Sphingosine-1-Phosphate Receptor-2 Function in Myeloid Cells Regulates Vascular Inflammation and Atherosclerosis

Athanasia Skoura, Jason Michaud, Dong-Soon Im, Shobha Thangada, Yuquan Xiong, Jonathan D. Smith, Timothy Hla

Objective—Sphingomyelin deposition and metabolism occurs in the atherosclerotic plaque, leading to the formation of sphingosine-1-phosphate (S1P), which activates G protein–coupled receptors to regulate vascular and immune cells. The role of S1P receptors in atherosclerosis has not been examined.

Methods and Results—We tested the hypothesis that S1P receptor-2 (S1PR2) regulates atherosclerosis. Apoe−/− S1pr2−/− mice showed greatly attenuated atherosclerosis compared with the Apoe−/− mice. Bone marrow transplant experiments indicate that S1PR2 function in the hematopoietic compartment is critical. S1PR2 is expressed in bone marrow–derived macrophages and in macrophage-like foam cells in atherosclerotic plaques. Reduced macrophage-like foam cells were found in the atherosclerotic plaques of Apoe−/− S1pr2−/− mice, suggesting that S1PR2 retains macrophages in atherosclerotic plaques. Lipoprotein profiles, plasma lipids, and oxidized low-density lipoprotein uptake by bone marrow–derived macrophages were not altered by the S1pr2 genotype. In contrast, endotoxin-induced inflammatory cytokine (interleukin [IL]-1β, IL-18) levels in the serum of S1PR2 knockout mice were significantly reduced. Furthermore, treatment of wild-type mice with S1PR2 antagonist JTE-001 suppressed IL-1β and IL-18 levels in plasma.

Conclusion—These data suggest that S1PR2 signaling in the plaque macrophage regulates macrophage retention and inflammatory cytokine secretion, thereby promoting atherosclerosis. (Arterioscler Thromb Vasc Biol. 2011;31:81-85.)

Key Words: atherosclerosis • coronary artery disease • lipids • macrophages • vascular biology

Risk factors for atherosclerosis, such as hyperlipidemia, smoking, and hypertension, injure the vascular endothelium, leading to lipoprotein deposition in the arterial vessel wall.1,2 Although cholesterol entry into the atherosclerotic plaque is well accepted to play a major role in atherogenesis, sphingomyelin (SM), which has an affinity for cholesterol in membranes, is also deposited. Metabolism of SM by the sphingomyelinase pathway produces sphingolipid metabolites—ceramide, sphingosine, and sphingosine-1-phosphate (S1P)3–5—whose function in vascular disease is not understood. Indeed, inflammatory cytokines stimulate the metabolism of SM by inducing the secretion of sphingomyelinase from endothelial cells and macrophages.6 However, suppression of SM synthesis with the fungal metabolite myriocin attenuates atherosclerosis in animal models, and SM and its metabolites are elevated in plasma of patients with coronary artery disease, suggesting that sphingolipid metabolites are important in atherosclerosis.7–9

Among the sphingolipid metabolites, extracellular S1P signals via G protein–coupled receptors (S1PR1 to S1PR5) and regulates vascular permeability, angiogenesis, and immune cell trafficking.10–13 S1P bound to high-density lipoprotein mediates the vascular protective functions of this lipoprotein via the S1PR1.14–16 On the other hand, clinical studies suggest that serum S1P could be a predictor of obstructive coronary artery disease.17 Interestingly, FYT720, a structural analog of myriocin and an S1P receptor modulator, inhibits atherosclerosis in mouse models even though the mechanisms involved remain unclear.18 However, the role of S1P in atherosclerosis is likely complex, because S1P receptor subtypes exhibit redundant, as well as antagonistic, signaling properties.10 For example, S1PR1 and S1PR2 activate the small GTPases Rac and Rho, respectively, and induce cytoskeletal changes to regulate vascular endothelial adherens junctions and permeability in both positive and negative manner.19–21 The role of the specific S1P receptors in atherosclerotic vascular disease is virtually unknown. In this report, we describe the proatherogenic role of S1PR2. In addition, we provide strong evidence that the atherogenic function of S1PR2 involves macrophage retention in the plaques and proinflammatory cytokine production.
Materials and Methods

A detailed expanded Materials and Methods section is available online at http://atvb.ahajournals.org).

Animals

Mice with targeted disruption of the S1pr2 gene were maintained on a mixed C57BL/6x129Sv genetic background. Experiments on knockout mice were performed with appropriate wild-type littermate controls. All procedures involving mice were approved by the University of Connecticut Health Center Animal Care Committee. Mice were fed a high-cholesterol diet (TD31857), and atherosclerosis was analyzed after 13 weeks.

Histology and Immunohistochemistry

Serial cross sections of the aortic leaflet were stained with the following antibodies: rat-anti-MOMA-2 (Serotec) and polyclonal anti-S1pr2 (1:200).

Bone Marrow Transplantation

Male Apoe−/− mice were lethally irradiated with 2 doses of 550 rads, 4 hours apart, and reconstituted with 5 × 10⁶ bone marrow cells from Apoe−/−S1pr2−/− and Apoe−/−S1pr1++ donors. Recipients were maintained on normal chow for 4 weeks and then placed on a high-fat diet for 13 weeks and analyzed for lesion development.

RNA Isolation and Reverse Transcription–Polymerase Chain Reaction Analysis

RNA was extracted from mouse aortas, and quantitative reverse transcription–polymerase chain reaction assay was done using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems).

Cytokine Measurements

Mouse serum levels of interleukin (IL)-1β, IL-18 (Invitrogen), and tumor necrosis factor-α (TNF-α) (e-Bioscience) were measured by ELISA.

Results

Requirement for S1PR2 in Atherosclerosis

To investigate the role of the S1PR2 in atherosclerosis, we placed Apoe−/−S1pr2−/− mice and Apoe−/−S1pr1++ littermate controls on a high-fat Western diet for 13 weeks. Mouse aortae were dissected and stained with Oil Red O to highlight the atherosclerotic plaques of aortic arch and the thoracic and abdominal aorta (Figure 1A). En face analysis of atheromatous lesions revealed significant and uniform reduction (≈70%) in Apoe−/−S1pr2−/− mice compared with Apoe−/−S1pr1++ litters (Figure 1B). We also performed hematoxylin-phloxin-saffron, Trichrome, and Oil Red O staining of serial cross-sections from the aortic sinus to examine the presence of fibrous caps, connective tissue, and lipid deposition, respectively (Figure 1C). As shown in Figure 1D, loss of S1PR2 led to a marked reduction (≈80%) in atheromatous plaque area (fibrous tissue and collagen content), as well as lipid deposition. Necrotic core from the plaques of the Apoe−/−S1pr2−/− mice was decreased to a similar extent (Supplemental Figure IA). In sharp contrast, nuclear-specific terminal deoxynucleotidyl transferase dUTP nick-end labeling staining did not reveal changes in apoptosis (Supplemental Figure IB).

Critical Role of Myeloid S1PR2 in Atheroma Development

Given that macrophages play major roles in driving atherosclerotic plaque inflammation and progression, we examined the requirement for the S1PR2 in the hematopoietic compartment. Immunostaining for the S1PR2 in the aortic sinus showed that S1PR2 is expressed by macrophage-like foam cells in atherosclerotic plaques (Figure 2A). Indeed, bone marrow–derived macrophages express high levels of S1PR2 and S1pr1 transcripts. Lack of S1PR2 in the knockout mice did not change the expression levels of S1pr1, S1pr3, and S1pr4 receptor transcripts (Figure 2B). Macrophage infiltration into the vessel wall was examined by immunostaining with a macrophage/monocyte specific antibody (MOMA-2). Apoe−/−S1pr2−/− mice showed a significant reduction in macrophage numbers (≈80%) (Figure 2C and 2D). These data suggest that macrophage retention in the atherosclerotic plaques is regulated by the S1PR2.

We generated bone marrow chimeras by transplanting lethally irradiated Apoe−/− mice with Apoe−/−S1pr2−/− or Apoe−/−S1pr1++ bone marrow. Following reconstitution of the hematopoietic system and after 13 weeks on a Western diet, en face analysis demonstrated a significant reduction in atherosclerotic lesion area throughout the aorta (≈65%) in mice receiving Apoe−/−S1pr2−/− bone marrow cells compared with Apoe−/−S1pr1++ counterparts (Figure 3A and 3B). Analysis of peripheral blood
monocytes (CD11b+), polymorphonuclear leukocytes (CD11b+, CD115+, Gr1+), and T- and B-lymphocytes (CD4+, CD8+, or B220+) revealed no differences between Apoe-/-S1pr2-/- and Apoe-/-S1pr2-/- mice (Supplemental Figure II). Atherosclerotic lesions at the aortic root showed decreased lipid and macrophage accumulation (Figure 3C and 3D), suggesting that the S1PR2 signaling in macrophages in the Apoe-/- mice is sufficient to promote atherosclerosis.

**S1PR2 Regulates Inflammatory Changes in Macrophages**

Body weight, plasma cholesterol, plasma triglyceride, and lipoprotein profiles were not different between Apoe-/-S1pr2-/- mice and littermate controls, suggesting that alterations in sterol and triglyceride metabolism are unlikely to account for S1PR2-induced atherosclerosis (Supplemental Figure III). In addition, foam cell differentiation in vitro was not altered in S1pr2-/- cells compared with S1pr2-/+ control cells (Supplemental Figure IV). These data suggest that S1PR2 signaling in macrophages rather than the changes in lipid metabolism or uptake is involved.

Although S1PR1 was proposed to regulate antiinflammatory events, the role of S1PR2 in macrophage-dependent inflammation is not known. To test whether S1PR2 regulates inflammatory pathways, we measured serum cytokine levels in wild-type or S1pr2-/- mice after treatment with lipopolysaccharide (LPS), which activates the toll-like recep-

**Discussion**

Despite the transformative nature of HMG CoA reductase inhibitors in the control of hypercholesterolemia and atherosclerosis, efforts continue to identify novel therapeutic agents. Sphingolipid signaling, which appears to be highly significant in atherogenesis, is poorly understood at the mechanistic level. In particular, enzymes in the sphingolipid metabolic cascade and the receptors for sphingolipid metabolites may regulate the complex process of atherosclerosis. As such, they represent novel opportunities in therapeutic development. Indeed, S1P receptor modulators were shown to be efficacious in clinical trials for the control of autoimmune inflammation in multiple sclerosis. S1P signaling is
complex and may have both pro- and antiatherosclerotic properties. The effect of S1P in atherosclerotic vascular disease might be different depending on the expression of different S1P receptors in the cells of the vessel wall or infiltrating hematopoietic cells. In addition, local production of S1P may also be important.

In this report, we demonstrate that the S1PR2 promotes atherosclerosis in the Apoe−/− mouse model. It is known that this receptor subtype, which potently activates the Rho GTPase, induces vascular permeability in endothelial cells. It is also associated with pathological angiogenesis and is required for abnormal vascular tuft formation in the retina ofopathy of prematurity in the mouse. Bone marrow transplant experiments in this report clearly indicate that the function of S1PR2 in the macrophage compartment is critical in the promotion of atherosclerosis. Although the function of S1PR2 in endothelial cells may also contribute to atherosclerosis, it appears that macrophage may be the important cell type in which S1PR2 signaling is critical.

The function of S1PR2 in hematopoietic cells is less well understood. Recent studies have shown that S1PR2 inhibits chemoattractant-induced motility in murine primary macrophages in vitro and may limit macrophage infiltration into the inflamed peritoneum. In these studies, we showed that S1PR2 induced the cAMP/protein kinase A pathway to inhibit macrophage motility in vitro. Given that macrophage accumulation in the atherosclerotic plaque is significantly reduced in S1pr2−/− mice, it is likely that S1PR2 in the macrophage regulates trafficking in and out of the plaque. Myeloid adhesion proteins (VLA-4 and CD18), which are important in monocyte-endothelial cell adhesion, were not altered in bone marrow–derived macrophages isolated from S1pr2−/− mice (Supplemental Figure V). In vitro studies do not support the hypothesis that S1P is a direct chemoattractant for myeloid cells. In fact, it is known that chemokine signals, i.e., those induced by CCL2 (monocyte chemoattractant protein 1 [MCP-1]) and CXCL11 (stromal derived factor [SDF-1]) regulate the recruitment of monocytes into atherosclerotic plaques. In contrast, S1P action on S1PR2 inhibits the motility of macrophages, suggesting that S1PR2 may retain these macrophage/foam cells in the atherosclerotic plaques. Interestingly, the related S1PR1 receptor is known to promote egress of immune cells (especially T and B cells) from lymphoid organs into lymph or blood. Thus, S1PR2 may be a retention-promoting receptor in myeloid cells.

In addition, we show that the S1PR2 is needed for TLR4-induced expression and secretion of the proinflammatory cytokines IL-1β and IL-18. It is known that these 2 cytokines are important for atherosclerosis. Thus, our findings suggest that specific inhibition of macrophage S1PR2 by pharmacological antagonists may be useful as an adjunct in the control of atherosclerotic vascular disease. Because of the pharmacokinetic and solubility properties of JTE-013, a pharmacological antagonist for S1PR2 that is not optimal for chronic administration, its effect on atherosclerosis cannot be assessed at present. However, acute administration of JTE-013 was able to inhibit TLR4-stimulated IL-1β and IL-18 release in vivo. Development of a specific S1PR2 antagonist with better in vivo properties will address pharmacological utility of this novel proatherogenic signaling pathway.

Interestingly, S1PR2 did not regulate TNF-α secretion. In contrast to IL-1β and IL-18, which are secreted by the caspase-dependent inflammasome pathway, TNF-α is produced by the classical endoplasmic reticulum/Golgi secretory pathway. Thus, S1PR2 signaling may selectively regulate the nonclassical cytokine secretion pathway.

In summary, the results of this study provide the first molecular genetic and pharmacological evidence to support the hypothesis that S1PR2 pathway is a crucial step in the development of atherosclerotic disease. We speculate that similar mechanisms may be involved in human cardiovascular disease. Thus, the specific inhibition of S1PR2 function could lessen lesion formation and facilitate plaque stability.

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Disclosures
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References


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Materials and Methods

Animals

C57BL/6x129Sv mice with targeted disruption of the \textit{SIpr}2 gene were generated as previously reported \cite{1}. Mice were maintained on a mixed C57BL/6x129Sv genetic background before crossing with \textit{Apo}e\textsuperscript{-/-} mice, the latter purchased from Jackson Laboratories (Bar Harbor, Maine). Experiments on KO mice were performed with appropriate WT littermate controls. All procedures involving mice were approved by the University of Connecticut Health Center Animal Care Committee. Homozygous \textit{SIpr}2\textsuperscript{-/-} were intercrossed with homozygous \textit{Apo}e\textsuperscript{-/-} mice, to generate heterozygous breeding pairs. Genomic DNA was isolated by tail biopsy. To confirm the mouse genotype PCR analysis was performed based on the genotyping conditions available at www.jax.org (Jackson Laboratories). Mice were fed with a high cholesterol diet (TD31857) and atherosclerosis was analyzed after 13 weeks. Mice were anesthetized and perfused with 4\% PFA for 5 min. Aortas were dissected out and processed for \textit{en face analysis} whereas the hearts were embedded in paraffin or frozen in OCT.

Histology and Immunohistochemistry

Atherosclerotic lesions were analysed in the aortic root, aortic arch and descending aorta. For the aortic root, cryosections (7 \(\mu\)m thick) throughout the aortic sinus were collected and stained for Hematoxylin Phloxin and Saffron (HPS), as well as Trichrome and Oil Red O to perform lesion composition analysis. For immunohistochemical analysis serial cross sections of the aortic leaflet were stained with the respective specific antibody. Antibodies used: rat-anti-MOMA-2 (Serotec), polyclonal anti-S1pr2 \cite{2,3} (1:200). Images of the sections were observed with a Zeiss Axioscop Microscope equipped for transmitted-light brightfield, and acquired and analyzed with digital
image processing software Axiovision. In addition, images were obtained with a Zeiss LSM 510 and/or Zeiss LSM 510 META confocal light microscopes and acquired and analyzed with the digital image processing software LSM Image Browser Software. Longitudinal preparations of fixed aortic arch and abdominal aorta were pinned-out on black wax and aortic lesions were stained with Oil Red O (ORO). TUNEL-apoptosis assay was performed as described

Bone Marrow Transplantation

For bone marrow transplantation, male Apoe\(^{-/-}\) mice were lethally irradiated with two 550rad doses, 4 hours apart, from a \(^{137}\)C source (Gammacell-40, MDS Nordion, Kanata, Canada). Apoe\(^{-/-}\) recipient mice were reconstituted, via the lateral tail vein, with 5x10\(^6\) unfractionated bone marrow cells from Apoe\(^{-/-}\)Slpr\(^{-/-}\) and Apoe\(^{-/-}\)Slpr\(^{2/-}\) donors. Recipients were maintained on normal chow for 4 weeks, then placed on high fat diet for 13 weeks and analyzed for lesion development.

Foam Cell Formation

Bone Marrow Derived Macrophages were plated on round glass coverslips in DMEM supplemented with 10% FBS and antibiotics. The next day, cells were treated with oxLDL (50\(\mu\)g/ml) for 3hrs, 5hrs and 24 hrs at 37\(^{0}\)C, followed by staining with Oil Red O and mounting with aqueous mounting medium. Images of random fields were captured and analysed with Image Pro Plus Analysis Software (Media Cybernetics).

RNA isolation and RT-PCR analysis
RNA was extracted (RNeasy kit;Qiagen) from mouse aortas or with RNAstat-60 (Tel-Test. B, Friendswood, TX) for macrophages. First-strand cDNA was synthesized using random hexamers, murine leukemia virus reverse transcriptase and accompanying reagents (Invitrogen Corp.) for 1hr at 37°C. Mouse RT-PCR primers shown in Table S1 were designed with Primer Express software (Applied Biosystems). Amplification and data analysis was performed in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Messenger RNA (mRNA) levels were quantified and corrected for β-actin.

**Cytokine ELISAs**

LPS (40mg/kg, *E.coli* serotype 0127:B8;Sigma) was intraperitoneally injected into WT and KO animals. After 3hrs or 5hrs treatment, animals were anesthetized and blood was collected by final cardiac puncture. Mouse serum levels of IL-1β, IL-18 (Invitrogen) and TNF-α (e-Bioscience) were measured by Enzyme Linked ImmunoSorbent assay (ELISA).

**Statistical Analysis**

Values are expressed as mean ± SEM. Statistical analyses were performed using unpaired, two-tailed Student's t test for comparison between two groups. One-way ANOVA test and subsequent Bonferroni’s multiple comparison test were used for groupwise comparisons. *P* values less than 0.05 were considered significant.

**References:**


Supplemental Figure Legends

Fig. I. (A) Hematoxylin-Phloxin-Saffron (HPS) staining of serial cross-sections from the aortic sinus to examine the presence of fibrous caps and connective tissue content. Representative cross-sections from Apoe−/−S1pr2−/− (n=5) mouse aortic sinus show significantly decreased anuclear and afibrotic areas (“necrosis”) compared to Apoe−/−S1pr2+/+ (n=5, P < 0.02). Scale bar, 100µm (B) Nuclear-specific TUNEL staining of serial cross-sections from the aortic sinus to study apoptosis. No significant difference was observed between Apoe−/−S1pr2+/+ (n=3) and Apoe−/−S1pr2−/− (n=3) mice. Scale bar, 50µm

Fig. II. Peripheral leukocyte counts in Apoe−/−S1pr2+/+ and Apoe−/−S1pr2−/− mice. Apoe−/−S1pr2+/+ and Apoe−/−S1pr2−/− were fed a high fat diet for 13 weeks, and peripheral blood leukocytes were analyzed by flow cytometry for CD11b, CD115, Gr-1, CD4, CD8, and B220. The numbers of monocytes (CD11b+, CD115+), polymorphonuclear leukocytes (CD11b+, CD115−, Gr1+), and CD4+, CD8+, or B220+ lymphocytes were determined. No significant differences were found between Apoe−/−S1pr2+/+ and Apoe−/−S1pr2−/− mice.
**Fig. III.** Metabolic parameters of Apoe<sup>−/−</sup>S1pr2<sup>−/−</sup> (n=5) and Apoe<sup>−/−</sup>S1pr2<sup>+/−</sup> (n=9) animals fed with “western” diet for 13 weeks. Body weight, cholesterol and triglyceride levels were not different between Apoe<sup>−/−</sup>S1pr2<sup>−/−</sup> mice and littermate controls. Plasma samples from each group of mice were subjected to fast-performance liquid gel-filtration chromatography.

**Fig. IV.** S1PR2 is dispensable for foam cell differentiation. After 3hrs incubation with oxLDL (50µg/ml), foam cell formation visualized by Oil Red O stained intracellular fat droplets is similar between S1pr2<sup>−/−</sup> and control S1pr2<sup>+/+</sup> bone marrow derived macrophages. Scale bar, 10µm

**Fig. V.** Expression of adhesion molecules in bone marrow-derived macrophages. S1pr2<sup>−/−</sup> and control S1pr2<sup>+/+</sup> bone marrow-derived macrophages were analyzed by FACS for myeloid markers CD11b/ F4/80 (top panel), CD18 (middle panel) or VLA4 (lower panel).

**Table I**

Primer sequences for quantitative real-time RT-PCR

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<th>Gene</th>
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| mS1pr1 | F: ATGGGTGTCCTACGACATCCC  
         | R: CGATGTTCAACCGCTGCTGATAG  |
| mS1pr2 | F: ATCGCCATCGAGAGACAAGT  
         | R: AGACAATTCCAGCCAGGGAT  |
| mS1pr3 | F: GCCTAGCGGGAGAGAAACCT  
         | R: CCGACTGCGGAAGAGAGGTG  |
mS1pr4
F: GCCCTCATCCTAGTGGCTATC
R: GCCCAGACATTAGAACCAAAGA

mS1pr5
F: TGTGCGCTCTATGCAAGGATT
R: CACGCTAAGGGTACGAAGCAG
Supplemental figure 1

A

HPS staining

Apo<sup>−/−</sup>S1pr<sup>2+/+</sup>

Apo<sup>−/−</sup>S1pr<sup>2−/−</sup>

Necrotic Area (%)

P<0.02

B

Tunel staining

Apo<sup>−/−</sup>S1pr<sup>2+/+</sup>

Apo<sup>−/−</sup>S1pr<sup>2−/−</sup>

TUNEL positive cells per mm<sup>2</sup> cellular lesion area

NS
supplemental figure II

![Bar chart showing % Cells for Mono, PMN, CD4, CD8, and B cells for Apo\textsuperscript{e/-}S1pr2\textsuperscript{++/} and Apo\textsuperscript{e/-}S1pr2\textsuperscript{-/-} genotypes.](image-url)
### Supplemental figure III

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![Graph showing cholesterol and triglyceride levels across elution fractions.](image-url)
supplemental figure IV

Oil Red O positive pixels/cell/Animal

- S1pr2^{++/+}
- S1pr2^{-/-}

NS