Anastellin, the Angiostatic Fibronectin Peptide, Is a Selective Inhibitor of Lysophospholipid Signaling

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Abstract

Angiogenesis is regulated by integrin-dependent cell adhesion and the activation of specific cell surface receptors on vascular endothelial cells by angiogenic factors. Lysophosphatidic acid (LPA) and sphingosine-1 phosphate (SIP) are bioactive lysophospholipids that activate G protein–coupled receptors that stimulate phosphatidylinositol 3-kinase (PI3K), Ras, and Rho effector pathways involved in vascular cell survival, proliferation, adhesion, and migration. Previous studies have shown that anastellin, a fragment of the first type III module of fibronectin, functions as an antiangiogenic peptide suppressing tumor growth and metastasis. We have previously shown that anastellin blocks serum-dependent proliferation of microvessel endothelial cells (MVEC) by affecting extracellular signal-regulated kinase (ERK)–dependent G1/S transition. However, the mechanism by which anastellin regulates endothelial cell function remains unclear. In the present study, we mapped several lysophospholipid-mediated signaling pathways in MVEC and examined the effects of anastellin on LPA- and SIP-induced MVEC proliferation, migration, and cytoskeletal organization. Both LPA and SIP activated PI3K, Ras/ERK, and Rho/Rho kinase pathways, leading to migration, G1/S cell cycle progression, and stress fiber formation, respectively. Stimulation of proliferation by LPA/SIP occurred through a G1-dependent Ras/ERK pathway, which was independent of growth factor receptors and PI3K and Rho/Rho kinase signaling. Although LPA and SIP activated both PI3K/Akt and Ras/ERK signaling through Gi, anastellin inhibited only the Ras/ERK pathway. Stress fiber formation in response to LPA was dependent on Rho/Rho kinase but independent of Gi and unaffected by anastellin. These results suggest that lysophospholipid mediators of Gi activation leading to PI3K/Akt and Ras/ERK signaling bifurcate downstream of Gi, and that anastellin selectively inhibits the Ras/ERK arm of the pathway.

Introduction

Angiogenesis is controlled by a complex series of coordinated signaling events that are regulated by integrin-dependent cell adhesion and the activation of specific cell surface receptors on vascular endothelial cells by angiogenic factors. The angiogenic response has both normal and pathologic roles, including tissue repair and regeneration, during wound healing and growth of primary and metastatic tumors. Integrin receptor ligation to an extracellular fibronectin matrix has long been recognized to play a critical role in the regulation of endothelial cell adhesion, migration, proliferation, and survival (reviewed in ref. 1). Lysophosphatidic acid (LPA) and sphingosine-1 phosphate (SIP) are membrane-derived bioactive lysophospholipids generated from phospholipid precursors of activated platelets, epithelial cells, macrophages, and some cancer cells with reported serum concentrations of 1 to 10 μmol/L and 0.2 to 0.5 μmol/L, respectively (2). LPA and SIP activate a variety of widely expressed G protein–coupled receptors of the endothelial differentiation gene (Edg) family that regulate a broad range of cellular functions, including survival, proliferation, adhesion, migration, and chemotaxis, suggesting potential roles in inflammation, wound healing, and tumor progression (3). LPA and SIP receptors couple to at least three distinct G protein subfamilies, including G12/13, Gq/11, and Gi. Effects of LPA and SIP on cell survival and proliferation have been linked to Gi-dependent activation of phosphatidylinositol 3-kinase (PI3K) and Ras effector pathways, whereas activation of the Rho/Rho kinase (ROCK) pathway, implicated in the regulation of cell morphology, adhesion, and migration, has been linked to activation of G12/13-coupled Edg receptors (4-8).

LPA is produced in vivo through the action of autotaxin, an exoenzyme that functions in serum to convert lysophosphatidylcholine into bioactive LPA (9). Studies using autotaxin-deficient mice indicate that autotaxin is a major regulator of plasma LPA levels. Autotaxin-deficient mice exhibit impaired vessel formation, suggesting that LPA production is essential for normal vascular development (10, 11). LPA regulates the barrier function of the endothelium and also stimulates endothelial cell migration and proliferation (reviewed in ref. 12). SIP is a proangiogenic factor that regulates endothelial cell proliferation and migration, tubulogenesis, and the homing of bone marrow–derived endothelial cell precursors to sites of neovascularization (reviewed in ref. 13). Mice in which SIP
Lysophospholipid-Mediated ERK Activation in MVECs Does Not Require EGFR or VEGF Receptor Transactivation

Previous studies have shown that activation of the ERK signaling pathway by LPA can occur through a mechanism that involves transactivation of EGFR receptor (EGFR) or platelet-derived growth factor receptor (20, 21). Similarly, another report has shown that S1P can activate ERK through transactivation of the VEGF receptor (VEGFR; ref. 22). To determine if growth factor receptor transactivation is required for LPA or S1P activation of ERK, MVECs were treated with inhibitors of EGFR and VEGFR kinase activity. As observed in Supplementary Fig. S4A, addition of LPA, S1P, and EGF each stimulated ERK activation. However, only EGF-mediated ERK activation was inhibited by the EGFR inhibitor AG1478, suggesting that LPA and S1P activation of ERK does not involve EGFR kinase activity. This concept was examined further by evaluating the effect of LPA on EGFR phosphorylation. In Supplementary Fig. S4B, MVECs were treated with AG1478 before stimulation with either LPA or EGF. Whereas EGF led to a significant increase in AG1478-sensitive EGFR phosphorylation, LPA treatment resulted in no detectable increase in EGFR phosphorylation. Similarly, we examined the role of VEGFR transactivation in LPA- and S1P-mediated
ERK activation. MVECs were treated with the VEGFR inhibitor SU5416 followed by addition of LPA, S1P, and VEGF. As shown in Supplementary Fig. S4C, addition of each led to a robust increase in ERK phosphorylation. Only VEGF activation of ERK was inhibited by treatment with SU5416. Additionally, MVECs treated with LPA resulted in no detectable increase in VEGFR phosphorylation, whereas VEGF was able to stimulate SU5416-inhibitable VEGFR tyrosine phosphorylation (Supplementary Fig. S4D). Similar results were seen using an inhibitor (AG1295) of platelet-derived growth factor receptor kinases, which had no effect on activation of ERK by either LPA or S1P (data not shown). Taken together, these data indicate that activation of ERK by LPA or S1P does not require transactivation of these growth factor receptor tyrosine kinases.

Lysophospholipid-Mediated Akt but not ERK Activation Depends on PI3K

Earlier studies have shown that lysophospholipids can activate PI3K/Akt survival pathways in endothelial cells (23, 24). To determine if lysophospholipids activate Akt in MVECs, cells treated with LPA and S1P were examined for phospho-Akt by Western blot analysis. As shown in Supplementary Fig. S5A and B, both LPA and S1P enhanced phosphorylation of Akt at Ser473 within 2.5 to 5 minutes of treatment. The kinetics of Akt phosphorylation paralleled those of ERK phosphorylation, peaking between 5 and 10 minutes and returning to basal levels within 60 minutes. To determine whether Akt phosphorylation depended on PI3K activity, MVECs were treated with the PI3K inhibitor LY294002 for 30 minutes before LPA and S1P stimulation. PI3K inhibition completely blocked the ability of both LPA and S1P to stimulate Akt phosphorylation at Ser473 but had no effect on ERK activation (Supplementary Fig. S5C). These results suggest that lysophospholipid-mediated Akt activation in microvessel cells occurs through a PI3K-dependent signaling mechanism, whereas ERK activation is independent of PI3K activity. To determine which G protein family member is required for PI3K/Akt activation, cells were treated with PTx (Supplementary Fig. S5D). These data show that PTx blocked both Akt and ERK activation, indicating that S1P/LPA activation of both Akt and ERK occurs through a Gi-dependent signaling pathway.

Effects of Rho/ROCK Signaling on Lysophospholipid-Mediated ERK Activation

Previous studies have shown that LPA is a strong inducer of Rho-dependent signaling pathways in many cell types and plays a central role in cell adhesion, migration, and cytoskeletal organization. Incubation of MVECs with either LPA or S1P resulted in an increase in phospho-Ser19 myosin light chain 2 (pMLC2), a downstream effector of Rho/ROCK signaling (Fig. 2A). These data suggest that both lysophospholipids activate Rho signaling in microvessel cells; however, the effects were more pronounced in LPA-treated cells. To test whether LPA signaling to Rho occurred through a Gi-dependent mechanism, MVECs were treated with PTx overnight, stimulated with LPA or S1P, and assessed for changes in MLC2 phosphorylation levels (Fig. 2B). As shown previously, PTx treatment completely blocked LPA- and S1P-mediated Akt and ERK activation. However, PTx had no effect on the ability of LPA or S1P to stimulate MLC2 phosphorylation, indicating that phospholipid-mediated Rho signaling proceeds through a Gi-dependent signaling pathway.
ERK signaling pathways were unaffected. In addition, inhibition of MEK with U0126 had no effect on either the PI3K/Akt or Rho/ROCK signaling pathways. In support of these data, C3 exoenzyme, a Rho inhibitor, had no effect on the ability of LPA to activate ERK (data not shown). These data indicate that the activation of ERK by LPA does not depend on Rho signaling pathways. Furthermore, the data show that ERK activation is not required for lysophospholipid activation of either Akt or Rho/Rho kinase, suggesting that in microvessel cells, the PI3K/Akt, MEK/ERK, and Rho/ROCK pathways are not interdependent.

Anastellin (III1C) Works Upstream of Ras to Block Lysophospholipid-Mediated ERK Activation and Transition to S Phase

To determine whether anastellin can alter lysophospholipid receptor signaling pathways, MVECs were treated with various concentrations of anastellin before LPA and S1P stimulation. As shown in Fig. 3A, anastellin reduced basal levels of phosphorylated ERK and blocked activation of ERK by LPA and S1P. The effects of anastellin on ERK activation were dose dependent and seen with as little as 5 μmol/L and reaching maximal inhibition at 20 μmol/L anastellin. The control fibronectin module (III13) had no effect on LPA or S1P activation of ERK. The effect of anastellin on ERK activation was seen within 10 minutes with nearly a complete loss of active ERK at 60 minutes (Fig. 3B). As shown in Fig. 3C, anastellin blocked LPA- or S1P-mediated [3H]thymidine uptake at concentrations similar to those required to block mitogen-activated protein kinase signaling.

To show a role for Ras in the activation of ERK by LPA/S1P, Ras activity was inhibited using an adenoviral construct of dominant-negative RasN17. Figure 4A shows that ERK activation in response to either LPA or S1P was completely attenuated in cells expressing RasN17. In contrast, Akt activation by LPA or S1P was unaffected by RasN17 expression, suggesting that Akt activation was not dependent on Ras. EGF-stimulated
ERK activation was also completely attenuated in RasN17-infected cells (data not shown). In contrast, there was no effect on either Akt or ERK activation in response to LPA or S1P stimulation in cells infected with control virus (adenovirus expressing green fluorescent protein alone).

To determine whether anastellin alters lysophospholipid-mediated Ras activation, anastellin-treated MVECs were stimulated with LPA and S1P and examined for changes in active Ras using a Ras-GTPase ELISA assay. EGF served as a positive control. LPA induced a significant increase in Ras activation; however, S1P did not increase Ras activity when compared with control cells (Fig. 4B). As shown in Fig. 4B, anastellin blocked the ability of LPA to induce Ras activation. Conversely, anastellin had no significant effect on EGF-stimulated Ras activation, which was consistent with control experiments showing that anastellin did not inhibit the activation of ERK by EGF (data not shown). These data indicate that the activation of ERK by LPA/S1P is dependent on Ras activity and that the inhibitory effect of anastellin on ERK lies upstream of Ras.

**Effects of Anastellin on Rho/ROCK Signaling and Cytoskeletal Organization**

To determine the effects of anastellin on lysophospholipid signaling through Rho, MVECs were treated with 20 μmol/L anastellin or the control module III13 before stimulation with LPA and S1P. Cell lysates were immunoblotted for anastellin-mediated effects on LPA-induced phosphorylation of ERK, Akt, and MLC2. Although anastellin treatment effectively blocked ERK activation, anastellin had no effect on S1P- or LPA-mediated phosphorylation of either Akt or MLC2 (Fig. 5A). These results suggest that anastellin does not interfere with the ability of S1P or LPA to initiate downstream signaling to both Rho/ROCK and PI3K/Akt signaling pathways. Rho pull-down assays confirmed that anastellin had no effect on the ability of LPA to activate Rho (data not shown).

We also examined the effects of LPA and S1P on the formation of stress fibers in confluent monolayers of endothelial cells (Fig. 5B). LPA stimulation dramatically induced the polymerization of actin into cortical stress fibers when compared with control cells (Fig. 5B, arrows) and enhanced immunodetection of pMLC2, consistent with the biochemical data presented in Figs. 2 and 5A. The increase in pMLC2 was predominantly associated with its colocalization to the newly organized cortical stress fibers following LPA stimulation (Fig. 5B, arrowheads and merge). S1P had little effect on stress fiber formation (data not shown), consistent with earlier results showing that S1P only weakly activated Rho/ROCK signaling (Fig. 2). Inhibition of Rho kinase with Y27632 dramatically reduced immunodetection of pMLC2 and blocked the formation of cortical stress fibers following LPA stimulation.

**FIGURE 3.** Anastellin inhibits lysophospholipid-mediated ERK activation and proliferation in MVECs. A, Serum-starved cells were treated with increasing concentrations of anastellin for 60 min before stimulation with 20 μmol/L LPA or 1 μmol/L S1P for 6 min. B, Cells were treated with 20 μmol/L anastellin for up to 60 min before the addition of 20 μmol/L LPA or 1 μmol/L S1P and lysed, and proteins were immunoblotted for changes in pERK. Membranes were stripped and reprobed with an anti-ERK2 antibody. An unrelated type III module of FN (III13) was used as negative control (A). To evaluate the effect of anastellin on lysophospholipid-mediated endothelial cell proliferation, serum-starved MVECs were treated with anastellin for 60 min before 20 μmol/L LPA and 1 μmol/L S1P stimulation and assessed for [3H]thymidine incorporation (C) as described in Materials and Methods. Statistical differences were measured by comparison with cells in the absence of III1C. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Y27632 or the MEK inhibitor U0126, before stimulation with LPA. Migration was PTx sensitive, suggesting that Gi activation migration, although S1P was a more potent stimulator than RpC (Fig. 6B). These results are consistent with the data presented in Figs. 2C and 5A and indicate that LPA activation of Rho/ROCK signaling leading to increased stress fiber formation is not affected by anastellin.

**PI3K Signaling Plays a Role in Lysophospholipid-Mediated Endothelial Cell Migration**

To determine whether lysophospholipid signaling through Ras/ERK, PI3K/Akt, or Rho/ROCK plays any role in cell migration, endothelial cells were seeded onto collagen-coated Transwell tissue culture inserts and stimulated with LPA or S1P. As shown in Fig. 6A, both lipids increased endothelial cell migration, although S1P was a more potent stimulator than LPA. Migration was PTx sensitive, suggesting that Gi activation is required (Fig. 6B). Cells pretreated with the ROCK inhibitor Y27632 or the MEK inhibitor U0126, before stimulation with LPA or S1P, exhibited no significant changes in migration (Fig. 6C and D, respectively), indicating that neither the Rho pathway nor the ERK pathway was required for LPA/S1P-induced migration. Conversely, LY294002 significantly reduced migration, suggesting a role for PI3K signaling in endothelial cell migration (Fig. 6E). Similar results were obtained with the PI3K inhibitor wortmannin (data not shown). To determine whether PI3K-mediated Akt activation was specifically required for migration, cells were treated with an Akt-selective inhibitor before stimulation with LPA and S1P. Although lysophospholipid-dependent phosphorylation of Akt was completely blocked (data not shown), migration was unaffected (Fig. 6E), suggesting that Gi-dependent activation of PI3K is required for lysophospholipid-mediated endothelial cell migration, whereas subsequent activation of Akt by PI3K is not. Anastellin had no significant effect on migration in response to LPA/S1P (Fig. 6F).

As shown in the schematic (Fig. 7), our results indicate that LPA/S1P activates both PI3K/Akt and ERK signaling through Gi-dependent pathways. Activation of Akt but not ERK required PI3K, suggesting that the ERK and Akt signaling pathways are independent. This was confirmed by studies showing that inhibitors of MEK and Ras blocked ERK phosphorylation but did not block Akt phosphorylation. Activation of PI3K led to an increase in cell migration, whereas ERK activation stimulated cell cycle progression. Anastellin had no effect on cell migration but completely inhibited cell proliferation. LPA and S1P (but to a lesser extent) activated the Rho signaling pathway, resulting in the phosphorylation of MLC2 and formation of stress fibers. This pathway was not dependent on Gi and did not affect the activation of either ERK or Akt. Activation of Rho by LPA resulted in an increase in stress fibers, which was unaffected by anastellin. These results suggest that the LPA/S1P signaling pathway initiated through Gi bifurcates downstream of Gi and that anastellin specifically inhibits the Ras/ERK arm of the pathway. The data further indicate that the site of anastellin action lies upstream of Ras but downstream of Gi.

**Discussion**

Anastellin, a peptide derived from the first type III domain of fibronectin, has been shown to exhibit antiangiogenic properties in vivo and to block serum-dependent growth of endothelial cells in vitro (19, 18, 25). Several studies have documented multiple cellular effects of anastellin, including changes in cell signaling pathways, reorganization of actin filaments, and conformational changes in extracellular fibronectin fibrils (26-28). In the present study, we address the mechanism by which anastellin might regulate the angiogenic phenotype. We show that anastellin inhibits signaling pathways initiated by both LPA and S1P, two bioactive phospholipids that have been implicated in the regulation of vascular development and angiogenesis (29, 30). Our studies indicate that in human MVECs, LPA and S1P regulate similar parallel pathways leading to the activation of ERK, Akt, and Rho kinase and that anastellin is a selective inhibitor of the pathway leading to ERK activation.

Both S1P and LPA bind to members of the Edg family of G protein–coupled receptors to affect several aspects of...
endothelial cell biology, including proliferation, migration, survival, and barrier function (reviewed in ref. 12). Our data indicate that LPA and S1P can stimulate ERK-dependent \([3H]\)thymidine incorporation in endothelial microvessel cells. Activation of ERK by lysophospholipids is Ras dependent and required for the movement of cells from G₁ into S phase. In several cell types, activation of ERK by LPA or S1P has been linked to transactivation of growth factor receptors or to Rho signaling (22, 31-33). We found no evidence to support a role for either growth factor receptors or Rho in the activation of ERK by LPA/S1P in human microvessel cells.

In agreement with this, anastellin had no effect on LPA-induced stress fiber formation, which was dependent on Rho kinase activity. LPA and S1P both stimulated cell migration, which was dependent on PI3K. Although both LPA and S1P stimulated phosphorylation on Akt by PI3K, Akt was not required for cell migration. This suggests that PI3K pathway bifurcates downstream into separate pathways regulating migration and other Akt-dependent pathways, such as cell survival. Anastellin had no effect on cell migration or Akt activation, suggesting that the antiangiogenic activity of anastellin does not result from effect of anastellin on migration or survival.

Activation of both ERK and Akt by LPA/S1P was inhibited by PTx, indicating a role for Gᵢ in the LPA/S1P signaling pathways. Although both ERK and Akt lie downstream of Gᵢ, anastellin inhibits only the activation of ERK, suggesting that anastellin acts on one arm of a signaling pathway that bifurcates downstream of Gᵢ. These data suggest that anastellin selectively inhibits only those biological effects that lie downstream of ERK, such as cell proliferation, without affecting other lysophospholipid-regulated activities, such as migration, survival, endothelial nitric oxide synthase activity, or endothelial barrier function, which lie downstream of PI3K, Akt, or Rho kinase (34-37).

Several studies have shown fragments of extracellular matrix molecules to be fairly potent regulators of angiogenesis. These endogenous angiogenic regulators may be presented to the cell either as matrix-derived fragments released through proteolysis or as unfolded cryptic domains that become exposed following changes in the organization of the extracellular matrix (38, 39). Steered molecular dynamics and nuclear magnetic resonance have shown that the fibronectin III-1 domain unfolds in response to cellular tension to a mechanically stable intermediate, which is similar to the structure of anastellin (40). Therefore, anastellin may regulate endothelial cell behavior by reproducing the biological effects of conformationally regulated sequences within matrix fibronectin. Alternatively, anastellin may mimic the effects of soluble peptides containing the III-1 domain of fibronectin. Truncated forms of fibronectin that contain the III-1 domain are synthesized by both tumors and tumor-associated stromal cells (41).

The molecular basis for the antiangiogenic action of anastellin is not well understood. One possibility is that anastellin...

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**FIGURE 5.** Effect of anastellin on lysophospholipid-mediated Rho activation. Serum-starved MVECs were treated with 20 μmol/L anastellin or 20 μmol/L III₁₃ as control for 60 min before stimulation with either 40 μmol/L LPA or 1 μmol/L S1P for 6 min. A, Cells were lysed and extracts were subjected to SDS-PAGE followed by immunodetection with antibodies directed against pMLC₂, pS473Akt, and pERK. Membranes were stripped and reprobed with an anti-ERK2 antibody. B, Serum-starved endothelial cells were treated with 2 μmol/L Y27632 (30 min) or 20 μmol/L anastellin (60 min) before 1-h stimulation with 20 μmol/L LPA. Green, cells were fixed and immunostained with phospho-specific antibodies to MLC₂; red, filamentous actin was visualized with Alexa Fluor 594–conjugated phalloidin. LPA-induced formation of cortical stress fibers (arrows) and regions of pMLC₂ colocalization (arrowheads) are indicated.
competes for cellular receptors that interact with the III-1 domain present in matrix fibronectin. Earlier studies have shown that \( \beta_1 \) integrin and proteoglycans mediate adhesion of smooth muscle cells to substrate-attached anastellin, which results in the activation of ERK (27). Additionally, our previous studies have shown that anastellin binds to matrix fibronectin causing conformational changes in the EDA domain (28), suggesting that anastellin may affect the association of \( \alpha_4 \beta_1 \) integrins with the EDA-containing fibronectin present in matrix (42, 43). Anastellin-induced changes in fibronectin matrix organization may also regulate VEGF activity, as recent studies have shown that VEGF binds to conformationally labile sites present in the fibronectin matrix (44, 45). In addition to its effects on angiogenesis, anastellin might exert anti-tumor effects by regulating lysophospholipid signaling in the tumor cell. LPA autocrine loops have been described in several tumor types (46-48). LPA present in ascites fluid contributes to the progression of both ovarian and pancreatic cancers (49, 50). It is also possible that anastellin may have direct effects on other cell types present in the tumor microenvironment, as earlier studies have shown that anastellin regulates p38 mitogen-activated protein kinase and Cdc42 activity in dermal fibroblasts (26, 28).

Anastellin triggers polymerization of soluble fibronectin into higher-order polymers termed “superfibronectin” (51). Superfibronectin exhibits distinct effects on cell behavior and seems to be important in mediating the antiangiogenic effects of anastellin (52). Numerous studies have indicated neosynthesis of fibronectin isoforms during tumor progression, and these isoforms are currently being evaluated as targets for tumor therapy (reviewed in ref. 53). Angiogenesis associated with solid tumor progression is marked by the presence of alternatively spliced forms of fibronectin, which contain extra type III modules, EDA and EDB (54-56), and \( \textit{in vivo} \) studies have shown the fibronectin matrix to be a critical component of angiogenic programs (57-60). The specific functions of these isoforms are not well understood, but recent studies have shown that deletion of the EDA/EDB modules from fibronectin results in embryonic lethality due to multiple cardiovascular defects, which include defects in vascular remodeling and angiogenesis (61). These findings provide compelling evidence that the EDA/EDB isoforms of fibronectin are necessary for...
appropriate vascular remodeling and modeling that the polymerizing fibronectin matrix may be a useful target for therapeutic modalities directed at controlling tumor progression.

Materials and Methods

Reagents

Unless indicated otherwise, reagents were obtained from Sigma-Aldrich Co. Fetal bovine serum (FBS) was from Hyclone. LPA and tyrophostin AG1478 were obtained from Biomol Research Laboratories. Rabbit polyclonal antibodies to ERK2 and Flk-1 (VEGFR-2) were obtained from Santa Cruz Biotechnology, Inc. Monoclonal antibody to phospho-ERK (pERK), pMLC2, and the MEK inhibitors U0126 and PD98059 were obtained from Cell Signaling Technology. Monoclonal antibody to phosphotyrosine (clone 4G10) was obtained from Bioclinica. Recombinant human VEGF and EGF were obtained from R&D Systems. Recombinant fragments of the ras oncogene were obtained from Q-Biogene. The green fluorescent protein adenovirus used as control was a generous gift of American Biologics. Dominant-negative RasN17 was a generous gift of Dr. Robert Weinberg (Whitehead Institute). As a control, adenovirus expressing wild-type Ras was obtained from Q-Biogene. The Akt-selective inhibitor VIII (AktI-1/2), PTx, SU5416, LY294002, and Y27632 were obtained from Calbiochem. Vitrogon-100 (type I collagen) was from Covance. Recombinant human VEGF and EGF were obtained from Upstate Biotechnology. Alexa Fluor 594 phalloidin and monoclonal antibody to p5437Akt were obtained from Invitrogen. Complete growth medium for MVECs was from Lonza. The Akt-selective inhibitor VIII (AktI-1/2), PTx, SU5416, LY294002, and Y27632 were obtained from Calbiochem. Vitrogon-100 (type I collagen) was from Covance. Recombinant human VEGF and EGF were obtained from Upstate Biotechnology. Alexa Fluor 594 phalloidin and monoclonal antibody to p5437Akt were obtained from Invitrogen. Complete growth medium for MVECs was from Lonza.

Results

Anastellin Blocks Phospholipid Signaling

Lysophospholipid-mediated signaling pathways in MVECs. LPA and S1P activate specific G protein–coupled receptors (LPA1-4 and S1P1-5, respectively) and initiate Ras/ERK, PI3K/Akt, and Rho/ROCK signaling pathways in MVECs. Results presented here have shown that both Ras/ERK and PI3K/Akt signaling are mediated by a G1-dependent pathway, whereas Rho signaling occurs through an alternate G protein, possibly G12/13 or Gq, as has been shown in previous studies (4-8). In addition, G1-dependent activation of Ras/ERK and PI3K/Akt occurs through independent pathways and is unaffected by inhibition of Rho signaling. These results are further supported by the finding that anastellin suppresses Ras activation by LPA and prevents ERK activation without affecting PI3K/Akt or Rho/ROCK signaling. The dashed line linking S1P receptors to G12/13 indicates that a weak induction by S1P was observed in MVECs.

Cell Culture

Human dermal MVECs were obtained from VEC Technologies, Inc. Endothelial cells were cultured in complete medium (MCDB-131, 20% defined FBS, 2 mmol/L GlutaMAX (Life Technologies), and EGM-2MV (Cambrex Corp.)) supplemented with 10 μg/mL heparin and cultured in a humidified incubator at 37°C/5% CO2 on collagen-coated (20 μg/mL) tissue culture dishes. For most experiments, endothelial cells were plated onto collagen-coated dishes in complete medium at a density of 8 × 10^4 per well (12-well tissue culture dish), cultured overnight, and serum starved in MCDB-131, 0.5% bovine serum albumin (BSA) for 24 h before treatment.

Immunoblot and Expression Analysis

Cell layers were washed twice with ice-cold PBS containing 1 mmol/L Na3VO4 before solubilization in radioimmunoprecipitation assay buffer [20 mmol/L Tris-Cl (pH 7.4), 1% Triton X-100, 0.1% NP40, 0.1 mmol/L NaCl, 40 mmol/L NaF, 30 mmol/L Na3P04, 2 mmol/L EGTA, 1 mmol/L Na3VO4, and 0.5 mmol/L phenylmethysulfonyl fluoride containing Complete Mini protease inhibitors (Roche Biochemical)]. Cell lysates were cleared by centrifugation (14,000 rpm for 10 min at 4°C) and stored at −80°C until use. Protein concentrations were determined with a bicinchoninic acid protein assay reagent kit (Pierce) using BSA as standard. In some experiments, whole-cell lysates were prepared. Briefly, cell layers were first washed thrice with ice-cold PBS containing 1 mmol/L Na3VO4 followed by solubilization in Laemmli buffer. Samples were separated on SDS polyacrylamide gels, transferred onto nitrocellulose membranes (Schleicher & Schuell Bioscience), blocked overnight in TBST [Tris-Cl (pH 7.4), 150 mmol/L NaCl, 0.1% Tween 20] containing 5% (w/v) BSA, and hybridized with primary antibodies. Bound antibodies were hybridized with horseradish peroxidase–conjugated secondary antibodies and detected using an enhanced chemiluminescence reagent (Amersham Biosciences). Blots were reprobed after stripping in 62.5 mmol/L Tris-Cl (pH 6.7) and 2% SDS containing 10 mmol/L β-mercaptoethanol for 30 min at 60°C.

Measurement of DNA Synthesis

MVECs were plated onto collagen-coated 24-well dishes at 1.25 × 10^4 per well in the presence of complete medium and allowed to adhere overnight. Cells were serum starved for 30 h and treated with various pharmacologic agents as indicated before stimulation with either 20 μmol/L LPA or 2 μmol/L S1P for an additional 16 h. S-phase nuclei were labeled by incubating cells with 1 μCi of [3H]thymidine for 6 h. Cells were treated with 10% trichloroacetic acid and recovered in 1N NaOH. Samples were neutralized with 1N HCl and transferred to Ecoscint A (National Diagnostics) scintillation fluid. Incorporation of [3H]thymidine was determined by liquid scintillation.

Ras Activation Assays

MVECs were plated onto collagen-coated 10-cm dishes (Ras pull-down) or 60-mm dishes (Ras activity) at a density of 1 × 10^5/cm^2 in complete medium and allowed to adhere overnight. Cells were then serum starved for 20 h before addition
of 40 mmol/L LPA, 4 mmol/L S1P, or 10 ng/mL EGF for 3 min. Precipitation of activated Ras (Ras-GTP) was carried out using EZ-Detect Ras Activation kit (Pierce) according to the manufacturer’s protocol. Alternatively, active Ras was measured using a Ras GTPase Chemiluminescent ELISA kit (Active Motif).

**Fluorescence Microscopy**

MEV Cs were cultured overnight on collagen-coated (20 μg/mL) glass coverslips, serum starved for 4 h (2% FBS, MCDB-131), and treated for 30 min with 2 μmol/L Y27632 or 60 min with 20 μmol/L anastatin before 1-h stimulation with 20 μmol/L LPA or 1 μmol/L S1P. Cells were fixed for 20 min in 3% paraformaldehyde, permeabilized in 0.5% Triton X-100 for 10 min, blocked in 1% BSA, and immunostained with polyclonal antibodies to pMLC2. Filamentous actin was visualized with Alexa Fluor 594–conjugated phalloidin. Cell nuclei were examined containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho. J Biol Chem 1999;274:5868–79.

**Cell Migration**

Transwell tissue culture inserts (6.5 mm diameter, 8.0 μm pore size; Costar) were coated with 20 μg/mL collagen for 1 h at 37°C, rinsed once, and blocked with 1% BSA. Microvessel cell layers were examined using an Olympus BMX-60 microscope equipped with a cooled charge-coupled device sensor-camera (Cooke) and images were acquired using Slidebook software (Intelligent Imaging Innovation).

**Data Analysis**

Unless indicated otherwise, results are presented as the mean ± SE of at least three independent experiments done in duplicate or triplicate. Statistical significance was determined by one-way ANOVA; P values of <0.05 were considered significant.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

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