

Modulation of P-glycoprotein function by sphingosine kinase-1 in brain endothelial cells

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Abstract

P-glycoprotein (P-gp), an ABC-transporter highly expressed in brain capillaries, protects the brain by extruding xenobiotics. However, its overexpression has also been associated with the multidrug resistance phenotype in tumors. Here, we have investigated the regulation of P-gp transport activity by sphingosine kinase 1 (SphK-1) in brain endothelial cells. We first demonstrated that SphK-1 is overexpressed in endothelial cells (EC) isolated from rat brain tumors compared with EC from normal brain. We also provide evidence that the overexpression of SphK-1 in the cerebral EC line RBE4 leads to the up-regulation of P-gp, both at the gene and protein levels, and that this modulation depends on the catalytic activity of SphK-1. Moreover, we determined the effect of sphingosine-1-

phosphate (S1P), the product of SphK-1, on P-gp function. S1P strongly stimulates P-gp transport activity, without modulating its expression. Finally, we found that the S1P-mediated stimulation of P-gp activity is mediated by S1P-1 and S1P-3 receptors at the RBE4 cell surface. Altogether, these results indicate that SphK-1 and its product S1P are involved in the control of P-gp activity in RBE4 cells. Since SphK-1 is overexpressed in EC from brain tumors, these data also suggest that this kinase and its product could contribute to the acquisition and the maintenance of the multidrug resistance phenotype in brain tumor-derived endothelial cells.

Keywords: brain endothelial cells, MDR phenotype, P-glycoprotein, sphingosine kinase, sphingosine-1-phosphate.

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P-glycoprotein (P-gp) was first studied in the context of cancer research where its overexpression in tumor cells has been associated with the multidrug resistance (MDR) phenotype (Henson *et al.* 1992). The MDR protein P-gp is encoded by the *MDR1* gene in humans, whereas two genes encode paralogous proteins in rodents, *mdr-1a* and *mdr-1b* (Roninson *et al.* 1986; Devault and Gros 1990). In cancer cells, P-gp acts as an efflux pump that extrudes chemotherapeutic agents out of the cells, decreasing their intracellular concentrations (See *et al.* 1974; Ozben 2006). High levels of P-gp expression have been observed in the endothelial cells (EC) of brain capillaries (Tanaka *et al.* 1994; Demeule *et al.* 2001), mainly at the luminal surface of the brain vascular endothelium (Beaulieu *et al.* 1997). P-gp expressed in brain EC presumably acts to protect the brain against xenobiotics that could affect normal brain functions. Several proteins, such as protein kinase C, caveolin-1 or HIV-Tat, have been shown to regulate P-gp function (Jodoïn *et al.* 2003; Hayashi *et al.* 2005; Tabe *et al.* 2006). However, a few studies have investigated the role of the sphingosine kinase (SphK), and

its product sphingosine-1-phosphate (S1P), in the control of P-gp function.

The lysophospholipid S1P is released following inflammation by activated platelets (Billich *et al.* 2005) and has been found to act as an *extracellular* mediator by binding to specific members of the Endothelial Differentiation Gene-family of G-protein coupled receptors (GPCRs, originally named Endothelial Differentiation Gene-1, -3, -5, -6, and -8 and now termed S1P-1 to -5 receptors). In addition to its function as a ligand for cell surface receptors, S1P also acts

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Abbreviations used: EC, endothelial cells; GFP, green fluorescent protein; HBSS, Hank's Buffered Salt Solution; MDR, multidrug resistance; P-gp, P-glycoprotein; PTX, pertussis toxin; S1P, sphingosine-1-phosphate; SphK, sphingosine kinase; Vb, vinblastine.

as an intracellular messenger (Chalfant and Spiegel 2005). SIP is produced by the phosphorylation of sphingosine, catalyzed by SphK, the most distal enzyme in the sphingomyelin pathway of sphingolipid degradation. To date two distinct SphK isoforms, SphK-1 and SphK-2, have been cloned and characterized (Maceyka *et al.* 2005). SphK-1 has been shown to be up-regulated in various tumors (French *et al.* 2003). The strong expression of SphK-1 is also associated with the sustained growth of tumor cells through SIP-mediated inhibition of tumor cell apoptosis (Maceyka *et al.* 2002). Recently, the expression of SphK-1 has been associated with poor survival prognosis for patients with brain tumors (Van Brocklyn *et al.* 2005). Moreover, it has been shown that SphK-1 impairs the efficacy of chemotherapy in prostate adenocarcinoma cells (Pchejetski *et al.* 2005). Since P-gp is also associated with a poor prognosis for patients with brain tumors and plays a major role in chemotherapy efficacy (Regina *et al.* 2001), we hypothesized that SphK-1, and its product SIP, could regulate P-gp transport activity.

In the present paper, we demonstrate that SphK-1 is up-regulated in brain EC isolated from brain tumors compared with normal brain EC. We also provide evidence that SphK-1 and its product SIP modulate P-gp transport activity in the rat brain endothelial cell line RBE4. Moreover, our data support the hypothesis that P-gp transport activity is regulated by SIP. Finally, we demonstrate that extracellular SIP mediates its effects on P-gp function by interacting with SIP-1 and SIP-3 on the RBE4 cell surface. Altogether, our results suggest that the SphK-1/SIP couple participates in the regulation of P-gp function in EC of the blood brain barrier.

Material and methods

Antibodies and chemicals

TRIZOL reagent, trypsin and geneticin (G418) were from Invitrogen (Burlington, ON, Canada). SIP, indomethacin, probenecid, verapamil, gentamycin, sodium dodecyl sulfate, trypsin for EC and bovine serum albumin were purchased from Sigma (Oakville, ON, Canada). *N,N'* dimethylsphingosine was from Cedarlane Laboratories (Hornby, ON, Canada). Cyclosporin A (CsA) and SDZ PSC833 were provided by Novartis Pharma Canada Inc. (Dorval, QC, Canada). The third generation P-gp inhibitor Zosuquidar was kindly provided by Kanisa Pharmaceuticals Inc. (San Diego, CA, USA). basic Fibroblast Growth Factor was from Upstate Biotechnology (Lake Placid, NY, USA). Epithelial growth factor was from BD Biosciences Labware (Bedford, MA, USA). Type-1 collagen was extracted from rat tail tendon by standard procedure (Silver and Trelstad 1980). The monoclonal antibody (mAb) against P-gp (namely mAb C219) was from ID Labs (London, ON, Canada). The rabbit polyclonal anti-SphK-1a antibody was a gift of Dr Yoshiko Banno (Gifu, Japan). Secondary antibodies, linked to horseradish peroxidase, were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and Enhanced Chemiluminescence

Reagent Plus (ECL) reagents were from NEN™ Life Science Products (Boston, MA, USA).

Cells and culture media

α -Minimum Eagle's Medium medium, Ham's F12 Nutrient Mixture and L-Glutamine were purchased from GIBCO BRL. Fetal calf serum was from HyClone Laboratories (Logan, UT, USA). RBE4 immortalized rat brain microvessel EC were the gift of Dr Françoise Roux (Paris, France). RBE4 cells were plated on type I collagen-coated plates (200 μ g/mL) and maintained in α -Minimum Eagle's Medium/Ham's F12 (1:1 v/v) containing 10% heat-inactivated fetal calf serum, 30 mg/mL geneticin, 50 mg/mL gentamycin and 100 ng/mL basic Fibroblast Growth Factor. All cells were incubated at 37°C, air/CO₂ (95%/5%).

Isolation of endothelial cells fractions from brain tissue

Brain tumor implantation and the isolation of EC from normal male Lewis rat (Charles River, QC, Canada) brains or gliomas was performed as previously described (Regina *et al.* 2003). Briefly, meninges-free cortex was homogenized in Ringer solution, mixed with Dextran T-70 and centrifuged to remove myelin. Intracerebral and subcutaneous glioblastomas were dissected and minced. Samples were then incubated with collagenase A (1 mg/mL) for 45 min at 37°C with agitation and the cell suspensions were passed through a Nitex filter (180 μ m) and then another Nitex filter (30 μ m). The pellets were washed three times in PBS containing 0.5% bovine serum albumin and 2 mmol/L ethylenediaminetetraacetic acid. The final pellets were resuspended in 80 μ L of PBS with 0.5% bovine serum albumin and 2 mmol/L ethylenediaminetetraacetic acid per 10⁷ cells. Microbeads linked to the anti-PECAM-1 antibody (20 μ L/10⁷ cells) were added to the cell suspension for 45 min at 4°C. The cells were washed by centrifugation (600 g, 10 min) and resuspended in a volume of 500 μ L. The EC, bound by the magnetic microbeads linked to the anti-PECAM-1 antibody, were selected with the separation unit. The cell fractions retained by the column were washed with PBS followed by centrifugation at 600 g for 5 min at 20°C. The final pellets containing EC were kept at -80°C until used. All animal experiments were evaluated and approved by the Institutional Committee for Good Animal Practices (UQAM, Montréal, QC, Canada). Magnetic cell sorting (MACS) was performed using microbeads, a MidiMacs separation unit and positive selection MACS columns from Miltenyi Biotec (Auburn, CA, USA). The mouse anti-PECAM-1 antibody which was linked to the microbeads was from Cedarlane Laboratories.

cDNA constructs and cell transfection

The plasmid encoding the mouse SphK-1-green fluorescent protein (GFP) chimera was generously provided by Dr Sarah Spiegel (Richmond, VA, USA). The dominant negative form of the human SphK-1-GFP (G82D) chimera was a gift of Dr Lina Obeid (Charleston, SC, USA). RBE4 cells were transiently transfected with cDNA plasmids by using the liposomal formulation TranspassD1 transfection reagent (New England Biolabs Inc., Pickering, ON, Canada). Transfection efficiency was confirmed by Western blotting. All experiments involving transfected cells were performed 36 h following transfection. Mock transfection of RBE4 cells with the empty pcDNA (3.1+) expression vector was used as a control.

Sphingosine-1-phosphate measurement

^{32}P was purchased from Amersham (Baie d'Urfé, QC, Canada). RBE4 cells were washed with phosphate-free medium and incubated with 2 mL of this buffer containing ^{32}P (40 $\mu\text{Ci}/\text{mL}$) for 16 h in a 6-well plate. Placing the dishes on ice, and rapidly removing the medium terminated the reactions. The cells were scraped from the dish in 1 mL of 0.1 N HCl. Samples were then supplemented with 2.5 volumes of chloroform/1-butanol/HCl (50/50/1 v/v), vortexed for 2 min, and incubated at 20°C for 10 min. Samples were centrifuged at 5000 g for 10 min. The organic phases were dried under nitrogen and analyzed by Thin Layer Chromatography. This single step extraction allowed the isolation of S1P with >90% recovery (Ancellin *et al.* 2002). Samples were resuspended in 50 μL of chloroform. [^{32}P]S1P was resolved by Thin Layer Chromatography on Silica Gel G60 using 1-butanol/acetic acid/water (60/20/20 v/v) buffer. Bands corresponding to S1P, revealed by the ninhydrine staining of purified S1P, were scraped from the plates and counted in a scintillation counter. Protein concentration was determined for each sample and radioactivity was normalized to the protein concentration.

Immunoblotting procedures

Cells were homogenized in lysis buffer. 30 μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All samples, aside from those used for P-gp detection, were boiled for 5 min at 100°C. After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked overnight at 4°C with 5% non-fat dry milk in Tris-buffered saline (150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.5) containing 0.3% Tween 20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies in TBST containing 3% bovine serum albumin, followed by a 1 h incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/5000 dilution) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence.

Total RNA isolation and reverse transcriptase-polymerase chain reaction

Total RNA was extracted from control and transfected RBE4 cells using TRIzol Reagent. Reverse transcriptase-polymerase chain reaction (RT-PCR) reactions were performed using the SuperScript™ One-Step RT-PCR with Platinum® Taq Kit from Invitrogen. RT-PCR reactions were performed with specific oligonucleotide primers derived from rat sequences and PCR conditions were optimized so that the gene products were obtained during the exponential phase of the amplification. PCR products were resolved on 2% (w/v) agarose gels containing 1 $\mu\text{g}/\text{mL}$ ethidium bromide.

Vinblastine accumulation assay

Cells were washed twice with Hank's Buffered Salt Solution (HBSS) (1.3 mmol/L CaCl_2 , 5.4 mmol/L KCl, 0.44 mmol/L KH_2PO_4 , 0.5 mmol/L MgCl_2 , 0.83 mmol/L MgSO_4 , 137 mmol/L NaCl, 4.2 mmol/L NaHCO_3 , 0.34 mmol/L Na_2HPO_4 , and 25 mmol/L D-glucose, pH 7.3) then incubated for 1 h at 37°C in HBSS containing 50 nmol/L [^3H]-Vb from Amersham. [^3H]-Vb accumulation was stopped by washing the cells five times with ice-cold HBSS. Cells were lysed with 0.1% Triton X-100 at 20°C overnight or with 0.1 N NaOH for 1 hour. As much as 200 μL of each sample were mixed with 10 mL scintillation cocktail and the radioactivity in

the cell lysate was measured. Protein concentration was determined for each sample and radioactivity was normalized to the protein concentration.

Treatment with antisense oligonucleotides

Antisense phosphorothioate oligodeoxyribonucleotides (PTO) against S1P-1, S1P-3 and their respective scrambled controls were custom synthesized based on published sequences (Liu *et al.* 2001; Son *et al.* 2006). The sequences of the oligonucleotides used are the following: S1P-1 antisense, 5'-GAC GCT GGT GGG CCC CAT-3'; scrambled S1P-1, 5'-TGA TCC TTG GCG GGG CCG-3'; S1P-3 antisense, 5'-CGG GAG GGC AGT TGC CAT-3'; scrambled S1P-3, 5'-ATC CGT CAA GCG GGG GTG-3'. To reduce S1P-1 and S1P-3 gene expression, RBE4 cells were grown to approximately 60% confluency and incubated with 200 nmol/L of either the scrambled controls or the S1P-1 or S1P-3 antisense oligonucleotides using Transpass D1 transfection reagent. Cells were used 72 h after transfection.

Results

Up-regulation of sphingosine kinase-1 in endothelial cells isolated from brain tumor

SphK-1 expression has been shown to be correlated with poor survival among patients with glioblastoma multiforme (Van Brocklyn *et al.* 2005). We therefore sought to measure the expression of SphK-1 in EC derived from normal brain and from glioma. By using a well-established protocol (Regina *et al.* 2003), EC from normal rat brain and glioma were isolated. The expression of SphK-1 in these lysates was evaluated by Western blot analysis. As shown in Fig. 1a, the expression of SphK-1 in glioma-derived EC is much higher than in normal brain-derived EC. By densitometric analysis, we found a threefold increase of SphK-1 expression in glioma-derived EC compared with normal brain EC (Fig. 1b).

Overexpression of sphingosine kinase-1-green fluorescent protein in RBE4 cells

The RBE4 cell line was used as a model of brain EC. These cells were transfected with either an empty plasmid or a plasmid encoding the wild-type, GFP-tagged form of SphK-1. Expression of the SphK-1-GFP protein in RBE4 cells was evaluated by fluorescence microscopy. As shown in Fig. 2a, the SphK-1-GFP plasmid transfection is barely effective in RBE4 cells. Afterwards, the concentrations of intracellular and extracellular S1P were measured, as described in the Materials and methods (Fig. 2b). Note that the vast majority of S1P remains within the cell, whereas a small percentage is excreted into the extracellular environment (Fig. 2b). When SphK-1-GFP was overexpressed in RBE4 cells, both the intracellular and the extracellular forms of S1P were increased (Fig. 2b). These results indicate that

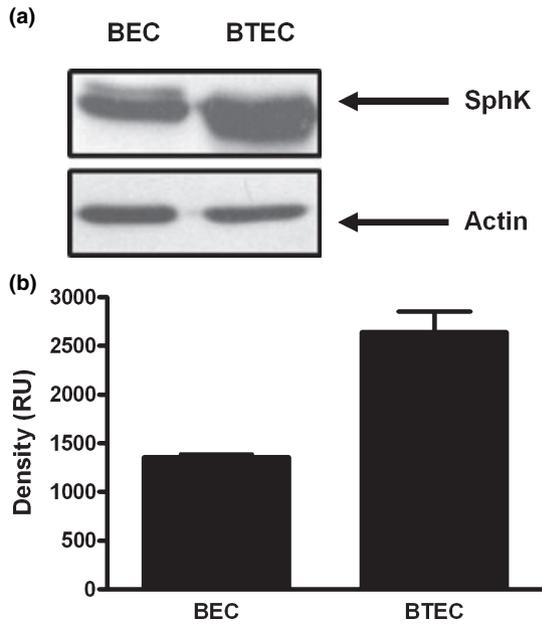


Fig. 1 Up-regulation of sphingosine kinase-1 (SphK-1) in brain endothelial cells isolated from brain tumors. (a) Endothelial cells were isolated from normal rat brain or from orthotopically implanted glioma, as described in the Material and Methods, and cells lysates were subjected to Western blot analysis for SphK-1 expression. β -actin was detected as a loading control. (b) The expression of SphK-1 was analyzed by densitometry using the IPLabGel software. Data represents means \pm SEM of results obtained with at least three gels.

this model, in spite of a transfection efficiency around 20%, can be used to assess the effects of SphK-1 on P-gp expression and activity.

Stimulation of P-glycoprotein expression in sphingosine kinase-1-transfected RBE4 cells

Because both SphK-1 and P-gp expression are associated with poor survival prognosis in patients with brain tumors, we investigated the effects of overexpression of SphK-1 on P-gp expression in RBE4 cells. The expression of SphK-1 in control and in SphK-1-GFP transfected RBE4 cells was determined by Western blot. In control and SphK-1-GFP transfected cells, a band at around 40 kDa, corresponding to the basal expression of SphK-1 in RBE4 cells (Fig. 3a) was detected. In SphK-1-GFP transfected cells, a second band, at around 70 kDa, corresponding to the GFP-tagged SphK-1 protein, was also observed (Fig. 3a). When P-gp was measured in the same lysates, we determined a twofold increase in the expression of this protein (Fig. 3a).

To better characterize this phenomenon, we performed RT-PCR analysis of the expression of both *mdr-1a* and *mdr-1b* genes in mock and transfected cells. As shown in Fig. 3b, the overexpression of the wild-type form of the SphK-1 protein led to the up-regulation of the *mdr-1b* gene by a sixfold factor, without affecting the expression of the *mdr-1a* gene.

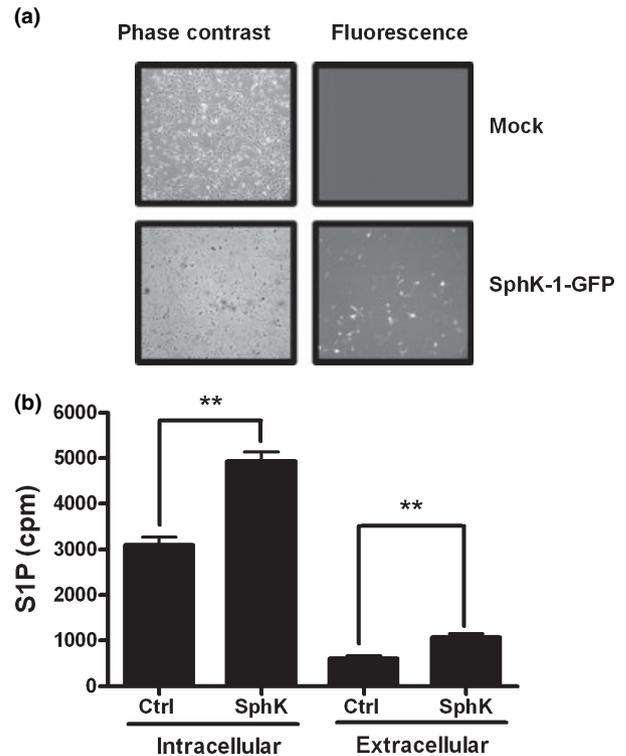


Fig. 2 Sphingosine kinase-1 (SphK-1)-mediated increase of intracellular and extracellular sphingosine-1-phosphate (S1P) in RBE4 cells. RBE4 cells were transiently transfected with a cDNA plasmid encoding SphK-1-GFP or with the empty pcDNA (3.1+) plasmid (mock). (a) Phase contrast (PC) and fluorescence views of mock or of SphK-1-GFP transfected RBE4 cells. (b) Extracellular and intracellular S1P was measured in control and RBE4 cells as described in the Material and Methods; **statistically significant differences ($p < 0.05$) compared with the control cells.

However, the expression of both *mdr-1a* and *mdr-1b* were unaffected by transfection of the dominant negative form of SphK-1 (G82D) (Fig. 3b). This indicates that the regulation of the P-gp expression by SphK-1 depends on the catalytic activity of SphK-1.

Stimulation of P-glycoprotein transport activity in sphingosine kinase-1 transfected RBE4 cells

Since the overexpression of SphK-1 in RBE4 cells induced P-gp expression, we further investigated the impact of SphK-1 transfection on P-gp transport activity by measuring the accumulation of vinblastine ($[^3\text{H}]\text{-Vb}$) in RBE4. As shown in Fig. 4a, blocking $[^3\text{H}]\text{-Vb}$ efflux by P-gp inhibitors (verapamil, cyclosporine A, PSC833 and zosuquidar) led to an increase in its intracellular accumulation. However, broad-spectrum inhibitors of MRPs (probenecid and indomethacin) did not affect the accumulation of $[^3\text{H}]\text{-Vb}$ in RBE4, suggesting that Vb, under these experimental conditions, is specifically extruded by P-gp in RBE4 cells. Following this, we investigated the effect of SphK-1

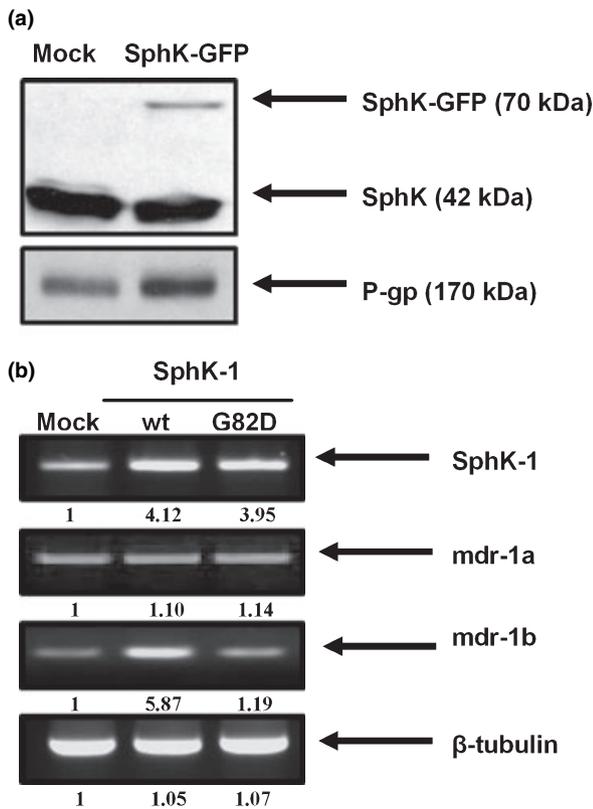


Fig. 3 Up-regulation of P-glycoprotein (P-gp) expression by the active form of sphingosine kinase-1 (SphK-1) in RBE4 cells. (a) RBE4 cells were transiently transfected with a cDNA plasmid encoding the wild-type form SphK-1-GFP or with the empty pcDNA (3.1+) plasmid (mock). SphK-1 and P-gp were detected by Western blot analysis. (b) RBE4 cells were transiently transfected with a cDNA plasmid encoding the wild-type form SphK-1-GFP, the dominant-negative form SphK-1-GFP (G82D) or with the empty pcDNA (3.1+) plasmid (mock). Total RNA was extracted as described in the Materials and Methods and the *SphK-1*, *mdr-1a* and *mdr-1b* cDNA fragments were amplified by RT-PCR using specific oligonucleotides. β -tubulin was amplified as a loading control. Results were subjected to a densitometric analysis, whose data are indicated under each band. Representative data of at least three independent experiments are presented.

overexpression on P-gp dependent [3 H]-Vb accumulation in RBE4 cells. When cells were transfected with the wild-type form of SphK-1, the accumulation of [3 H]-Vb within the cells was significantly lower than in the mock-transfected cells (Fig. 4b). However, overexpression of the dominant negative form of SphK-1 (G82D) did not modify [3 H]-Vb accumulation in RBE4 cells (Fig. 4b). These results indicate that overexpression of SphK-1 induces the expression of functional P-gp. By using specific inhibitors of P-gp, we were able to antagonize the efflux of [3 H]-Vb in RBE4 cells. This indicates that the SphK-1-mediated stimulation of Vb efflux is specifically related to the increase of P-gp transport activity.

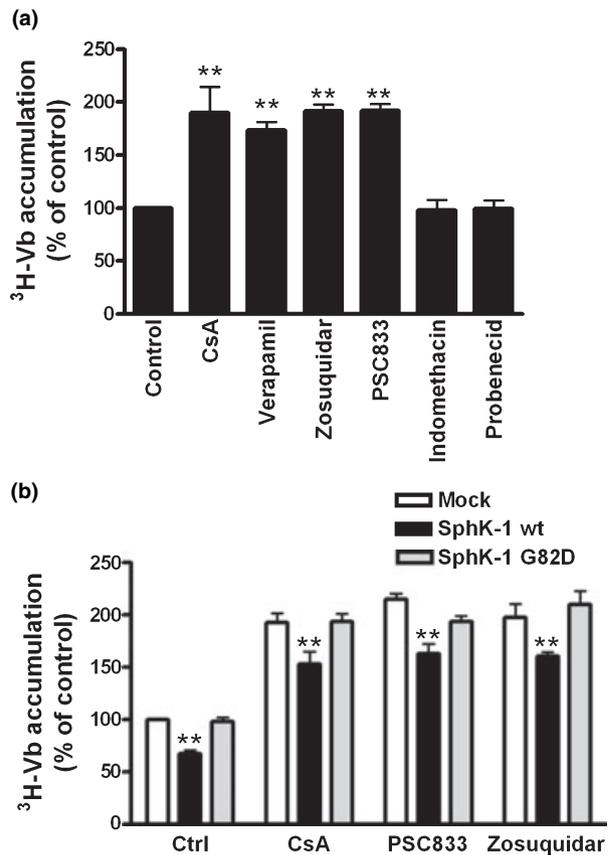


Fig. 4 Sphingosine kinase-1 (SphK-1)-mediated decrease of [3 H]-vinblastine (Vb) accumulation in RBE4 cells. (a) [3 H]-Vb accumulation was measured in the presence and absence of various P-glycoprotein (P-gp) inhibitors (CsA: 2.5 μ mol/L; Verapamil: 10 μ mol/L; PSC833: 5 μ mol/L and Zosuquidar: 2.5 μ mol/L) or MRP inhibitors (Probenecid: 1 mmol/L and Indomethacin: 10 μ mol/L). (b) RBE4 cells were transiently transfected with a cDNA plasmid encoding the wild-type form SphK-1-GFP, the dominant-negative form SphK-1-GFP (G82D) or the empty pcDNA (3.1+) plasmid (mock). [3 H]-Vb accumulation was measured as described in the Method section in presence or absence of various P-gp inhibitors (CsA: 2.5 μ mol/L; PSC833: 5 μ mol/L and Zosuquidar: 2.5 μ mol/L). Results were expressed as the percentage of [3 H]-Vb accumulation measured in the absence of P-gp inhibitors. The data represent means \pm SEM of at least three independent experiments; **statistically significant differences ($p < 0.05$) compared with the control cells.

Stimulation of P-glycoprotein transport activity by sphingosine-1-phosphate in RBE4 cells

We also determined the effect of S1P on P-gp-mediated [3 H]-Vb efflux. RBE4 cells were incubated with 1 μ mol/L S1P for 1 hour and [3 H]-Vb accumulation was assayed. As shown in Fig. 5, treatment with S1P stimulated [3 H]-Vb efflux from cells. By Western blot analysis, we determined that P-gp expression was unaffected by the treatment of RBE4 cells with S1P (data not shown).

We next determined whether overexpression of SphK-1 could also stimulate P-gp transport activity through the

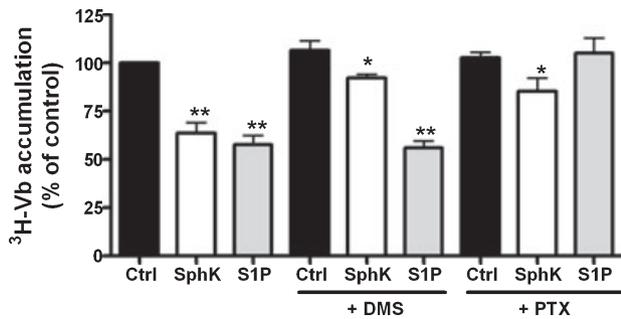


Fig. 5 Modulation of P-glycoprotein (P-gp) activity by sphingosine kinase-1 (SphK-1) and sphingosine-1-phosphate (S1P) in RBE4 cells. RBE4 cells were transiently transfected with a cDNA plasmid encoding the wild-type form SphK-1-GFP or with the empty pcDNA (3.1+) plasmid (mock), or treated with 1 μ mol/L S1P for 1 h and [³H]-vinblastine (Vb) accumulation was measured. Cells were pre-treated with either 20 ng/mL PTX for 16 h or with 20 μ mol/L DMS for 1 h. The data represent means \pm SEM of at least three independent experiments; **statistically significant differences ($p < 0.05$) compared with the control cells.

formation of S1P in the RBE4 cell line. RBE4 cells were transfected with SphK-1-GFP plasmid or were treated with 1 μ mol/L S1P, in the presence or absence of 10 μ mol/L DMS, a specific inhibitor of SphK-1 (Fig. 5). The addition of DMS partially antagonized the effect of overexpressing SphK-1 on [³H]-Vb accumulation. The partial effect of DMS on transfected cells can be explained by the SphK-1-mediated up-regulation of P-gp expression in RBE4 cells. However, S1P-mediated stimulation of P-gp function in RBE4 cells was unaffected by the addition of DMS (Fig. 5).

To further characterize the SphK-1-mediated regulation of P-gp, RBE4 cells were also pre-treated for 16 h with pertussis toxin (PTX) to block the activity of S1P receptors at the cell surface. The PTX treatment partially antagonized SphK-1-mediated stimulation of the [³H]-Vb efflux in RBE4 cells (Fig. 5). This suggests that SphK-1 modulates P-gp function through the up-regulation of the protein but also through S1P-mediated stimulation of P-gp transport activity. The PTX treatment fully antagonized the S1P-mediated stimulation of P-gp function in RBE4 cells.

Stimulation of P-glycoprotein activity through sphingosine-1-phosphate-1 and sphingosine-1-phosphate-3 receptors in RBE4 cells

Because PTX inhibited S1P-mediated modulation of P-gp transport activity, we evaluated which of the two Gi-associated S1P receptors (i.e. S1P-1 and S1P-3) were involved in the regulation of this phenomenon. To identify which receptor is responsible for the effect of S1P on P-gp transport activity, RBE4 cells were treated with antisense oligonucleotides raised against S1P-1 or S1P-3. As shown in Fig. 6, the scrambled oligonucleotides did not affect the S1P-mediated stimulation of the [³H]-Vb transport in RBE4 cells.

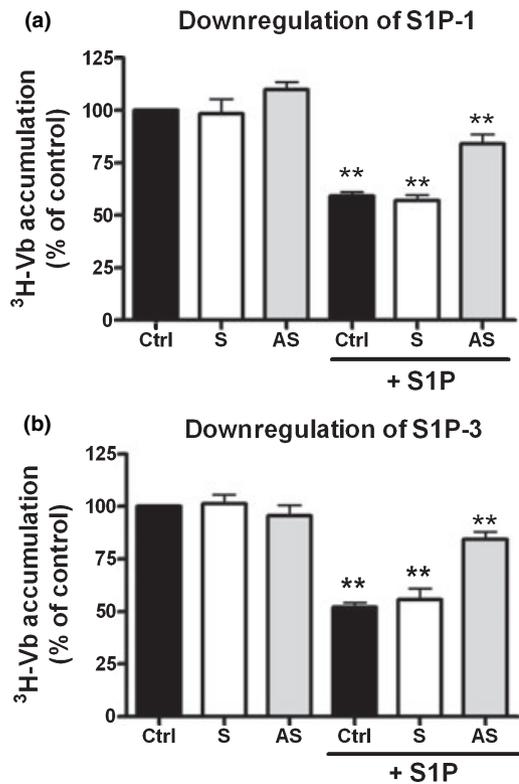


Fig. 6 Sphingosine-1-phosphate (S1P)-mediated stimulation of P-glycoprotein (P-gp) transport activity in RBE4 cells through S1P-1 and S1P-3 receptors. [³H]-vinblastine (Vb) accumulation was measured in RBE4 cells 72 h after transient transfection with 200 nmol/L antisense (AS) oligonucleotides against S1P-1 and S1P-3, or with their scrambled controls (S). (A) S1P-1 (S) and (AS) or (B) S1P-3 (S) and (AS) transfected cells were treated (or not) with 1 μ mol/L S1P. The data represent means \pm SEM of at least three independent experiments; **statistically significant differences ($p < 0.05$) compared with the control cells.

However, both S1P-1 and S1P-3 antisense oligonucleotides partially antagonized the S1P-mediated stimulation of the P-gp activity in RBE4 cells. These results indicate that the S1P-mediated stimulation of P-gp transport activity is mediated by both S1P-1 and S1P-3 receptors in RBE4 cells.

Discussion

The ATP-dependent P-gp present on luminal membranes of the brain capillary EC that form the blood-brain barrier (BBB) is a key element in the extrusion of structurally dissimilar compounds from the brain EC into the blood vessel lumen. P-gp also plays a crucial role in the acquisition and maintenance of the MDR phenotype in brain EC (Regina *et al.* 2001). Here, we report that SphK-1, which is also involved in the development of brain tumors (Van Brocklyn *et al.* 2005), regulates P-gp expression and transport activity through the generation of both intracellular and extracellular S1P in brain EC.

Since the up-regulation of P-gp expression is a common feature in the acquisition of the MDR phenotype (Henson *et al.* 1992), the increase in P-gp expression and activity induced by SphK-1 suggests that this kinase plays a crucial role in development of the MDR phenotype. This is in agreement with other studies that have also demonstrated the importance of SphK-1 for the MDR phenotype. For instance, it has been shown that doxorubicin-sensitive HL-60 cells display SphK-1 inhibition coupled with ceramide generation. In contrast, chemoresistant HL-60 cells, which express P-gp, have sustained SphK-1 activity and do not produce ceramide (Bonhoure *et al.* 2005). Moreover, camptothecin-resistant PC3 cells exhibit higher SphK-1 activity and also elevated expression of the S1P receptors S1P-1 and -3 than do camptothecin-sensitive cells (Akao *et al.* 2006). Altogether, these data and our own results confirm a major role for SphK-1 in the MDR phenotype acquisition. However, our data are the first results showing that SphK-1 could be involved in the regulation of P-gp at the BBB.

Interestingly, the overexpression of SphK-1 has been shown to be strongly associated with inflammation (Baumruker *et al.* 2005; Billich *et al.* 2005; Wang *et al.* 2005). The higher expression of SphK-1 in brain EC isolated from brain tumors, compared with normal brain EC, suggests that brain tumor-derived EC undergo chronic inflammation, as previously described (Deininger and Schluesener 1999). Cyclooxygenase-2 (COX-2) is one of the major inflammatory factors (Zha *et al.* 2004; Monnier *et al.* 2005). COX-2 has been shown to be up-regulated by SphK-1 (Pettus *et al.* 2003) and to stimulate P-gp expression (Patel *et al.* 2002). As our results clearly show that the overexpression of SphK-1 leads to the up-regulation of P-gp, this stimulation of P-gp expression in RBE4 could be dependent on COX-2 activity. This possibility remains to be established and is currently under investigation in our laboratory.

Through the use of RT-PCR, we demonstrated that overexpression of SphK leads to overexpression of the *mdr1-b* gene in RBE4 cells. The up-regulation of *mdr1-b* can be associated with a cell differentiation phenotype. Thus, undifferentiated alveolar epithelial type II cells do not express the *mdr1-b* gene, whereas differentiated alveolar epithelial type II cells do (Campbell *et al.* 2003). We have already demonstrated that the brain tumor endothelium displays a different molecular phenotype than does the normal brain endothelium, including the up-regulation of *mdr1-b* (Regina *et al.* 2003). Therefore, the brain-tumor mediated SphK overexpression in brain EC might, in part, lead to tumoral differentiation of the cerebral endothelium characterized by the up-regulation of *mdr1-b* gene.

It has been clearly demonstrated that the current use of chemotherapy to treat brain tumors is limited by the expression of P-gp in cancer cells (Ozben 2006). The expression of P-gp in EC from the BBB (Beaulieu *et al.* 1997) also limits the penetration of chemotherapeutic drugs

into the brain, decreasing the efficacy of brain tumor treatments. In this paper, we have demonstrated that SphK-1 and its product S1P stimulate P-gp transport activity in EC from brain capillaries. This suggests that SphK-1 could affect the efficacy of current brain tumor treatments through the modulation of P-gp expression. The use of SphK-1 inhibitors could therefore favor the penetration of drugs into the CNS, by inhibiting the SphK-1-mediated up-regulation of P-gp. Moreover, they could also affect brain tumors themselves. Indeed, the overexpression of SphK-1 observed in brain tumor cells (Van Brocklyn *et al.* 2005) and in brain tumor-derived EC leads to increased S1P concentration in the brain. Because S1P stimulates both the invasiveness and the proliferation of brain tumor cells (Van Brocklyn *et al.* 2002, 2003), SphK-1 inhibitors could block the development of brain tumors by decreasing the overall S1P concentration in the brain. The specific targeting of SphK-1 in brain EC and brain tumors may thus reduce the development of brain tumors through a decrease in S1P and facilitate the penetration of chemotherapeutic drugs in the brain by inhibiting the up-regulation of P-gp in brain EC. Consequently, SphK-1 inhibitors represent new potential antitumor molecules that can be of great help for the treatment of brain tumors.

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