Overlapping and distinct roles for PI3Kβ and γ isoforms in S1P-induced migration of human and mouse endothelial cells

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Received 19 October 2007; revised 3 June 2008; accepted 6 June 2008; online publish-ahead-of-print 16 June 2008

Time for primary review: 22 days

Aims Sphingosine-1-phosphate (S1P), a key regulator of vascular homeostasis and angiogenesis, promotes endothelial cell migration via stimulation of phosphoinositide 3-kinase (PI3K). The aim of this study was to identify the role of PI3Kβ and γ isoforms and their downstream effector pathways in mediating endothelial cell migration induced by S1P.

Methods and results Experiments were performed in human umbilical vein endothelial cells (HUVEC) and murine lung endothelial cells (MLEC). A combination of specific inhibitors, RNA interference, and PI3Kγ2/2 mice were used to investigate the role of PI3Kβ and γ isoforms in endothelial cell migration. Both PI3Kβ and γ isoforms are required for full migration induced by S1P, with Rac1 being a major mediator downstream of both isoforms. In addition, PI3Kβ but not PI3Kγ mediates migration via Akt but independent of Rac1 and endothelial NO synthase (eNOS). Further, a S1P-mediated activation of extracellular signal-regulated kinases (Erk) 1/2 contributes to the chemotactic response independent of PI3Kβ or PI3Kγ.

Conclusions Our data demonstrate that both PI3Kβ and PI3Kγ isoforms are required for S1P-induced endothelial cell migration through activation of Rac1. In addition, PI3Kβ initiates an Akt-sensitive chemotactic response which is independent of Rac1 and eNOS. Thus, PI3Kβ and PI3Kγ have both overlapping and distinct roles in regulating endothelial cell migration, which may underlie S1P-triggered angiogenic differentiation.

KEYWORDS Angiogenesis; Endothelial function; Lipid signalling; Protein kinases; Signal transduction

1. Introduction

Sphingosine-1-phosphate (S1P), a biologically active sphingolipid metabolite, plays a key role in the regulation of vascular homeostasis.1,2 In endothelial cells, S1P participates in processes that are important in angiogenesis such as proliferation, migration, and barrier integrity.1,2 S1P stimulates endothelial migration and subsequent angiogenic differentiation through the G12/G13-coupled receptor, S1P1, and the G13/G12-coupled receptor, S1P3, leading to activation of the Rho GTTPases Cdc42, Rac, and Rho.3-10

S1P-induced migration is also mediated via activation of class I phosphoinositide 3-kinases (PI3Ks).7-9 These enzymes produce phosphatidylinositol 3,4,5-trisphosphate (PIP3), which recruits pleckstrin homology domain-containing molecules such as Akt and guanine nucleotide exchange factors (GEFs) to the membrane. Importantly, several lines of evidence support a role for Akt and GEFs in mediating migration.11

Class I PI3Ks are classified into two subclasses: class IA, which has three members, PI3Kα, β, and δ; and class IB, which has one member, PI3Kγ. PI3Kα and δ isoforms are activated by receptor tyrosine kinases, while PI3Kγ is activated by G protein βγ subunits (Gβγ), which are usually derived from Gt-coupled receptors. In contrast, the PI3Kβ isoform is regulated by receptor tyrosine kinases and by Gβγ-subunits.11-13

Although a role for PI3K in S1P-induced endothelial cell migration has been proposed, the responsible isoforms have not been characterized. Further, the contribution and
interrelationship of the downstream effectors, Rac1 and Akt and its substrate endothelial NO synthase (eNOS), in mediating migration is not fully understood.

In the present study, we have focused on the role of the two Gₛ-sensitive PI3K isoforms, β and γ, in mediating migration of endothelial cells to S1P. Importantly, both isoforms have been shown to control chemotaxis in leukocytes. The present observations demonstrate differential functions for the two isoforms in regulating endothelial cell migration upstream of Rac1 and Akt following activation by S1P. The results may have important consequences for the role of the two PI3K isoforms in regulation endothelial cell migration in vivo.

2. Methods

2.1 Materials

Endothelial mitogen was obtained from Biomedical Technologies (Stoughton, MA, USA). Rabbit polyclonal antibodies reacting with Akt1 or with the PI3Kβ catalytic subunit p110β were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal PI3Kγ antibody (clone H1) recognizing the sequence aa 97–335 of human and mouse PI3Kγ (catalytic subunit p110γ) was generated as described and is distributed by Jena Bioscience GmbH (Jena, Germany). Monoclonal antibodies against human eNOS (clone 2) and Rac1 (clone 102) or against mouse CD102 were from BD Transduction or BD Pharmingen, respectively (Heidelberg, Germany). The phosphospecific antibodies recognizing phosphorylated Akt (serine 473), eNOS (serine 1177), or Erk1/2 (threonine 202/tyrosine 204 of Erk1, threonine 185/tyrosine 187 of Erk2) and the antibodies against Erk1/2 and Akt were from Cell Signaling Technologies (Frankfurt, Germany).

2.2 Animals

PI3Kβ⁻/⁻ mice were obtained as previously reported. Experiments were performed with sex-matched 3-month-old wild-type (PI3Kβ⁺/⁺) and AS-252424 (selective for PI3Kγ), both synthesized as described, were kind gifts from the Baker Heart Institute. S1P, PD-98059, Akt inhibitor VIII, and the Ral1 inhibitor NCST-2366 were purchased from Merck Chemicals Ltd. (Darmstadt, Germany). The protease inhibitor cocktail Complete was from Roche Diagnostics (Frankfurt, Germany). Wortmannin, TGX-221, AS-252424, genistein, and Akt inhibitor VIII were dissolved in dimethyl sulfoxide. Control cells received the same volume of solvent and the final concentration did not exceed 0.1%. The S1P stock solution was prepared in methanol and, after evaporation of the solvent, reconstituted with HEPES/albumin buffer [20 mM HEPES (pH 7.4), 150 mM NaCl, 0.25 mM bovine serum albumin].

2.4 Small interfering RNA (siRNA)-mediated knockdown of PI3K isoforms, Akt1, and eNOS

siRNA duplex oligonucleotides used in this study are based on the human cDNA sequences encoding PI3Kβ and γ (catalytic subunits), Akt1, and eNOS. PI3Kγ-specific siRNA duplexes were custom-made by Sigma-Proligo (Hamburg, Germany). Sense and antisense strands of the siRNA targeting PI3Kβ had the following sequences: 5’-GCAUAAUCCUAAGCUAUUUA-dTdT-3’ and 5’-UAAACGCUAGGAUUGCdTdT-3’. Validated PI3Kβ-siRNA (Catalog number SI0622Z214), Akt1-siRNA (Catalog number SI00299145), and non-silencing siRNA (5’-UUCCGAGAAGUGACUGCUdTdT-3’ (sense)) and 5’-GCUAGACCCGU UCGGAGAAdTdT-3’ (antisense) were obtained from Qiagen (Hilden, Germany). ON-TARGETplus SMARTpool siRNA for downregulation of eNOS (L-006490-00-0005) and ON-TARGETplus Non-targeting Pool (D-001810-10-0005) as control were obtained from Thermo Fisher Scientific (Lafayette, CO, USA).

Transfection was performed using the amphotillic delivery system SAINT-RED (Synvolux Therapeutics B.V., Groningen, The Netherlands) as described earlier. The transfection efficiency of fluorescence-labelled siRNA was >99%.

2.5 Transwell migration assay

Migration assays were performed in the absence of serum or growth factors. Cells were seeded on membrane inserts (8 μm pore size, BD Falcon™) coated with 0.2% gelatin and placed into 12-well plates (2.5 × 10⁵ cells/well). Inhibitors were added to insert and bottom well, and following pre-incubation, S1P was applied to the lower chamber. After 4 h, cells were fixed with 4% parafomaldehyde and stained with haematoxylin. Non-migrated cells were wiped away and cells migrated to the lower surface of the membrane were scored under a microscope in 10 randomly selected fields. Basal migration was very low (~5 cells per field) and not significantly changed by the used inhibitors or siRNAs. S1P-induced migration is typically shown as the difference from stimulated and corresponding unstimulated cells and expressed as average cell number per field.

2.6 Western blot analysis

Cells were lysed in ice-cold Tris buffer [50 mM Tris (pH 7.4), 2 mM EDTA, 1 mM EGTA] containing 1% Triton X-100, 0.1% SDS, 50 mM NaF, 10 mM Na₂HPO₄, 1 mM Na₂VO₄, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μL/mL protease inhibitor cocktail. For the detection of PI3Kβ, cell lysates were subjected to immunoprecipitation with a polyclonal anti-PI3Kβ antibody and protein A sepharose. Lysate proteins or immune complexes were solubilized in Laemmli buffer and separated by SDS–polyacrylamide gel electrophoresis. Blots were immunostained with primary antibodies overnight and peroxidase-conjugated secondary antibodies for 1 h. Protein bands were visualized by chemiluminescence and evaluated by densitometry. Phosphospecific signals were normalized against the amount of total protein. Changes in phosphorylation were evaluated by comparing the differences between basal and
stimulated values of specifically treated samples and their respective controls.

2.7 Rac activity assay

The activation of Rac was measured in pull-down assays, in which the glutathione S-transferase-tagged Cdc42- and Rac-interacting binding domain of p21-activated kinase (GST-PAK) was used to isolate the active GTP-bound form of Rac. Cells were lysed on ice in a buffer consisting of 50 mM Tris (pH 7.4), 50 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 1% NP-40, 10% glycerol, 100 μM GDP, 10 μL/mL proteinase inhibitor cocktail, and 20 μg/mL GST-PAK. Lysates (200 μg protein) were incubated with glutathione sepharose beads (40 μL of 50% slurry, 30 min, 4°C). The beads were washed three times with lysis buffer. The bead bound protein was eluted with Laemmli buffer and analysed by immunoblotting using Rac1 antibody.

2.8 Statistical analysis

Data represent means ± SEM of three to five independent experiments. For evaluation of statistical significance, analyses of variance with Bonferroni's correction for multiple comparisons, or t-tests were performed. A P-value of <0.05 was accepted as statistically significant.

3. Results

3.1 S1P induces endothelial cell migration via a G₁ protein and PI3K-dependent pathway

S1P-stimulated migration of both HUVEC and MLEC as assayed in transwell filter systems (Figure 1A). The effect was dose-dependent with a threshold at 0.01 μM and a maximal response at 1 μM (Figure 1B). S1P-induced cell migration was blocked by pertussis toxin (0.1 μg/mL) and decreased by wortmannin (0.1 μM), a general inhibitor of PI3K, by almost 50% demonstrating a role for heterotrimeric G₁ proteins and PI3K activity, respectively (Figure 1C). In contrast, general tyrosine kinase inhibition by genistein did not affect S1P-induced migration suggesting that transactivation of receptor tyrosine kinases was not involved (data not shown).

3.2 PI3Kβ and PI3Kγ mediate S1P-induced endothelial cell transwell migration

PI3Kβ and PI3Kγ, which are both candidates for the wortmannin-sensitive component of S1P-induced migration, are expressed in HUVEC and MLEC (Supplementary material online, Figure S1). Further, the level of PI3Kβ is not altered in MLEC isolated from PI3Kγ-/- mice, which as expected lack detectable PI3Kγ (Supplementary material online, Figure S1B).

To investigate the role of the two PI3K isoforms in S1P-induced endothelial migration, we employed TGX-221 and AS-2924X, which have been shown to inhibit PI3Kβ and PI3Kγ, respectively, with high specificity. Both TGX-221 (0.1 μM) and AS-2924X (1 μM) decreased S1P-induced migration in HUVEC significantly more than 50% (Figure 1D). Further, the combined effect of TGX-221 and AS-2924X (52% inhibition) was comparable to that of wortmannin indicating that PI3Kβ and γ were the major isoforms in mediating migration. In line with the results in HUVEC, MLEC derived from PI3Kγ-/- mice exhibited a reduced migratory response towards S1P when compared with wild-type cells (48% inhibition) (Figure 1E). Furthermore, the PI3Kβ inhibitor TGX-221 reduced S1P-induced migration in wild-type MLEC by approximately 56% and in PI3Kγ-/- MLEC by about 52% (Figure 1E). In contrast, AS-2924X inhibited S1P-induced migration in wild-type MLEC to a similar degree to that seen in HUVEC while it had no effect in the absence of PI3Kγ (data not shown). Taken together, these results reveal that both PI3Kβ and PI3Kγ are required for the full migratory response of endothelial cells to S1P.

3.3 PI3Kβ but not PI3Kγ mediates S1P-induced Akt phosphorylation

One of the downstream effectors of PI3K implicated in cell migration is the serine/threonine kinase Akt. Stimulation of HUVEC with 1 μM S1P resulted in a time-dependent phosphorylation of Akt (Supplementary material online, Figure S2A). As expected, the response was completely blocked by pertussis toxin (0.1 μg/mL) and wortmannin (0.1 μM) (Figure 2A). Interestingly, the PI3Kβ inhibitor TGX-221 (0.01–0.1 μM) decreased S1P-stimulated Akt phosphorylation in a concentration-dependent manner with 90% reduction at 0.1 μM (Figure 2A and Supplementary material online, Figure S3A), whereas AS-2924X (1 μM) had no effect (Figure 2A). Pre-treatment of HUVEC with inhibitors of the vascular endothelial cell growth factor (VEGF) receptor-2 (SU5614, 1 μM), of the epidermal growth factor (EGF) receptor (AG1478, 10 μM), or of tyrosine kinases in general (genistein, 10 μM) did not affect the S1P response indicating that a transactivation of receptor tyrosine kinases by S1P was not involved (Supplementary material online, Figure S3B).

To confirm these results, we used a specific siRNA treatment leading to a downregulation of PI3Kβ or PI3Kγ expression in HUVEC by 74 ± 2% (n = 3) or 88 ± 7% (n = 3), respectively (Supplementary material online, Figure S1A). S1P-induced Akt phosphorylation was significantly reduced in cells deficient in PI3Kβ (60% inhibition) but was not altered in cells treated with PI3Kγ siRNA (Figure 2B). Similarly, no difference was found between S1P-induced Akt phosphorylation in MLEC from wild-type and PI3Kγ-/- mice (Figure 2C). Thus, these data confirm that S1P-induced phosphorylation of Akt is mediated via PI3Kβ but not via PI3Kγ.

3.4 S1P-induced Akt activation mediates endothelial cell migration

To investigate whether PI3Kβ-mediated Akt activation contributes to the chemotactic response of HUVEC towards S1P, we employed Akt inhibitor VIII, an Akt1/2 inhibitor, or a specific Akt1 siRNA in our study. Akt inhibitor VIII (20 μM) decreased S1P-induced Akt-phosphorylation (1 μM, 2 min) by 98 ± 1% (n = 4). Since Akt2 is not expressed in endothelial cells, these data strongly suggest that Akt1, the primary Akt isoform in endothelial cells, is stimulated by S1P. As a second approach, we reduced the level of expression of Akt1 by 77.9 ± 1.9% (n = 5) using siRNA (Supplementary material online, Figure S4A).

S1P-induced migration (1 μM) measured in the presence of the Akt1/2 inhibitor or following pre-treatment of HUVEC with Akt1 siRNA was decreased by 54 or 55%, respectively (Figure 2D), pointing to a role of Akt1 in mediating the migratory response. Interestingly, Akt seems to affect S1P-induced migration independent of the small GTPase
Rac1 since neither inhibition nor downregulation of Akt1 decreased S1P-triggered activation of Rac1 (Figure 2E and F).

3.5 S1P-stimulated eNOS phosphorylation does not contribute to S1P-induced migration

Akt has been reported to phosphorylate eNOS at serine 1177 and thereby promote NO formation and support growth factor-stimulated migration.\(^8,23\) eNOS phosphorylation at serine 1177 was time-dependently stimulated by 1 μM S1P (Supplementary material online, Figure S2B). The effect of S1P was blocked by pertussis toxin, inhibited by wortmannin and TGX-221 (42 and 38%, respectively), but not affected by AS-252424 (Figure 3A). Accordingly, pre-treatment of HUVEC with PI3Kβ-specific siRNA significantly decreased S1P-induced eNOS phosphorylation at serine 1177 (60%), whereas PI3Kγ deficiency in HUVEC or the absence of PI3Kγ in MLEC was without effect (Figure 3B and C). These data indicate that S1P stimulates eNOS phosphorylation partially via a PI3Kβ/Akt-dependent pathway whereas PI3Kγ is not involved.
Figure 2 Role of PI3Kγ isoform in S1P-stimulated Akt phosphorylation and the function of Akt1 in migration and Rac1 activation. (A–C) HUVEC or MLEC from wild-type or PI3Kγ-deficient mice were stimulated with S1P (1 μM, 2 min). Akt was analysed using antibodies against phosphorylated Akt (serine 473) and total Akt. (A) HUVEC were pre-incubated with pertussis toxin (0.1 μg/mL, 3 h) or with PI3K inhibitors (wortmannin and TGX-221: 0.1 μM, AS-252424: 1 μM, 30 min). (B) HUVEC were pre-treated with PI3Kβ- or PI3Kγ-specific siRNA or non-silencing control siRNA (1.0 μg/30 mm dish, 72 h). (D) HUVEC were seeded on the top of migration chambers, pre-treated with Akt1/2 inhibitor (20 μM, 30 min), and stimulated with S1P applied to the bottom (1 μM, 4 h). Alternatively, cells were transfected with Akt1-specific or control siRNA (1 μg/30 mm dish, 72 h) before migration experiments. S1P-induced response was calculated as difference of non-stimulated and stimulated migrated cells (means ± SEM). Cells with and without inhibitor or siRNA pre-treatment (n = 5 or n = 3, respectively) were compared. *P < 0.05. (E and F) HUVEC were pre-treated with Akt1/2 inhibitor or with Akt1 siRNA (D), stimulated with S1P (1 μM, 1 min), and lysed in the presence of GST-PIK (see Methods). GTP-bound Rac was precipitated with glutathione-sepharose beads and immunostained using Rac1 antibody. Total Rac1, phospho-Akt, total Akt and Akt1 were stained in cell lysates. (A–C, E, and F) Immunoblot data are shown as representative experiments and densitometry analyses [means ± SEM, n = 3 (C, E, and F) or n = 4 (A and B)]. Phospho-Akt or GTP-Rac1 signals in S1P-stimulated cells with or without inhibitor pre-treatment, RNA interference, or PI3Kγ-knockdown were compared. *P < 0.05.
impaired Rac1 activation induced by S1P (1 μM, 1 min) by 45 and 41%, respectively (Figure 4B). Further, Rac activation was reduced by 56% in MLEC derived from PI3Kβ−/− mice relative to controls (Figure 4C) confirming an essential role of PI3Kβ. In addition, pre-treatment with TGX-221 decreased S1P-induced Rac1 activation in MLEC from both PI3Kγ−/− and PI3Kβ−/− mice emphasizing the importance of PI3Kβ (Figure 4C). Together, these data identify Rac1 as a common downstream effector for both PI3K isoforms. In contrast, Rac1 does not seem to act upstream of Akt, since Rac1 inhibition by NSC-23766 (100 μM) did not affect S1P-induced Akt phosphorylation in HUVEC (Figure 4D).

3.7 S1P-induced activation of Erk1/2 is independent of PI3Kβ or γ and contributes to endothelial cell migration

S1P stimulation of HUVEC with 1 μM S1P leads to time-dependent stimulation of Erk1/2 (Supplementary material online, Figure S2D). The effect was blocked by pertussis toxin (0.1 μg/mL), but not affected by TGX-221 (0.1 μM) or AS-252424 (1 μM) (Figure 5A) and not altered in MLEC from PI3Kγ−/− mice (Figure 5B). Interestingly, inhibition of Erk1/2 activation by PD-98059 (10 μM), an inhibitor of the upstream kinase mitogen activated kinase kinase (MEK), reduced S1P-triggered endothelial migration by 49% indicating a role of Erk1/2 activation in this process (Figure 5C).

4. Discussion

Our study in human and mouse endothelial cells demonstrates that both G protein sensitive PI3K species β and γ contribute to S1P-stimulated migration. Specific inhibition of either isoform or genetic ablation of PI3Kγ resulted in a significantly reduced activation of Rac1 and an impaired capacity of endothelial cells to migrate in response to S1P. In addition, our data reveal that PI3Kβ but not PI3Kγ stimulates Akt and that the PI3Kβ/Akt pathway contributes to S1P-induced migration independent from Rac1 and eNOS activation. S1P-induced Erk1/2 activation, on the contrary, is not mediated by PI3Kβ or γ but participates in the migratory response.

Both PI3Kβ and PI3Kγ isoforms have been identified in endothelial cells.25–28 But, so far, their functions are poorly characterized. PI3Kβ has been thought to stimulate the Akt/eNOS pathway,26 whereas PI3Kγ has been shown to be required for selectin-mediated neutrophil adhesion27 and for TNFα-induced NADPH oxidase activation and ICAM-1 expression.28 To our knowledge, our study is the first to describe a coordinated role of PI3Kβ and PI3Kγ in endothelial cell migration towards a chemotactic gradient. The requirement of both isoforms points to differential cellular functions of these enzymes as described in other cells types. PI3Kγ, for example, has been shown to define the direction of migration in leukocytes and epithelial cells,29,30 whereas PI3Kβ has been proposed to regulate cell adhesion via integrins in platelets.17 Our study has used pharmacological and siRNA approaches to demonstrate that PI3Kβ has a distinct function in mediating S1P-triggered Akt phosphorylation and Akt-mediated migration. Previous investigations have already shown that PI3Kβ is able to transmit signals from G protein-coupled receptors to Akt31,32 and that S1P stimulates PI3Kβ activity and Gβγ-dependent Akt activation in bovine

To investigate the possible role of NO in mediating migration of endothelial cells, we inhibited eNOS using l-NAME (1 mM). l-NAME had no effect on the migration of HUVEC induced by S1P (1 μM) (Figure 3D). Moreover, downregulation of eNOS in HUVEC using RNA interference led to an inhibition of S1P-triggered endothelial migration by 72% (Figure 3D) and 55%, respectively (Figure 3F). Further, Rac activation in HUVEC induced by S1P (1 μM) was reduced by 50% in the Rac1 inhibitor NSC-23766 (100 μM) (Figure 3E) and 49% in AS-252424 (1 μM) (Figure 3E).

3.6 PI3Kβ and PI3Kγ mediate S1P-induced activation of the small GTPase Rac

Rac has been proposed to play a major role in mediating S1P-induced cell motility.24,25 Indeed, a time-dependent Rac1 activation by S1P (1 μM) was observed in HUVEC in our study (Supplementary material online, Figure S2C). Moreover, the Rac1 inhibitor NSC-23766 (100 μM) led to an inhibition of endothelial migration towards S1P by 72% (Figure 4A).

To understand the role of PI3Kβ or PI3Kγ in Rac activation, we performed pull-down assays in which GTP-bound Rac was isolated from HUVEC pre-treated with isoform-specific inhibitors and stimulated with S1P. Inhibition of PI3Kβ by TGX-221 (0.1 μM) and of PI3Kγ by AS-252424 (1 μM) significantly
aortic endothelial cells (which lack PI3K$\gamma$). Our data extend the latter observations and show a direct link between PI3K$\beta$ and Akt phosphorylation using PI3K$\beta$-specific tools. In addition, our results indicate that PI3K$\beta$ is activated via a G$\beta\gamma$-dependent pathway rather than via transactivation of receptor tyrosine kinases since neither specific inhibition of the EGF receptor or of the VEGF receptor-2 nor general tyrosine kinase inhibition affected S1P-induced Akt phosphorylation.

Prevention of Akt phosphorylation by an Akt1/2 inhibitor or downregulation of Akt1 by specific siRNA led to a significant decrease of S1P-induced migration underlying the importance of the PI3K$\beta$/Akt pathway and pointing to Akt1 as the responsible isoform. Interestingly, although eNOS was partially phosphorylated via the PI3K$\beta$/Akt pathway in our study, it did not mediate the effect of Akt on S1P-induced migration. Neither eNOS inhibition by L-NAME nor downregulation of eNOS by specific siRNA impaired the

Figure 3 Role of the PI3K$\beta$ isoform in S1P-stimulated eNOS phosphorylation and the function of eNOS in migration. (A–C) HUVEC or MLEC from wild-type or PI3K$\gamma$-deficient mice were stimulated with S1P (1 $\mu$M, 2 min). eNOS was analysed using antibodies against phosphorylated eNOS (serine 1177) and total eNOS. (A) HUVEC were pre-incubated with pertussis toxin (0.1 $\mu$g/mL, 3 h) or with PI3K inhibitors (wortmannin and TGX-221: 0.1 $\mu$M, AS-252424: 1 $\mu$M, 30 min). (B) HUVEC were pre-treated with PI3K$\beta$- or PI3K$\gamma$-specific or control siRNA (1.0 $\mu$g/30 mm dish, 72 h). (A–C) Typical experiments and densitometry analyses are shown [mean ± SEM, n = 4 (A and B) or n = 3 (C)]. Phospho-eNOS signals in S1P-stimulated cells with or without inhibitor pre-treatment, RNA interference, or PI3K$\gamma$-knockdown were compared. *P < 0.05. (D) HUVEC were seeded on upper wells of migration chambers, pre-treated with L-NAME (1 mM, 30 min), and stimulated by adding 1 $\mu$M S1P to the bottom. Alternatively, cells were transfected with eNOS-specific or control siRNA (1 $\mu$g/30 mm dish, 72 h) before measuring migration. Four hours after seeding, migrated cells were counted and S1P-induced migration was calculated by subtracting the respective control values (means ± SEM). Cells with and without L-NAME or siRNA pre-treatment (n = 6 or n = 4, respectively) were compared. *P < 0.05.
The chemotactic effect of S1P. This is in agreement with a previous study and supports the view that in contrast to its role in VEGF-induced migration the eNOS/NO pathway is of minor importance in promoting S1P-induced chemotaxis. Akt effects in S1P-induced migration were also independent of Rac1. In contrast, a previous report in CHO cells transfected with the receptor S1P1 suggested that Akt mediates Rac activation via phosphorylation of S1P1.7 Our data demonstrate that neither pharmacological inhibition nor downregulation of Akt1 via RNA interference inhibited Rac1 activation in endothelial cells. Thus, other Akt targets involved in cytoskeleton remodelling (Girdin, PAK1, glycogen synthase kinase 3) or integrin transport may be involved in the chemotactic response towards S1P.33

Our study confirms that Rac1, activated via Akt-independent pathways, is a major effector molecule in S1P-triggered endothelial migration. Inhibition of Rac1 by NSC-23766 resulted in a significant inhibition of the chemotactic response towards S1P. Interestingly, Rac1 appears to be a downstream target of both PI3Kβ and PI3Kγ since specific inhibition of either isoform as well as knockout of PI3Kγ suppresses S1P-induced Rac1 activation. These findings are in line with previous studies showing that Rac1 activation was dependent on PI3K stimulation.24,25 Conversely, inhibition of Rac1 by NSC-23766 did not affect S1P-induced Akt phosphorylation indicating that Rac activation is not a proximal event in S1P-mediated PI3K/Akt signalling as suggested recently.34 PI3Kβ and PI3Kγ may stimulate Rac1 via the activation of PI3K-sensitive GEFs35 including common and/or distinct pathways. Notably, Rac activation by S1P has been shown to be mediated via Tiam1 or P-Rex2b24,25,34 and the latter was suggested to involve PI3Kγ.24 S1P-induced migration was only partially blocked by PI3K inhibition in our study suggesting the involvement of PI3K-independent pathways. One of these mechanisms might include Erk1/2 activation which was not dependent on PI3Kβ or γ activities but contributed to the chemotactic effect of S1P. Inhibition of MEK, the upstream kinase of Erk1/2, led to a significant inhibition of S1P-stimulated cell migration in our experiments. A role of Erk1/2 in promoting S1P effects on cell movement has not been observed in an earlier study,36 but likely involves the substrates myosin light chain kinase, calpain, and/or focal adhesion kinase as suggested recently.37

In summary, our study shows that both PI3Kβ and PI3Kγ are required for endothelial migration towards S1P but their downstream signalling pathways and functions differ. PI3Kβ mediates the phosphorylation of Akt and initiates an Akt-sensitive part of cell migration which is independent from eNOS and Rac1 activation. In addition, both PI3Kβ and PI3Kγ mediate endothelial migration via the activation of Rac1. The characterization of these pathways may

Figure 4 Role of PI3Kβ and γ isoforms in S1P-stimulated Rac activation and the effect of Rac inhibition on Akt activation and migration. (A) HUVEC were pre-treated with NSC-23766 (100 μM, 4 h) and seeded on upper wells of migration chambers. NSC-23766 was re-added to both compartments and S1P (1 μM, 4 h) was applied to the bottom. Migrated cells were counted and S1P-induced migration was calculated by subtracting the respective control values (means ± SEM, n = 4). Cells with and without NSC-23766 pre-treatment were compared. *P < 0.05. The insert shows the inhibitory effect of NSC-23766 on S1P-induced GTP-loading of Rac measured as described below. (B and C) HUVEC or MLEC from wild-type or PI3Kγ-deficient mice were pre-treated with 0.1 μM TGX-221 or 1 μM AS-252424 for 30 min, stimulated with S1P (1 μM, 1 min), and lysed in the presence of GST-PAK (see Methods). GTP-bound Rac was precipitated with glutathione-sepharose beads and immunostained using Rac1 antibody. Total Rac1 was stained in cell lysates. (D) HUVEC were pre-treated with NSC-23766 (100 μM, 4 h) and stimulated with 1 μM S1P at the indicated times. Akt was analysed using antibodies against phosphorylated Akt (serine 473) and total Akt. (B–D) Typical experiment and densitometry analyses (means ± SEM, n = 4) are shown. Rac1-GTP or phospho-Akt signals in S1P-stimulated cells with or without inhibitor pre-treatment or PI3Kγ-knockdown were compared. *P < 0.05.
reveal novel approaches for the modulation of the S1P response and therapies affecting angiogenesis.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

We thank Gunda Guhr, Elke Teuscher, and Gabriele Riehl for their excellent technical assistance. We are grateful to Michael Gruen and Christian Koenig for supporting mice experiments and to Nadine Stahmann for flow cytometric analyses.

Conflict of interest: none declared.

Funding

Deutsche Forschungsgemeinschaft (SFB 604 to R.W., KU-1206 (1-2) to P.K.). Interdisziplinäres Zentrum für Klinische Forschung (IZKF Jena, TP 4.6 to R.H., IZKF Würzburg to P.K.).

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