Sphingosine-1-Phosphate and Lysophosphatidic Acid Stimulate Endothelial Cell Migration

Tracee Scalise Panetti, Julie Nowlen, Deane F. Mosher

Abstract—Endothelial cell migration is necessary for the formation of new blood vessels. We investigated the effects of 2 lysophospholipid mediators, sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA), on endothelial cell migration. S1P and LPA stimulated migration of fetal bovine heart endothelial cells (FBHEs) in a 3D-modified Boyden chamber assay with concentrations as low as 15 nmol/L stimulating a 2-fold change and concentrations in the 1- to 2-μmol/L range stimulating 14- to 20-fold changes. S1P specifically stimulated the migration of several endothelial cell strains but did not stimulate the migration of tumor cells or smooth muscle cells. LPA stimulated some endothelial and nonendothelial cell types to migrate. For FBHEs, S1P and LPA were mostly chemokinetic in checkerboard assays. S1P and LPA stimulated extracellular signal-regulated kinase 1/2 phosphorylation and enhanced paxillin localization to focal contacts, with no discernible change in the actin cytoskeleton in FBHEs. To characterize responsible receptor-dependent signaling pathways, we investigated the involvement of G_i, Rho, and phosphoinositide 3-OH kinase in S1P- and LPA-stimulated migration. Although perturbation of all 3 signaling molecules resulted in decreased migration, the mechanisms underlying the decreased migration were different. Pertussis toxin treatment, to target G_i, caused endothelial cells to develop dense bundles of F-actin and distribute paxillin staining to the cell periphery in response to S1P or LPA. Modification of Rho with C3 toxin disrupted the actin cytoskeleton. Inhibition of extracellular signal-regulated kinase kinase with PD98059 caused a loss of phosphorylation of extracellular signal–regulated kinase 1/2, similar to pertussis toxin, but only a minimal decrease in migration. These results indicate that S1P and, for some cells, LPA stimulate migration of endothelial cells through a mechanism that likely requires a balance between G_i and Rho signaling to achieve the cytoskeletal remodeling necessary for cell migration. (Arterioscler Thromb Vasc Biol. 2000;20:1013-1019.)

Key Words: sphingosine-1-phosphate ■ lysophosphatidic acid ■ endothelial cells ■ cell migration

Sphingosine-1-phosphate (S1P) is a lysophospholipid mediator released on platelet activation with a serum concentration of 0.5 μmol/L, ~2-fold higher than the plasma concentration.1 S1P stimulates DNA synthesis by fibroblasts, neurite retraction, stress fiber formation,1,2 fibronectin deposition,3 and suppression of T-cell apoptosis.4 Lysophosphatidic acid (LPA) is the major lysophospholipid growth factor in serum generated as a result of platelet activation.2 The concentration of LPA in serum is estimated at 1 to 10 μmol/L, whereas the concentration in plasma is considerably less.1 LPA has numerous biological effects on cells, including stimulation of cell proliferation, increased tight junction permeability in brain endothelial cells, stress fiber formation, increased binding and deposition of fibronectin by fibroblasts, smooth muscle cell contraction, and platelet aggregation.2

Several G-protein–coupled receptors of the endothelial cell differentiation gene (Edg) family that bind and mediate biological responses to S1P and/or LPA have been identified (reviewed in References 1 and 5). LPA and S1P stimulate the Rho, Ras/mitogen-activated protein kinase, and phospholipase C pathways and perhaps others through G proteins.1,2,5 Rho is a small GTPase downstream from G_i that stimulates phospholipase C downstream from platelet-activating factor (PAF) and G_s,7 that stimulates phospholipase C downstream from Clostridium botulinum.6 G_i, the pertussis toxin–sensitive heterotrimeric G protein, stimulates the Ras pathway that leads to extracellular signal–regulated kinase (Erk) 1/2 activation through multiple intermediates, including extracellular signal-regulated kinase (Erk)-activated protein kinase (Mek-1).1,2 G_i activation of Ras may include phosphoinositol 3-OH kinase (PI3-kinase) as an intermediate.3 Stimulation of the G_i pathway may also inhibit cAMP generation by cells.1 Finally, lysophospholipids stimulate phospholipase C downstream from G_s.1

New blood vessel formation, angiogenesis, is required for normal development and wound healing.7,8 Aberrant angiogenesis contributes to many diseases, including diabetic retinopathy, psoriasis, tumor growth, and arthritis.8 Directed migration of endothelial cells is one of the critical steps in...
angiogenesis. The present studies were initiated to examine the migratory response of endothelial cells in response to S1P and LPA. LPA has been shown to induce cell migration of nonendothelial cells by pathways that use G, Rh activation, 10, 11 Ca mobilization, 12 Rac activation, 13 and Ras activation 14 but do not require activation of Erk1/2. 13, 14 S1P, in contrast, has been shown to inhibit cell migration of smooth muscle cells, 15 mouse melanoma cells, 16 breast cancer cells, 17 neutrophils, 18 and fibroblasts. 10 We found that endothelial cells derived from several sources migrated in response to S1P. The responses to LPA were more variable. Inactivation of G, inactivation of Rho, and inhibition of PI3-kinase all decreased S1P- and LPA-induced endothelial cell migration independent of their effects on the activation of Erk1/2 but with characteristic effects on the cytoskeleton.

Methods

Materials

SIP was purchased from Alexis Corp and solubilized in methanol. LPA (1-oleoyl-LPA) was purchased from Avanti Polar Lipids and solubilized as previously described. 19 Lyso phosphatidylcholine, lyso phosphatidylserine, and sphingomyelin were purchased from Sigma Chemical Co. Vascular endothelial growth factor (VEGF) and epidermal growth factor were purchased from R&D Systems and Upstate Biotechnology, respectively. Cells were pretreated with pertussis toxin (List Biologicals) or C3 toxin overnight at the indicated concentrations. C3 toxin was expressed in the pGex vector (generous gift of Dr Larry Feig, Tufts University Medical School, Boston, Mass) as a glutathione S-transferase fusion protein, and purification of the fusion protein was performed by glutathioneagarose affinity chromatography as described. 6 The fusion protein was cleaved with thrombin while bound to glutathione-agarose, and C3 toxin was recovered in the supernatant after removal of thrombin by benzamidine-Sepharose. L2Y94002 (Sigma) and Wortmannin (Sigma) were dissolved in dimethyl sulfoxide at 5 to 10 mmol/L and stored at -20°C. The Mek-1 inhibitor, PD98059 (Calbiochem), was dissolved in dimethyl sulfoxide at 6.5 mg/mL and stored at -20°C. The inhibitors were preincubated with cells for 30 to 60 minutes before the cells were used in experiments.

Cell Culture and Cell Migration Assays

Fetal bovine heart endothelial cells (FBHEs) and ECV (T-24 variant) bladder carcinoma cells were obtained from American Type Culture Collection and cultured in DME containing 10% FBS. Bovine vascular smooth muscle cells and bovine aortic endothelial cells (BAEs) were obtained from the Coriell Institute for Medical Research. FBHES were supplemented with 20 ng/mL recombinant basic fibroblast growth factor (bFGF, generous gift from Scios, Mountain View, Calif). BAEs were cultured in DME containing 20% FBS. Human umbilical vein endothelial cells (HUVECs), bovine adrenal microvascular endothelial cells (BAMECs), bovine lung microvessel endothelial cells (BLMVECs), and bovine pulmonary artery endothelial cells (BPAEs) were obtained from VEC Technologies, Inc, and cultured in MCD-131 complete media (VEC Technologies, Inc). Cells were grown in a humidified incubator at 37°C with 5% CO. BFs (FBHES, ECVs, and BAEs) or 5% CO (HUVECs, BAMECs, BLMVECs, BPAEs, and vascular smooth muscle cells) and passedaged twice a week. For migration assays, newly confluent cells in growth media were lifted with trypsinization in the presence of EDTA; the media were lifted with trypsinization in the presence of EDTA; the

Results

SIP or LPA stimulated FBHE migration in a dose-dependent fashion over a wide concentration range (Figure 1). Migration, 5- to 8-fold greater than baseline, was stimulated with SIP or LPA concentrations as low as 15 nmol/L SIP, with a 14- to 20-fold increase above baseline at 0.5 to 2 μmol/L SIP or LPA. Maximum migration was somewhat higher for SIP than for LPA. The migration was not stimulated above baseline by 1 μmol/L lyso phosphatidylcholine, lyso phosphatidylserine, or sphingomyelin (data not shown). The SIP and LPA responses were similar on fibronectin- or vitronectin-coated filters for FBHES, although background migration in the absence of growth factor was higher on vitronectin than on fibronectin (data not shown). VEGF (10 ng/mL) stimulated FBHE migration to ~50% of the value seen with 1 μmol/L SIP, whereas epidermal growth factor did not stimulate migration above baseline (data not shown). Endothelial cells from other sources, including BAEs, HUVECs, BLMVECs, and BPAEs, migrated to SIP, whereas vascular smooth muscle cells and ECV bladder carcinoma cells did not migrate to SIP (Table). BAMECs were tested in a 2D cell motility assay and showed migration in response to SIP. LPA stimulated the migration of BAEs but not HUVECs, BAMECs, BPAEs, or BLMVECs. LPA caused bladder carcinoma cells (but not vascular smooth muscle cells) to migrate.
To examine the directional component of migration, we examined lysophospholipid-induced migration of FBHEs by using a checkerboard analysis. S1P or LPA was required in the bottom chamber for maximum migration of cells to the bottom of the filter (Figure 2). High concentrations of S1P in the top, however, stimulated some cell migration to the bottom of the filter, albeit there was a 4-fold less migration than with S1P in the bottom chamber. S1P and LPA enhanced migration in the absence of a gradient (equal concentrations in the top and bottom), showing that there is a large chemokinetic component to the migration.

To understand the downstream signaling pathways stimulated by S1P or LPA that lead to FBHE migration, we tested agents that perturb signal transduction. Pertussis toxin modification of Gi is known to block stimulation of Ras and the induction of mitogenesis in cells treated with S1P or LPA. Overnight pretreatment with pertussis toxin, at doses as low as 2.5 ng/mL (Figure 1A, published online only at http://atvb.ahajournals.org/cgi/content/full/20/4/1013/DC1), caused a loss of ability of FBHEs to migrate in response to S1P or LPA (Figure 3). Pertussis toxin was not globally deleterious to the endothelial cells, inasmuch as the cells attached and spread on tissue culture plastic in the presence of 10% FBS (data not shown) and assembled a fibronectin matrix (data not shown). To learn whether the effects of pertussis toxin are mediated downstream from mitogen-activated protein kinase, a Mek-1 inhibitor, PD98059, was tested. A 25 μmol/L concentration of PD98059 had little effect on S1P- or LPA-induced endothelial cell migration (Figure 3) and was not dose dependent (Figure IB). Treatment with either pertussis toxin or PD98059, however, caused equivalent loss of Erk1/2 activation in response to S1P or LPA (Figure 4). These results suggest that the Gi-mediated pathway important for cell migration diverges upstream from Mek-1.

Cell movement requires the reorganization of the actin cytoskeleton as the cell establishes a leading edge and migrates forward. Therefore, we examined the effect of modifiers of S1P- and LPA-induced cell migration on the actin cytoskeleton and paxillin staining in focal contacts. In untreated cells, there was no detectable change in the actin cytoskeleton after lysophospholipid stimulation (Figure 5A); however, paxillin immunofluorescence was altered (Figure 5B). Paxillin staining was found in fine fibrillar streaks in serum-starved cells but was more intense, with shorter thicker patches, after lysophospholipid stimulation. Inhibition of Gi with pertussis toxin did not have a dramatic effect on the actin cytoskeleton or paxillin accumulation in the focal contacts in nonstimulated cells. S1P or LPA, however, induced cellular contraction of pertussis toxin–treated cells: the actin cytoskeleton was more cortical, and the cells were less flattened so that the cytoskeleton was present in several planes with only a small portion in focus in any single plane (Figure 5A). The paxillin staining was also rearranged and present only at the cell periphery and was not distributed throughout the cell, as in the non–pertussis toxin-treated cells (Figure 5B).

Two additional signaling molecules, Rho and PI3-kinase, were perturbed to characterize better the processes involved in endothelial cell migration stimulated by lysophospholipids. PI3-kinase links the activation of Gi to Ras and the activation of Ras to other small G proteins, particularly Rac, with downstream activation of Rho. Furthermore, S1P or LPA, signaling through Gi1/2, causes direct activation of Rho1. FBHE migration in response to S1P or LPA was decreased after an overnight preincubation with C3 toxin, the Rho inactivator, to ~25% of control at a concentration of 10 μg/mL (Figure 3). Preincubation with increasing doses of toxin was not associated with further inhibition (Figure II, published online only at http://atvb.ahajournals.org/cgi/content/full/20/4/1013/DC1). The toxin inhibited migration with the same efficacy regardless of the LPA dose used to stimulate cell migration (Figure II). The toxin did not have deleterious effects on the cells, as shown by cell attachment to tissue culture plastic in DME containing 10% FBS (data not shown). Pretreatment with wortmannin (10 μmol/L) or LY294002 (50 μmol/L), 2 inhibitors of PI3-kinase, resulted in a decrease in migration in response to S1P, LPA, or serum to ~30% in the absence of the inhibitor (Figure III, published online only at http://atvb.ahajournals.org/cgi/content/full/20/4/1013/DC1, and Figure 3). C3 toxin and the PI3-kinase

![Figure 1. Dose-response of S1P and LPA stimulation of FBHE migration. Cells (1x10^6 cells per 50 μL) were added to the top of the fibronectin-coated polycarbonate filter and migrated through in response to S1P or LPA added to the bottom. After 6 hours, nonmigrated cells were scraped from the top of the filter, and migrated cells were fixed and stained. Each condition was performed in triplicate, and 3 fields from each well were counted at ×400 magnification (n=9). Data are expressed as mean±SEM. Data are an average of 3 experiments.](http://atvb.ahajournals.org/cgi/content/full/20/4/1013/DC1)
inhibitor LY294002 each caused a modest inhibition of Erk1/2 phosphorylation in response to S1P or LPA (Figure 4). The effects of C3 toxin, wortmannin, LY294002, or PD98059 on actin cytoskeleton were compared with the effects of pertussis toxin (Figure 6). Despite serum starvation, the cells exhibited an extensive actin cytoskeleton, as detected by rhodamine-phalloidin fluorescence in the absence of modifiers (Figure 6). C3 toxin caused loss of the actin cytoskeleton, such that in the majority of C3 toxin–treated cells, no filamentous actin was detected. Wortmannin also disrupted the actin cytoskeleton with an intermediate phenotype of fewer stress fibers than control cells but not a complete loss of actin cytoskeleton, as was caused by C3 toxin. LY294002 had the same effect as wortmannin, with some loss of stress fibers (data not shown). PD98059 did not effect the actin cytoskeleton. The actin cytoskeleton remained unchanged after stimulation with S1P or LPA in the presence of these modifiers (data not shown).

Discussion
We found that S1P stimulates the migration of FBHEs, BAEs, HUVECs, BLMVECs, BPAEs, and BAMECs. This response was specific inasmuch as S1P did not stimulate the migration of vascular smooth muscle cells or bladder carcinoma cells. S1P inhibits migration or does not stimulate the migration of a number of other non–endothelial cell types, including vascular smooth muscle cells, mouse melanoma cells, BALB/c3T3 fibroblasts, human HT1080 fibrosarcoma cells, MC63 osteosarcoma cells, breast cancer cells, and human neutrophils. T-lymphoma cells have been shown to invade a fibroblast monolayer after S1P treatment but only after transfection of activated Tiam1, a Rac guanine nucleotide exchange factor. Endothelial cells, therefore, are unique in that S1P stimulates cell migration in nontransformed cells. LPA stimulated the migration of FBHEs and BAEs but not HUVECs, BPAEs, BLMVECs, or BAMECs. Related phospholipids, lysophosphatidylcholine, lysophosphatidic acid, and sphingosine 1-phosphate, also stimulated migration of endothelial cells.

Figure 2. Checkerboard assay of FBHE migration in response to LPA (A) or S1P (B). The migration assay was performed as described for Figure 1, except that LPA or S1P was added to the top and/or bottom wells as indicated. The data are expressed as mean of 9 determinations, with the SEM being <5% of the mean. The data are from a representative experiment that was repeated 2 times.

Figure 3. Comparison of effects of modifiers on S1P- and LPA-induced FBHE migration. The data are expressed as percentage of control ± SEM (n = 9), where control indicates cell migration in the absence of the modifiers (100%). Background migration, in the absence of LPA or S1P, was subtracted from all values. The concentrations of the modifiers were as follows: pertussis toxin (PTX), 5 ng/mL; C3 toxin (C3), 50 μg/mL; LY294002 (LY), 50 μmol/L; and PD98059 (PD), 25 μmol/L. Data are expressed as mean ± SD (n = 9) and are from a representative experiment that was repeated 4 times. All mediators were statistically different from control, as determined by t test.

Figure 4. Western blot detection of active Erk1/2 in FBHEs after treatment with modifiers of Rho, Gi, PI3-kinase, and Mek-1. Cells were plated in DME with 10% FBS, attached for 4 to 6 hours, serum-starved overnight, and pretreated with the different substances at the indicated concentrations: untreated (control); C3 toxin, 50 μg/mL; pertussis toxin, 5 ng/mL; LY294002, 50 μmol/L; and PD98059, 25 μmol/L. Cells were stimulated for 5 minutes with 1 μmol/L LPA or S1P as indicated, put on ice, and solubilized in lysis buffer. Erk1 and Erk2 are designated as p44 and p42, respectively. Data are from a representative experiment that was repeated 2 times.
lysocephatidylserine, or sphingomyelin did not stimulate endothelial cell migration. Other cell types have been shown to migrate in response to LPA, including human Rat-1 fibroblasts, \(^1\) \(\beta\)1A-GD25 fibroblasts, \(^1\) hepatoma cells, \(^2\) breast carcinoma cells, \(^1\) and Dictyostelium discoideum slime mold. \(^2\) However, LPA does not stimulate the migration of vascular smooth muscle cells or osteosarcoma cells (M. Magnusson and D.F. Mosher, unpublished data, 2000), suggesting that whether a cell migrates in response to LPA is also cell type specific.

Endothelial cell migration to lysospholipids can be contrasted with VEGF- and bFGF-stimulated migration of endothelial cells in 3 ways: First, S1P and LPA are primarily chemokinetic, with a small chemotactic component, similar to bFGF but unlike VEGF, which is primarily chemotactic. \(^2\) Second, S1P and LPA stimulate migration over a broad dose

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**Figure 5.** Effect of S1P and LPA on actin stress fiber formation and paxillin localization. FBHEs were plated on fibronectin-coated glass coverslips, attached for 4 to 6 hours, and serum-starved overnight in the absence or presence of PTX (5 ng/mL). Cells were untreated (control) or stimulated for 15 minutes with 1 \(\mu\)mol/L LPA or S1P, fixed, and permeabilized. A, Actin cytoskeleton was visualized by rhodamine-phalloidin staining. B, Paxillin localization was examined by anti-paxillin antibodies followed by a fluorescein-labeled secondary antibody. Cells are representative of changes observed in 3 different experiments.

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**Figure 6.** Rhodamine-phalloidin staining of FBHEs after treatment with modifiers of Rho, PI3-kinase, and Mek-1. Cells were plated on glass coverslips in DME with 10% FBS, attached for 4 to 6 hours, serum-starved overnight, and pretreated with C3 toxin (50 \(\mu\)g/mL), wortmannin (10 \(\mu\)mol/L), or PD98059 (25 \(\mu\)mol/L), as indicated in Methods. Cells are representative of changes observed in 2 different experiments.
range (200-fold) that is distinct from the narrow dose response (10-fold) that results in the bell-shaped curve characteristic of VEGF or bFGF. Third, the magnitude of migration is greater, with a 10-fold increase found with lysophospholipids compared with the few-fold change found with VEGF (herein) or bFGF. As evidence that S1P and LPA are acting through G-protein-coupled receptors, we found that treatment with pertussis toxin ablates S1P- or LPA-stimulated endothelial cell migration. Pertussis toxin and the Mek-1 inhibitor PD98059 caused Erk1/2 activation by S1P or LPA to fall to undetectable levels; however, PD98059 had minimal effects on S1P- and LPA-stimulated endothelial cell migration. Therefore, Erk1/2 activation is not required for S1P- and LPA-stimulated endothelial cell migration, consistent with the previous report indicating that PD98059 does not affect LPA-stimulated migration of Rat-1 fibroblasts. Examination of the actin cytoskeleton and paxillin localization in pertussis toxin–treated cells suggests that inactivation of G1 uncovers a lysophospholipid-induced contractile phenotype (ie, cortical localization of actin and peripheral localization of paxillin) that is incompatible with cell migration. The phenotype is consistent with the contractile response of fibroblasts and osteosarcoma cells to LPA, which has been shown to be dependent on Rho activation. Nobes and Hall have shown that microinjection of activated Rho does indeed prevent fibroblast cell migration in a wound assay. Treatment of endothelial cells with C3 toxin to inactivate Rho had a deleterious effect on cell migration that was probably due to disruption of the actin cytoskeleton. C3 toxin disrupted the actin cytoskeleton and resulted in loss of migration by the majority of the cells. C3 toxin has been shown to decrease hepatoma cell invasion in response to LPA and closure of wounds in fibroblast monolayers. Therefore, a balance between the activation of Rho, presumably by G1-21, and activation of G, is important for endothelial cell migration stimulated by S1P and LPA. Rac, a member of the Rho family, stimulates the formation of lamellipodia and may activate Rho. PI3-kinase is an intermediate in the activation of Rac by Ras. T-lymphoma cells with activated Rac or activated Tiam1, a Rac guanine nucleotide exchange factor, require an additional signal from LPA or S1P to migrate. We found that PI3-kinase inhibitors decreased lysophospholipid-stimulated endothelial cell migration by a 50% to 70% and partially disrupted the actin cytoskeleton, with a minimal inhibition of Erk1/2 phosphorylation. The effect of PI3-kinase inhibitors may be explained by the ability of PI3-kinase to activate Ras downstream from G123,22 of PI3-kinase to activate Rac downstream from Ras,22 and of integrin receptors to activate Rac through PI3-kinase.23 Our data do not distinguish among these possibilities, although we have demonstrated that disruption of the actin cytoskeleton is the important end point, rather than disruption of Erk phosphorylation, for inhibition of cell migration. Lysophospholipids activate numerous cell processes through the Edg family of 7 transmembrane G-protein–coupled receptors. The Edg family of receptors fall into 2 homology clusters, with the S1P homology cluster containing Edg-1, Edg-3, and Edg-5 and the LPA homology cluster containing Edg-2 and Edg-4. Edg family members may be coupled to 3 heterotrimeric G proteins, G12/13, and G. We found that downstream effectors of G and G12/13 have an important role in endothelial cell migration. An important question is how each of the Edg receptors contributes to the effect. Reverse transcriptase–polymerase chain reaction with human primers in a bovine system indicates that at least 2 Edg receptor family members, Edg1 and Edg3, are present on FBHEs to mediate the biological response to S1P (O. Peyruchaud and T. Panetti, unpublished data, 1999). Therefore, there are multiple readouts for Edg-1, Edg-3, and probably other Edg receptors through G1, G12/13, and G. A balance of these downstream signaling pathways may account for the different responses of different cells to S1P and LPA as migratory agents over such a wide concentration range.

Endothelial cell migration is a critical component of angiogenesis and wound repair. S1P and LPA are released from platelets at sites of blood coagulation. LPA increases vascular permeability of brain endothelial cells, and endothelial cell proliferation, and endothelial cell migration as described herein. It may be especially noteworthy that S1P stimulates endothelial cell migration in contrast to its inhibitory effect on platelet-derived growth factor–stimulated smooth muscle cell migration. In vivo, the variable responses of endothelial and smooth muscle cells may aid in tissue repair. For example, after balloon angioplasty, endothelial cell migration and repair of the denuded endothelium are necessary to limit further platelet activation and stimulation of smooth muscle cell proliferation and migration into the site of injury, leading to restenosis and occlusion of the vessel. Therefore, S1P may provide a way to stimulate endothelial cell migration and repair while limiting the influx of smooth muscle cells, even in the presence of platelet-derived growth factor.

Acknowledgments

This study was supported by grants HL-21644 and HL-54462 from the National Institutes of Health (to Dr Mosher) and a grant from the Wisconsin Affiliate of the American Heart Association (to Dr Panetti). We thank Drs Qinghong Zhang and Olivier Peyruchaud for many helpful discussions. We would like to dedicate this article to the memory of Prof Russell Ross.

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doi: 10.1161/01.ATV.20.4.1013
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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