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Distribution of sphingosine kinase activity and mRNA in rodent brain

Nicolas Blondeau^{*,1}, Yushuan Lai^{*}, Sarah Tyndall^{*}, Margherita Popolo^{*,3}, Kamil Topalkara^{*,2}, James K. Pru[†], Ling Zhang[†], HyungHwan Kim[‡], James K. Liao[‡], Kan Ding^{*}, and Christian Waeber^{*}

^{*}Stroke and Neurovascular Regulation Laboratory, Department of Radiology, Massachusetts General Hospital, Charlestown, Massachusetts, USA

[†]Department of Obstetrics and Gynecology, Vincent Center for Reproductive Biology, Massachusetts General Hospital, Charlestown, Massachusetts, USA

[‡]Vascular Medicine Research, Brigham & Women's Hospital and Harvard Medical School, Cambridge, Massachusetts, USA

Abstract

Sphingosine-1-phosphate (S1P) is a lipid mediator that exerts multiple cellular functions through activation of a subfamily of G-protein-coupled receptors. Although there is evidence that S1P plays a role in the developing and adult CNS, little is known about the ability of brain parenchyma to synthesize this lipid. We have therefore analyzed the brain distribution of the enzymatic activity of the S1P synthesizing enzyme, sphingosine kinase (SPHK) [EC:2.7.1.91], as well as mRNA distribution for one of the two isoforms of this enzyme, sphingosine kinase 2. SPHK activity, measured by the conversion of [³H]sphingosine to [³H]S1P, is highest in cerebellum, followed by cortex and brainstem. Lowest activities were found in striatum and hippocampus. Sensitivity to 0.1% Triton-X suggests that this activity is accounted for by SPHK2. RT-PCR and *in situ* hybridization studies show that mRNA for this isoform has a distribution similar to that of SPHK activity. *In vivo* and *in vitro* ischemia increase SPHK activity and SPHK2 mRNA levels. These results indicate that SPHK2 is the predominant S1P-synthesizing isoform in normal brain parenchyma. Its heterogeneous distribution, in particular laminar distribution in cortex, suggests a neuronal localization and a possible role in cortical and cerebellar functions, in normal as well as ischemic brain.

Keywords

cerebral ischemia; glial cells; neurons; sphingo-lipid metabolism; sphingosine-1-phosphate

Sphingosine-1-phosphate (S1P) exerts a wide variety of biological activities in many vertebrate cell types. It was initially proposed to act as a second messenger, based on the ability of extracellular growth factors to activate sphingosine kinase (SPHK) [EC:2.7.1.91] and increase

Address correspondence and reprint requests to Christian Waeber, Stroke and Neurovascular Regulation Laboratory, Department of Radiology, Massachusetts General Hospital, Charlestown, MA 02129, USA. E-mail: waeber@helix.mgh.harvard.edu.

¹The present address of Nicolas Blondeau is the Institut de Pharmacologie Moléculaire et Cellulaire (IPMC), UMR 6097, CNRS-UNSA, 660 route des lucioles - Sophia Antipolis, 06560 Valbonne, France.

²The present address of Kamil Topalkara is the Department of Neurology, Faculty of Medicine, Cumhuriyet University, Sivas, 58140 Turkey.

³The present address of Margherita Popolo is the Service de Neurologie, Hôpital Pasteur, BP69, Av de la voie Romaine, 06002 Nice Cedex 1, France.

intracellular S1P levels. However, the putative site that mediates these intracellular actions of S1P has not been identified. The discovery and cloning of five S1P receptors (S1P₁/EDG₁, S1P₂/EDG₅, S1P₃/EDG₃, S1P₄/EDG₆, and S1P₅/EDG₈) has stimulated the notion that S1P acts as an extracellular signaling molecule, regulating a host of cellular functions such as proliferation, immunomodulation, apoptosis, migration, cytoskeletal organization, and differentiation/morphogenesis (Hla 2003).

Sphingosine-1-phosphate concentrations in different tissues are regulated at the level of its synthesis from sphingosine by the activity of SPHK (Olivera and Spiegel 2001), and by its degradation by S1P phosphatases (Sciorra and Morris 2002) and endoplasmic reticulum-associated S1P lyase (Van Veldhoven and Mannaerts 1991; Zhou and Saba 1998). Basal intracellular levels of S1P are generally low (Spiegel *et al.* 1998), but can increase rapidly and transiently when cells are exposed to various agonists. A number of growth factors have been shown to activate SPHK, including tumor necrosis factor (Xia *et al.* 1998, 1999), vascular endothelial growth factor (Shu *et al.* 2002), platelet-derived growth factor (Olivera and Spiegel 1993; Olivera *et al.* 1999), nerve growth factor (Edsall *et al.* 1997; Rius *et al.* 1997), and basic fibroblast growth factor (Rius *et al.* 1997). Protein kinase C has been implicated in the activation of SPHK in some, but not all, receptor-mediated events leading to SPHK activation (Buehrer *et al.* 1996; Cuvillier *et al.* 1996, 1998; Kleuser *et al.* 1998; Shu *et al.* 2002).

Activated platelets represent a major source of S1P (English *et al.* 2000, 2001; Yatomi *et al.* 2000). S1P levels in serum are considerably higher than in plasma (0.4 vs. 0.1 $\mu\text{mol/L}$, respectively). However, the interaction of S1P with low-density lipoprotein (Kimura *et al.* 2001) might reduce the free concentration of S1P, possibly as a protection mechanism from full activation of vascular S1P receptors.

The fact that the brain is the organ containing the largest amount of S1P [6 $\mu\text{mol/kg}$ wet weight (Edsall and Spiegel 1999)] suggests that neurons may represent a significant source of S1P. In the present study, we have found that one of the two enzymes synthesizing S1P, SPHK2 accounts for most of the SPHK activity in brain. SPHK2 mRNA and SPHK activity showed a heterogeneous distribution, suggesting a neuronal localization and a possible role in cortical and cerebellar function in adult brain. In addition, the distribution of SPHK2 mRNA in embryo brain indicates that S1P might play a role in neurogenesis. Finally, the increased neuronal SPHK2 mRNA expression following *in vivo* or *in vitro* ischemic insult, taken together with the fact that SPHK activation mediates ischemic pre-conditioning in murine heart (Jin *et al.* 2004), suggests that S1P might be protective following brain ischemia.

Materials and methods

Animals

Experiments were performed on male Sprague-Dawley rats (200-300 g), male SV129 mice weighing 20-25 g (Charles River Laboratories, Wilmington, MA, USA), or female ICR mice (6 weeks of age, Charles River Laboratories). The animals were maintained on a 12/12 h light/dark cycle and given food and water *ad libitum*. Animals were acclimated to the animal facility for at least 2 days prior to beginning experiments. Animal experiments were approved by the institutional animal care and use committee and conducted according to the National Institutes of Health guide for the Care and Use of Laboratory Animals.

Cell cultures

Primary neurons - E15-16 embryos were collected from pregnant rats deeply anaesthetized with 4% isoflurane and their brains were harvested in ice-cold phosphate-buffered saline (PBS). The region of interest was dissected, freed from meninges and choroid plexus, minced

and digested in trypsin-EDTA at 37°C (20-25 min). Trypsin action was stopped with 3 mL fetal calf serum and the tissue was homogenized by gentle trituration with a pipette, passed through a cell strainer and spun down (3 min, 4°C, 300 g). The pellet was resuspended in Neurobasal medium with glutamine, B27 supplement and Penicillin/Streptomycin, centrifuged, re-suspended in Neurobasal medium (10^6 cells/mL), and plated onto polylysine-coated dishes. Cells were grown in the presence of 25 μ mol/L Glu for 3 days and 10 μ mol/L β -mercapto-ethanol for 7 days. 10 μ mol/L AraC was added between day 3 and 4 to kill non-neuronal cells.

Primary glial cultures - Primary cultures of glial cells were generated using a method similar to the primary neuronal culture method, except that 2-day-old rat pups were used. Pups were killed with fluothane and decapitation, their cerebral hemispheres were dissected aseptically and placed in PBS. Cells were plated at a density of $1-3 \times 10^6$ cells in Dulbecco's Modified Eagle's Medium/5% fetal calf serum per 10-cm plate. When the culture became confluent, cells were washed once, detached and dissociated with trypsin/EDTA solution and re-plated.

Cell line - The mouse brain endothelial cell line bEnd.3 was cultured in Dulbecco's Modified Eagle's Medium/10% fetal calf serum. Cells were seeded into uncoated culture plates at $2-3 \times 10^5$ cells/well and grown to confluence in complete culture medium before use.

Sphingosine kinase activity

Sphingosine kinase activity was assessed by measuring the conversion of [3 H]sphingosine (17 Ci/mmol; Amersham Biosciences, Piscataway, NJ, USA) to [3 H]S1P. Mice were killed by isoflurane anesthesia/decapitation, brains were collected, various brain regions were dissected on ice and homogenized in protein extraction buffer (50 mmol/L K_3PO_4 , pH 7.4, 1 mmol/L EDTA, 20% glycerol, 1 mmol/L dithiothreitol, 1% Sigma cocktail inhibitors). Protein concentration was measured using the Bradford assay. Samples (15 μ L) were mixed with 45 μ L assay buffer (50 mmol/L K_3PO_4 , 0.267% fatty acid free bovine serum albumin, 3 mmol/L $MgCl_2$, 40 mmol/L NaF, 2 mmol/L semicarbazide, 1 mmol/L DTT, 50 mmol/L phosphoenolpyruvate, 10 mmol/L ATP, 100 U/mL pyruvate kinase) containing 0.01 mmol/L sphingosine and 16 μ Ci/mL [3 - 3 H] sphingosine (Amersham Biosciences). The reaction was performed at 37°C for 30 min and stopped on ice. The reaction products were extracted with 2 volumes of $CHCl_3$ /methanol/HCl (100 : 200 : 1), to which another volume of $CHCl_3$ and of 1 mol/L KCl were added. After vortexing, the samples were centrifuged at 2000 g for 10 min and the organic phase was evaporated in a Speedvac centrifuge. Samples were dissolved in methanol, spotted onto Silica gel 60 thin layer chromatography plates, resolved using 1-butanol/acetic acid/water (3 : 1 : 1) and visualized by fluorography with En 3 Hance (PerkinElmer Life Sciences, Boston, MA, USA). Radioactive spots were scraped from the plates and counted by liquid scintillation. Authentic sphingosine and S1P were applied with the samples for identification of [3 H]S1P and [3 H]sphingosine. Sphingosine (Rf 0.67) and S1P (Rf 0.51) were visualized with ninhydrin (staining sphingosine and S1P).

Because 0.1% Triton stimulates the SPHK1 activity, leading to incorrect estimation of SPHK2 activity, we used in a separate series of experiments the conditions described by Hait *et al.*, in an attempt to better differentiate SPHK1- from SPHK2-mediated activity (Hait *et al.* 2005). Briefly, striata and cortices from three mice were homogenized as described above. SPHK activity was determined in the presence of 20 μ mol/L sphingosine, 10 mmol/L $MgCl_2$, 1 mmol/L ATP, and 10 μ Ci/mL [3 - 3 H] D-erythro-sphingosine (American Radiolabeled Chemicals, St. Louis, MO, USA). SPHK1 activity was determined in the presence of 0.25% Triton X-100, which inhibits SPHK2. SPHK2 activity was determined with sphingosine added as a complex with 4 mg/mL BSA and 1 mol/L KCl, conditions in which SPHK2 activity is optimal and SPHK1 is strongly inhibited. The reaction was performed at 37°C for 30 min and stopped on ice. The reaction products were extracted, resolved and quantified as described above.

***In situ* hybridization**

Brains were frozen in isopentane at 40°C. Cryostat sections (12 µm) were post-fixed by immersion in 4% paraformaldehyde/PBS, treated with proteinase K (3 mg/mL, 20 min at 37°C) and acetylated in 0.1 mol/L triethanolamine (3 min, RT) followed by 0.1 mol/L triethanolamine/acetic anhydride (100/0.25, 10 min, RT).

Hybridization of [³³P]-labeled-cDNA probe, synthesised by PCR amplification of SPHK2 cDNA, was performed at 59°C overnight in the presence of 50% formamide. The sections were then washed in 2× saline-sodium citrate (SSC) (20'), 1× SSC (15'), 0.5× SSC (10'), 0.5× SSC (20' at 50°C), 0.5× SSC (5'), 0.1× SSC (30") and H₂O (30"), dehydrated in ethanol series (70%, 85%, 100%), air dried and exposed to X-ray film at 4°C for 3 days. Hybridization specificity was assessed by pre-treating sections with 40 mg/mL RNase in Tris-EDTA buffer for 30 min at 37°C followed by two washes in 0.5× SSC.

Real time PCR

Total RNA extraction of mouse tissues (around 100 mg), primary neuron (day 12), glial, and bEnd3 cells (35 mm plate) were performed with Trizol Reagent according to the manufacturer's protocol. cDNA was synthesized from total RNA using Super-ScriptIII reverse transcriptase. Real-time PCR reactions were completed in triplicates using an ABI Prism 7000 from Applied Biosystems (Foster City, CA, USA) with 100 ng/mL cDNA aliquots as template, together with primers and probes for Sphk1, Sphk2, and 18S ribosomal RNA (used to normalize mRNA expression). Primers and probes were from Applied Biosystems. The gene expression data were analyzed using Qgene, a publicly available Excel script package (Muller *et al.* 2002).

Northern blotting

Gene expression of SPHK2 in primary cultures of glial cells and neurons, as well as several organs was determined using northern blot analysis. After isolation using TriReagent (Sigma Chemical Co., St Louis, MO, USA), 10 micrograms of total cellular RNA was denatured, electrophoresed, and transferred to nylon membranes by capillary blotting. After ultraviolet cross-linking, membranes were pre-hybridized (50% formamide, 5× SSC, 50 mmol/L NaPO₄, 5× Denhardt solution, 0.1% sodium dodecyl sulphate (SDS), 0.1 mg/mL salmon sperm DNA) for 3 h at 65°C. Blots were then hybridized (15 h, 65°C) with an SPHK2 anti-sense cRNA probe (3 × 10⁶ cpm/mL; NM_020011; 5'-CCCACAGTGCCCCAGAAGCCT-3'; 5'-CCCAGTGAGCAGCGTGGCGAG-3'). Blots were washed (65°C, 1× SSC, 0.1% SDS, 15 min, 65°C; 0.1× SSC, 0.1% SDS, 30 min) and exposed to X-ray film for 48 h and then reprobed with radiolabeled cDNA for murine 18S rRNA (Ambion, Austin, TX, USA) to ensure equal loading.

Western blotting

Protein lysates were collected from postnatal day 25 female mouse organs for western blotting as described elsewhere (Pru *et al.* 2003). Proteins (20 µg/lane, *n* = 3 for each organ) were separated by SDS-PAGE and then transferred (100 V, 1 h) to polyvinylidene difluoride membranes. Non-specific binding was blocked with 5% fat free milk in Tris-buffered saline-Tween 20 [50 mmol/L Tris-HCl (pH 7.5), 0.15 mol/L NaCl, 0.05% Tween-20] for 1 h at 20°C. Membranes were incubated with polyclonal anti-SPHK1 antibody [250 µg/mL; kindly provided by Dr. Yoshiko Banno, Gifu University School of Medicine, Gifu Japan (Murate *et al.* 2001)], washed three times 10 min each in TBST and incubated an additional 30 min with anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing as before, bound antibody was detected using enhanced chemiluminescence reagents using the manufacturer's specifications (Santa Cruz Biotechnology). X-ray film was exposed to membranes for 2 min and developed using standard procedures.

Middle cerebral artery (MCA) occlusion

Anaesthesia was induced with 3% isoflurane mixed with 30% oxygen and 70% nitrous oxide, and maintained with 1.5% isoflurane. The left MCA was occluded with a coated monofilament as previously described (Endres *et al.* 1998). The filament was withdrawn at the indicated times and reperfusion was confirmed by laser-doppler flowmetry. The MCA occlusion and sham operated mice were perfused 24 h later with saline under isoflurane anaesthesia and their brains were quickly extracted for further processing.

Oxygen Glucose deprivation (OGD)

Primary neurons were placed into an anaerobic chamber ($37 \pm 1^\circ\text{C}$) containing 5% $\text{CO}_2/95\%$ N_2 . The culture medium was replaced with deoxygenated glucose-free Hanks' Balanced Salt Solution, and the cells were kept in the hypoxia chamber for 2 h. Cells recovered for 24 h in Neurobasal medium before SPHK activity was assayed.

Results

SPHK activity is heterogeneously distributed in mouse brain regions

$[\text{}^3\text{H}]$ sphingosine to $[\text{}^3\text{H}]$ S1P converting activity was examined in different brain regions. The levels of SPHK activity varied among the different brain areas (Fig. 1a) and were highest in cerebellum, followed by cortex ($81 \pm 3\%$ of cerebellum) and brainstem ($67 \pm 1\%$). Lower activities were found in striatum ($59 \pm 2\%$) and hippocampus ($39 \pm 6\%$).

SPHK2 is the major contributor to mouse brain SPHK activity

$[\text{}^3\text{H}]$ sphingosine to $[\text{}^3\text{H}]$ S1P conversion assay does not distinguish between SPHK1 and SPHK2 activity. Although there are no known isoform-specific inhibitors, comparison of recombinant SPHK1 and SPHK2 activities shows that Triton X-100 concentrations above 0.005% drastically inhibit SPHK2 activity and stimulate SPHK1 activity (Liu *et al.* 2000). Similarly, BSA inhibits SPHK2 activity without affecting SPHK1. 0.1% Triton X (Fig. 1b) inhibited $[\text{}^3\text{H}]$ sphingosine to $[\text{}^3\text{H}]$ S1P conversion by $72 \pm 2\%$ (hippocampus), $73 \pm 2\%$ (cortex), and $75 \pm 1\%$ (striatum), suggesting that SPHK2 accounts for most of this activity. Moreover, increasing BSA or total protein concentration caused a concentration-dependent inhibition of SPHK activity (data not shown). Experiments performed in the presence of 1 mol/L KCL (that optimizes SPHK2 activity and strongly inhibits SPHK1 activity) showed comparable ratios of SPHK1 to SPHK2 activity: SPHK1 activity was $19 \pm 8\%$ and $23 \pm 1\%$ of SPHK2 activity in striatum and cortex, respectively.

Sphingosine kinase gene expression

In order to confirm the predominance of the SPHK2 isoform in brain, we compared the tissue distribution of SPHK1 protein and *SPHK2* mRNA using western and northern blot analyses, respectively (Fig. 2). Whereas SPHK1 protein immunoreactivity predominated in kidney and lung (Fig. 2a), the brain abundantly expresses *SPHK2* mRNA (Fig. 2b). *SPHK2* mRNA signal was particularly intense in cerebellar tissue and primary glial cultures, and was weaker in brainstem and cortical neuron cultures. Furthermore, real time RT-PCR studies (Fig. 2c) showed more than 10-fold excess of *SPHK2* over *SPHK1* mRNA levels in glial, neuronal, and endothelial cell cultures. In order to rule out the possibility that these differences in the *SPHK1* and *SPHK2* mRNA amounts resulted from different efficiencies of PCR amplification for the respective primers, we examined the linearity of amplification efficiency plot for rat *SPHK1*, *SPHK2*, and ribosomal 18S RNA (Fig. 2d). Serial dilutions of cDNA were amplified in an ABI 7000 Prism thermal cycler (Applied Biosystems). The threshold CT values were then plotted versus the logarithm of the relative concentration of cDNA. Based on the slopes, the calculated efficiencies were: 96% (18S), 101% (*SPHK1*), and 84% (*SPHK2*).

Distribution of SPHK2 mRNA in the mouse brain

Because our results suggested a predominance of the SPHK2 isoform in brain, we further analyzed *SPHK2* mRNA distribution using real time RT-PCR and *in situ* hybridization. As shown in Fig. 3a, *SPHK2* mRNA was most abundant in cortex, followed by hippocampus and cerebellum. Lower amounts of *SPHK2* mRNA were seen in the striatum. Using a probe synthesized by PCR amplification of *SPHK2* cDNA in the presence of [³³P]dCTP, we performed *in situ* hybridization studies on adult (Fig. 3b) and E17 (Fig. 3c) embryonic mouse brain sections. Highest densities of *SPHK2* mRNA were found in adult cerebellum and deep cortical layers, as well as pyramidal layer of hippocampus. Lower densities were observed in striatum (Fig. 3b), in agreement with RT-PCR and enzyme activity data obtained in different brain regions. In E17 mouse embryo brain, highest density of labeling was observed in the cortical plate, ventricular/subventricular zone and ganglionic eminence area, while lower densities were seen in differentiating areas such as the striatum. Hybridization signals were not different from film background in sections treated for 30 min with RNase (negative control, not shown).

Effect of *in vivo* ischemia and oxygen-glucose deprivation

Tumor necrosis factor- α , which is rapidly increased in most brain cell types after stroke (Hallenbeck 2002), up-regulates SPHK activity in astrocytes (Vann *et al.* 2002). In addition, S1P synthesis is significantly increased in vascular smooth muscle cells subjected to hypoxia (Yun and Kester 2002). We therefore examined whether SPHK activity and *SPHK2* mRNA expression were increased following *in vivo* and *in vitro* models of brain ischemia. At 24 h after a 30-min MCA occlusion, ipsi- and contralateral mouse brain regions were harvested and homogenized. SPHK activity (assayed by the conversion of [³H]sphingosine to [³H]S1P) was examined in five regions on the ischemic side (cortical core and penumbra, striatal core and penumbra, hippocampus) and compared in each mouse to the activity in the corresponding region on the contralateral side. Statistical differences were assessed using paired t-tests. SPHK activity was markedly increased with respect to the contralateral side in the cortical and striatal penumbras ($p < 0.01$), and to a lesser extent in the striatal core and hippocampus ($p < 0.05$) (Fig. 4a).

Similarly, *SPHK2* mRNA expression was examined in three regions on the ischemic side (cortical penumbra, striatal penumbra, hippocampus) and compared in each mouse to the activity in the corresponding region on the contralateral side (statistical differences were assessed using paired t-tests). Using real-time RT-PCR, we found significantly increased *SPHK2* mRNA expression in mouse cortical and striatal penumbra, but not in hippocampus, following 20 min ischemia/24 h reperfusion (Fig. 4b). In contrast, following 2 h ischemia/24 h reperfusion, *SPHK2* mRNA expression was increased in hippocampus, but not in cortex or striatum (Fig. 4b). *in situ* hybridization studies showed increased *SPHK2* mRNA expression in the pyramidal layer of ipsilateral hippocampus 24 h after 20 min MCA occlusion (Fig. 4c). A similar 4.1 ± 0.3 fold increase in *SPHK2* mRNA expression was observed *in vitro* in primary cultures of striatal neurons 22 h following exposure to a 2-h episode of oxygen glucose deprivation. In contrast, 2-, 4- and 12-h OGD had no effect on *SPHK2* mRNA expression in bEnd3 brain endothelial cells (three independent experiments, not shown).

Discussion

The role of SPHK has been investigated in various isolated cell types, but few studies have focused on this enzyme at the organ or systemic level. Although the brain generates more S1P than any other organ [6 μ mol/kg wet weight (Edsall and Spiegel 1999)], little is known about the ability of brain parenchyma to synthesize S1P or the mechanisms that regulate SPHK activity. To begin to investigate the physiological functions of SPHK and S1P signaling in

brain we studied the spatial and cellular distribution of this enzyme in this tissue. The major findings of the present study is that SPHK activity shows regional-specific distribution in brain, most of which can be accounted for by the SPHK2 isoform, and that ischemic damage elevates enzyme activity. Our results suggest that SPHK2 plays an important role in development and in pathological situations such as brain ischemia. Furthermore, we find that a correlation exists in SPHK2 activity and gene expression.

A previous study reported that SPHK1 is the major SPHK isoform in many tissues, especially brain and heart, based on immunodepletion analysis with a SPHK1 antibody (Fukuda *et al.* 2003). It is likely that the presence of Triton X-100 [reported to stimulate SPHK1 and inhibit SPHK2 activity (Liu *et al.* 2000)] in the assay inhibited SPHK2 activity, accounting for the discrepancy between this study and our findings. Indeed, SPHK activity and S1P levels were recently assayed in various tissues of knockout mice lacking the SPHK1 isoform (Allende *et al.* 2004). In the absence of Triton X-100, SPHK activity was significantly decreased in kidney, spleen, and testis, but not in brain, liver, and platelets. This study suggests that the SPHK2 isoform predominates in some organs, including brain. It also confirms our northern blot data as well as previous reports, which have found SPHK2 to be widely distributed among mouse tissues (Liu *et al.* 2000). SPHK2 knockout mice have recently been described (Kharel *et al.* 2005; Mizugishi *et al.* 2005). However, S1P concentrations in various tissues of adult SPHK2 knockout mice were not reported, except for plasma S1P levels, which were 25% lower than in wild type mice. Interestingly, S1P levels were reduced in whole E11.5 embryos of both SPHK1 and SPHK2 knockout mice, but both single knockout developed normally (Mizugishi *et al.* 2005). In contrast, double knockouts showed severe defects in neurogenesis. It is interesting to note that although SPHK activity was reduced in various organs of SPHK1 knockout mice, S1P levels remained unaltered in all tissues examined with the exception of serum (Allende *et al.* 2004). Because no compensatory changes were observed in other enzymes involved in S1P metabolism, it is possible that studies of *SPHK1/SPHK2* mRNA or protein distribution, or enzyme assays using Triton X-100 underestimate the relative contribution of the SPHK2 isoform to the functional regulation of S1P levels.

Triton X-100 or 1 mol/L KCl inhibit SPHK activity in all brain regions investigated and the spatial distribution of SPHK activity matches that of *SPHK2* mRNA. The overlapping distribution of SPHK activity and *SPHK2* mRNA argues against the possibility that the regional variations in SPHK activity are accounted for by different levels of S1P degrading enzymes. Furthermore, SPHK activity was assayed in the presence of NaF and semicarbazide, known to inhibit sphingosine phosphatase and sphingosine-phosphate lyase, respectively (Gijsbers *et al.* 2001). The highly heterogeneous distribution of *SPHK2* mRNA suggests that S1P is involved in the functions of specific neurons or pathways, rather than in a housekeeping role common to different cell types or brain areas.

The brain is composed of a wide diversity of cells types. Because no SPHK2 antibody suitable for immunohistochemistry is currently available, double-labeling immunohistochemistry cannot be used to determine which cell type expresses SPHK2 protein. We therefore used real time RT-PCR to quantify *SPHK2* mRNA levels in cultured cells and found *SPHK2* mRNA in endothelial cells, glia, and cortical neurons. Although the protein expression pattern of these cells might differ in culture and *in vivo*, these results suggest that multiple brain cell types express SPHK2.

Several studies have documented an effect of SPHK activity and/or S1P in neuronal cell functions. S1P was originally shown to play a role in the differentiation and survival effects induced by nerve growth factor in PC12 cells (Edsall *et al.* 1997, 2001; Rius *et al.* 1997). In NG108-15 neuroblastoma, inhibition of SPHK accelerates thapsigargin-induced cell death, and exogenous S1P protects from carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone-

induced cell death (Chin *et al.* 2002). Similarly, S1P protects cultured mesencephalic neurons against glutamate-induced neurotoxicity (Shinpo *et al.* 1999). S1P also exerts fast actions on neurons or neuron-related cells, inducing neurite retraction and soma rounding in N1E-115 (Postma *et al.* 1996) and PC12 cells (Sato *et al.* 1997), and evoking a transient rises in intracellular Ca^{2+} in dorsal root ganglion neurons (Pollock *et al.* 2002). Interestingly, the distribution of SPHK2 mRNA in proliferation-associated areas of embryo brain matches the distribution of S1P₁ receptors (McGiffert *et al.* 2002) and the pattern of G-protein coupled S1P receptor activation (Harada *et al.* 2004) at this stage of development. Because we have shown that S1P stimulates the proliferation of neural precursor cells (Harada *et al.* 2004), it is tempting to speculate that the early expression of SPHK2 in brain areas with active neurogenesis is associated with a important role of this enzyme in brain development.

The increase in SPHK activity and *SPHK2* mRNA following OGD or ischemia is worthy of note. S1P is commonly thought of as proliferation-inducing and antiapoptotic agent [for review see (Spiegel *et al.* 1998)]. We have shown that S1P induces proliferation of neural progenitor cells (Harada *et al.* 2004), protects primary cortical neurons (Ding *et al.*, in preparation) and brain endothelial cells against OGD-induced cell death (Ding *et al.* 2004). However, SPHK2 has also been suggested to enhance apoptosis (Maceyka *et al.* 2005). These findings, taken together with the increased neuronal *SPHK2* mRNA expression *in vivo* and *in vitro* following ischemic insult, and the fact that S1P₁ receptor mRNA is dramatically up-regulated as early as 1 h following 30-min occlusion of a mouse MCA (Hayashi *et al.* 2003) suggest that S1P might play a role in recovery mechanisms following brain ischemia. It is unclear at this stage whether this role is beneficial or deleterious. Interestingly, the promoter regions of SPHK1 and SPHK2 both possess putative high-probability binding sites for two different networks of transcription factor, one consisting of EGRF, HEAT, and PAX5 families, and the other consisting of ETSF and NF- κ B families, suggesting that both gene products are co-expressed under various conditions [see (Simard and Gerzanich 2006) for a discussion]. Importantly, the promoter regions for both proteins have multiple consensus sequences for the transcription factor Egr-1 (which is induced by hypoxia and strongly up-regulated in cerebral ischemia) and for the transcription factor Sp1 (which is known to translocate to the nucleus following cerebral ischemia). It is therefore quite likely that both isoforms are unregulated under ischemic conditions.

In conclusion, this study shows that SPHK2 is highly expressed in rodent brain in a region-specific fashion, and indicates that this enzyme may be implicated in the interactions between different cell types (e.g. cerebrovascular blood flow regulation, cell proliferation, trophic effects) in normal adult brain, as well as during development or pathological conditions.

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Abbreviations used

BSA, bovine serum albumin; MCA, middle cerebral artery; OGD, oxygen glucose deprivation; PBS, phosphate-buffered saline; S1P, sphingosine-1-phosphate; SDS, sodium dodecyl sulphate; SPHK, sphingosine kinase.

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Fig. 1. Sphingosine kinase activity, assessed by measuring the conversion of [3H]sphingosine to [3H] sphingosine-1-phosphate (S1P) and thin layer chromatography (TLC) separation of the reaction products, was examined in different brain regions. (a) The levels of sphingosine kinase (SPHK) activity in different brain areas are expressed as percent of cerebellum activity (± SEM). (b) Representative fluorography image of a TLC plate showing the effect of 0.1% Triton X-100 on [3H]sphingosine to [3H]S1P converting activity of hippocampus and cortex homogenates. (c) Representative fluorography image of a TLC plate in which SPHK1 activity was determined in the presence of 0.25% Triton X-100, which inhibits SPHK2, whereas SPHK2 activity was determined with sphingosine added as a complex with 4 mg/mL BSA and

1 mol/L KCl, conditions in which SPHK2 activity is optimal and SPHK1 is strongly inhibited (Hait *et al.* 2005).

Fig. 2.

Tissue distribution of sphingosine kinase (SPHK)1A protein (a) and *SPHK2* mRNA (b) studied by western and northern blotting, respectively. (a) Proteins, collected from postnatal day 25 female mouse organs, were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, which were incubated with a polyclonal anti-sphingosine kinase 1 antibody. Intense SPHK1A-immunoreactive signal was observed in lung and spleen, while weak signal was found in brain tissue. In contrast, brain was among the tissue types expressing the largest amounts of *SPHK2* mRNA (b). Primary glial cells express more *SPHK2* mRNA than primary cortical neurons. This finding was confirmed using real time RT-PCR studies in which we examined the expression of *SPHK2* and *SPHK1* mRNA in primary cultures of mouse glial cells, brain endothelial cells and cortical neurons (c). mRNA contents are normalized for the amount of 18s mRNA in each cell type. All cell types contain at least 10 times more *SPHK2* than *SPHK1* mRNA. (d) Amplification efficiency plot for rat *SPHK1*, *SPHK2* and ribosomal 18S RNA. Serial dilutions of cDNA were amplified in an ABI 7000 Prism thermal cyclor. The threshold CT values were then plotted versus the logarithm of the relative concentration of cDNA. Based on the slopes, the calculated efficiencies were: 18S, 96%; *SPHK2*, 84%, *SPHK1*, 101%.

Fig. 3.

Sphingosine kinase (*SPHK*)2 mRNA levels were analyzed using real-time RT-PCR (a). Total RNA was extracted from various mouse brain regions; cDNA was synthesized using SuperScriptIII reverse transcriptase. Real-time PCR reactions were completed in triplicates using an ABI Prism 7000 from Applied Biosystems with 100 ng/mL cDNA aliquots as template, together with primers and probes for *Sphk2* and 18S ribosomal RNA (used to normalize mRNA expression). Highest mRNA concentrations were found in cortex. Lower amounts were seen in cerebellum and hippocampus, while mRNA was least abundant in striatum. In adult mouse brain, *in situ* hybridization (b) shows highest densities of *SPHK2* mRNA in cerebellum (granular layer: Cb_{Gr}) and internal cortical layers (IV and VI), as well as pyramidal layer of hippocampus (Hp_{Pyr}) and granular layer of dentate gyrus (DG_{Gr}). Lower densities are observed in striatum (Str). *SPHK2* mRNA distribution in mouse embryo brain strikingly differed from the adult mRNA pattern. (c) shows four coronal sections corresponding to levels 4, 8, 10, and 11 of the *Atlas of the Prenatal Mouse Brain* (Schambra *et al.* 1992) at gestational day 17. High levels of *SPHK2* mRNA were found in periventricular areas known to contain proliferating cells (ventricular/subventricular zone: VZ/SVZ), the ganglionic eminence (GE), as well as in the cortical plate (CP). Low levels of hybridization signal were seen in differentiating areas such as the striatum (Str).

Fig. 4.

(a) *in vivo* ischemia [middle cerebral artery (MCA) occlusion] enhances SPK activity in the ipsilateral brain regions. The MCA of 6 SV129 mice were occluded for 30 min as described in Methods. 24 h after reperfusion, ipsi- and contralateral brain regions were harvested and homogenized. Sphingosine kinase (SPHK) activity of the extracts was assayed by the conversion of [3 H]sphingosine to [3 H]sphingosine-1-phosphate. SPHK activity was markedly increased with respect to the contralateral side in the cortical and striatal penumbras (** $p < 0.01$), and to a smaller extent in the striatal core and hippocampus (* $p < 0.05$). (b) real-time RT-PCR analysis of *SPHK2* mRNA expression in mouse hippocampus, and cortical or striatal penumbra, following 20-min ischemia/24-h reperfusion, or 2-h ischemia/24-h reperfusion (n

= 8 for each group). 24 h after 20-min MCA occlusion, *SPHK2* mRNA levels were increased with respect to the contralateral side in cortical and striatal penumbras ($***p < 0.001$). 24 h after 2-h MCA occlusion, ipsilateral *SPHK2* mRNA levels were increased with respect to the contralateral hippocampus ($**p < 0.01$); *SPHK2* mRNA levels were decreased in striatal penumbra ($**p < 0.01$). (c) *in situ* hybridization labeling showing increased *SPHK2* mRNA expression in the pyramidal layer of ipsilateral CA1 field of hippocampus 24 h after 20 min MCA occlusion.