112-120.
- [3] S. Spiegel, S. Milstien. Sphingosine-1-phosphate: an enigmatic signalling lipid, *Nat. Rev. Mol. Cell. Biol.* 4(2003)397-407.
- [4] I. Ishii, N. Fukushima, X. Ye, J. Chun. Lysophospholipid receptors: signaling and biology, *Annu. Rev. Biochem.* 73(2003)321-354.
- [5] T. Hla. Physiological and pathological actions of sphingosine 1-phosphate, *Semin. Cell. Dev. Biol.* 15(2004)513-520.
- [6] S. Spiegel, S. Milstien. Functions of the multifaceted family of sphingosine kinases and some close relatives, *J Biol Chem.* 282(2007)2125-2129.
- [7] A. Kihara, Y. Igarashi. Production and release of sphingosine 1-phosphate and the phosphorylated form of the immunomodulator FTY720, *Biophys. Biochem. Acta.* (2008) In press.
- [8] K. Venkataraman, S. Thangada, J. Michaud, M.L. Oo, Y. Ai, Y.M. Lee, M. Wu, N.S. Parikh, F. Khan, R.L. Proia, T. Hla. Extracellular export of sphingosine kinase-1a

contributes to the vascular S1P gradient, *Biochem. J.* 397(2006)461-471.

[9] K. Mizugishi, C. Li, A. Olivera, J. Bielawski, A. Bielawska, C.X. Deng, R.L. Proia .
Maternal disturbance in activated sphingolipid metabolism causes pregnancy loss in mice. *J. Clin. Invest.* 117(2007)2993-3006.

[10] M. Kohno, M. Momoi, M.L. Oo, J.H. Paik, Y.M. Lee, K. Venkataraman, Y. Ai, A.P. Ristimaki, H. Fyrst, H. Sano, D. Rosenberg, J.D. Saba, R.L. Proia, T. Hla. Intracellular role for sphingosine kinase 1 in intestinal adenoma cell proliferation, *Mol. Cell. Biol.* 26(2006)7211-723.

[11] A. Olivera, K. Mizugishi, A. Tikhonova, L. Ciaccia, S. Odom, R.L. Proia, J. Rivera. The sphingosine kinase-sphingosine-1-phosphate axis is a determinant of mast cell function and anaphylaxis, *Immunity.* 26(2007)287-297.

[12] Y. Liu, R. Wada, T. Yamashita, Y.Mi, C.X. Deng, J.P. Hobson, H.M. Rosenfeldt, V.E. Nava, S.S. Chae, M.J. Lee, C.H. Liu, T. Hla, S. Spiegel, R.L. Proia. Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation, *J Clin Invest.* 106(2000)951-961.

[13] K. Mizugishi, T. Yamashita, A. Olivera, G.F. Miller, S. Spiegel, R.L. Proia.

Essential role for sphingosine kinases in neural and vascular development, *Mol Cell Biol.* 24(2005)11113-11121.

[14] H. Rosen, M.G. Sanna, S.M. Cahalan, P.J. Gonzalez-Cabrera. Tipping the gatekeeper: S1P regulation of endothelial barrier function, *Trends Immunol.* 28(2007)102-107.

[15] V. Brinkmann. Sphingosine 1-phosphate receptors in health and disease: mechanistic insights from gene deletion studies and reverse pharmacology, *Pharmacol Ther.* 115(2007)84-105.

[16] M.J. Lee, S. Thangada, K.P. Claffey, N. Ancellin, C.H. Liu, M. Kluk, M. Volpi, R.I. Shaafi, T. Hla. Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate, *Cell.* 99(1999)301-312.

[17] Y. Ryu, N. Takuwa, N. Sugimoto, S. Sakurada, S. Usui, H. Okamoto, O. Matsui, Y. Takuwa. Sphingosine-1-Phosphate, a Platelet-Derived Lysophospholipid Mediator, Negatively Regulates Cellular Rac Activity and Cell Migration in Vascular Smooth Muscle Cells, *Circ. Res.* 90(2002)325-332.

[18] P.A. Singleton, S.M. Dudek, E.T. Chiang, J.G. Garcia. Regulation of sphingosine

1-phosphate-induced endothelial cytoskeletal rearrangement and barrier enhancement by S1P1 receptor, PI3 kinase, Tiam1/Rac1, and alpha-actinin, *FASEB J.* 19(2005)1646-1656.

[19] E. Kupperman, S. An, N. Osborne, S. Waldron, D.Y. Stainier. A sphingosine-1-phosphate receptor regulates cell migration during vertebrate heart development, *Nature.* 406(2000)192-195.

[20] M. Kono, Y. Mi, Y. Liu, T. Sasaki, M.L. Allende, Y.P. Wu, T. Yamashita, R.L. Proia. The sphingosine-1-phosphate receptors S1P1, S1P2, and S1P3 function coordinately during embryonic angiogenesis, *J. Biol Chem.* 279(2004)29367-29373.

[21] A.J. MacLennan, S.J. Benner, A. Andringa, A.H. Chaves, J.L. Rosing, R. Vesey, A.M. Karpman, S.A. Cronier, N. Lee, L.C. Erway, M.L. Miller. The S1P2 sphingosine 1-phosphate receptor is essential for auditory and vestibular function, *Hear Res.* 220(2006)38-48.

[22] M. Kono, I.A. Belyantseva, A. Skoura, G.I. Frolenkov, M.F. Starost, J.L. Dreier, D. Lidington, S.S. Bolz, T.B. Friedman, T. Hla, R.L. Proia. Deafness and stria vascularis defects in S1P2 receptor-null mice, *J Biol Chem.* 282(2007)10690-10696.

- [23] I. Inoki, N. Takuwa, N. Sugimoto, K. Yoshioka, S. Takata, S. Kaneko, Y. Takuwa. Negative regulation of endothelial morphogenesis and angiogenesis by S1P2 receptor, *Biochem Biophys Res Commun.* 346(2006)293-300.
- [24] T. Sanchez, S. Thangada, M.T. Wu, C.D. Kontos, D. Wu, H. Wu, T. Hla. PTEN as an effector in the signaling of antimigratory G protein-coupled receptor, *Proc Natl Acad Sci USA.* 102(2005)4312-4317.
- [25] A. Skoura, T. Sanchez, K. Claffey, S.M. Mandala, R.L. Proia, T. Hla. Essential role of sphingosine 1-phosphate receptor 2 in pathological angiogenesis of the mouse retina, *J Clin Invest.* 117(2007)2506-2516.
- [26] T. Sanchez, A. Skoura, M.T. Wu, B. Casserly, E.O. Harrington, T. Hla. Induction of vascular permeability by the sphingosine-1-phosphate receptor-2 (S1P2R) and its downstream effectors ROCK and PTEN, *Arterioscler Thromb Vasc Biol.* 27(2007)1312-1318.
- [27] S.L. Peters, A.E. Alewijnse. Sphingosine-1-phosphate signaling in the cardiovascular system, *Curr Opin Pharmacol.* 7(2007)186-192.
- [28] I. Ishii, B. Friedman, X. Ye, S. Kawamura, C. McGiffert, J.J. Contos, M.A.

Kingsbury, G. Zhang, J.H. Brown, J. Chun. Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP(B3)/EDG-3, *J Biol Chem.* 276(2001)33697-33704.

[29] M. Forrest, S.Y. Sun, R. Hajdu, J. Bergstrom, D. Card, G. Doherty, J. Hale, C. Keohane, C. Meyers, J. Milligan, S. Mills, N. Nomura, H. Rosen, M. Rosenbach, G.J. Shei, I.I. Singer, M. Tian, S. West, V. White, J. Xie, R.L. Proia, S. Mandala. Immune cell regulation and cardiovascular effects of sphingosine 1-phosphate receptor agonists in rodents are mediated via distinct receptor subtypes, *J Pharmacol Exp Ther.* 309(2004)758-768.

[30] R. Ochi, Y. Momose, K. Oyama, W.R. Giles. Sphingosine-1-phosphate effects on guinea pig atrial myocytes: Alterations in action potentials and K⁺ currents, *Cardiovasc Res.* 70(2006)88-96.

[31] J.R. Nofer, M. van der Giet, M. Tölle, I. Wolinska, K. von Wnuck Lipinski, H.A. Baba, U.J. Tietge, A. Gödecke, I. Ishii, B. Kleuser, M. Schäfers, M. Fobker, W. Zidek, G. Assmann, J. Chun, B. Levkau. HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3, *J Clin Invest.* 113(2004)569-581

- [32] B. Levkau, S. Hermann, G. Theilmeyer, M. van der Giet, J. Chun, O. Schober, M. Schäfers. High-density lipoprotein stimulates myocardial perfusion in vivo, *Circulation*. 110(2004)3355-3359.
- [33] A.E. Alewijnse, S.L. Peters, M.C. Michel. Cardiovascular effects of sphingosine-1-phosphate and other sphingomyelin metabolites, *Br J Pharmacol*. 143(2004)666-684.
- [34] S. Usui, N. Sugimoto, N. Takuwa, S. Sakagami, S. Takata, S. Kaneko, Y. Takuwa. Blood lipid mediator sphingosine-1-phosphate potently stimulates platelet derived growth factor-A and -B chain expression through S1P1-Gi-Ras-MAPK-dependent induction of krüppel-like factor 5, *J Biol Chem* 279(2004)12300-12311.
- [35] D.A. Lauffenburger, A.F. Horwitz. Cell migration: a physically integrated molecular process, *Cell*. 84(1996) 359-369.
- [36] H. Okamoto, N. Takuwa, T. Yokomizo, N. Sugimoto, S. Sakurada, H. Shigematsu, Y. Takuwa. Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3, *Mol Cell Biol*. 20(2000)9247-9261.

[37] N. Sugimoto, N. Takuwa, H. Okamoto, S. Sakurada, Y. Takuwa, Y. Inhibitory and stimulatory regulation of Rac and cell motility by the G12/13-Rho and Gi pathway integrated downstream of a single G protein-coupled sphingosine-1-phosphate receptor isoform, *Mol. Cell. Biol.* 23(2003)1534-1545.

[38] R.T. Windh, M.J. Lee, T. Hla, S. An, A.J. Barr, D.R. Manning. Differential coupling of the sphingosine 1-phosphate receptors Edg-1, Edg-3, and H218/Edg-5 to the G(i), G(q), and G(12) families of heterotrimeric G proteins, *J Biol Chem.* 274(1999)27351-27358.

[39] S. Fukumoto, H. Chikumi, J.S. Gutkind. RGS-containing RhoGEFs: the missing link between transforming G proteins and Rho, *Oncogene.* 20(2001)1661-1668.

[40] S.Y. Nagasawa, N. Takuwa, N. Sugimoto, H. Mabuchi, Y. Takuwa. Inhibition of Rac activation as a mechanism for negative regulation of actin cytoskeletal reorganization and cell motility by cAMP, *Biochem J.* 385(2005)737-744.

[41] S.S. Chae, J.H. Paik, H. Furneaux, T. Hla. Requirement for sphingosine-1-phosphate receptor-1 in tumor angiogenesis demonstrated by in vivo RNA interference, *J Clin Invest.* 114(2004)1082–1089.

[42] B. Visentin, J.A. Vekich, B.J. Sibbald, A.L. Cavalli, K.M. Moreno, R.G. Matteo, W.A. Garland, Y. Lu, S. Yu, H.S. Hall, V. Kundra, G.B. Mills, R.A. Sabbadini. Validation of an anti-sphingosine-1-phosphate antibody as a potential therapeutic in reducing growth, invasion, and angiogenesis in multiple tumor lineages, *Cancer Cell*. 9(2006)225-238.

[43] J.F. Lee, Q. Zeng, H. Ozaki, L. Wang, A.R. Hand, T. Hla, E. Wang, M.J. Lee. Dual roles of tight junction-associated protein, zonula occludens-1, in sphingosine 1-phosphate-mediated endothelial chemotaxis and barrier integrity, *J Biol Chem*. 281(2006)29190-29200.

[44] X. Peng, P.M. Hassoun, S. Sammani, B.J. McVerry, M.J. Burne, H. Rabb, D. Pearce, R.M. Tuder, J.G. Garcia. Protective effects of sphingosine 1-phosphate in murine endotoxin-induced inflammatory lung injury, *Am J Respir Crit Care Med*. 169(2004)1245-1251.

[45] T. Sanchez, A. Skoura, M.T. Wu, B. Casserly, E.O. Harrington, T. Hla. Induction of vascular permeability by the sphingosine-1-phosphate receptor-2 (S1P2R) and its downstream effectors ROCK and PTEN, *Arterioscler Thromb Vasc Biol*.

27(2007)1312-1318.

[46] P.A. Singleton, S.M. Dudek, S.F. Ma, L.G. Garcia. Transactivation of sphingosine 1-phosphate receptors is essential for vascular barrier regulation. Novel role for hyaluronan and CD44 receptor family, *J Biol Chem.* 281(2006)34381-34393.

[47] N. Ferrara, R.S. Kerbel. Angiogenesis as a therapeutic target, *Nature.* 438(2005)967-974.

[48] O. Oyama, N. Sugimoto, X. Qi, N. Takuwa, K. Mizugishi, J. Koizumi, Y. Takuwa. The lysophospholipid mediator sphingosine-1-phosphate promotes angiogenesis in vivo in ischaemic hindlimbs of mice, *Cardiovasc Res.* 2008. In Press

[49] J.F. Lee, Q. Zeng, H. Ozaki, L. Wang, A.R. Hand, T. Hla, E. Wang, M.J. Lee. Dual roles of tight junction-associated protein, zonula occludens-1, in sphingosine 1-phosphate-mediated endothelial chemotaxis and barrier integrity, *J Biol Chem.* 281(2006)29190-29200.

[50] J.H. Paik, A. Skoura, S.S. Chae, A.E. Cowan, D.K. Han, R.L. Proia, T. Hla. Sphingosine 1-phosphate receptor regulation of N-cadherin mediates vascular stabilization, *Genes Dev.* 18(2004)2392-2403.

[51] D.H. Walter, U. Rochwalsky, J. Reinhold, F. Seeger, A. Aicher, C. Urbich, D.H.

Walter, U. Rochwalsky, J. Reinhold. Sphingosine-1-phosphate stimulates the functional capacity of progenitor cells by activation of the CXCR4-dependent signaling pathway via the S1P3 receptor, *Arterioscler Thromb Vasc Biol.* 27(2007)275-282.

[52] P. Robert, P. Tsui, M.P. Laville, G.P. Livi, H.M. Sarau, A. Bril, I. Berrebi-Bertrand.

EDG1 receptor stimulation leads to cardiac hypertrophy in rat neonatal myocytes, *J Mol Cell Cardiol.* 33(2001)1589-1606.

[53] L.K. Landeen, N. Aroonsakool, J.H. Haga, B.S. Hu, W.R. Giles.

Sphingosine-1-phosphate receptor expression in cardiac fibroblasts is modulated by in vitro culture conditions, *Am J Physiol.* 292(2007)H2698-2711.

[54] J. Zhang, N. Honbo, E.J. Goetzl, K. Chatterjee, J.S. Karliner, M.O. Gray. Signals

from type 1 sphingosine 1-phosphate receptors enhance adult mouse cardiac myocyte survival during hypoxia, *Am J Physiol.* 293(2007)H3150-3158.

[55] G. Theilmeyer, C. Schmidt, J. Herrmann, P. Keul, M. Schafers, I. Herrgott, *et al.*

High-density lipoproteins and their constituent, sphingosine-1-phosphate, directly protect the heart against ischemia/reperfusion injury in vivo via the S1P3

lysophospholipid receptor, *Circulation*. 114(2006)1403-1409.

[56] C.K. Means, C.Y. Xiao, Z. Li, T. Zhang, J.H. Omens, I. Ishii, J. Chun, J.H. Brown. Sphingosine 1-phosphate S1P2 and S1P3 receptor-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury, *Am J Physiol*. 292(2007)H2944-2951.

[57] Z.Q. Jin, J. Zhang, Y. Huang, H.E. Hoover, D.A. Vessey, J.S. Karliner. A sphingosine kinase 1 mutation sensitizes the myocardium to ischemia/reperfusion injury, *Cardiovasc Res*. 76(2007)41-50.

[58] D.H. Deutschman, J.S. Carstens, R.P. Klepper, W.S. Smith, M.T. Page, T.R. Young, L.A. Gleason, N. Nakajima, R.A. Sabbadini. Predicting obstructive coronary artery disease with serum sphingosine-1-phosphate, *Am Heart J*. 146(2003)62-68.

[59] P. Xia, J.R. Gamble, L. Wang, S.M. Pitson, P.A. Moretti, B.W. Wattenberg, R.J. D'Andrea, M.A. Vadas. An oncogenic role of sphingosine kinase, *Curr Biol*. 10(2000)1527-1530.

[60] J.H. Brown, D.P. Del Re, M.A. Sussman. The Rac and Rho hall of fame: a decade of hypertrophic signaling hits, *Circ Res*. 98(2006)730-742.

Figure legend

Figure 1. S1P signaling and actions through S1P₁, S1P₂ and S1P₃ receptors in the cardiovascular system. S1P, which is synthesized by membrane sphingolipid metabolism and final phosphorylation of sphingosine by SPHKs, binds to S1P₁, S1P₂ and S1P₃ in vascular endothelial cells, VSMCs, and cardiomyocytes. These three receptors activate partially overlapping but distinct sets of signaling pathways, to exert complex effects on cardiovascular cells, which include stimulation or inhibition of cell migration and angiogenesis, a decrease or increase in vascular permeability, vascular contraction or relaxation, and protection from myocardial ischemia. SPP, sphingosine-1-phosphate phosphatase; SPL, sphingosine-1-phosphate lyase; VSM, vascular smooth muscle.

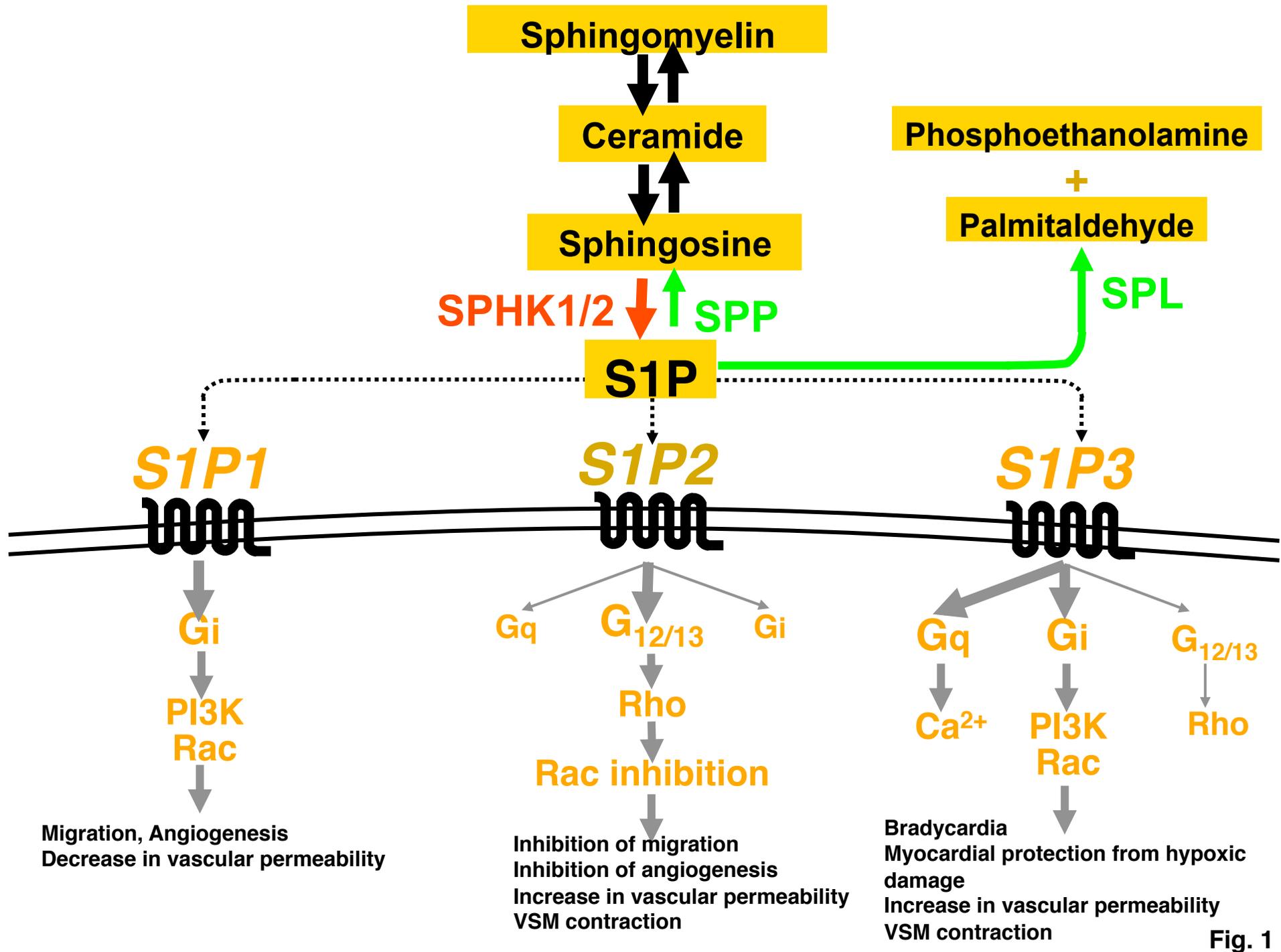


Fig. 1