Sphingosine-1-Phosphate
A Novel Nonhypoxic Activator of Hypoxia-Inducible Factor-1 in Vascular Cells

Maude D. Michaud, Geneviève A. Robitaille, Jean-Philippe Gratton, Darren E. Richard

Objective—Sphingosine-1-phosphate (SIP) is a potent bioactive phospholipid responsible for a variety of vascular cell responses. Hypoxia-inducible factor-1 (HIF-1) is a transcriptional activator of genes essential for adaptation to low oxygen. SIP and HIF-1 are both important mediators of vascular cell responses such as migration, proliferation, and survival. Studies have shown that nonhypoxic stimuli can activate HIF-1 in oxygenated conditions. Here, we attempt to determine whether SIP can modulate the vascular activation of HIF-1.

Methods and Results—We show that in vascular endothelial and smooth muscle cells, activation of the SIP type-2 receptor by SIP strongly increases HIF-1α protein levels, the active subunit of HIF-1. This is achieved through pVHL-independent stabilization of HIF-1α. We demonstrate that the HIF-1 nuclear complex, formed on SIP stimulation, is transcriptionally active and specifically binds to a hypoxia-responsive element. Moreover, SIP activates the expression of genes known to be closely regulated by HIF-1.

Conclusion—Our results identify SIP as a novel and potent nonhypoxic activator of HIF-1. We believe that understanding the role played by HIF-1 in SIP gene regulation will have a strong impact on different aspects of vascular biology.

Keywords: sphingosine 1-phosphate ■ hypoxia-inducible factor-1 ■ gene expression ■ endothelial cells ■ vascular smooth muscle cells

The main cellular components of the vasculature are endothelial (ECs) and smooth muscle cells (VSMCs). Normally quiescent, these cells proliferate and migrate in response to vessel wall injury, atherosclerosis, and during angiogenesis associated with a variety of different conditions and diseases.1–3 Hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcription factor, has been shown to be involved in various functional responses in ECs and VSMCs.4,5 HIF-1 controls the expression of genes involved in adaptation responses to hypoxic stress.6 HIF-1 is formed after the interaction of a constitutive HIF-1β subunit with a tightly regulated HIF-1α subunit. In normal oxygen conditions, HIF-1α is hydroxylated through the action of HIF prolyl-hydroxylases. Hydroxylation of HIF-1α allows the binding of the product of the von Hippel-Lindau tumor suppressor gene (pVHL). As the recognition component of a E3 ubiquitin ligase complex, pVHL allows for HIF-1α polyubiquitination and subsequent proteasomal degradation.7 Although hypoxia is the ubiquitous inducer of HIF-1, accumulating evidence has revealed that under normal oxygen tension many other extracellular stimuli can also strongly induce the HIF-1 nuclear complex in a cell-specific manner.8 A clear understanding of the mechanisms regulating HIF-1 in vascular cells is fundamental and especially important in the endothelium where the loss of HIF-1 inhibits many aspects of EC behavior.4

Mainly released into circulation by activated platelets and erythrocytes, sphingosine-1-phosphate (SIP) is a potent and multifunctional phospholipid exerting a broad range of vascular cell responses including migration, proliferation and survival SIP has important impacts on physiological and pathological events such as wound healing, hemostasis, thrombosis, tumor progression, inflammation, and atherosclerosis.9–12

Given the importance of SIP and HIF-1 in a number of biological events, especially their central role in angiogenesis and concomitant implications in pathologies like atherosclerosis and tumor progression, the goal of the current study is to evaluate the potential role of SIP on HIF-1 activation. Here, we demonstrate that the treatment of vascular cells with SIP stabilizes HIF-1α protein levels in a time- and dose-dependent manner. The HIF-1 complex, formed on SIP stimulation, is transcriptionally active, specifically binds to hypoxia-response elements (HRE), and drives HIF-1–depen
dent gene expression. Taken together, these results identify S1P as a novel and potent normoxic activator of the HIF-1 transcriptional complex and HIF-1 as a mediator of S1P cellular responses.

Materials and Methods
A detailed description of all methods is available in the supplemental materials (available online at http://atvb.ahajournals.org).

Cell Culture
The murine lung endothelial 1G11 cell line was originally obtained from Alberto Mantovani and Annunciata Vecchi (Istituto Ricerche Farmacologiche Mario Negri, Milan, Italy). VSMCs were isolated from thoracic aortas of young Wistar rats (Charles River). Bovine aortic endothelial cells (BAECs) were isolated from calf thoracic aortas (obtained from a local slaughterhouse). For all experiments, cells were rendered quiescent by overnight (16-hour) serum starvation. Hypoxic conditions were obtained by placing cells in a sealed hypoxic workstation (Ruskinn Technology Ltd).

RNA Interference
All siRNAs oligonucleotides were obtained from Applied Biosystems. To downregulate HIF-1α protein expression in VSMCs, a siRNA duplex targeting rat HIF-1α (accession no. NM_024359) was transfected by CaPO₄ precipitation: sense: 5′-AGGACAGGU CACCAGAGGAAU-3′. As a control oligonucleotide, the same siRNA duplex containing 2 mismatches was used: sense: 5′-AG GACAAGGCAUCAGAGGAAU-3′. To downregulate S1P2 protein expression in BAECs, a siRNA duplex targeting bovine S1P2 receptor (accession no. NM_001081541) was transfected by CaPO₄ precipitation: sense: 5′-GCUCUACGGCAGGCA AGGt-3′. As a control oligonucleotide, the Silencer Negative Control #2 siRNA was used.

Western Blot Analysis
Cell extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore). Proteins were visualized with an enhanced chemiluminescence system (ECL; GE Healthcare Life Sciences). HIF-1α and anti–HIF-2α antibodies were purchased from Santa Cruz Biotechnology and anti-HIF-2α antibody was purchased from Abcam. Protein levels were assayed by Western blotting as previously described. Experiments are an average ± SEM of at least 3 independent experiments.

Northern Blot Analysis
RNA was isolated with TRIzol reagent (Invitrogen), resolved on 1% agarose/6% formaldehyde gels, and transferred to Hybond N nylon membrane (GE Healthcare Life Sciences). HIF-1α and anti–HIF-2α antibodies were purchased from Santa Cruz Biotechnology and anti-HIF-2α antibody was purchased from Abcam. Protein levels were assayed by Western blotting as previously described. Experiments are an average ± SEM of at least 3 independent experiments.

pVHL Capture Assay
1G11 cytoplasmic extracts (250 μg) were incubated with sepharose-bound GST–HIF-1α. GST pull-down assays for pVHL were performed as previously described. Experiments are representative of at least 3 independent experiments.

Transcription Factor Enzyme-Linked Immunoassay
Preparation of nuclear extracts and transcription factor enzyme-linked immunoassay (TFEIA) were performed as previously described. Experiments are an average ± SEM of at least 3 independent experiments performed in triplicate.

Luciferase Assays
Transient transfections were performed using 2 μg per well pGL3 (R2.2) 3HRE-tk-LUC or CMV-luc-HIF-1α-ODDD luciferase reporter vectors. Renilla reniformis luciferase expression vector (250 ng/well) was used as a control for transfection efficiency. Luciferase assays were performed as previously described. Experiments are an average ± SEM of at least 3 independent experiments performed in triplicate.

SIP Induces HIF-1α Protein Levels
Nonhypoxic stimulations have been shown to strongly increase HIF-1α protein levels. Because S1P and HIF-1 share common downstream activities in the vasculature, we attempted to determine whether vascular cell stimulation with S1P could induce HIF-1 leading to activation and gene regulation. ECs were incubated in the presence of different concentrations of S1P followed by the evaluation of HIF-1α protein levels by Western blot (Figure 1A). Interestingly, the treatment of cells with S1P for 4 hours strongly increased
HIF-1α protein levels. Treatment with 0.1 μmol/L S1P led to a detectable increase in HIF-1α protein levels, whereas maximal induction was attained at 2 μmol/L S1P. Time-course studies were then performed on these same cell lines treated with 2 μmol/L S1P (Figure 1B). An increase in HIF-1α protein levels was detected after 1 hour of S1P treatment, and a maximal induction was attained after 4 hours in the presence of S1P. After 6 hours, HIF-1α levels subsequently decreased. Phosphorylation of p42/p44 MAP kinase, a pathway known to be activated through S1P in vascular cells, was used as a control for S1P receptor activation. Similar results for HIF-1α induction by S1P treatment were also observed in VSMCs (supplemental Figure I, available at http://atvb.ahajournals.org) and in bovine aortic endothelial cells (BAECs) (results not shown). Taken together, these results demonstrate that S1P increases the expression of the inducible subunit of HIF-1 in vascular cells.

Because hypoxia is the ubiquitous activator of HIF-1, we compared the hypoxic induction of HIF-1α protein with the normoxic induction elicited after S1P treatment. As shown in Figure 1C, the level of HIF-1α protein during S1P treatment of 1G11 cells is similar to the level induced by a 4-hour incubation in hypoxic conditions. This result demonstrates the potency of S1P for inducing HIF-1α in these cells. Similar results were observed in BAECs and VSMCs (results not shown). Interestingly, S1P was unable to induce HIF-2α, a close homolog of HIF-1α also induced under hypoxic conditions in 1G11 cells (Figure 1C). This result indicates that S1P specifically targets the induction of the HIF-1 complex. Finally, the treatment of cells with S1P in the presence of hypoxia had an additive effect on HIF-1α induction (results not shown). This result suggests that different mechanisms are responsible for induction/activation of HIF-1 by S1P and hypoxia.

In vascular cells, S1P mediates its intracellular effects through S1P1, S1P2, and S1P3 receptors.17 We attempted to determine which S1P receptor led to HIF-1α induction. The treatment of cells with pertussis toxin, an inhibitor of Gi protein–coupled S1P receptors, did not block S1P-mediated HIF-1α induction, precluding the implication of S1P1, which couples exclusively through Gi (supplemental Figure IIIB).17 Pertussis toxin treatment did indeed block the phosphorylation of p42/p44 MAP kinase, a pathway known to be activated through Gi-coupled S1P receptors (supplemental Figure IIIB). However, the use of JTE-013, a potent S1P2-selective antagonist, strongly decreased HIF-1α levels after S1P treatment (Figure 2A; supplemental Figure IIA). Additionally, BAECs transfected with specific S1P2 siRNA oligonucleotide blocked HIF-1α levels after S1P treatment as compared to a control siRNA oligonucleotide (Figure 2B; supplemental Figure IIIB). Effective silencing of S1P2 in BAECs is shown in the lower panel of Figure 2B. It is important to note that JTE-013 and S1P2 siRNA had no effect on hypoxic HIF-1α induction (Figure 2A and 2B). Finally, antagonists for S1P1 and S1P3, such as W146 (a S1P1 antagonist), VPC23019 (a S1P1/S1P3 antagonist), and suramin (a S1P3 antagonist), had no effect on HIF-1α protein levels (supplemental Figure IIIA). These results indicate that S1P acts through S1P2 to increase HIF-1α protein levels.
SIP Activates the HIF-1 Nuclear Complex

We next attempted to determine whether increases in HIF-1α during SIP treatment led to the formation of an active HIF-1 complex with the constitutive nuclear subunit, HIF-1β. To perform these studies, we used a HIF-1 TFEIA. TFEIA uses a specific dsDNA oligonucleotide sequence (W26) fixed on a 96-well plate which corresponds to the sequence of a known hypoxic response element (HRE). Nuclear extracts from 1G11 cells maintained in hypoxic conditions or in the presence of SIP both demonstrated increased DNA-binding activity for HIF-1α and HIF-1β (Figure 4A). To control the specificity, we substituted the W26 dsDNA oligonucleotide sequence with a sequence mutated on 2 essential residues of the HIF-1-binding sequence (M26). In this case, very little HIF-1 binding could be observed (results not shown). These results demonstrate that endothelial HIF-1α protein induced by SIP can form the HIF-1 complex and bind to a HIF-1-specific promoter sequence. Similar results were obtained in VSMCs (results not shown).

We then determined whether the HIF-1 complex induced by SIP is transcriptionally active. We measured HIF-1–dependent transcription in BAECs transiently transfected with a luciferase reporter gene driven by 3 HRE sequences (pGL3 [R2.2] 3HRE-tk-LUC). As shown in Figure 4B, SIP generates an unstable form of luciferase when transfected into cells. The half-life of this luciferase construct is increased by oxygen deprivation and can be quantified by luciferase assays. As seen in Figure 3B, when BAECs were transiently transfected with the CMV-luc-HIF-1α-ODDD vector and treated with MG132, an inhibitor of proteasomal degradation, increased luciferase activity was observed (2.2-fold over basal levels). More interestingly, the stimulation of BAECs by SIP also increased luciferase activity (1.5-fold over basal levels). This result indicates that SIP targets the HIF-1α ODDD to increase HIF-1α protein stabilization.

A crucial event leading to HIF-1α protein degradation is its binding to the product of the von Hippel–Lindau tumor suppressor gene (pVHL), which is a direct consequence of HIF-1α hydroxylation. To determine the effect of SIP treatment on pVHL binding, a pVHL capture assay was used. A GST–HIF-1α fusion protein, comprising amino acids 344 to 582 from human HIF-1α, was subjected to modification by protein degradation is its protein stability. The treatment of cells with SIP (2 μmol/L) for 4 hours led to the formation of an active HIF-1 complex with the constitutive nuclear subunit, HIF-1β. To perform these studies, we used a HIF-1 TFEIA. TFEIA uses a specific dsDNA oligonucleotide sequence (W26) fixed on a 96-well plate which corresponds to the sequence of a known hypoxic response element (HRE). Nuclear extracts from 1G11 cells maintained in hypoxic conditions or in the presence of SIP both demonstrated increased DNA-binding activity for HIF-1α and HIF-1β (Figure 4A). To control the specificity, we substituted the W26 dsDNA oligonucleotide sequence with a sequence mutated on 2 essential residues of the HIF-1-binding sequence (M26). In this case, very little HIF-1 binding could be observed (results not shown). These results demonstrate that endothelial HIF-1α protein induced by SIP can form the HIF-1 complex and bind to a HIF-1-specific promoter sequence. Similar results were obtained in VSMCs (results not shown).
pared to control siRNA oligonucleotides, which were
beled probes against the indicated genes. Immunoblotting was
hours. Northern blotting was performed using specific radiola-
HSP90 for binding to HIF-1
complex and could ultimately lead to the activation of
lead to the formation of a transcriptionally active HIF-1
complex and could ultimately lead to the activation of
different HIF-1 target genes.

treatment increased reporter activity in BAECs by 2.5-fold
over basal levels. In the same cells, hypoxia increased
reporter activity by 3.6-fold over basal levels. Additionally,
significant activation of HIF-1 transcriptional activity was
observed at S1P concentrations as low as 0.5 μmol/L (results
not shown). Similar results were also obtained in VSMCs
(results not shown). These results indicate that S1P treatment
leads to the formation of a transcriptionally active HIF-1
complex and could ultimately lead to the activation of
different HIF-1 target genes.

Role of HIF-1 in S1P-Mediated Gene Expression

We next investigated the role of HIF-1 induction in the
expression of known HIF-1 target genes. As seen in the upper
panels of Figure 5, VSMCs treated with S1P increased the
expression of VEGF, GLUT-1, and PAI-1 mRNA (2.9±0.3, 5.3±0.5, and 5.5±0.6-fold over basal levels respectively); 3
transcripts shown to be upregulated during HIF-1 activation.21–23 Similar results were observed with 1G11 ECs
(supplemental Figure IVA). Increased expression of HIF-1
responsive gene induction was also seen with S1P concentrations as low as 0.5 μmol/L (supplemental Figure IVB). To
determine the implication of HIF-1 in these responses, VSMCs were transfected with specific HIF-1α siRNA oligo-
nucleotides. During S1P treatment, HIF-1α siRNAs significa-
cantly decreased the expression of all 3 transcripts as com-
pared to control siRNA oligonucleotides, which were
mismatched by 2 base pairs (Figure 5, upper panel; supple-
mental Figure V). Effective silencing of HIF-1α in VSMCs is
shown in the lower panels of Figure 5. These results indicate
that HIF-1 is involved in activating downstream target genes
during cell stimulation with S1P and suggest a functional
importance of these pathways in the vascular activity of S1P.

Discussion

The past decade has seen great advances in understanding the
time of biology and vascular actions of lysosphospholipids. Precisely
because of its proliferative, invasive, and migrative effects on
vascular cells, S1P has been implicated in the pathophysiology
of cancer and certain cardiovascular diseases.24–25 Because the
modulation of HIF-1 is also implicated in the progression of
aforementioned diseases,26–28 and recent studies have shown
that a number of nonhypoxic stimuli can activate HIF-1 in a
cell-specific manner,8 the present study set out to investigate
the possible interactions between HIF-1 and S1P in vascular
cells and subsequent activation of HIF-1 target genes. Here, we
show that in vascular cells, HIF-1α protein levels are strikingly
increased on S1P stimulation, which leads to HIF-1 complex
formation and transcriptional activity.

This study identifies S1P as an activator of the HIF-1
complex in oxygenated conditions and demonstrates that
HIF-1 can activate hypoxia-responsive genes after S1P stimu-
lation. Indeed, we report that in VSMCs, HIF-1 is involved
in the expression of VEGF, GLUT-1, and PAI-1 mRNA elicted by S1P, because all 3 transcripts are diminished when
HIF-1α protein levels are reduced by RNA interference. As
previously mentioned, ECs and VSMCs respond very
strongly to the bioactive lipid S1P to promote a range of
crucial intracellular events through multiple signaling path-
ways.9 Moreover, in these 2 cell types, a tight regulation in
the expression of the HIF-1 transcription factor is very
important for a variety of responses that can lead to cell
survival or cell death.4–27 We believe that during S1P treat-
ment, along with other pathways and transcription factors,
HIF-1 will permit the maximal activation of certain genes,
leading to key cellular responses.

When cells are subjected to hypoxia, HIF-1α protein is
stabilized and rapidly accumulated to permit a rapid adapta-
tive response. Our results demonstrate that S1P increases
HIF-1α protein stability through a pathway which is indepen-
dent of HIF-1α hydroxylation and pVHL binding. Recent
studies have also revealed that HIF-1α protein stabilization
can occur through pathways independent of pVHL under
normal oxygen conditions. Two studies are of particular
interest. The first involves HSP90, a molecular chaperone
that protects client proteins from misfolding and degradation,
and the receptor of activated protein kinase C (RACK1). By
compeiting with HSP90 for binding to HIF-1α, RACK1 medi-
ates a proteasomal degradation pathway that is mechanistically
similar to the pVHL pathway but independent of O2, hydroxy-
lation, and pVHL binding.29 Using a specific inhibitor of HSP90,
17-(allylamo)-17-demethoxygeldanamycin (17-AAG), we
were unable to block HIF-1α induction or stabilization in our
cell models, indicating that HSP90 is not implicated in HIF-1α
stabilization by S1P (results not shown). A second study in-
volves glycogen synthase kinase 3 (GSK-3) which appears to
exert its effect on HIF-1α in such a way that the activation of GSK-3 downregulates HIF-1α protein levels. The activation of signaling pathways, such as the phosphatidylinositol 3-kinase (PI3K) pathway, leads to an inhibition of GSK-3 activity during the treatment of cells with various growth factors and thus the stabilization of HIF-1α protein.35 In vascular cells, S1P is a known activator of the PI3K pathway leading to the phosphorylation and inactivation of GSK-3β (4a and unpublished results). We are now evaluating the possible role of the PI3K/ GSK-3 pathway in the regulation of HIF-1α by S1P.

In our studies, the strong induction of HIF-1α protein levels elicited by S1P was specific to vascular cells. We were unable to increase HIF-1α in other S1P-responsive cell models such as macrophages, fibroblasts, HEK293, and HeLa (results not shown). However, recent evidence also indicates that S1P may be involved in the expression of HIF1α isoforms in lymphocytes.32 Whereas hypoxia is the ubiquitous HIF-1 activator, most nonhypoxic stimuli seem to function in a more cell type–specific manner.4 It is possible that given the importance of HIF-1 gene induction in a variety of cellular functions and the lack of controlled stimuli, cells specifically develop mechanisms for HIF-1 induction to suit their needs. Because S1P is a key cardiovascular signaling molecule in the regulation of vascular function and homeostasis and HIF-1 is the master regulator of various genes in vascular cells that are particularly related to cell survival and angiogenesis, specific interactions between these 2 components in vascular cells are likely to play an important role in different physio- and pathological functions in the vessel wall.

It is also interesting to note that HIF-1 is induced by S1P concentrations that are in the physiological range. Because high levels of S1P are found in blood, this bioactive lipid is in an effective position to regulate the vascular endothelium, which is a major S1P-responsive cell type. S1P concentrations in serum, estimated to be around 0.4 μmol/L, can be twice as elevated in plasma, attaining concentrations similar to those used in the present study.32 Moreover, in conditions of platelet activation, such as in injured vessels during wound healing, the concentration of S1P could be increased considerably.30 This could also be the case in various atherosclerotic diseases where the levels of lipids and lipoproteins are altered and endothelial functions are disturbed.31

Our studies also demonstrate that the effects of S1P on HIFs are specific to HIF-1 and not to HIF-2. This finding concurs with studies demonstrating that effects of HIF on EC migration are critically dependent on HIF-1α and not HIF-2α.3,4 S1P receptors (S1PR) are expressed in almost every cell type, but in the vascular system, S1P1, S1P2, and S1P3 are predominant.37 In our EC model, HIF-1α protein expression is abrogated by pretreatment of cells with JTE-013, a specific antagonist of S1P2, or a specific S1P2 siRNA. On the other hand, different inhibitors and antagonists for S1P1 and S1P3 (pertussis toxin, W146, VPC23019 and suramin) were ineffective. Therefore, our results indicate that S1P induces HIF-1 through S1P2. It is interesting to note that human umbilical vesel endothelial cells (HUVECs), which express very low levels of S1P2, do not increase HIF-1α levels after S1P treatment (results not shown).39 In line with our results, S1P increases PAI-1 expression after S1P2 activation in glioblastoma cells.38 S1P also increases vascular permeability through S1P2.39 Because VEGF has a central role in the regulation of vascular permeability and is highly regulated through HIF-1, the activation of HIF-1 (and subsequently VEGF) by S1P2 activation could collaborate with direct S1P2 signaling events in the regulation of vascular permeability by S1P. Finally, an interesting study has shown that in the developing limbs of S1P1 knockout animals, HIF-1α protein levels and VEGF expression is increased, suggesting a repressive role of S1P on HIF-1 expression.40 However, severe hypoxia exists in the limbs of these animals. It is likely that defective vascular development induced by lack of S1P1 caused this hypoxia, leading to enhanced HIF-1 and VEGF induction.

In conclusion, our work identifies S1P as a novel nonhypoxic activator of the HIF-1 transcription factor in vascular cells. The elevation of HIF-1α protein levels by S1P leads to the formation of an active HIF-1 complex and to the expression of downstream target genes. These events are likely to have important physiological implications given the wide spectrum of genes possibly activated through this pathway and their roles in various areas of vascular biology.

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Disclosures
None.

References


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Supplement Material.

Supplementary Figure Legends

**Figure I:** HIF-1α induction by S1P in vascular smooth muscle cells. A, Quiescent VSMC were maintained under control conditions or in the presence of different concentrations of S1P for 4 hours. B, Quiescent VSMC were maintained under control conditions or in the presence of S1P (2 µM) for different periods of time of up to 6 hours. Total cell extracts (25 µg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8% gel) and immunoblotted using an anti-HIF-1α antiserum, an anti-phospho-p44/p42 MAPK monoclonal antibody or an anti-α-tubulin monoclonal antibody.

**Figure II:** S1P2 regulates HIF-1α protein expression by S1P in EC. Western blots in Fig.2A and 2B were quantified and normalized using α-tubulin as an internal loading control for total protein levels. Results are an average ± SEM of at least three independent experiments. Statistical significance was evaluated using a Student t-test.

**Figure III:** Use of specific inhibitors against S1P1 and S1P3 receptors. A, Quiescent 1G11 were maintained under control conditions or pretreated with either W146 (10 µM), VPC23019 (10 µM) or suramin (1 mg/mL) for a period of 20 minutes prior to stimulation with S1P (2 µM) for 4 hours. B, Quiescent 1G11 were maintained under control conditions or pretreated with pertussis toxin (0.5 µg/mL) for a period of 16 hours prior to stimulation with S1P (2 µM) for 4 hours. Total cell extracts (25 µg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8% gel) and immunoblotted using an anti-HIF-1α polyclonal antibody, or an anti-α-tubulin monoclonal antibody.
**Figure IV:** HIF-1 target gene induction by S1P in EC. A, Quiescent 1G11 were maintained under control conditions or in the presence of S1P (2 µM) for 4 hours. B, Quiescent 1G11 were maintained under control conditions or in the presence of different S1P concentrations for 4 hours. Total RNA was extracted and resolved on formaldehyde/agarose gels. Northern blotting was performed using specific radiolabeled probes. Total cell extracts (25 µg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8% gel) and immunoblotted using an anti-HIF-1α antiserum or an anti-α-tubulin monoclonal antibody.

**Figure V:** S1P increases the expression of HIF-1 target genes in VSMC. Northern blots in Fig.5A were quantified and normalized using total ribosomal 18S RNA. Results are an average ± SEM of at least three independent experiments. Statistical significance was evaluated using a Student t-test.
Supplement Material.

Expansion of Methods

Reagents – S1P, MG132, suramin sodium salt and JTE-013 were from Calbiochem. Cobalt chloride, pertussis toxin and cycloheximide were from Sigma. VPC23019 and W146 were from Avanti Polar Lipids.

Cell Culture - 1G11 cells were cultured on gelatin-coated culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% inactivated fetal bovine serum (FBS), antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin), 30 μg/ml endothelial cell growth supplement (Becton Dickinson) and 50 U/ml heparin. VSMC were isolated from thoracic aortas of 6-week-old male Wistar rats and cultured in DMEM containing 10% FBS and antibiotics. All cells were cultured in a humid atmosphere (5% CO₂, 95% air) and serially passaged upon reaching confluence. Quiescent cells were obtained by total deprivation of serum for 16 hours. Hypoxic conditions were obtained by placing cells in a sealed hypoxic workstation (Ruskin, Bridgend UK). The oxygen level in this workstation was maintained at 1% with the residual gas mixture containing 94% nitrogen and 5% carbon dioxide. Media for cell culture were from Invitrogen unless otherwise indicated.

RNA Interference - Cells were plated in 6-well plates at a density of $6 \times 10^5$ cells/ml. 24 h after plating, siRNA oligonucleotides (20 nM) were transfected by CaPO₄ precipitation. 12 hours post-transfection, the medium was changed and the cells were allowed to
recover. 24 or 48 hours post-transfection, cells were then deprived of FBS for 16 hours prior to stimulation.

*Western Blot Analysis and antibodies* - Cells were lysed in 2× Laemmli sample buffer. Protein concentration was determined by Lowry assay. Total cell extracts (25 µg) were loaded on 8% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (Immobilon-P, Millipore Corp, Billerica, MA) or nitrocellulose membranes (Hybond C Extra, GE Healthcare Life Sciences, Piscataway, NJ). Anti-HIF-1α and anti-HIF-2α antiserum were raised in rabbits immunized against the last 20 amino acids of the C termini of each human protein. Polyclonal anti-S1P2 was from Santa Cruz Biotechnology. Monoclonal anti-β-actin and anti-α-tubulin antibodies were from Sigma. Monoclonal anti-HIF-1α, anti-GST and polyclonal anti-HIF-1β antibody were from Novus Biologicals (Littleton, CO). Monoclonal HA.11 antibody was from Convance (Emeryville, CA). Total polyclonal p42/p44 MAPK antibody was from Upstate (Lake Placid, NY) and used as a loading control. Horseradish peroxidase-coupled anti-mouse and anti-rabbit antibodies were from Promega (Madison, WI). Proteins were visualized with an enhanced chemiluminescence (ECL) system (GE Healthcare Life Sciences) or with the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). Western blots were quantified using Odyssey quantification software or ImageJ (http://rsb.info.nih.gov/ij).

*Northern blot analysis and probes* - RNA was isolated with TRIzol reagent (Invitrogen), resolved on 1% agarose / 6% formaldehyde gels and transferred to Hybond N+ nylon membrane (GE Healthcare Life Sciences) before hybridization with a radioactive cDNA
probes comprising either the total coding sequence of the mouse VEGF gene, the rat plasminogen activator inhibitor-1 (PAI-1), the human GLUT1 gene or the 900-bp coding sequence of the human HIF-1α gene. An oligonucleotide probe against 18S rRNA was used as a loading control. Northern blots were quantified using a STORM phosphoimaging system equipped with ImageQuant software (GE Healthcare Life Sciences). Experiments are an average ± SEM of at least 3 independent experiments performed in triplicate.

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*Transcription Factor Enzyme-Linked Immunoassay* - High-bind NeutrAvidin-coated 96-well strip plates (Pierce Biotechnology) were incubated with 33 nM of a 5'-biotinylated 26-base pair dsDNA oligonucleotide sequence for 1 hour at room temperature. This
sequence contains wild-type or mutant (bold underlined) HIF-1 binding motif ². The sequences used here were: 5'-GATCGCCCTACGTGCTGTCTCAGATC-3' for wild-type sequence (W26) and 5'-GATCGCCCTAAAGCTGTCTCAGATC-3' for mutant sequence (M26). Preparation of nuclear extracts was performed as described previously ². DNA-binding reactions were carried out in a total volume of 50 µL containing 10 µg nuclear protein extract in a buffer containing 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.9), 50 mM NaCl, 5% glycerol, 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM dithiothreitol (DTT), and 3% nonfat milk for 1 hour at room temperature. Specific antibodies were then added at a 1:1000 concentration in phosphate-buffered saline (PBS) containing 3% nonfat milk for 1 hour at room temperature, followed by the addition of the corresponding HRP-coupled secondary antibody. Between each addition, wells were extensively washed in PBS containing 0.1% Tween-20. HRP activity was then detected by the addition of 100 µL TMB-One solution (Promega). After a 5-minute incubation period, the reaction was arrested by the addition of 0.5 M H₂SO₄. Color intensity was detected at 450 nM using a UVmax microplate reader (Molecular Devices). HRP activity was normalized to control values (i.e., non-stimulated cells). Experiments are an average ± SEM of at least 3 independent experiments performed in triplicate.

**Luciferase Assay** - Cells were seeded in 6-well plates at a density of 6 × 10⁵ cells/ml. The next day, transient transfections were performed using 2 µg/well pGL3 (R2.2) 3HRE-tk-LUC luciferase reporter vector 2. *Renilla reniformis* luciferase expression vector (250 ng/well) was used as a control for transfection efficiency. Transfection was performed
using the Lipofectamine 2000 reagent (Invitrogen Life Technologies) at a 2:5 DNA:reagent ratio. 4 hours post-transfection, cells were washed, and fresh medium was added. 24 hours post-transfection, cells were deprived of FBS for 16 hours. Stimulation with S1P (2 μM) or hypoxia was performed for 6 hours. Cells were then washed with cold phosphate-buffered saline and luciferase assays were performed with the Dual-Luciferase Reporter Assay System (Promega). Results were quantified with a Luminoskan Ascent microplate reader with integrated injectors (Thermo Scientific). Results are expressed as a ratio of firefly luciferase activity over *Renilla reniformis* luciferase activity. Experiments are an average ± SEM of at least 3 independent experiments performed in triplicate.

**References**


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- HIF-1α
- Phospho-p42/p44 MAPK
- α-tubulin

### B

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- HIF-1α
- Phospho-p42/p44 MAPK
- α-tubulin

*Online data supplement Fig. I*
Online data supplement Fig. II

A

Fold over untreated cells with control siRNA

S1P   Hypoxia   JTE013
-    -    -    -    -    -    -    +    -    -    -    -    -    +    +    +

2.6  1.3  4.0  3.9

B

Fold over untreated cells with control siRNA

S1P   Hypoxia
-    -    -    -    +    +    -    -    +    -    -    +    +    +    +

S1P2   Control   siRNA

0.8  2.9  2.9  3.0

**p < 0.01
***p < 0.001
† † p < 0.01
† † † p < 0.001

Online data supplement Fig. II
Online data supplement Fig. III

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HIF-1α
α-tubulin

B

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HIF-1α
α-tubulin

Online data supplement Fig. III
Online data supplement Fig. IV

[A] Comparison of VEGF, GLUT-1, HIF-1α, and β-actin (18S) expression levels between control and S1P-treated samples.

[B] Western blot analysis showing S1P-induced changes in GLUT-1, HIF-1α, and β-actin (18S) levels at various concentrations (0.5, 1, 2 μM) compared to control.
Mismatch siRNA / Control
Mismatch siRNA / S1P
HIF-1α siRNA / Control
HIF-1α siRNA / S1P

VEGF
GLUT-1
PAI-1

Fold over Mismatch siRNA / Control

**p < 0.01
***p < 0.001
† p < 0.05
††† p < 0.001

Online data supplement Fig. V
Supplement Material.

Supplementary Figure Legends

Figure I: HIF-1α induction by S1P in vascular smooth muscle cells. A, Quiescent VSMC were maintained under control conditions or in the presence of different concentrations of S1P for 4 hours. B, Quiescent VSMC were maintained under control conditions or in the presence of S1P (2 µM) for different periods of time of up to 6 hours. Total cell extracts (25 µg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8% gel) and immunoblotted using an anti-HIF-1α antiserum, an anti-phospho-p44/p42 MAPK monoclonal antibody or an anti-α-tubulin monoclonal antibody.

Figure II: S1P2 regulates HIF-1α protein expression by S1P in EC. Western blots in Fig.2A and 2B were quantified and normalized using α-tubulin as an internal loading control for total protein levels. Results are an average ± SEM of at least three independent experiments. Statistical significance was evaluated using a Student t-test.

Figure III: Use of specific inhibitors against S1P1 and S1P3 receptors. A, Quiescent 1G11 were maintained under control conditions or pretreated with either W146 (10 µM), VPC23019 (10 µM) or suramin (1 mg/mL) for a period of 20 minutes prior to stimulation with S1P (2 µM) for 4 hours. B, Quiescent 1G11 were maintained under control conditions or pretreated with pertussis toxin (0.5 µg/mL) for a period of 16 hours prior to stimulation with S1P (2 µM) for 4 hours. Total cell extracts (25 µg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8% gel) and immunoblotted using an anti-HIF-1α polyclonal antibody, or an anti-α-tubulin monoclonal antibody.
**Figure IV:** HIF-1 target gene induction by S1P in EC. A, Quiescent 1G11 were maintained under control conditions or in the presence of S1P (2 µM) for 4 hours. B, Quiescent 1G11 were maintained under control conditions or in the presence of different S1P concentrations for 4 hours. Total RNA was extracted and resolved on formaldehyde/agarose gels. Northern blotting was performed using specific radiolabeled probes. Total cell extracts (25 µg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8% gel) and immunoblotted using an anti-HIF-1α antiserum or an anti-α-tubulin monoclonal antibody.

**Figure V:** S1P increases the expression of HIF-1 target genes in VSMC. Northern blots in Fig.5A were quantified and normalized using total ribosomal 18S RNA. Results are an average ± SEM of at least three independent experiments. Statistical significance was evaluated using a Student t-test.
Supplement Material.

Expansion of Methods

Reagents – S1P, MG132, suramin sodium salt and JTE-013 were from Calbiochem. Cobalt chloride, pertussis toxin and cycloheximide were from Sigma. VPC23019 and W146 were from Avanti Polar Lipids.

Cell Culture - 1G11 cells were cultured on gelatin-coated culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% inactivated fetal bovine serum (FBS), antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin), 30 μg/ml endothelial cell growth supplement (Becton Dickinson) and 50 U/ml heparin. VSMC were isolated from thoracic aortas of 6-week-old male Wistar rats and cultured in DMEM containing 10% FBS and antibiotics. All cells were cultured in a humid atmosphere (5% CO₂, 95% air) and serially passaged upon reaching confluence. Quiescent cells were obtained by total deprivation of serum for 16 hours. Hypoxic conditions were obtained by placing cells in a sealed hypoxic workstation (Ruskinn, Bridgend UK). The oxygen level in this workstation was maintained at 1% with the residual gas mixture containing 94% nitrogen and 5% carbon dioxide. Media for cell culture were from Invitrogen unless otherwise indicated.

RNA Interference - Cells were plated in 6-well plates at a density of 6 × 10⁵ cells/ml. 24 h after plating, siRNA oligonucleotides (20 nM) were transfected by CaPO₄ precipitation. 12 hours post-transfection, the medium was changed and the cells were allowed to
recover. 24 or 48 hours post-transfection, cells were then deprived of FBS for 16 hours prior to stimulation.

*Western Blot Analysis and antibodies* - Cells were lysed in 2× Laemmli sample buffer. Protein concentration was determined by Lowry assay. Total cell extracts (25 μg) were loaded on 8% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (Immobilon-P, Millipore Corp, Billerica, MA) or nitrocellulose membranes (Hybond C Extra, GE Healthcare Life Sciences, Piscataway, NJ). Anti-HIF-1α and anti-HIF-2α antiserum were raised in rabbits immunized against the last 20 amino acids of the C termini of each human protein \(^1\). Polyclonal anti-S1P2 was from Santa Cruz Biotechnology. Monoclonal anti-β-actin and anti-α-tubulin antibodies were from Sigma. Monoclonal anti-HIF-1α, anti-GST and polyclonal anti-HIF-1β antibody were from Novus Biologicals (Littleton, CO). Monoclonal HA.11 antibody was from Convance (Emeryville, CA). Total polyclonal p42/p44 MAPK antibody was from Upstate (Lake Placid, NY) and used as a loading control. Horseradish peroxidase-coupled anti-mouse and anti-rabbit antibodies were from Promega (Madison, WI). Proteins were visualized with an enhanced chemiluminescence (ECL) system (GE Healthcare Life Sciences) or with the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). Western blots were quantified using Odyssey quantification software or ImageJ ([http://rsb.info.nih.gov/ij](http://rsb.info.nih.gov/ij)).

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