Sphingosine-1-Phosphate-Induced Inflammation Involves Receptor Tyrosine Kinase Transactivation in Vascular Cells Upregulation in Hypertension

Alvaro Yogi, Glaucia E. Callera, Anna B. Aranha, Tayze T. Antunes, Delyth Graham, Martin McBride, Anna Dominiczak, Rhian M. Touyz

Abstract—Sphingosine-1-phosphate (S1P), a multifunctional phospholipid, regulates vascular cell function. Whether S1P influences vascular inflammatory responses, particularly in hypertension, is unclear. We tested the hypothesis that S1P is a proinflammatory mediator signaling through receptor tyrosine kinase transactivation and that responses are amplified in vascular smooth muscle cells from stroke-prone spontaneously hypertensive rats (SHRSPs), a model in which we demonstrated Edg1 (S1P1 receptor) to be a candidate gene for salt-sensitive hypertension. Vascular smooth muscle cell from Wistar-Kyoto rats and SHRSPs were studied. S1P receptor subtypes, S1P1 and S1P2, were similarly expressed in Wistar-Kyoto rats and SHRSPs. S1P induced phosphorylation of epidermal growth factor receptor and platelet-derived growth factor receptor by angiotensin II has been well described, and recent data suggest that S1P may also influence signaling through receptor tyrosine kinases. We identify a novel pathway linking S1P/S1P1 receptors to specific proinflammatory signaling pathways through epidermal growth factor receptor and platelet-derived growth factor transactivation, a process that is upregulated in SHRSPs. Such molecular events may contribute to vascular inflammation in hypertension. (Hypertension. 2011;57:809-818.) ● Online Data Supplement

Key Words: sphingosine-1-phosphate ■ SHRSP ■ EGFR ■ PDGFR ■ inflammation

Sphingosine 1-phosphate (S1P), an important structural entity of biological membranes, is also a bioactive lipid mediator involved in cell proliferation, survival, migration, and inflammation in various cell types, including vascular smooth muscle cells (VSMCs).1-3 S1P serves as a ligand for a subset of G protein–coupled receptors, of which 5 mammalian S1P receptors (S1P1 through S1P5) have been identified. VSMCs, in culture, express S1P1, S1P2, and S1P3 receptors.4-8 The downstream targets of S1P signaling include adenylate cyclase, Ras, mitogen-activated protein kinase (MAPK), phospholipase C, c-Src tyrosine kinase, hypoxia-inducible factor 1, the small GTPases Rac and Rho, phospholipase D, p125FAK, phosphatidylinositol 3-kinase/AKT, and calcium.7,8 Transactivation of receptor tyrosine kinase (RTK) by ligand binding to G protein–coupled receptors is involved in pathophysiological processes associated with vascular inflammation and injury in hypertension.9-12 Transactivation of epidermal growth factor receptor (EGFR), platelet-derived growth factor (PDGFR), insulin-like growth factor receptor 1, and vascular endothelial growth factor receptor by angiotensin II has been well described, and recent data suggest that S1P may also influence signaling through EGFR and PDGFR in VSMCs.13

Sphingolipids, vasoactive compounds produced and secreted by platelets, endothelial cells, and VSMCs, are implicated in vascular contraction/dilation, hypertrophy, and fibro-
sis and have been suggested to influence inflammatory responses and, accordingly, may be involved in endothelial dysfunction and vascular remodeling in hypertension. In fact, the Edg1 (S1P1 receptor) gene is a candidate to control salt sensitivity and hypertension in the stroke-prone spontaneously hypertensive rat (SHRSP).14

Molecular mechanisms underlying S1P vascular effects remain unclear, particularly in hypertension. We hypothesized that S1P, through RTKs, influences proinflammatory vascular signaling, an effect that is upregulated in hypertension. Here we examined molecular mechanisms and functional effects of S1P in VSMCs from SHRSPs, focusing on the roles of EGFR and PDGFR in vascular inflammation.

**Methods**

Detailed methods and protocols are available in the online Data Supplement at http://hyper.ahajournals.org.

**Cell Culture**

The study was approved by the animal ethics committee of the University of Ottawa and performed according to the recommendations of the Canadian Council for Animal Care. VSMCs from adult male Wistar-Kyoto rats (WKYs), Wistar rats, and SHRSPs were studied. VSMCs from mesenteric arteries were isolated, characterized, and cultured. Low-passage cells (4 to 8) were studied.

**Protocols for Cell Stimulation**

VSMCs were stimulated with S1P (10^{-6} mol/L) for short (1, 5, and 30 minutes) or long term (1 to 24 hours). In some experiments, cells were pre-exposed (30 minutes) to VPC23019 (S1P1 antagonist, 10^{-6} mol/L), AG1478 (EGFR inhibitor, 10^{-6} mol/L) or AG1296 (PDGFR inhibitor, 10^{-6} mol/L).

**Western Blotting**

Total protein from S1P-stimulated VSMCs was extracted, separated by electrophoresis on a polyacrylamide gel, and transferred onto a nitrocellulose membrane.15 Membranes were incubated with specific antibodies: S1P1, S1P2, and S1P3 receptors; EGFR [Y\(_{245}\)]; PDGFR [Y\(_{490/497}\)]; p38MAPK [T\(_{180/182}\)]; stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) [T\(_{183/189}\)]; intercellular adhesion molecule (ICAM) 1; vascular cell adhesion protein (VCAM) 1; and cyclooxygenase (COX) 2. Non-phosphoproteins= β-actin or β-tubulin were used as housekeeping controls.

**Adhesion Assay**

Growth-arrested VSMCs from WKYs and SHRSPs were stimulated with S1P (10^{-6} mol/L; 6 hours) in the absence and presence of AG1478, AG1296 (10^{-7} mol/L; 30 minutes), and VPC23019 (10^{-6} mol/L; 30 minutes), and adhesion of macrophages to VSMCs was evaluated.

**Statistical Analysis**

Results are presented as mean±SEM and compared by Student t test and ANOVA 1 or 2 way. P<0.05 was considered significant.

**Results**

SIP1 and SIP2 But Not SIP3 Receptors Are Expressed in VSMCs From WKYs and SHRSPs

SIP receptor types 1 and 2 are expressed in VSMCs from WKYs and SHRSPs (Figure S1). There were no differences in expression profiles of SIP1 and SIP2 receptors between WKYs and SHRSPs. SIP3 could not be detected in WKY or SHRSP cells.

Basal phosphorylation levels of the signaling molecules and/or expression of proinflammatory markers evaluated in this study were assessed in VSMCs from WKYs and SHRSPs. We did not observe any significant difference in the basal phosphorylation of EGFR, PDGFR, p38MAPK, and SAPK/JNK and expression of ICAM-1, VCAM-1, and COX-2 in VSMCs from normotensive and hypertensive animals (Figure S2). SIP effects were evaluated in VSMCs from 2 normotensive rat strains, WKY and Wistar, as normotensive controls. SIP induced a similar magnitude of increase in EGFR, PDGFR, p38MAPK, and SAPK/JNK phosphorylation, as well as ICAM-1, VCAM-1 and COX-2 expression (Figure S3).

**SIP Induces Transactivation of EGFR and PDGFR via SIP1**

Figures 1 and 2 demonstrate that SIP induces phosphorylation of EGFR and PDGFR with greater effects in VSMCs from SHRSPs compared with WKYs. VPC 23019, an SIP1 receptor antagonist, blocked SIP-induced phosphorylation of EGFR and PDGFR in VSMCs from WKYs and SHRSPs.

**SIP-Induced Phosphorylation of p38MAPK and JNK Is Mediated by EGFR and PDGFR Transactivation**

Figure 3 demonstrates that SIP induces p38MAPK phosphorylation in both WKYs and SHRSPs, with increased responses in cells from hypertensive rats. This effect was blocked by VPC 23019 (Figure 3B and 3C). AG1478 and AG1296 inhibited SIP-mediated phosphorylation of p38MAPK in WKYs (Figure 3D). AG1478 inhibited SIP-induced p38MAPK phosphorylation in SHRSPs. AG1296 only partially inhibited activation of p38MAPK in SHRSP cells (Figure 3E).

SIP stimulates SAPK/JNK phosphorylation in VSMCs from WKYs and SHRSPs (Figure S4). Effects in cells from SHRSPs were enhanced compared with WKY cells. VPC 23019 inhibited SIP-mediated SAPK/JNK phosphorylation in both strains. In VSMCs from WKYS, both AG1478 and AG1296 blocked SIP-induced SAPK/JNK phosphorylation. In SHRSP VSMCs, EGFR and PDGFR inhibitors only partially blocked SIP-mediated SAPK/JNK phosphorylation.

**Differential Role of RTK Transactivation in SIP-Induced Expression of Proinflammatory Mediators in SHRSPs But Not WKYs**

Long-term exposure of cells to SIP increased expression of ICAM-1 (Figure 4) and VCAM-1 (Figure S5) in VSMCs from WKYs and SHRSPs with enhanced effects in cells from hypertensive rats. VPC 23091 blocked SIP-induced actions. AG1478 and AG1296 inhibited SIP-induced expression of ICAM-1 and VCAM-1 in VSMCs from SHRSPs, with partial inhibition in WKY cells.

**SIP-Induced COX-2 Expression Does Not Involve EGFR and PDGFR Transactivation in SHRSPs and WKYs**

In VSMCs from WKYs and SHRSPs, SIP stimulated increased expression of COX-2 (Figure 5A), an effect abro-
Figure 1. Effect of S1P1 receptor antagonism on S1P-induced EGFR phosphorylation in VSMCs from WKYs and SHRSPs. Top and right, Representative immunoblots for EGFR [Tyr845] and EGFR. Corresponding bar graphs demonstrate the following: (A) the time-course (1, 5, and 30 minutes) stimulation of S1P (1 μmol/L) on EGFR phosphorylation in VSMCs from WKYs and SHRSPs; the effect of VPC 23091 in S1P-induced EGFR phosphorylation in VSMCs from WKYs (B) and SHRSPs (C). Results are mean ± SEM of 4 experiments. *P<0.05 vs vehicle (ANOVA 1 way); **P<0.05, corresponding S1P stimulation times in WKYs vs SHRSPs (ANOVA 2 way).

Figure 2. Effect of S1P1 receptor antagonism on S1P-induced PDGFR phosphorylation in VSMCs from WKYs and SHRSPs. Top and right panels, Representative immunoblots for PDGFRα/β [Tyr849/857] and PDGFR. Corresponding bar graphs demonstrate the following: (A) the time-course (1, 5, and 30 minutes) stimulation of S1P (1 μmol/L) on PDGFR phosphorylation in VSMCs from WKYs and SHRSPs; the effect of VPC23091 in S1P-induced PDGFR phosphorylation in VSMCs from WKYs (B) and SHRSPs (C). Results are mean ± SEM of 4 experiments. *P<0.05 vs vehicle (ANOVA 1 way); **P<0.05, corresponding S1P stimulation times in WKYs vs SHRSPs (ANOVA 2 way).
Figure 3. Effect of EGFR, PDGFR inhibition, or S1P1 receptor antagonism on S1P-induced p38MAPK phosphorylation in VSMCs from WKYs and SHRSPs. Top and right panels, Representative immunoblots for p38MAPK [Thr180/Tyr182] and p38MAPK. Corresponding bar graphs demonstrate the following: (A) the time course (1, 5, and 30 minutes) stimulation of S1P (1 μmol/L) on p38MAPK phosphorylation in VSMCs from WKYs and SHRSPs; the effect of VPC23091 in S1P-induced p38MAPK phosphorylation in VSMCs from WKYs (B) and SHRSPs (C); the effect of AG1478 or AG1296 in S1P-induced p38MAPK phosphorylation in VSMCs from WKYs (D) and SHRSPs (E). Results are mean±SEM of 4 experiments. *P<0.05 vs vehicle (ANOVA 1 way); **P<0.05, corresponding S1P stimulation times in WKYs vs SHRSPs (ANOVA 2 way).
Figure 4. Effect of EGFR, PDGFR inhibition, or S1P1 receptor antagonism on S1P-induced ICAM-1 expression in VSMCs from WKYs and SHRSPs. Top and right panels, Representative immunoblots for ICAM-1 and β-tubulin. Corresponding bar graphs demonstrate the following: (A) the time course (4, 8, and 24 hours) stimulation of S1P (1 μmol/L) on ICAM-1 expression in VSMCs from WKYs and SHRSPs; the effect of VPC 23091 in S1P-induced ICAM-1 expression in VSMCs from WKYs (B) and SHRSPs (C); and the effect of AG1478 or AG1296 in S1P-induced ICAM-1 expression in VSMCs from WKYs (D) and SHRSPs (E). Results are mean ± SEM of 4 to 8 experiments. *P<0.05 vs vehicle (ANOVA 1 way); **P<0.05, corresponding S1P stimulation times in WKYs versus SHRSPs (ANOVA 2 way).
Figure 5. Effect of EGFR, PDGFR inhibition, or S1P1 receptor antagonism on S1P-induced COX-2 expression in VSMCs from WKYs and SHRSPs. Top and right panels, Representative immunoblots for COX-2 and β-tubulin. Corresponding bar graphs demonstrate the following: (A) the time course (4, 8, and 24 hours) stimulation of S1P (1 μmol/L) on COX-2 expression in VSMCs from WKYs and SHRSPs; the effect of VPC23091 in S1P-induced COX-2 expression in VSMCs from WKYs (B) and SHRSPs (C); and the effect of AG1478 or AG1296 in S1P-induced COX-2 expression in VSMCs from WKYs (D) and SHRSPs (E). Results are mean ± SEM of 4 to 8 experiments. *P<0.05 vs vehicle (ANOVA 1 way); **P<0.05, corresponding S1P stimulation times in WKYs vs SHRSPs (ANOVA 2 way).
gated by S1P1 inhibition (Figure 5B and 5C). Unlike the other proinflammatory markers studied, neither AG1478 nor AG1296 prevented S1P-induced expression of COX-2 in WKYs (Figure 5D) and SHRSPs (Figure 5E).

**S1P-Induced Responses in VSMCs From WKY and Wistar Rats Are Similar**

To evaluate whether differences between WKYs and SHRSPs may be strain related, we compared S1P-induced effects in VSMCs derived from 2 normotensive rat strains, namely WKY and Wistar. As shown in Figure S3A through S3G (available in the online Data Supplement at http://hyper.ahajournals.org), the magnitude of S1P-induced responses between WKY and Wistar groups was similar for EGFR, PDGFR, p38MAPK, and SAPK/JNK phosphorylation and ICAM-1, VCAM-1, and COX-2 expression.

**S1P-Induced Monocyte Adhesion Is Mediated via RTK Transactivation**

To further confirm the functional significance of PDGFR and EGFR in S1P proinflammatory actions, we assessed monocyte adhesion to VSMCs by fluorimetry (Figure 6A and 6B) and by imaging (Figure 6C) using RTK inhibitors. S1P increased monocyte adhesion to VSMCs from WKYs and SHRSPs, an effect blocked by VPC 23091. EGFR and PDGFR inhibition blocked S1P-induced cell adhesion in WKYs. In SHRSPs, AG1296 inhibited S1P-mediated monocyte adhesion.

**Discussion**

Major findings from the present study demonstrate the following: (1) in VSMCs, S1P induces activation of p38MAPK and JNK/SAPK and induction of inflammatory...
mediators, ICAM-1, VCAM-1, and Cox; (2) S1P stimulates inflammatory signaling pathways via RTK transactivation through S1P1 receptors; and (3) responses to S1P are augmented in SHRSPs possibly because of increased phosphorylation of PDGFR and EGFR. These data indicate that S1P/S1P1 is a potent inducer of proinflammatory signaling pathways, an effect that involves differential transactivation of EGFR and PDGFR. Upregulation of S1P/S1P1/RTK/MAPK in VSMCs from SHRSPs may contribute to vascular inflammation in hypertension. We believe that the significant differences observed for S1P-induced effects in VSMCs from WKYs and SHRSPs are not simply related to strain differences, because responses between 2 normotensive strains, WKY and Wistar, were similar. Our data provide new insights into sphingolipid signaling through RTKs and suggest that augmentation of such molecular processes may contribute to vascular changes in SHRSPs.

Growing evidence supports an important role for S1P and its receptors in the development and regulation of the vascular system and in the pathogenesis of vascular diseases.5,16–19 S1P is generated from membrane phospholipids in various cell types, and its production is stimulated by many vasoactive factors, including angiotensin II.20 In the vasculature, the precise distribution of S1P receptors has still not been clearly established, although S1P1, S1P2, and S1P3 seem to be expressed in vascular cells, with endothelial cells expressing primarily S1P1 and S1P3 and VSMCs expressing S1P1, S1P2, and S1P3.4–8,21–26 In the present study, we found that S1P1 and S1P2 but not S1P3 receptors are present in VSMCs from WKYs and SHRSPs. Expression profiles of S1P receptors were similar in VSMCs from WKYs and SHRSPs, suggesting that increased signaling evoked by S1P in cells from hypertensive animals is probably attributable to postreceptor phenomena. We previously identified the S1P1 gene (Edg1) as a candidate gene in salt sensitivity in SHRSPs and demonstrate here increased signaling through the S1P1 receptor in VSMCs from SHRSPs.

In addition to classic second messenger-regulated mechanisms, G protein–coupled receptors activate several signaling cascades via transactivation of RTKs.9–12,27–30 This has been very well characterized in the vascular system for EGFR activation by angiotensin II.31–33 Whether S1P receptors also signal through RTKs is less clear, although there is some evidence in cancer cell lines that S1P1 receptors form complexes with vascular endothelial growth factor receptors, which signal through extracellular signal–regulated kinase 1/2 pathways.34 Here we show that, in VSMCs, S1P induces rapid phosphorylation of EGFRs and PDGFRs in VSMCs from 2 different strains of normotensive rats, WKY and Wistar, with exaggerated responses in cells from hypertensive animals. Although not studied here, it may be possible that other RTKs, such as insulin-like growth factor receptor 1 and vascular endothelial growth factor receptor, are also modulated by S1P.

To evaluate the receptor subtype through which S1P induces RTK transactivation, VSMCs were pretreated with an S1P receptor antagonist, VPC23019.35 VPC23019, which has potent S1P1 receptor inhibitory actions, abrogated phosphorylation of PDGFR and EGFR, indicating the role of S1P1 receptors in this process. Protein kinases, second messengers, chaperone molecules, and adaptor proteins through which S1P/S1P1 receptors induce RTK transactivation are not established but may involve the S1P1 receptor:RTK complex formation as demonstrated in cancer cell lines.35 Mechanisms responsible for increased phosphorylation of EGFRs and PDGFRs in SHRSPs most likely involve posttranslational events, because protein content of the RTK was similar in cells from WKYs and SHRSPs.

EGFR and PDGFR activation triggers complex signaling events, including activation of MAPKs, a family of serine/threonine protein kinases that transduces signals from the cell membrane to the nucleus in response to growth factors to influence cellular responses, including vascular inflammation.36–38 Here we demonstrate that S1P, through S1P1 receptors, induces rapid activation of p38MAPK and JNK, via EGFRs and PDGFRs, because AG1478 and AG1296 inhibited S1P actions. Second messengers and molecular pathways linking RTKs to MAPKs involve tyrosine kinases, such as c-Src, and Pyk2; transcription factors, such as hypoxia-inducible factor 1; and generation of reactive oxygen species.13,39,40 Although MAPK phosphorylation was completely blocked by growth factor receptor inhibitors in WKY cells, effects were only partial in SHRS cells, suggesting the importance of pathways other than through EGFRs and PDGFRs in this response.

Hypertension-associated vascular injury and target organ damage are inflammatory processes,41 involving activation of proinflammatory signaling pathways, including p38MAPK and JNK; increased expression of adhesion molecules; production of cytokines, chemokines, and growth factors42,43; and recruitment and adhesion of inflammatory cells. It is also possible that, in this context, vascular cells produce and secrete S1P, which may itself modulate vascular function. S1P treatment stimulated expression of the adhesion molecules ICAM-1, VCAM-1, and Cox-2 in VSMCs from WKYs and Wistar rats, with amplified responses in SHRSPs. AG1478 and AG1296 variably blocked S1P-mediated actions on ICAM-1 and VCAM-1, without effect on COX-2 expression. These findings indicate that S1P vascular signaling via RTKs is not a generalized phenomenon and that inflammatory pathways regulated through receptor transactivation are highly specific.

To evaluate functional cellular responses as an inflammatory end point of S1P signaling, we examined adhesion of monocytes to VSMCs in response to S1P. Adhesion and migration of circulating inflammatory cells, including monocytes and macrophages, represent initial events in vascular inflammation and have been shown to be increased in hypertension, both in vivo and in vitro studies.44 To our knowledge the data here are the first to show increased monocyte adhesion to VSMCs by S1P/S1P1 receptors through RTK transactivation. Increased monocyte adhesion by S1P in SHRSPs may be related, at least in part, to increased phosphorylation of EGFRs and PDGFRs, with associated upregulation of proinflammatory signaling pathways involving p38MAPK, JNK, and induction of ICAM-1 and VCAM-1. Involvement of other pathways, independent of tyrosine kinases and MAPKs, may also influence vascular
function by S1P, such as generation of reactive oxygen species, activation of transcription factors, and stimulation of cation channels.\(^5\)

In addition to facilitating molecular and cellular events underlying vascular inflammation, as shown in our study, S1P influences various responses in VSMCs that impact on vascular remodeling and endothelial dysfunction in hypertension. Although we focused on inflammation, it is very possible that transactivation of EGFRs and PDGFRs, and possibly other RTKs, by S1P may also stimulate VSMC growth, contraction, and migration, especially in cells from SHRSPs, that have been shown to exhibit enhanced mitogenic and stress responses. It is possible that receptors other than S1P1 may play a role in these effects. Murakami et al\(^{46}\) showed that S1P influences VSMC contraction through S1P3 receptors, and Takashima et al\(^{47}\) demonstrated that VSMC migration involves S1P2 receptors.

In summary our data indicate that molecular processes underlying vascular inflammation and cell adhesion in SHRSPs involve S1P-induced phosphorylation of RTKs, mediated through S1P1 receptors. We identify a novel pathway linking S1P/S1P1 receptors to specific proinflammatory signaling pathways through EGFR and PDGFR transactivation, a process that is augmented in SHRSPs. Such molecular events may contribute to vascular inflammation in hypertension.

**Perspectives**

Originally, sphingolipids were thought to serve only as structural components of the plasma membrane, but current evidence suggests that sphingolipids are pleiotropic molecules participating in the regulation of numerous cellular functions. Here we have extended this notion by demonstrating the importance of S1P in molecular processes underlying vascular inflammation. Moreover, we have identified a novel pathway linking S1P/S1P1 receptors to inflammatory responses through EGFR and PDGFR transactivation, a process that is upregulated in SHRSPs, probably because of hyperphosphorylation of RTKs. Such events may have significant pathophysiological implications in vascular remodeling and endothelial dysfunction in hypertension considering the importance of inflammation and cell growth in these processes.

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**Disclosures**

None.

**References**


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SUPPLEMENTAL DATA

Sphingosine-1-phosphate-induced vascular inflammation involves receptor tyrosine kinase transactivation in SHRSP

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Methods

Cell Culture

The study was approved by the Animal Ethics Committee of the University of Ottawa and performed according to the recommendations of the Canadian Council for Animal Care. VSMCs from adult male Wistar-Kyoto rats (WKY), Wistar and stroke-prone spontaneously hypertensive rats (SHRSP) were studied. Rats (16 weeks) were anaesthetized and euthanized by decapitation. VSMCs derived from mesenteric arteries were isolated and characterized as we previously described. Briefly, arteries were cleaned of adipose and connective tissue; VSMCs were dissociated by digestion of vascular arcades with enzymatic solution (collagenase, elastase, soybean trypsin inhibitor, and bovine serum albumin type I) for 60 minutes at 37°C. After incubation vessel fragments were washed and cells dispersed mechanically by passage 4 times through a 1-inch 20-gauge needle followed by debris filtration. Cell suspension was centrifuged and resuspended in Dulbecco modified Eagle medium containing 10% fetal calf serum, 2 mmol/L glutamine, 20 mmol/L HEPES (pH 7.4), and antibiotics. At subconfluence, the culture medium was replaced with serum-free medium for 24 hours to render the cells quiescent. Low-passage cells (4 to 8) were studied.

Protocols for cell stimulation

VSMCs were stimulated with S1P (10^{-6} mol/L) for short (1, 5, 30 min) or long term (1-24 hrs). In some experiments cells were pre-exposed for 30 minutes to: VPC23019 (S1P1 antagonist, 10^{-6} mol/L), AG1478 (EGFR inhibitor, 10^{-6} mol/L) or AG1296 (PDGFR inhibitor, 10^{-6} mol/L).

Western blotting

Total protein (30 µg) from S1P-stimulated VSMCs was extracted, separated by electrophoresis on a polyacrylamide gel (7.5 % or 10%), and transferred onto a nitrocellulose membrane as previously described. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with Tween for 1 hour at 24°C. Membranes were then incubated with specific antibodies overnight at 4°C. Antibodies were as follows: S1P1, S1P2 and S1P3 receptors (Abcam Inc), EGFR [Tyr^{845}], PDGFR [Tyr^{849/857}], p38 MAPK [Thr^{180}/Tyr^{182}], SAPK/JNK [Thr^{183}/Tyr^{185}] (Cell Signaling Technology), ICAM-1, VCAM-1 (Santa Cruz Biotechnology, Inc) and Cox2 (Cayman Chemical). After incubation with secondary antibodies, signals were revealed with chemiluminescence, visualized by autoradiography and quantified densitometrically. Antibodies to non phosphoproteins, β-actin or β-tubulin were used as internal housekeeping controls.

Adhesion Assay

VSMCs were cultured to confluence in 6-well culture plates. Growth-arrested VSMCs from WKY and SHRSP rats were stimulated with S1P (10^{-6} mol/L, 6 hr). AG1478, AG1296 and VPC23019 were used respectively to determine the role of EGF, PDGF and S1P1 receptors in adhesion of macrophages to VSMCs. VSMCs treated with vehicle served as controls. Rat-derived NR8383 monocyte/macrophage cell line was obtained
from the American Type Culture Collection (Manassas, VA). Mixed NR8383 cells, adherent and suspension, were cultured in growth medium (Ham's F12K with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate and 15% heat inactivated fetal bovine serum). For cell fluorescent labeling, macrophages (10^5 cells/mL) were suspended in 1% BSA supplemented phosphate buffered saline containing 1 µmol/L calcein-AM (Molecular Probes, Eugene, OR) and incubated for 20 min at 37 °C. Labeled macrophages were washed with phosphate-buffered saline and suspended in Hanks’ buffered salt solution. Fluorescence-labeled cells (10^5 cells/mL) were added to both unstimulated and stimulated VSMC layers and allowed to adhere for 30 min at 37 °C and in 5% CO₂. After the incubation, non-adherent cells were removed by gently washing with pre-warmed Hanks’ buffered salt solution. The quantitative measure of adhered cells was determined by lysing cells with 0.1 M NaOH. The cell lysate was transferred to a 96 well plate and the fluorescence intensity was measured with using a fluorescence multiwell plate reader (excitation wavelength 485 nm, emission wavelength 535 nm, Cary Eclipse, Varian). VSMCs lysate was used as a blank. Experiments were performed in duplicates. In some experiments the number of adherent cells was counted by fluorescence microscopy. The adhesion assay was carried out with VSMCs cultured on glass coverslips. The fixation procedure with 4% paraformaldehyde for 15 min at room temperature was performed after the incubation of VSMCs with NR8383 fluorescence-labeled cells and the removal the non-adherent cells. The number of NR8383 cells adhering to VSMCs was determined by counting (40x oil immersion objective) on a fluorescence microscope. Ten fields were evaluated per experiment. Imaging was acquired with the Stallion Digital Hi-Speed Multi-Channel Imaging System (Zeiss, Germany).

Statistical analysis

Results are presented as mean ± SEM and compared by t test and ANOVA one or two way. Values of P<0.05 were considered to be significant.

References


Figure legends

**Figure S1.** S1P receptors expression in VSMCs from WKY and SHRSP. Top panels, representative immunoblots for S1P1, S1P2 and β-actin. Corresponding bar graphs demonstrate the expression of S1P1 (A) and S1P2 (B) receptors. Results are mean ± SEM of 3 experiments.

**Figure S2.** Basal phosphorylation levels of signaling molecules and basal expression of pro-inflammatory markers in VSMC from WKY and SHRSP. Top panels, representative immunoblots for EGFR, SAPK/JNK, p38MAPK, PDGFR, COX-2, ICAM-1, VCAM-1 and GAPDH. Corresponding bar graphs demonstrate the phosphorylation of EGFR (A),
SAPK/JNK (B), p38MAPK (C), PDGFR (D) and expression of COX-2 (E), ICAM-1 (F) and VCAM-1 (G). Results are mean ± SEM of 8 experiments.

**Figure S3.** S1P-induced PDGFR, EGFR, p38MAPK and SAPK/JNK phosphorylation and VCAM-1, ICAM-1 and COX-2 expression in VSMC from WKY and Wistar. Top panels, representative immunoblots for PDGFR α/β [Tyr^{849/857}] and PDGFR (A), EGFR [Tyr^{845}] and EGFR (B), p38MAPK [Thr^{180}/Tyr^{182}] and p38MAPK (C), SAPK/JNK [Thr^{183}/Tyr^{185}] and SAPK/JNK (D), VCAM-1 (E), ICAM-1 (F) and COX-2 (G). Corresponding bar graphs demonstrate: the time course (1, 5, 30 min or 4, 8, 24 hours) stimulation of S1P (1 µmol/L) on PDGFR, EGFR, p38MAPK and SAPK/JNK phosphorylation and VCAM-1, ICAM-1, COX-2 in VSMCs from WKY and Wistar. Results are mean ± SEM of 3 to 6 experiments. *P<0.05 vs vehicle.

**Figure S4.** Effect of EGFR, PDGFR inhibition or S1P1 receptor antagonism on S1P-induced SAPK/JNK phosphorylation in VSMCs from WKY and SHRSP. Top and right panels, representative immunoblots for SAPK/JNK [Thr^{183}/Tyr^{185}] and SAPK/JNK. Corresponding bar graphs demonstrate: the time course (1, 5, 30 min) stimulation of S1P (1 µmol/L) on SAPK/JNK phosphorylation in VSMCs from WKY and SHRSP (A); the effect of VPC 23091 in S1P-induced SAPK/JNK phosphorylation in VSMCs from WKY (B) and SHRSP (C); the effect of AG1478 or AG1296 in S1P-induced SAPK/JNK phosphorylation in VSMCs from WKY (D) and SHRSP (E). Results are mean ± SEM of 4 experiments. *P<0.05 vs vehicle; **P<0.05, corresponding S1P stimulation times in WKY vs SHRSP.

**Figure S5.** Effect of EGFR, PDGFR inhibition or S1P1 receptor antagonism on S1P-induced VCAM-1 expression in VSMCs from WKY and SHRSP. Top and right panels, representative immunoblots for VCAM-1 and β-tubulin. Corresponding bar graphs demonstrate: the time course (4, 8, 24 hr) stimulation of S1P (1 µmol/L) on VCAM-1 expression in VSMCs from WKY and SHRSP (A); the effect of VPC 23091 in S1P-induced VCAM-1 expression in VSMCs from WKY (B) and SHRSP (C); the effect of AG1478 or AG1296 in S1P-induced VCAM-1 expression in VSMCs from WKY (D) and SHRSP (E). Results are mean ± SEM of 4 to 8 experiments. *P<0.05 vs vehicle; **P<0.05, corresponding S1P stimulation times in WKY vs SHRSP.
Figure S1

A

S1P1 expression (β-actin ratio)

WKY          SHRSP

B

S1P2 expression (β-actin ratio)

WKY          SHRSP
EGFR phosphorylation (Phospho/Total)

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p38 phosphorylation (Phospho/Total)

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PDGFR phosphorylation (Phospho/Total)

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JNK phosphorylation (Phospho/Total)

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ICAM-1 expression (ICAM-1/GAPDH)

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VCAM-1 expression (VCAM-1/GAPDH)

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Cox-2 expression (Cox-2/GAPDH)

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SAPK/JNK [Thr^183/Tyr^185] 54/46 kDa

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p38 [Thr^180/Tyr^182] 38 kDa

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PDGFR α/β [Tyr^840/857] 190 kDa

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Figure S2
Figure S3
Figure S4

(A) Western blot analysis showing phosphorylation of SAPK/JNK (Thr183/Tyr185) in WKY and SHRSP mice. S1P treatment induced a time-dependent increase in phosphorylation levels.

(B) Bar graph depicting the percentage of SAPK/JNK phosphorylation in WKY and SHRSP mice. S1P treatment showed a significant increase in phosphorylation compared to vehicle control.

(C) Western blot analysis showing the effect of VPC23019 on SAPK/JNK phosphorylation in SHRSP mice. VPC23019 inhibited S1P-induced phosphorylation.
Figure S4

SAPK/JNK phosphorylation (% vehicle)

**WKY**

- **Vehicle**
  - 1
  - 5
  - 30

- **S1P 10^{-6} mol/L**
  - +
  - +
  - +
  - +

- **AG1478 10^{-6} mol/L**
  - -
  - -
  - +
  - +

- **AG1296 10^{-6} mol/L**
  - -
  - -
  - -
  - +

**SHRSP**

- **Vehicle**
  - +
  - -
  - -

- **S1P 10^{-6} mol/L**
  - +
  - +
  - +

- **AG1478 10^{-6} mol/L**
  - -
  - -
  - -

- **AG1296 10^{-6} mol/L**
  - -
  - -
  - -

*Significant differences compared to vehicle.*
Figure S5

**Panel A**

WKY and SHRSP cells were treated with S1P and VPC23019 for 4, 8, or 24 hours. The expression of VCAM-1 and β-tubulin was analyzed by Western blotting.

**Panel B**

VCAM-1 expression was measured in WKY cells treated with vehicle, S1P, and S1P + VPC23019 at various time points.

**Panel C**

VCAM-1 expression was measured in SHRSP cells treated with vehicle, S1P, and S1P + VPC23019 at various time points.
**D**

VCAM-1 expression (% vehicle)

Vehicle + - - - - - - - - - -
S1P 10^-6 mol/L - + + + - + + + - + + +
AG1478 10^-6 mol/L - - - - + + + + - - - -
AG1296 10^-6 mol/L - - - - - - - - + + + +

**E**

VCAM-1 expression (% vehicle)

Vehicle + - - - - - - - - - -
S1P 10^-6 mol/L - + + + - + + + - + + +
AG1478 10^-6 mol/L - - - - + + + + - - - -
AG1296 10^-6 mol/L - - - - - - - - + + + +

Figure S5