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Src REGULATES SPHINGOSINE-1-PHOSPHATE MEDIATED SMOOTH MUSCLE CELL MIGRATION

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Abstract

Background—Sphingosine-1-phosphate (S-1-P) is a bioactive sphingolipid released from activated platelets at sites of arterial injury that stimulates migration of smooth muscle cells (SMC). The kinase src is a significant focal point in transmembrane signaling. This study examines the role of src during smooth muscle cell migration in response to S-1-P.

Methods—Human coronary arterial SMCs were cultured in vitro. Boyden microchemotaxis assays of migration were performed in response to S-1-P in the presence and absence the src inhibitor (PP2, 10 μ M) and a dominant negative src construct (DNsrc). siRNA to S-1-P receptors was used to downregulate the S-1-P receptors. Western blotting was performed for src and MAPK phosphorylation.

Results—Inhibition of src with PP2 but not PP3 partially blocked S-1-P-mediated cell migration. S-1-P induced time-dependent activation of src, which was inhibited by PP2 and adenoviral DNsrc. PP3 or an empty vector had no effect. Activation of src by S-1-P was inhibited by siRNA to S-1-PR1 and S-1-PR3 but not by S-1-PR2. When the VSMC were transfected with adenovirus containing β ARK_{CT}, an inhibitor to G β γ , src activation was significantly attenuated. Src inhibition with PP2 reduced p38^{MAPK} and JNK activation but did not alter ERK1/2 activation.

Conclusion—S-1-P mediated VSMC migration is modulated by a G-protein-coupled src pathway partially through src-mediated p38^{MAPK} and JNK signaling and requires S-1-PR1 and S-1-PR3 receptors.

Keywords

S-1-P; cell signaling; cell migration; src; reactive oxygen species

INTRODUCTION

Multiple factors are responsible for vessel remodeling in response to external forces (atherogenesis, flow and injury). In human atheroma, cell migration as opposed to cell proliferation is an important if not pivotal cellular event during remodeling. While agonists

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for both receptor-linked tyrosine kinases and G-protein-coupled receptors have been implicated, little is known about the role of phospholipids in the vascular smooth muscle cell (VSMC) response during remodeling. S-1-P is a bioactive sphingolipid, released in large amounts from activated platelets, that has been shown to have numerous biological effects in a large number of vascular cell types (1). Our previous data has suggested that S-1-P is a moderate chemo-attractant for vascular smooth muscle cells expressing the G-protein-coupled receptors S-1-PR1, S-1-PR2 and S-1-PR3 (2, 3), while others have shown that S-1-P1 is necessary for S-1-P to stimulate VSMC migration (4, 5). More recently, Reidy et al have demonstrated the differential effects of the S-1-PR1, S-1-PR2 and S-1-PR3 receptors in VSMC migration using a murine vessel wall model (6, 7). Owens et al have shown that while S-1-PR1 and S-1-PR3 receptors promote, S-1-PR2 receptors antagonize phenotypic modulation in vivo after vascular injury (8). These data support a role for S-1-P in vascular remodeling.

We have examined the role of cell signaling in S-1-P-mediated VSMC migration and determined that G-protein-coupled ERK and p38^{MAPK} activation are required (9–12). While S-1-PR1 and S-1-PR3 are promigratory, S-1-PR2 is not. The intermediate signaling molecules Ras, Rho and PI3K influence both ERK and p38^{MAPK} activation (2, 3, 9, 12, 13). We have also demonstrated that G-protein-coupled oxygen free radical mediated activation of Phospholipase C (PLC) is also involved (14). Alterations in the balance between proteases, protease inhibitors, integrins and extracellular matrix molecules allow cells to migrate during vessel remodeling in response to atherogenesis, altered flow and injury (15–17). The aim of this study is to examine the role of src during smooth muscle cell migration in response to S-1-P.

METHODS

Experimental Design

Human coronary arterial VSMCs were cultured in vitro (passage 3–6; Clontech, Mountain View, CA). Boyden microchemotaxis assays of migration were performed in response to S-1-P (100 nM) in the presence and absence of the src inhibitor PP2 (10 μ M) and its inactive analogue, PP3 (10 μ M). The inhibitory concentration of PP2 used was calculated after a dose response curve of src phosphorylation was performed. Additional experiments were performed following transfection with an adenovirus containing the G $\beta\gamma$ inhibitor β ARK_{CT} and the src inhibitor dominant negative src (DNsrc). Western blotting was performed for src, ERK1/2, p38^{MAPK} and JNK phosphorylation.

Materials

D-erythro-sphingosine-1-phosphate was purchased from Avanti Polar-Lipids (Alabaster, AL). SB203580 and SP600125 were purchased from Sigma Chemical Co. (St. Louis, MO). PP2 and PP3 were purchased from Calbiochem (La Jolla, CA). Peroxidase-conjugated anti-rabbit IgG antibody (raised in goat) and peroxidase-conjugated anti-mouse IgG antibody (raised in goat) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Phospho-ERK1/2, Phospho-JNK, Phospho-src and Phospho-p38^{MAPK} antibodies were purchased from Biosource (Camarillo, CA). MMP2, total src, total ERK1/2, total p38^{MAPK} and total JNK antibodies were purchased from Cell Signaling (Beverly, MA). Empty adenovirus and adenoviral β ARK_{CT} were purchased from Welgen, Inc. (Worcester, MA). Dominant negative src (DNsrc) was a gift from Dr. Brad Berk (University of Rochester, NY). Dulbecco's Minimum Essential medium (DMEM) and Dulbecco's phosphate buffered saline (dPBS) were purchased from Cellgro (Manassas, VA).

Boyden Chamber

Chemotaxis was measured using a 48-well Boyden chamber (Neuro Probe, Inc., Gaithersburg, MD) and polycarbonate filters (Neuro Probe, Inc., 10 μ m pore size, 25 $\times$ 80 mm, PVP free) with VSMC as previously described (18, 19). S-1-P (100 nM) was added to the lower wells. For trials with the inhibitors, the inhibitor was added to 2 mL of the cell suspension 1 hour prior to addition of cells to the upper wells. Trials included eight or twelve wells per reagent or inhibitor per trial, and were repeated no fewer than three times.

Western Blotting

Cells were allowed to grow to 80% confluence and starved for 48 hours. Cells were then stimulated with S-1-P alone and in the presence of pharmacological inhibitors (see Results) and harvested at time points from 0 to 30 minutes. Western blotting was performed as previously described (9). Total protein was determined using antibodies against each intact kinase.

siRNA Transfection

Pre-designed HPLC purified siRNA for gene knockdown for S1PR1, S1PR2 and S1PR3 proteins were purchased from Ambion, Inc. (Austin, TX). VSMC of 50% confluence in 60 mm plates were starved overnight in 4 mL of Opti-MEM reduced serum medium (Gibco). siRNA was transfected using Lipofectamine 2000 from Invitrogen, Inc. (Carlsbad, CA) following the product protocol. Briefly, 22 μ L of Lipofectamine 2000 was first incubated in a total volume of 250 μ L of Opti-MEM for 5 minutes at room temperature. It was then added to 250 μ L of Opti-MEM containing 440 pmoles of siRNA. The solution was mixed gently and incubated for 20 minutes at room temperature, after which it was added to the starved plates. The medium was changed after 4–6 hours of incubation. The cells could then be used between 24–72 hours later for transfection for cell assays. Scrambled siRNA served as a control. Using the methodologies described, we conducted concentration-dependent experiments with siRNA against S1PR1, S1PR2 and S1PR3 and demonstrated a concentration-dependent decrease in protein expression that was specific for the protein targeted without altering the expression of the other proteins.

Adenoviral Infection

Adenoviral vectors were constructed by Welgen, Inc. (Worcester, MA) using purified plasmid encoding β ARK_{CT}, obtained from Guthrie cDNA Resource Center (Sayre, PA) and plasmid encoding dominant negative src (DNsrc) from Dr. Brad Berk (Univ. Rochester, NY). VSMC were plated at 70% confluence in 100 mm dishes and allowed to grow overnight. Recombinant adenovirus was then added at the appropriate concentrations (β ARK_{CT}: 50 MOI; DNsrc: 30 MOI) in a reduced volume of media (1.5 – 2 mL). After 48 hr incubation, the media was changed and cells grown for an additional 24 hr. The cells were then used for assays. Empty vector served as a control. Transfection was performed as previously described (14).

Data and Statistical Analysis

All data are presented as the mean $\pm$ standard error of the mean (s.e.m.) and statistical differences between groups were tested with a Kruskal-Wallis nonparametric test with post hoc Dunn's multiple comparison correction, where appropriate. A p-value less than 0.05 was regarded as significant.

RESULTS

Cell migration

To examine the role of src in S-1-P-mediated cell migration, VSMC were pre-incubated with the src inhibitor PP2 and its inactive equivalent, PP3, for one hour. Inhibition of src with PP2 (active src inhibitor) but not PP3 (inactive src inhibitor) partially blocked cell migration in response to S-1-P in the Boyden chamber assays (Fig 1). Application of a DNsrc virus but not the empty virus produced similar effects (Fig 1).

src pathway

S-1-P induced time-dependent src phosphorylation in the VSMC, which was inhibited by PP2 and adenoviral DNsrc (Fig 2). PP3 and an empty virus had no effect. Following incubation with siRNA to S-1-PR1, S-1-PR2 and S-1-PR3 for 24 hrs which resulted in a ~60% reduction in expression of S-1-PR1, 2 and 3, activation of src by S-1-P was inhibited by siRNA to S-1-PR1 and S-1-PR3 but not by S-1-PR2 (Fig 3). When the VSMC were transfected with adenovirus containing β ARK_{CT}, an inhibitor to G β γ , src activation in response to S-1-P was significantly attenuated (Fig 4). Src inhibition with PP2 reduced both p38^{MAPK} and JNK activation but did not alter ERK1/2 activation as determined by western blotting (Fig 5). A similar response was seen with adenoviral DNsrc. PP3 and an empty virus had no effect.

DISCUSSION

This study demonstrates that exposure of human VSMC to S-1-P activates the intermediate kinase, src. Activation of src is G β γ -dependent and is upstream of the stress MAPK p38^{MAPK} and JNK but not ERK1/2. Interruption of src leads to a decrease in S-1-P-mediated human VSMC migration (Figure 6A).

Src activity is known to be required for the migration of a number of cell types. It is also involved in the Focal Adhesion Complex. Intracellular injection of a src-blocking antibody will block VSMC migration, suggesting that activation of src is necessary for VSMC migration (20, 21). We have shown evidence that S-1-P can induce cell migration through G α i-coupled receptors (9, 10). S-1-P has been shown to induce membrane ruffling and an increase in cell motility of human endothelial cells through the activation of G α i-coupled receptor(s) and activation of src (22, 23). In VSMC, we saw a similar linkage of src to the stress kinases, with inhibition of src decreasing both p38^{MAPK} and JNK activation. Src is known to be upstream of these kinases in many cell types and these kinases are linked to cytoskeletal changes necessary for migration.

The receptors S-1-PR1 and S-1-PR3 were linked to src phosphorylation by S-1-P. Reidy et al have demonstrated the differential effects of the S-1-PR1, S-1-PR2 and S-1-PR3 receptors in VSMC migration, with S-1-PR1 and S-1-PR3 being promigratory and S-1-PR2 being antimigratory (6, 7). Others have shown that focal adhesion formation and activation of FAK, Src, and stress-activated protein kinase 2, p38^{MAPK}, are dysregulated in the absence of S-1-PR1. It appears that S-1-PR1 also functions as an integrator linking the PDGF receptor to lamellipodia extension and cell migration (24). S-1-PR2 appears to inhibit these processes. Activation of PDGF induces production and release of S-1-P into the pericellular space, permitting S-1-P receptor activation. In addition, transactivation of EGFR and PDGFR β by S-1-P is dependent on Src, reactive oxygen species, and cholesterol-rich membranes in VSMC (25). This transmembrane signaling pathway requires G-protein-coupled receptors to transactivate tyrosine kinase receptors, which in turn require src activation via a G β γ pathway. Expression of β ARK_{CT} and thus inhibition of G β γ prevents src-mediated tyrosine phosphorylation of caveolin-1 and dynamin-2 and the resultant

phosphorylation-dependent association of dynamin-2 and caveolin-1 (26). The current study supports the work on G-proteins and demonstrates that G $\beta\gamma$ is upstream of src and is necessary for its activation in VSMC. It also offers a second possible mechanism of src inhibition that is through interruption of G-protein-coupled receptor endocytosis.

In conclusion, S-1-P mediated VSMC migration is modulated by a G-protein-coupled src pathway partially through src-mediated p38^{MAPK} and JNK signaling and requires S-1-PR1 and S-1-PR3 receptors. Our current understanding of S-1-P signaling in human VSMC is shown in Figure 6B. Defining key pathways to interrupt cell kinetics will allow for targeted therapeutics.

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REFERENCES

- Goetzl EJ, An S. Diversity of cellular receptors and functions for the lysophospholipid growth factors: lysophosphatidic acid and sphingosine 1-phosphate. *FASEB J*. 1998; 12:1589–1598. [PubMed: 9837849]
- Tanski WJ, Roztocil E, Davies MG. Sphingosine-1-phosphate induces G α_i -coupled, PI3K/ras dependent smooth muscle cell migration. *J Surg Res*. 2002; 108(1):98–106. [PubMed: 12443721]
- Fegley AJ, Tanski WJ, Roztocil E, Davies MG. Sphingosine-1-phosphate stimulates smooth muscle cell migration through G α_i - and PI3-kinase-dependent p38MAPK activation. *J Surg Res*. 2003; 113:32–41. [PubMed: 12943808]
- Kluk MJ, Hla T. Role of the Sphingosine-1-phosphate receptor -1 in vascular smooth muscle cell proliferation and migration. *Circ Res*. 2001; 89:496–502. [PubMed: 11557736]
- Hobson JP, Rosenfeldt HM, Barak LS, Olivera A, Poulton S, Caron MG, et al. Role of the sphingosine-1-phosphate receptor -1 in PDGF-induced cell motility. *Science*. 2001; 291:1800–1803. [PubMed: 11230698]
- Shimizu T, Nakazawa T, Cho A, Davastan F, Shilling D, Daum G, et al. Sphingosine 1-phosphate receptor 2 negatively regulates neointimal formation in mouse arteries. *Circ Res*. 2007; 101(10):995–1000. [PubMed: 17872461]
- Inoue S, Nakazawa T, Cho A, Davastan F, Shilling D, Daum G, et al. Regulation of arterial lesions in mice depends on differential smooth muscle cell migration: a role for sphingosine-1-phosphate receptors. *J Vasc Surg*. 2007; 46(4):756–763. [PubMed: 17903653]
- Wamhoff BR, Lynch K, Macdonald TL, Owens G. Sphingosine-1-Phosphate Receptor Subtypes Differentially Regulate Smooth Muscle Cell Phenotype. *Arterioscler Thromb Vasc Biol*. 2008
- Tanski WJ, Nicholl SM, Kim D, Fegley AJ, Roztocil E, Davies MG. Sphingosine-1-Phosphate-induced smooth muscle cell migration involves the Mammalian Target of Rapamycin. *J Vasc Surg*. 2005; 39:91–98. [PubMed: 15696050]
- Tanski WJ, Roztocil E, Davies MG. Sphingosine-1-phosphate induces G α_i -coupled, PI3K/ras dependent smooth muscle cell migration. *J Surg Res*. 2002; 108(1):98–106. [PubMed: 12443721]
- Fegley AJ, Tanski WJ, Roztocil E, Davies MG. Sphingosine-1-phosphate stimulates smooth muscle cell migration through G α_i - and PI3-kinase-dependent p38^{MAPK} activation. *J Surg Res*. 2003; 113:32–41. [PubMed: 12943808]
- Galaria, Fegley AJ, Nicholl SM, Roztocil E, Davies MG. Differential regulation of ERK1/2 and p38^{MAPK} by components of the rho signaling pathway during sphingosine-1-phosphate-induced smooth muscle cell migration. *J Surg Res*. 2004; 22(2):173–179. [PubMed: 15555614]
- Roztocil E, Nicholl SM, Davies MG. Mechanisms of sphingosine-1-phosphate-induced akt-dependent smooth muscle cell migration. *Surgery*. 2009; 145(1):34–41. [PubMed: 19081473]

14. Roztocil E, Nicholl SM, Davies MG. S-1-P induced VSMC migration via activation of NAD(P)H oxidase requires Gα_{12/13} protein mediated phospholipase C activation. *J Vasc Surg.* 2007; 46:1253–1259. [PubMed: 18155002]
15. Nagase H, Woessner JF. Matrix Metalloproteinases. *J Biol Chem.* 1999; 274:21491–21494. [PubMed: 10419448]
16. Birkedal-Hansen H. Proteolytic remodeling of extracellular matrix. *Curr Opin Cell Biol.* 1995; 7:728–735. [PubMed: 8573349]
17. Galis ZS, Khatri JJ. MMPs in vascular remodeling and atherogenesis: the good, the bad and the ugly. *Circ Res.* 2002; 90:251–262. [PubMed: 11861412]
18. Nicholl SM, Roztocil E, Davies MG. Urokinase (uPA) induced smooth muscle cell responses require distinct signaling pathways: a role for the epidermal growth factor receptor. *J Vasc Surg.* 2005; 41:672–681. [PubMed: 15874933]
19. Tanski WJ, Fegley AJ, Roztocil E, Davies MG. Domain dependent actions of urokinase on smooth muscle cell responses. *J Vasc Surg.* 2004; 39:214–222. [PubMed: 14718842]
20. Mureebe L, Nelson PR, Yamamura S, Lawitts J, Kent KC. Activation of pp60c-src is necessary for human vascular smooth muscle cell migration. *Surgery.* 1997; 122(2):138–145. [PubMed: 9288116]
21. Kyaw M, Yoshizumi M, Tsuchiya K, Kagami S, Izawa Y, Fujita Y, et al. Src and Cas are essentially but differentially involved in angiotensin II-stimulated migration of vascular smooth muscle cells via extracellular signal-regulated kinase 1/2 and c-Jun NH₂-terminal kinase activation. *Mol Pharmacol.* 2004; 65(4):832–841. [PubMed: 15044612]
22. Endo A, Nagashima K, Kurose H, Mochizuki S, Matsuda M, Mochizuki N. Sphingosine 1-phosphate induces membrane ruffling and increases motility of human umbilical vein endothelial cells via vascular endothelial growth factor receptor and CrkII. *J Biol Chem.* 2002; 277(26):23747–23754. [PubMed: 11956190]
23. Lamalice L, Houle F, Huot J. Phosphorylation of Tyr1214 within VEGFR-2 triggers the recruitment of Nck and activation of Fyn leading to SAPK2/p38 activation and endothelial cell migration in response to VEGF. *J Biol Chem.* 2006; 281(45):34009–34020. [PubMed: 16966330]
24. Rosenfeldt HM, Hobson JP, Maceyka M, Olivera A, Nava VE, Milstien S, et al. EDG-1 links the PDGF receptor to Src and focal adhesion kinase activation leading to lamellipodia formation and cell migration. *FASEB J.* 2001; 15(14):2649–2659. [PubMed: 11726541]
25. Tanimoto T, Lungu AO, Berk BC. S-1-P transactivates the PDGFβ receptor and EGFR in VSMC. *Circ Res.* 2004; 94:1050–1058. [PubMed: 15044318]
26. Shajahan AN, Tiruppathi C, Smrcka AV, Malik AB, Minshall RD. Gβγ activation of Src induces caveolae-mediated endocytosis in endothelial cells. *J Biol Chem.* 2004; 279(46):48055–48062. [PubMed: 15345719]

Figure 1. Cell migration

VSMC migration in response to S-1-P in the Boyden chamber assay of migration is partially inhibited by src inhibitor PP2 and dominant negative src but not by its inactive equivalent PP3 or the empty vector (EV). Values are the mean±SEM (n=6, **p<0.01 compared to control).

S-1-P induced src phosphorylation

Figure 2. src activation

S-1-P induces time-dependent src phosphorylation in the VSMC (A) and src phosphorylation was inhibited by PP2 and adenoviral DNsrc (B). PP3 and an empty virus had no effect. Values are the mean $\pm$ SEM (n=6, **p<0.01 compared to control). Relevant western blots are shown above the figures.

Figure 3. src pathway and S-1-P receptors

Incubation with siRNA to the receptors S-1-PR1, S-1-PR2 and S-1-PR3 demonstrated that S-1-PR1 and S-1-PR3 were linked to src phosphorylation by S-1-P. Values are the mean $\pm$ SEM (n=6, **p<0.01 compared to control). Relevant western blots are shown above the figures.

A

S-1-P induced src phosphorylation

Figure 4. src pathway and $G\beta\gamma$

Incubation with adenovirus containing $\beta\text{ARK}_{\text{CT}}$, an inhibitor to $G\beta\gamma$, blocked the activation of src. Values are the mean $\pm$ SEM (n=6, **p<0.01 compared to control). Relevant western blots are shown above the figures.

Figure 5. MAPK pathways

Src inhibition with PP2 reduced p38^{MAPK} and JNK activation but did not alter ERK1/2 activation. Values are the mean $\pm$ SEM (n=6, **p<0.01 compared to control). Relevant western blots are shown above the figures.

6A

6B

Figure 6.

(A) Summary of S-1-P src activation pathways in VSMC: S-1-P-mediated src phosphorylation is mediated by S-1-PR1 and S-1-PR3 through a Gβγ-dependent pathway that leads to activation of p38^{MAPK} and JNK. The inhibitors at each level are shown in the dark boxes. **(B) Summary of S-1-P signaling in VSMC:** S-1-P interacts with G-protein-coupled receptors S-1-PR1, S-1-PR2 and S-1-PR3 to induce MAPK-mediated cell migration. Activation of PI3-K by S-1-P leads to activation of akt, ERK1/2 and p38^{MAPK} and mTOR. S-1-P can mediate ras and rho activation and modulate ERK1/2 and p38^{MAPK} activity. src is activated by S-1-P through a Gβγ pathway and can in turn activate p38^{MAPK} and JNK. S-1-P can induce the activation of PLCβ, the production of oxygen free radicals through NAD(P)H oxidase and the activation of the EGF receptor. The incremental knowledge derived from this report is shown in the dark boxes.