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ROLE OF S-1-P RECEPTORS AND HUMAN VASCULAR SMOOTH MUSCLE CELL MIGRATION IN DIABETES AND METABOLIC SYNDROME

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Abstract

Background—Sphingosine-1-phosphate (S-1-P) is a bioactive sphingolipid released from activated platelets that stimulates migration of vascular smooth muscle cells (VSMC) in vitro. S-1-P is associated with oxidized low-density lipoprotein (oxLDL) and is important in vessel remodeling. S-1-P will activate multiple G protein-coupled receptors (S-1-PR 1 to 5) which can regulate multiple cellular functions, including cell migration. The aim of this study is to examine the role of S-1-PR signaling during smooth muscle cell migration in response to S-1-P.

Methods—Human VSMCs were cultured in vitro. Expression of S-1-PR 1 to 5 was determined in conditions mirroring diabetes (40 mM glucose) and metabolic syndrome (25 mM glucose with 20 μ M linoleic acid and 20 μ M oleic acid). Linear wound and Boyden microchemotaxis assays of migration were performed in the presence of S-1-P with and without siRNA against S-1-PR 1 to 5. Assays were performed for activation of ERK1/2, p38^{MAPK} and JNK.

Results—Human VSMCs express S-1-PR1, S-1-PR2 and S-1-PR3. There was no significant expression of S-1-PR4 and S-1-PR5. The expression of S-1-PR1 and S-1-PR3 is enhanced under high glucose conditions and metabolic syndrome conditions. Migration of VSMC in response to S-1-P is enhanced 2 fold by diabetes and 4 fold by metabolic syndrome. In diabetes, S-1-PR1 expression is enhanced, while S-1-PR2 and S-1-PR3 expression are both maintained. In metabolic syndrome, S-1-PR1 and 3 expression are enhanced and that of S-1-PR2 is reduced. siRNA to S-1-PR1 results in a 2-fold reduction in S-1-P-mediated cell migration under all conditions. siRNA to S-1-PR2 enhanced cell migration only under normal conditions, while siRNA S-1-PR3 decreased migration in metabolic syndrome only. Down regulation of S-1-PR1 reduced ERK1/2 activation in response to S-1-P, while that of S-1-PR2 had no effect under normal conditions. In diabetes, down regulation of S-1-PR1 reduced activation of all three MAPKs. In metabolic syndrome, down regulation of S-1-PR1 and S-1-PR3 reduced activation of all three MAPKs.

Conclusion—S-1-PR 1, 2 and 3 regulate human VSMC migration and their expression level and function are modulated by conditions simulating diabetes and metabolic syndrome.

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Keywords

S-1-P; S-1-P receptors; cell signaling; cell migration; src; diabetes; metabolic syndrome

INTRODUCTION

Changes in circulating sphingolipids may serve as biomarkers for type 1 diabetes, as evidenced by an increase in sphingosine-1-phosphate (S-1-P) levels and a reduction in cardioprotective omega-9 esterified sphingolipids (1). S-1-P is a bioactive sphingolipid released from activated platelets that stimulates migration of vascular smooth muscle cells (VSMC) in vitro. S-1-P is associated with oxidized low-density lipoprotein (oxLDL) and is important in vessel remodeling. S-1-P will activate multiple G protein-coupled receptors (S-1-PR 1 to 5) which can regulate multiple cellular functions, including cell migration. Our previous data has suggested that S-1-P is a moderate chemo-attractant for VSMC 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-PR1 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 S-1-PR1 and S-1-PR3 receptors promote while S-1-PR2 receptors antagonize phenotypic modulation in vivo after vascular injury (8). Wound healing, a biological process similar to vessel remodeling in non-diabetic mice is not significantly decreased upon S-1-P injection compared to S-1-P-untreated controls. However, S-1-P injection alone into the wound showed significant promotion of wound healing in diabetic mice compared to no S-1-P treatment. The combination of S-1-P and S-1-PR2 antagonist administration induced maximal wound healing in diabetic mice (9). These data support a role for S-1-P receptors in vascular wall remodeling. Whetzel and colleagues have demonstrated that S-1-P prevents endothelial induction of vascular cell adhesion molecule-1 (VCAM-1) in diabetic mice and that this activity is dependent on S-1-PR1 (10). Activation of S-1-PR1 by S-1-P decreases NF- κ B nuclear translocation and p65 phosphorylation, resulting in reduced monocyte adhesion to diabetic NOD endothelium (11). The influence of diabetic conditions on vascular smooth muscle cells is not well defined. The aim of this study is to examine the role of S-1-PR signaling during smooth muscle cell migration in vitro in response to sphingosine-1-phosphate (S-1-P) under normal, diabetic and metabolic syndrome conditions.

MATERIALS AND METHODS

Experimental design

Human VSMCs were cultured in vitro. These cells did not come from diabetic (insulin or non-insulin dependent) patients; however, the metabolic syndrome status was not defined. Expression of S-1-PR 1 to 5 was determined under conditions mirroring normal conditions (5.5mM glucose) diabetes (40 mM glucose) and metabolic syndrome (25 mM glucose with oleic acid (20 μ M 18:1, ω -9) and linoleic acid (20 μ M 18:2, ω -6)). The combinations of 5.5 mM L-glucose with 19.5 mM D-glucose or 5.5 mM L-glucose with 34.5 mM D-glucose were used as osmotic controls. Linear wound and Boyden microchemotaxis assays of migration were performed in the presence of S-1-P with and without siRNA against S-1-PR1, S-1-PR2 and S-1-PR3. Assays were performed for ERK1/2, p38^{MAPK} and JNK activation in the presence or absence of S-1-PR siRNA.

Wound Assay

The wound assay was performed with VSMC as previously described (12, 13). Trials with each reagent or inhibitor were performed in six separate dishes, and the results were averaged. Cells were then allowed to migrate over 24 hours at 37 °C in Dulbecco's modified Eagle's medium (DMEM) with or without S-1-P (100 nM). In a second series of experiments, migration in response to S-1-P was examined in the presence or absence of siRNA.

Boyden Chamber

Chemotaxis was measured using a 48-well Boyden chamber (Neuro Probe, Inc., Gaithersburg, MD) and polycarbonate filters (Neuro Probe, Inc., 10 µm pore size, 25 × 80 mm, PVP free) with VSMC as previously described (12, 13). S-1-P (100 nM) was added to the lower wells. For trials with siRNA, the siRNA 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.

RT-PCR

Reverse transcription (RT)-PCR amplification of RNA was performed using total RNA (5 µg) isolated from vessels. RT was performed using the Superscript enzyme system with oligo-dT primers (Gibco BRL, Gaithersburg, MD). The complementary cDNA was subsequently amplified by PCR (BIO-RAD thermocycler, Hercules, CA) using Taq polymerase (Gibco BRL). We have optimized these primers and have determined the linear range of amplification of various amounts of first-strand cDNA. The PCR fragments were compared to α -actin and GAPDH. The PCR products were separated on a 1.5% agarose gel containing ethidium bromide. Gels were photographed under UV illumination using Kodak 1D imaging software (Eastman Kodak, Rochester, NY).

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 siRNA and harvested at time points ranging from 0 to 30 minutes. Western blotting was performed as previously described (14). 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 expression were procured commercially. 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 µL of Lipofectamine 2000 was first incubated in a total volume of 250 µL of Opti-MEM for 5 minutes at room temperature. It was then added to 250 µL 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 after 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.

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

S-1-P receptor expression

Human VSMCs expressed S-1-PR1, 2 and 3. There was no significant expression of S-1-PR4 and S-1-PR5 (Figure 1A and 1B). Under high glucose conditions mimicking diabetes, S-1-PR1 expression was enhanced, while S-1-PR2 and S-1-PR3 expression were both maintained (Figure 1A and 1B). Under metabolic syndrome conditions, S-1-PR1 and S-1-PR3 expression were enhanced and S-1-PR2 expression was reduced (Figure 1A and 1B). This change in responses was not seen in the presence of the osmotic control conditions. siRNA to S-1-PR1, S-1-PR2 and S-1-PR3 reduced the specific receptor protein expression by 70% without compromising expression of the other receptors (Figure 1C).

Cell Migration

In the wound assay, migration of VSMC in response to S-1-P was enhanced 1.5 fold by diabetes and 2 fold by metabolic syndrome (Figure 2A). Migration of VSMC following exposure to S-1-P was enhanced 2 fold by diabetes and 4 fold by metabolic syndrome in the Boyden Chamber assay (Figure 2B). siRNA to S-1-PR1 resulted in a 2-fold reduction in S-1-P-mediated cell migration in all conditions (Figures 2C, 2D and 2E). siRNA to S-1-PR2 enhanced cell migration in control conditions only (Figures 2C, 2D and 2E), while siRNA to S-1-PR3 decreased migration in metabolic syndrome only (Figures 2C, 2D and 2E).

MAPK Activation

S-1-P activated ERK1/2, p38^{MAPK} and JNK under normal conditions (Figure 3A and 3B). Down regulation of S-1-PR1 reduced the activation of each of the MAPKs in response to S-1-P, while down regulation of S-1-PR3 had no effect (Figures 3C, 3D and 3E). Down regulation of S-1-PR2 enhanced ERK1/2 activation without affecting the two stress kinases (Figures 3C, 3D and 3E). Diabetic conditions induced enhanced ERK1/2 activation in response to S-1-P compared to control conditions (Figure 3A). S-1-P activation of the stress kinases, p38^{MAPK} and JNK, was also enhanced under diabetic conditions compared to normal conditions (Figure 3A). Under diabetic conditions, siRNA against S-1-PR1 reduced the activation of each of the MAPKs in response to S-1-P, while down regulation of S-1-PR2 had no effect (Figures 3C, 3D and 3E). Activation of p38^{MAPK} and JNK was enhanced under metabolic syndrome conditions with these stress kinases significantly increased compared to diabetes (Figure 3A). ERK1/2 activation was equivalent to control conditions (Figure 3A). Down regulation of S-1-PR1 and S-1-PR3 resulted in a decrease in the activation of all three kinases under metabolic syndrome conditions (Figures 3C, 3D and 3E). siRNA to S-1-PR2 had no effect under metabolic syndrome conditions (Figures 3C, 3D and 3E).

DISCUSSION

Metabolic syndrome is a constellation of well-defined metabolic risk factors: insulin resistance, atherogenic dyslipidemia, central abdominal obesity, hypertension and development of vascular pro-thrombotic and pro-inflammatory states (15). It is characterized by high plasma levels of free fatty acids in association with high blood glucose levels. Clinically, metabolic syndrome increases the likelihood of cardiovascular disease, stroke,

diabetes, kidney disease and ultimately premature death. Patients with metabolic syndrome have a prothrombotic state and appear to fare less well in response to injury. Diabetes and insulin resistance have been shown to be independent predictors of early restenosis after coronary stenting (16–19). Muangman et al (20) have demonstrated that in an in vitro environment with high glucose concentrations (40 mM) and the unsaturated fatty acids oleic (40 μ M 18:1) and linoleic acid (40 μ M 18:2), they can mimic changes seen in vivo within endothelial cells in animal models of metabolic syndrome. Hamilton et al have demonstrated that the combination of high glucose and free fatty acids results in enhanced oxidative stress within VSMC (21). We adopted a similar set of conditions for our experiments.

This study demonstrates that the S-1-PR1 and S-1-PR3 are necessary for S-1-P-mediated vascular smooth muscle cell migration through activation of ERK1/2, p38^{MAPK} and JNK. S-1-PR2 limits S-1-P induced migration and its suppression results in increased migration. Diabetic conditions increased S-1-PR1 expression and enhanced S-1-P mediated migration. This cell migration was associated with increased ERK1/2, p38^{MAPK} and JNK activation, which was shown to be mediated in large part by S-1-PR1. Under diabetic conditions, interruption of S-1-PR2 did not augment the migratory response. Metabolic syndrome conditions enhanced cell migration and were associated with increased S-1-PR1 and S-1-PR3 expression and a decrease in S-1-PR2 expression. ERK1/2, p38^{MAPK} and JNK activation was significantly enhanced. Under these circumstances, down regulation of S-1-PR1 and S-1-PR3 resulted in a decrease in MAPK activation. siRNA to S-1-PR2 had no effect. This is the first description of changes in S-1-PR expression and downstream pathways under conditions of diabetes and metabolic syndrome in VSMC.

S-1-P is a bioactive sphingolipid released in large amounts from activated platelets that mediates VSMC migration (22). Spiegel et al have shown that the PDGF-mediated migratory response, which is a principal mechanism of cell migration in the injured artery, is due to stimulation of sphingosine kinase leading to intracellular S-1-P production (23). S-1-P thus produced diffuses out of the cell and binds S-1-PR1, allowing for rac activation and VSMC chemotaxis. We have previously shown that S-1-P is a chemo-attractant for vascular smooth muscle cells expressing the G protein-coupled receptors S-1-PR1, S-1-PR2 and S-1-PR3 [Tanski, 2002 #1859; Fegley, 2003 #2059], while others have shown that S-1-PR1 is necessary for S-1-P to stimulate VSMC migration (4, 5). 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 pro-migratory and S-1-PR2 being anti-migratory (6, 7). Our data under control conditions validates these findings. VSMC function is known to be highly sensitive to glucose concentrations (24–28). Our data demonstrates that VSMC migration is enhanced under diabetic conditions. The further enhancement under the conditions of metabolic syndrome is likely a combination of elevated glucose and a pro-inflammatory condition induced in the VSMC. We have demonstrated that the metabolic conditions we used lead to an increased expression of inflammatory cytokines. The increases in cell migration are associated with changes in S-1-PR, with increased expression of promigratory receptors (S-1-PR1 and S-1-PR3) and a lack of change in the anti-migratory receptor (S-1-PR2). 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 (29). S-1-PR2 will inhibit these processes. Owens et al have shown that while S-1-PR1 and S-1-PR3 receptors promote, S-1-PR2 antagonizes phenotypic modulation in vivo after vascular injury (8). The current data support the role for S-1-PR1 and S-1-PR3 and demonstrate that diabetes and metabolic syndrome differentially modulate increased expression of these receptors that is associated with enhanced migration.

S-1-P activates mitogen-activated protein kinases (MAPK) in VSMC. Through a G α i-mediated, ras-dependent mechanism, S-1-P activates MEK1/2 and ERK1/2; MKK3/6 and p38^{MAPK} are activated by a G α i-mediated phosphatidylinositol 3' kinase (PI3-K)-dependent mediated pathway in vascular smooth muscle cells (2, 3). S-1-P can also activate the small GTPase, rho, and induce the generation of reactive oxygen species (30, 31). S-1-P activated ERK1/2, p38^{MAPK} and JNK primarily through S-1-PR1 under normal conditions in the human VSMC. ERK1/2 activation was negatively modulated by S-1-PR2 only. Diabetic conditions induced enhanced ERK1/2 activation in response to S-1-P, which was still mediated in whole by S-1-PR1, but in contrast to the findings, under normal conditions S-1-PR2 did not appear to regulate the ERK1/2 responses. S-1-P activation of the stress kinases, p38^{MAPK} and JNK, was also enhanced response under diabetic conditions and also mediated by S-1-PR1. A similar observation has been seen after stimulation of VSMC with Angiotensin II under elevated glucose concentration in which ERK1/2 and p38^{MAPK} activation is enhanced (32). Activation of the stress kinases p38^{MAPK} and JNK was enhanced under metabolic syndrome conditions, was significantly increased compared to diabetes and was mediated by both S-1-PR1 and S-1-PR3. ERK1/2 activation was mediated by S-1-PR1 and S-1-PR3 and was equivalent to non-diabetic conditions.

This study does have limitations in that it is not always possible to extrapolate in vitro data to in vivo conditions. One caveat in the conditions for metabolic syndrome that we must make is that the VSMC were not subjected to any mechanical force that would mimic hypertension, which is a necessary component of metabolic syndrome. Several authors have however used the in vitro conditions that we used to mimic the extracellular milieu that VSMC see in either diabetes (high glucose, high osmolarity) or metabolic syndrome (high glucose, high osmolarity and free fatty acids). Muangman et al (20) have demonstrated that in an in vitro environment with high glucose concentrations (40 mM) and the unsaturated fatty acids oleic (40 μ M 18:1, ω -9) and linoleic acid (40 μ M 18:2, ω -6), they can mimic changes seen in vivo in animal models of metabolic syndrome. Previous studies by this group demonstrated that this combination of substrates (high glucose, oleic and linoleic acids) was the most appropriate to mimic metabolic syndrome in vitro (20). Hamilton et al have demonstrated that the combination of high glucose and free fatty acids results in enhanced oxidative stress within VSMC (21). Extrapolating in vitro data to in vivo data can be a limitation of any study. To answer this question we have performed some preliminary in vivo studies. We have examined S-1-PR expression in the aortae of mice that are considered to be normal (C57B), to have insulin-dependent diabetes (NOD) and to suffer from metabolic syndrome (RCS10) (unpublished observations). In these mice, S-1-PR 1-3 expression is identified in the normal animals. S-1-PR1 expression is elevated in both diabetes and metabolic syndrome. S-1-PR2 expression is unchanged in both diabetes and metabolic syndrome, while S-1-PR3 expression is enhanced in metabolic syndrome only.

Conclusion

S-1-PR1, 2 and 3 regulate human VSMC migration and VSMC migration is modulated by conditions of diabetes and metabolic syndrome. The S-1-PR expression levels and MAPK kinase activation were also modulated by conditions of diabetes and metabolic syndrome and likely represent in part the pathway altered by conditions of diabetes and metabolic syndrome.

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A

Figure 1. S-1-P Receptor Expression in VSMCs

(A) Expression of S-1-PR 1 through 5 in human VSMCs cultured under normal conditions (5.5 mM glucose), high glucose conditions simulating diabetes (40 mM glucose), or conditions simulating metabolic syndrome (25 mM glucose, 20 μ M oleic acid, 20 μ M linoleic acid). (B) A representative western blot of the S-1-PR expression. (C) Expression of S-1-PR 1 through 3 with or without receptor-specific siRNA or scrambled (scr) control siRNA. Values are the mean $\pm$ SEM of 5 experiments. * $p < 0.05$, ** $p < 0.01$ compared to control conditions. ¶ $p < 0.05$, ¶¶ $p < 0.01$ compared to control within the diabetic or metabolic syndrome group.

A

C

D

Figure 2. Cell Migration in Response to S-1-P
VSMC migration in response to S-1-P under normal, diabetes, and metabolic syndrome culture conditions as assessed by (A) linear wound and (B) Boyden chamber assays. (C, D, and E) Boyden chamber assays of S-1-P-mediated cell migration with or without receptor-specific siRNA under normal, diabetes, or metabolic syndrome conditions. Scr siRNA, scrambled siRNA control. Values are the mean±SEM of 5 experiments. * p<0.05, ** p<0.01 compared to control. ¶ p<0.05, ¶¶ p<0.01 compared to control within the diabetic or metabolic syndrome group.

A

B

C

Figure 3. MAPK Activation by S-1-P

(A) S-1-P activation of ERK1/2, p38^{MAPK} and JNK under normal, diabetes, and metabolic syndrome culture conditions. (C, D, and E). (B) is a representative western blot of the peak active ERK1/2, p38^{MAPK} and JNK expression with respective total controls. S-1-P activation of ERK1/2 (C), p38^{MAPK} (D) and JNK (E) with or without receptor-specific

siRNA under normal, diabetes, or metabolic syndrome conditions. * $p < 0.05$, ** $p < 0.01$ compared to control. ¶ $p < 0.05$, ¶¶ $p < 0.01$ compared to control within the diabetic or metabolic syndrome group.