ONLINE SUPPLEMENT

MATERIALS AND METHODS

Cell culture and reagents. Rat aortic SMCs were isolated and cultured (passage 11 to 15) as previously described[1]. For all experiments, SMCs were grown to full confluence and growth arrested in serum-free media prior to stimulation. Sphingosine-1-phosphate (Avanti Polar Lipids); JTE013 (Tocris); VPC44116, VPC01091, VPC25239 were synthesized at the University of Virginia[2-4]; Y27632 gift from Mitsubishi Pharma Corp, Koyata, Japan; (*s*)FTY720 a gift from Novartis.

Rat carotid artery balloon injury. Male Sprague-Dawley rats (350–400 g) were anesthetized, and acute injury to the left common carotid artery was made with a 2F Fogarty balloon catheter as described previously[5]. At the indicated times after injury, animals were euthanized, and the injured left carotid and uninjured right carotid were removed and processed accordingly for mRNA. Total RNA was prepared from cultured TRIzol reagent (Invitrogen) and Q-BIOGene lysing matrix D, cDNA synthesized using the iScript cDNA Synthesis Kit (BioRad) and real-time polymerase chain reaction (PCR) analysis (iCycler, BioRad) was performed on cDNA using SybrGreen. Each time point represents the mean ± SE of the injured (S1PR:18S) vessel normalized to the uninjured (S1PR:18S) vessel. In drug treatment studies, animals were euthanized 14 days after injury, perfusion fixed with 4% PFA and vessel were prepped for routine histology. Images were acquired of 6-8 histological sections at the anatomical midpoint of the balloon injury and neointimal hyperplasia (NI) determined using ImagePro. Using the region of interest function in ImagePro, the areas (mm^2) of the following were determined: 1) area contained by the EEL (inclusive of the media, neointima and lumen), 2) area contained by the IEL (inclusive of the neointima and lumen), 3) area contained by the lumen. The medial area was determined by subtracting the IEL area from the EEL area. The area of the neointima was determined by subtracting the lumen area from the IEL area. The neointima to media ratio (NI/M) was calculated by dividing the neointimal area by the media area. All animal procedures were approved by the University of Virginia Animal Care and Use Committee.

Sphingosine-1-phosphate primer sequences rS1P1 *for* 5-ggaatctactccttggtgaggactc-3, *rev* 5-ccagcaggcaatgaagacactc-3; rS1P2 *for* 5-gcaagttccactcagccatgt-3, *rev* 5-gggagttaaggacagggtgaca-3; rS1P3 *for* 5-tcatcagcatcttcacagccattc-3, *rev* 5-atggatctctcggagttgtggtt-3; rS1P4 *for* 5-ctattccttccgcagccgtg-3, *rev* 5-cagtctccagggcctctcag-3; rS1P5 *for* 5-aaacgctggaagacacagggatc-3, *rev* 5-cacggctctgcctcactctg-3.

Transient transfections. SMCs were transfected with plasmids using FuGENE6 (Roche) when the cells were ≈80% confluent. Cells were grown to confluence and changed to SFM at 2 days, at which time cells were treated with various drugs as described in the Results section. The total amount of DNA per well was kept constant (500 ng). Luciferase activity (Promega) was measured and normalized to cellular protein concentrations (Pierce). Each sample was examined in duplicate with a minimum of 3

biological replicates per treatment. CMV-S1P1 (rat), CMV-S1P2 (rat) and pcDNA3.1 were contransfected at 100 ng plasmid DNA with SMα-actin luciferase (400 ng).

Quantitative real-time PCR and chromatin immunoprecipitation assays (ChIP) Total RNA was prepared from cultured rat aortic SMCs using TRIzol reagent (Invitrogen), cDNA synthesized using the iScript cDNA Synthesis Kit (BioRad) and real-time polymerase chain reaction (PCR) analysis (iCycler, BioRad) was performed on cDNA using SybrGreen, as previously described[1,6]. All results were normalized to 18S rRNA and expressed as a percent of control. Chromatin immunoprecipitation (ChIP) was performed as previously described[1,6]. Real-time PCR was performed on 1 ng genomic DNA from ChIP. Real-time PCR primers were designed to flank the 5'-CArG elements of SMα-actin and SMMHC, the c-fos CArG[1,6]. Quantification of protein:DNA interaction/enrichment was determined by the following $2^{Ct(Ref)-Ct(IP)}$-$2^{Ct(Ref)-Ct(No\ antibody\ control)}$.

Primer sequences were as follows: *SM α-actin* 5-agcagaacagaggaatgcagtggaagagac-3., 5-cctcccactcgcctcccaaacaaggagc-3; *SMMHC* 5.-ctgcgcgggaccatatttagtcaggggggag-3, 5-ctgggcgggagacaacccaaaaaggccagg-3; *c-fos* 5-cggttcccccccctgcgctgcaccctcagag-3, 5-agaacaacagggaccggccgtggaaacctg-3.

Statistics. Data are expressed as mean±SE. Statistical significance among treatment groups was confirmed with a 1-way ANOVA when appropriate. Statistical significance between specific groups was determined by a post-hoc multiple comparison Student-Newman-Keuls test ($P<0.05$).

Supplemental Figure 1. S1P receptor mRNA profiles in cultured rat aortic SMCs and rat carotid arteries. A, relative S1P receptor mRNA levels in cultured aortic SMCs as determined by quantitative RT-PCR. Cells were serum starved for 72 hrs. Data are normalized to 18S mRNA levels. **B**, relative S1P receptor mRNA levels in the right carotid artery (N=6). Data are normalized to 18S mRNA levels.

Supplemental Figure 2. A, Transcriptional activation of various promoter-reporter constructs in cultured SMCs by S1P. Transiently transfected SMCs were treated with S1P for 24 hours and assayed for luciferase activity. Data are expressed as percent of control (vehicle treated cells) ± SEM. **B,** SMCs were treated with 100 nM or 1.0 μM S1P for 24 hours and expression of SMα-actin, SMMHC, myocardin, and 18S rRNA mRNA levels were quantified by real-time RT-PCR.

Supplemental Figure 3. Overexpression of S1P1 attenuated S1P induction of SMα-actin luciferase whereas overexpression of S1P2 potentiated S1P-induced activation of SMα-actin luciferase activity.

Supplemental Figure 4. Dose response to (*s*)FTY720 in transiently transfected SMCs.

References

1. Wamhoff BR, Bowles DK, McDonald OG, Sinha S, Somlyo AP, Somlyo AV, Owens GK. L-type Voltage-Gated Ca2+ Channels Modulate Expression of Smooth Muscle Differentiation Marker Genes via a Rho Kinase/Myocardin/SRF-Dependent Mechanism. *Circ Res* 2004;95:406-414.

2. Davis MD, Clemens JJ, Macdonald TL, Lynch KR. Sphingosine 1-Phosphate Analogs as Receptor Antagonists. *J Biol Chem* 2005;280:9833-9841.

3. Zhu R, Snyder AH, Kharel Y, Schaffter L, Sun Q, Kennedy PC, Lynch KR, Macdonald TL. Asymmetric Synthesis of Conformationally Constrained Fingolimod Analogues-Discovery of an Orally Active Sphingosine 1-Phosphate Receptor Type-1 Agonist and Receptor Type-3 Antagonist. *J Med Chem* 2007.

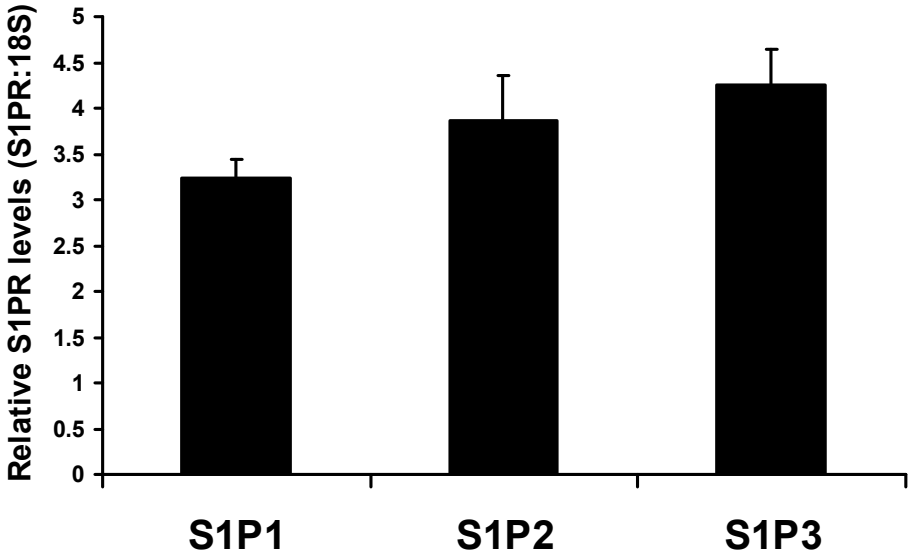
4. Foss FW, Jr., Snyder AH, Davis MD, Rouse M, Okusa MD, Lynch KR, Macdonald TL. Synthesis and biological evaluation of gamma-aminophosphonates as potent, subtype-selective sphingosine 1-phosphate receptor agonists and antagonists. *Bioorg Med Chem* 2007;15:663-677.

5. Clowes AW, Reidy MA, Clowes MM. Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Lab Invest* 1983;49:327-333.

6. McDonald OG, Wamhoff BR, Hoofnagle MH, Owens GK. Control of SRF binding to CArG box chromatin regulates smooth muscle gene expression in vivo. *J Clin Invest* 2006;116:36-48.

Supplemental Figure 1. Wamhoff et al, ATVB.

A.



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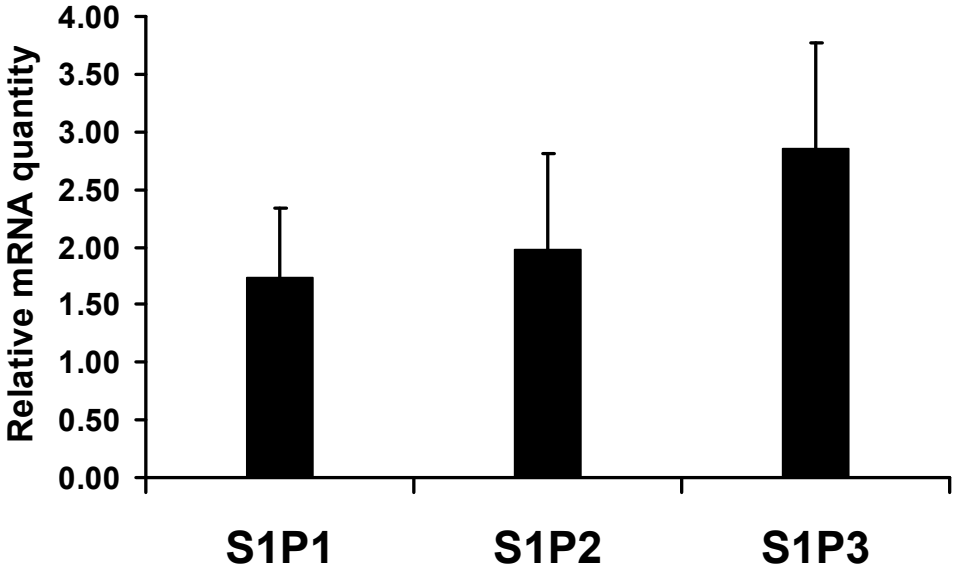
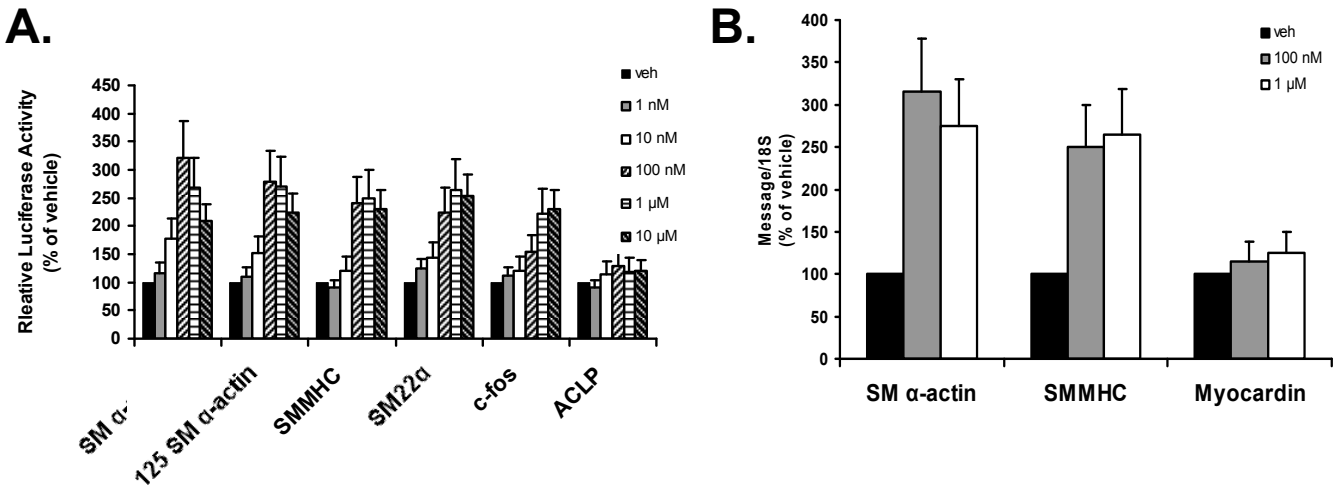
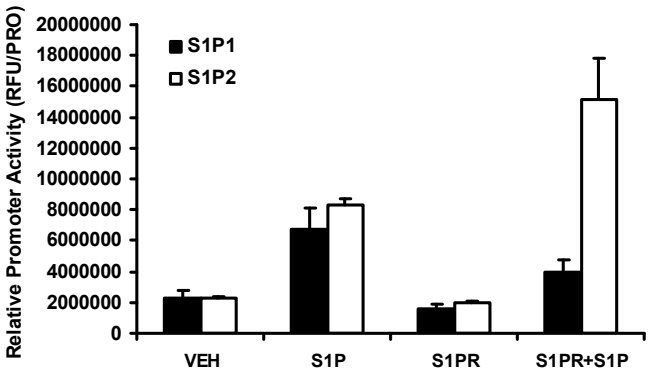


Supplemental Figure 2. Wamhoff et al, ATVB.



Supplemental Figure 3. Wamhoff et al, ATVB.



Supplemental Figure 4. Wamhoff et al, ATVB.