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Involvement of Sphingosine Kinase in TNF-α-stimulated Tetrahydrobiopterin Biosynthesis in C6 Glioma Cells*

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In C6 glioma cells, the sphingolipid second messenger ceramide potentiates expression of inducible nitric-oxide synthase (iNOS) induced by tumor necrosis factor α (TNF-α) without affecting GTP cyclohydrolase I (GTPCH), the rate-limiting enzyme in the biosynthesis of 6(*R*)-5,6,7,8-tetrahydrobiopterin (BH_4), a cofactor required for iNOS activity. TNF-α also stimulates sphingosine kinase, the enzyme that phosphorylates sphingosine to form sphingosine-1-phosphate (SPP), a further metabolite of ceramide. Several clones of C6 cells, expressing widely varying levels of sphingosine kinase, were used to examine the role of SPP in regulation of GTPCH and BH_4 biosynthesis. Overexpression of sphingosine kinase, with concomitant increased endogenous SPP levels, potentiated the effect of TNF-α on GTPCH expression and activity and BH_4 biosynthesis. In contrast, enforced expression of sphingosine kinase had no effect on iNOS expression or NO formation. Furthermore, *N*,*N*-dimethylsphingosine, a potent sphingosine kinase inhibitor, completely eliminated the increased GTPCH activity and expression induced by TNF-α. Surprisingly, we found that, although C6 cells can secrete SPP, which is enhanced by TNF-α, treatment of C6 cells with exogenous SPP or dihydro-SPP had no affect on BH_4 biosynthesis. However, both SPP and dihydro-SPP markedly stimulated ERK 1/2 in C6 cells, which express cell surface SPP receptors. Interestingly, although this ERK activation was blocked by PD98059, which also reduced cellular proliferation induced by enforced expression of sphingosine kinase, PD98059 had no effect on GTPCH activity. Collectively, these results suggest that only intracellularly generated SPP plays a role in regulation of GTPCH and BH_4 levels.

6(*R*)-5,6,7,8-Tetrahydrobiopterin (BH_4)[1] is the obligate cofactor for the aromatic L-amino hydroxylases and is also required for activity of all nitric-oxide synthase isoforms (reviewed in Ref. 1). It has been proposed that BH_4 may also have cofactor-independent roles (2), including inhibition of cytokine-induced apoptosis (3) and stimulation of dopamine release (4). Cellular levels of BH_4 are regulated by the activity of GTP cyclohydrolase I (GTPCH), the first and rate-limiting enzyme in the BH_4 biosynthetic pathway (5). Its expression is increased by proinflammatory cytokines, such as IFN-γ and TNF-α, and is coordinately regulated with cytokine-inducible nitric-oxide synthase (iNOS) (6). BH_4 binds to NOS monomers, promoting their dimerization and subsequent activation (7), and recent crystallographic analysis suggests that it may play a direct role in the NOS reaction in a radical form (8).

GTPCH, a homodecamer of 30-kDa subunits arranged as two pentamers facing one another (9), catalyzes the rearrangement of GTP to dihydroneopterin triphosphate. This intermediate is then converted to BH_4 in two subsequent reactions catalyzed by 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase, respectively, neither of which are rate-limiting. The signal transduction pathways that regulate induction of GTPCH are not well understood, although transcription factors such as NF-κβ and AP-1 are required for GTPCH mRNA expression (10) and recently, 5.8 kb of the rat GTPCH promoter have been cloned and found to contain a number of potential cytokine responsive elements (11). Although it is well established that iNOS induction is dependent on the redox-sensitive transcription factor NF-κB, we previously showed that NF-κβ was not required for induction of GTPCH in rat C6 glioma cells, a convenient astrocyte model, when stimulated with combinations of proinflammatory cytokines (12). Furthermore, whereas increases in levels of the sphingolipid metabolite ceramide induced by TNF-α played a role in iNOS induction, ceramide did not mimic the stimulatory effect of TNF-α on GTPCH expression. This led us to suggest that TNF-α regulates iNOS and GTPCH expression by divergent pathways and that TNF-α stimulates GTPCH transcription in C6 cells via a ceramide- and NF-κB-independent pathway.

Sphingolipid metabolites, especially ceramide (*N*-acyl-sphingosine) and its further metabolite, sphingosine-1-phosphate (SPP), have been implicated as mediators of TNF-α actions (reviewed in Refs. 13–15). In many cell types, TNF-α increases ceramide levels by activating sphingomyelinase, which hydrolyzes plasma membrane sphingomyelin (14, 16). However, in certain cells, such as human umbilical vein endothelial cells

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[1] The abbreviations used are: BH_4, 6(*R*)-5,6,7,8-tetrahydrobiopterin; DMEM, Dulbecco's modified Eagle's medium; EDG, endothelial differentiation gene; Egr-1, early growth response-1; ERK, extracellular signal regulated kinase; GTPCH, GTP cyclohydrolase I; IL-1β, interleukin-1β; IFN-γ, interferon-γ; iNOS, inducible nitric-oxide synthase; RT, reverse transcriptase; SPP, sphingosine-1-phosphate; TNF-α, tumor necrosis factor α; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ceramide, *N*-acyl-sphingosine; EB, extraction buffer; DTT, dithiothreitol; SPHK, sphingosine kinase; DMS, *N*,*N*-dimethylsphingosine; LDH, lactate dehydrogenase; FGF-2, fibroblast growth factor 2; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; GPCR, G protein-coupled receptor.

(17, 18), hepatocytes (19), and neutrophils (20), TNF-α stimulates sphingosine kinase, the enzyme that catalyzes the phosphorylation of sphingosine to form SPP.

It is now well established that the bioactive lipid SPP regulates vital biological processes, including growth, survival, cell locomotion, angiogenesis, vascular maturation, inflammation, neurite retraction, and soma rounding (reviewed in Refs. 21–24). SPP has been suggested to have novel dual modes of action: It is the ligand of G protein-coupled receptors (GPCRs), known as the Endothelial Differentiation Gene-1 (EDG-1) family, which includes EDG-1, -3, -5, -6, and -8 (21–24), and it functions intracellularly, acting through as yet unidentified targets (25). The EDG-1 receptors are highly specific and are only activated by SPP and dihydro-SPP, which has the same structure as SPP but lacks the 4,5-*trans* double bond. Dihydro-SPP, however, does not mimic all of the effects of SPP, especially those related to cell survival (26), supporting the notion that SPP also has non-EDG receptor-mediated actions.

As TNF-α stimulates GTPCH expression via a ceramide-independent pathway (12) and increases SPP levels (17–19), it was of interest to investigate the role of sphingosine kinase and SPP in the regulation of GTPCH and BH_4 biosynthesis. To this end, we generated several clones of stably transfected C6 cells that express varying levels of SPHK with concomitant increased endogenous SPP levels. TNF-α stimulated BH_4 synthesis to a much greater extent in C6 cells expressing the highest sphingosine kinase activity. Surprisingly, we found that, although C6 cells can secrete SPP, only SPP generated intracellularly in response to TNF-α stimulated GTPCH activity and BH_4 biosynthesis.

EXPERIMENTAL PROCEDURES

Reagents—Murine recombinant IL-1β was purchased from Invitrogen (Gaithersburg, MD). Recombinant rat TNF-α was from R&D Systems (Minneapolis, MN). Bovine intestinal alkaline phosphatase was purchased from Calbiochem (La Jolla, CA). Mouse macrophage polyclonal iNOS antibody and mouse macrophage-positive controls were purchased from Transduction Laboratories (Ann Arbor, MI). Peroxidase-labeled goat anti-rabbit IgG was from KPL (Gaithersburg, MD). All electrophoresis and Western blot materials were from NOVEX (Invitrogen, Carlsbad, CA).

Cell Culture—C6 cells were obtained from ATCC (Manassas, VA) and cultured in Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml) (Sigma Chemical Co.) at 37 °C in a humidified atmosphere of 95% air, 5% CO_2. Murine SPHK1 (GenBank™ accession number AF068748) was subcloned by PCR into pcDNA3.1 (Invitrogen, Carlsbad, CA) with an N-terminal c-Myc epitope tag as previously described (27). Plasmids were transfected into cells using LipofectAMINE Plus (Invitrogen, Gaithersburg, MD) according to the manufacturer's instructions, and stable transfectants were selected in growth medium containing 1 g/L G418 (Invitrogen).

In preliminary studies, we found that multiple passages of C6 cells altered their responses to cytokines. Thus, all experiments were performed on a freshly thawed batch of cells that had been passaged only twice, then aliquoted and stored frozen in liquid nitrogen vapor. Prior to each experiment, cells were defrosted and grown in 175-cm^2 flasks until 80–90% confluent, trypsinized, and then seeded at 100,000 cells/well in 6-well plates (3 ml of medium). After 48 h, cells were serum-starved for 4 h prior to treatments. All cytokine solutions were prepared according to the suppliers' instructions.

Measurement of Sphingosine Kinase Activity—After various treatments, cells were washed twice with PBS and harvested by scraping in buffer A (0.1 M Tris-HCl (pH 7.4) containing 20% (v/v) glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM Na_3VO_4, 15 mM NaF, 10 μg/ml leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxypyridoxine). Cells were lysed by freeze-thawing three times, and cytosolic fractions were prepared by centrifugation at 13,000 $\times$ *g* for 20 min. 100 μl of cytosol (20–50 μg) was incubated with 10 μl of sphingosine (1 mM), prepared as a sphingosine-BSA complex (4 mg/ml BSA), and [γ-^{32}P]ATP (Amersham Biosciences, Inc., Arlington Heights, IL) and sphingosine kinase activity measured as previously described (28).

Measurement of SPP—C6 cells were washed with PBS and scraped in 1 ml of methanol containing 2.5 μl of concentrated HCl. Lipids were extracted by adding 2 ml of chloroform/1 M NaCl (1:1, v/v) and 100 μl of 3 N NaOH, and phases were separated. The basic aqueous phase containing SPP, and devoid of sphingosine, ceramide, and the majority of phospholipids, was transferred to a siliconized glass tube. The organic phase was re-extracted with 1 ml of methanol/1 M NaCl (1:1, v/v) plus 50 μl of 3 N NaOH. Mass levels of SPP in the aqueous phase were measured as described previously (29). Total phospholipids in the organic phase were determined exactly as previously described (28).

Measurement of [^{32}P]SPP Release—C6 cells were incubated with 40 μCi/ml [^{32}P]orthophosphate (Amersham Biosciences, Inc.) in serum-containing medium for 48 h to label phospholipid pools to isotopic equilibrium. Cells were then washed and incubated in serum-free medium for the indicated times. Cells and media were extracted in alkaline conditions as described above, followed by back extraction into chloroform/methanol/conc. HCl (100:100:1, v/v). [^{32}P]SPP was resolved by TLC in chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v) and quantified with a Molecular Dynamics Storm PhosphorImager (Sunnyvale, CA).

Extraction and Analysis of Lipids—After metabolically labeling cells with $^{32}P_i$, lipids were extracted as described before (28) and separated by two dimensional-TLC on Silica Gel 60-G. Plates were developed in the first dimension with chloroform/methanol/ammonium hydroxide (13:7:1, v/v) and with chloroform/methanol/water/acetic acid (30:30:2:5, v/v) in the second dimension. Phospholipids were located by autoradiography and quantified with a PhosphorImager.

Measurement of BH_4 and GTPCH Activity—Cells were washed with ice-cold PBS (pH 7.4), detached by trypsinization, then pelleted in a microcentrifuge at 10,000 $\times$ *g* for 3 min. Cell pellets were washed twice with 1 ml of ice-cold PBS and resuspended in 250 μl of extraction buffer (EB) containing 50 mM Tris, pH 7.4/1 mM EDTA/1 mM DTT. Cell suspensions were sonicated on ice with a fine-tipped probe sonicator for 15 s, lysates were cleared at 10,000 $\times$ *g* for 3 min, supernatants were collected, and the protein concentrations were determined using Coomassie Blue Plus reagent (Pierce, Rockville, IL).

BH_4 was measured as previously described (12) with minor modifications. In brief, 50 μl of cell lysate was diluted to 80 μl with EB buffer, then mixed with 20 μl of 1 M H_3PO_4/1.5 M $HClO_4$ (1:1, v/v). Approximately 10 mg of MnO_2 was added to oxidize reduced pterins to their fluorescent aromatic forms. After 20 min at room temperature, samples were centrifuged at maximum speed in a microcentrifuge for 5 min. Supernatants were removed, and biopterin measured by reverse-phase high performance liquid chromatography with fluorescence detection (30).

GTPCH activity was measured essentially as previously described (30). In brief, to 30 μl of lysate was added 5 μl of 0.5 M Tris-HCl (pH 7.4), 5 μl of 10 mM DTT, 5 μl of 10 mg/ml BSA, and 5 μl of 10 mM GTP. Samples were incubated for 2 h at 37 °C and placed on ice, and the reactions were terminated by the addition of 5 μl of 1 M HCl, followed by 5 μl of iodine reagent (1% I_2/2% potassium iodide (w/v)). After 20 min at room temperature in the dark, 5 μl of 2% ascorbic acid (w/v) was added followed by 10 μl of 2 M Tris base. Neopterin triphosphate was then dephosphorylated by incubation for 30 min at 37 °C with 10 units of bovine intestinal alkaline phosphatase, followed by addition of 50 μl of 1 M H_3PO_4 to terminate the reactions. Samples were cleared by centrifugation, and neopterin was measured by reverse phase high performance liquid chromatography with fluorometric detection (30).

RT-PCR—Total RNA was isolated from confluent cultures with TRIzol reagent (Amersham Biosciences, Inc.) according to the manufacturer's directions. RNA (1 μg) was converted to cDNA with random hexamers and Omniscript reverse transcriptase according to the manufacturer's instructions (Qiagen, Valencia, CA). cDNA was amplified by PCR as previously described (12). The following forward and reverse PCR primers were used (predicted product size): GTPCH fwd, 5′-GGATACCAGGAGACCATCTCA-3′; GTPCH rev, 5′-TAGCATCCTGCTAGTGACAGT-3′ (372 bp); actin fwd, 5′-TTGTAACCAACTGGGACGATATGG-3′; actin fwd, 5′-GATCTTGATCTTCATGGTGCTAGG-3′ (743 bp).

PCR products were resolved on 2% agarose gels containing ethidium bromide and visualized by UV fluorescence. Reaction conditions were optimized in preliminary experiments so that amplifications were within the logarithmic phase and yields were approximately linear with input cDNA concentration. To ensure that contaminating genomic DNA was not being amplified, PCR was also performed without reverse transcriptase treatment.

Nitrite Determination—Nitrite, the stable oxidation product of NO and an index of iNOS activity, accumulates in the media and was measured essentially as described previously (12).

Western Analysis—Aliquots of cell lysates containing 20 μg of protein were concentrated using solvent phase partition. In brief, lysates were diluted to 450 μl with H_2O and mixed with 1 ml of chloroform:methanol (1:1) to give a final ratio of 1:1:0.9. The mixture was vortexed and then centrifuged in a microcentrifuge at maximum speed for 3 min to separate the phases. With this solvent combination, the proteins aggregate at the interphase. 700 μl of the upper phase was removed without disturbing the interphase, discarded, and an equivalent volume of methanol added back to the lower phase. The protein aggregates were pelleted at maximum speed for 10 min in a microcentrifuge. Supernatants were carefully aspirated, and the pellets were dried at 50 °C. Pellets were resuspended in 25 μl of 1× LDS NuPAGE sample buffer (NOVEX) containing 50 mM DTT, then heated at 100 °C for 10 min. Proteins were resolved on 4–12% Bis/Tris NuPAGE gels for 90 min at 175 V and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) at 100 V for 60 min in NOVEX transfer buffer at 4 °C. NuPAGE antioxidant (1:1000, v/v) was added to assist with transfer of high molecular weight proteins. Membranes were blocked with 10% (w/v) nonfat dry milk/0.02% (v/v) azide/0.05% (v/v) Tween 20 for at least 1 h at room temp, or overnight at 4 °C. After extensive washing with wash buffer (KPL), membranes were incubated with iNOS antibody (1:7500) for 2 h in milk diluent (KPL) and then with peroxidase-conjugated secondary antibody (1:3000) for 1 h. Membranes were extensively washed, and bands were visualized by enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA). For analysis of total and phosphorylated ERK1/2, 80–90% confluent cultures of C6 cells in 10-cm dishes were washed with ice-cold PBS and scraped into 500 μl of lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM sodium orthovanadate, 4 mM sodium pyrophosphate, 100 mM NaF, 1:500 protease inhibitor mixture (Sigma)). Samples were incubated on ice for 20 min and centrifuged at 14,000 × *g* for 15 min. Equal amounts (20 μg) of cytosol proteins were separated by SDS-PAGE, and ERK activation was determined with phospho-ERK1/2 antibodies (New England BioLabs).

Analysis of Cell Growth—Stably transfected C6 cells (1000 cells) were plated in 24-well plates in DMEM containing 10% fetal bovine serum. After 24 h, cells were washed with DMEM and then grown in serum-free medium. At the indicated times, cells were washed with PBS, fixed with methanol for 10 min, and stained with crystal violet. Incorporated dye was dissolved in 700 μl of 0.1 M sodium citrate (pH 4.0) in 50% ethanol, and the absorbance was measured at 540 nm.

RESULTS

Overexpression of Sphingosine Kinase in C6 Cells Increases SPP Levels—Previously, we found that, although TNF-α-induced expression of iNOS and subsequent NO production were mediated in part by increases in ceramide levels, GTPCH expression and activity and levels of the iNOS cofactor BH_4 were not increased in parallel (12). Because ceramide can be readily converted to sphingosine and then SPP, it was of interest to determine whether a shift in the balance of cellular sphingolipid metabolites toward SPP might play a role in regulating the levels of BH_4. To examine the possibility of a link between SPP levels and BH_4 synthesis, C6 cells were stably transfected with c-*Myc*-tagged SPHK1 and multiple clones were analyzed for their respective SPHK activities and SPP levels. Three clonal lines were selected for further study with low, intermediate, and high levels of recombinant SPHK1 expression, designated C6-A, C6-B and C6-C, respectively (Fig. 1*A*). Basal SPP levels in these clones somewhat mirrored the differences in SPHK activities (Fig. 1*B*). Moreover, in the C6-C high SPHK1 cells, sphingosine levels were concomitantly reduced to 0.13 ± 0.01 from 0.2 ± 0.01 pmol/nmol phospholipid in the vector transfectants. Western blot analysis with anti-c-*Myc* antibody revealed that the different levels in SPP and SPHK activity resulted from differing levels of SPHK protein expression (Fig. 1*C*).

Surprisingly, enforced expression of SPHK1 did not result in any changes in ceramide levels, although treatment with TNF-α increased ceramide levels by 50% in C6-C cells. In agreement, TNF-α induced a significant reduction in ^{32}P-labeled sphingomyelin in these cells. However, two-dimensional TLC analysis did not reveal any differences in ^{32}P-labeled phospholipids comigrating with phospholipids of C6 vector cells compared with C6-C cells, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and sphingomyelin (data not shown).

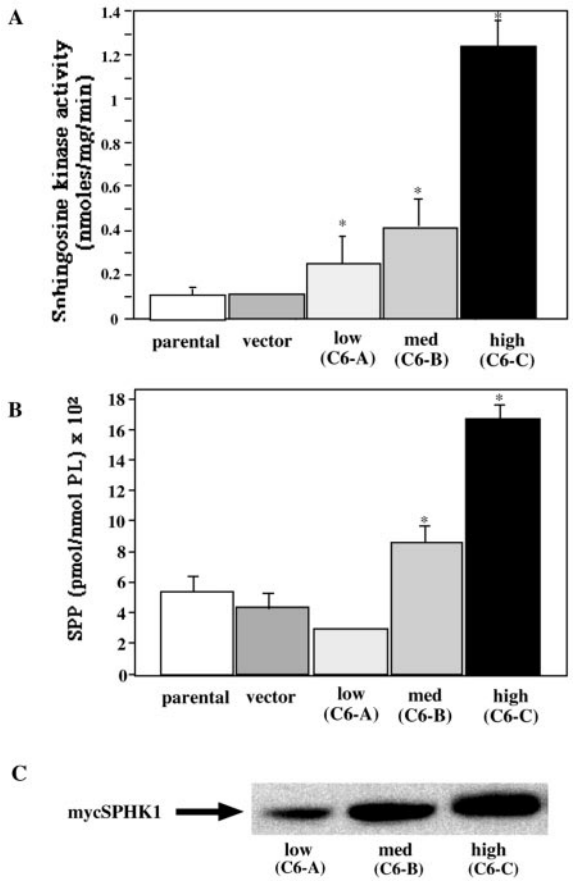

FIG. 1. **Expression of SPHK1 increases sphingosine kinase activity and SPP levels.** Parental or C6 cells stably transfected with empty vector or with c-*myc*-SPHK1. Stable clones expressing low (*C6-A*), medium (*C6-B*), and high (*C6-C*) levels of SPHK1 expression were selected. *A*, sphingosine kinase activity; *B*, SPP levels; and *C*, c-*myc*-SPHK1 protein expression were determined as described under "Experimental Procedures."

TNF-α, a proinflammatory cytokine, has previously been shown to stimulate SPHK activity and SPP levels (17), as well as to stimulate BH_4 biosynthesis in various types of cells (10, 31, 32), including C6 cells (6, 33). In agreement, TNF-α increased SPHK activity and further increased SPP (data not shown) in the transfected C6 cell lines (Fig. 2, *A* and *B*). Treatment with TNF-α activated endogenous SPHK activity in empty vector-transfected cells after 15 min, which returned to basal levels by 30 min, typical of changes in SPHK activity previously observed in other cell types following stimulation with various agonists (17, 18, 34). TNF-α also rapidly induced a robust 3-fold activation of SPHK activity in C6-C cells. After stimulation with TNF-α, SPHK activity in C6-C cells peaked at around 5 min, remained elevated for up to 30 min, an decreased thereafter (Fig. 2*B*). Thus, in agreement with previous results in transfected HEK 293 cells (35), recombinant SPHK1 is also activated by TNF-α in C6 cells.

Effect of TNF-α on BH_4 Biosynthesis in C6 Cells Overexpressing SPHK1—Previously, in accordance with other studies (6, 10, 32), we showed that TNF-α was not able to maximally stimulate BH_4 biosynthesis in C6 cells unless added together with other proinflammatory cytokines, such as IFN-γ and IL-1β (12). In agreement, in vector-transfected C6 cells, TNF-α only slightly stimulated BH_4 biosynthesis. Surprisingly, we

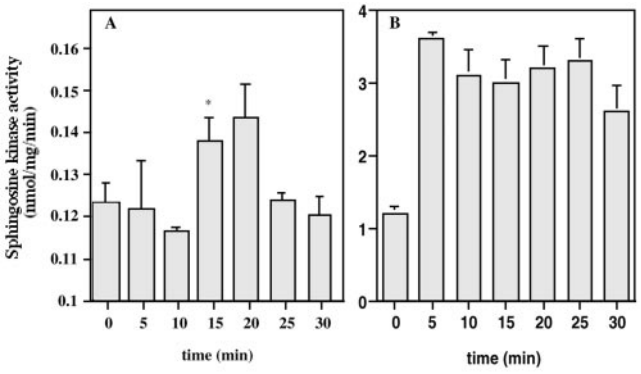

FIG. 2. **TNF-α increases SPHK activity in vector and SPHK1-transfected cells.** Cells expressing (*A*) C6 vector or (*B*) C6-C high SPHK1 were stimulated with TNF-α (10 ng/ml) for the indicated times. *In vitro* sphingosine kinase activity was determined by measurement of the conversion of sphingosine to SPP as described under "Experimental Procedures." The data are expressed as nanomoles of SPP formed/mg/min and are means ± S.E. of two independent experiments carried out in triplicate. *Asterisks* indicate statistically significant differences compared with controls ($p \leq 0.05$).

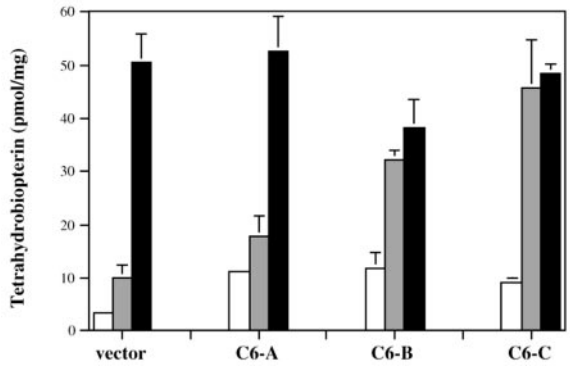

FIG. 3. **Effect of SPHK1 overexpression on TNF-α-induced BH_4 biosynthesis.** Stable clones of vector transfected C6 cells (*vector*) and cells expressing low (*C6-A*), medium (*C6-B*), and high (*C6-C*) levels of SPHK1 expression were treated for 16 h without (*open bars*) or with TNF-α (*hatched bars*, 10 ng/ml) or with TNF-α in the presence of IFN-γ (40 units/ml) and IL-1β (8 units/ml) (*filled bars*). Cells were harvested and cellular BH_4 levels were measured as described under "Experimental Procedures." The data are expressed as means ± S.E. of three independent experiments carried out in triplicate.

found that TNF-α alone significantly increased BH_4 biosynthesis in SPHK1-expressing C6 cells, and the stimulation was proportional to the intracellular levels of SPP. Thus, when C6-C cells, the clone with the highest level of SPP, were treated with TNF-α alone, the BH_4 levels increased to the maximum level found with combinations of cytokines. Whereas the response was somewhat lower in C6-B cells, and much lower in the low SPHK1-expressing C6-A cells and in vector-transfected cells, both of which do show a significant increase in SPP in response to TNF-α (Fig. 3). This result suggests that activation of SPHK and formation of SPP may play a role in regulating BH_4 synthesis. Interestingly, the combination of IFN-γ and IL-1β with TNF-α, which maximally stimulates GTPCH and BH_4 synthesis, does not further activate SPHK or increase SPP levels, indicating that these cytokines can potentiate GTPCH by non-SPP-dependent pathways.

SPHK1 Increases GTPCH Activity and Expression without Affecting iNOS—Because the response to TNF-α was greatest in C6-C cells, this clone was used to examine the mechanism whereby SPHK1 overexpression potentiates the effect of TNF-α on BH_4 levels. A large body of evidence indicates that cellular BH_4 levels are regulated by the activity and expression of GTPCH, the rate-limiting enzyme of *de novo* BH_4 biosynthesis (5). Thus, levels of GTPCH activity were next examined. Although basal GTPCH activity was the same in vector-transfected and in C6-C cells, and was similar to the activity previously reported in untransfected C6 cells (12), TNF-α treatment increased GTPCH activity by more than 4-fold in C6-C cells, yet had little effect on GTPCH activity in the vector or parental cells (Fig. 4*A*). The GTPCH activity in TNF-α-treated C6-C cells was increased nearly to the maximum attainable in parental cells after stimulation with a combination of TNF-α, IFN-γ, and IL-1β (12). This observation correlates well with the measured changes in BH_4 levels after stimulation with TNF-α (Fig. 3), in agreement with its role as the rate-limiting enzyme in BH_4 biosynthesis.

We previously found that GTPCH mRNA is constitutively expressed at low levels in C6 cells and that its expression is increased by IFN-γ/IL-1β, in combination with TNF-α, but not by TNF-α alone (12). To investigate whether SPHK1 increases GTPCH activity by increasing its expression in response to TNF-α, empty vector-transfected and C6-C cells were stimulated with TNF-α and then GTPCH mRNA was measured by RT-PCR. As shown in Fig. 4*B*, TNF-α treatment only slightly

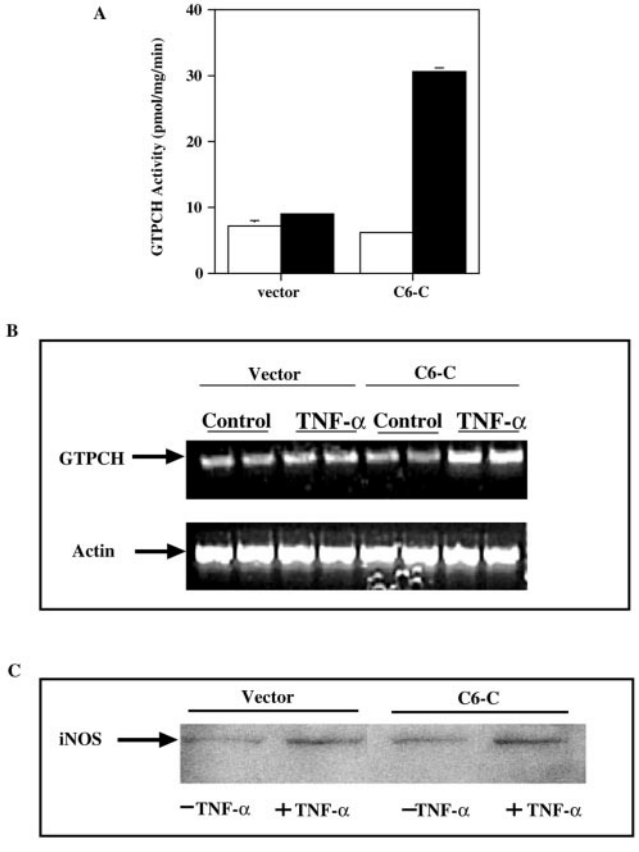

FIG. 4. **SPHK1 overexpression regulates TNF-α-induced GTPCH activity and mRNA expression but not iNOS.** Vector and C6-C SPHK1-transfected cells were stimulated without (*open bars*) or with TNF-α (*filled bars*, 10 ng/ml). Cells were harvested after 16 h and (*A*) GTPCH activity, (*B*) GTPCH and actin mRNA expression, and (*C*) iNOS protein were determined as described under "Experimental Procedures." Similar results were obtained in at least two independent experiments.

increased GTPCH expression in empty vector cells. However, GTPCH expression was greatly enhanced in the TNF-α-stimulated C6-C cells overexpressing high levels of SPHK1. Taken together, these data indicate that sustained activation of SPHK and high intracellular levels of SPP potentiate the effect of TNF-α on GTPCH expression.

Synthesis of BH_4, a required cofactor for iNOS activity, is

usually coordinately regulated with iNOS expression (36). Moreover, we and others (12, 37) have found that ceramide, a sphingolipid metabolite upstream of SPP, is involved in the regulation of iNOS expression. Because TNF-α has been demonstrated to increase ceramide levels (38) and iNOS expression in C6 cells (12, 33), we investigated whether iNOS expression was also enhanced in SPHK1-expressing C6-C cells. In agreement with previous reports (10, 12, 37), iNOS protein, determined by immunoblotting, is expressed at only very low levels in empty vector and C6-C cells (Fig. 4*C*). The basal expression could only be detected when blots were exposed for extended periods. Crucially, however, iNOS protein levels were only slightly enhanced to a similar extent by TNF-α in both cell lines and thus did not parallel the stimulatory effect of SPHK on GTPCH expression and activity (Fig. 4, *A* and *B*). In agreement, we were unable to detect formation of nitrite, an oxidation product of NO, in the media of vector or C6-C cells even after stimulation with TNF-α (<1 μM). Whereas treatment with the combination of TNF-α, IL-1β, and IFN-γ induces a nitrite production of $\sim$30 μM (data not shown). Taken together, these results indicate that SPHK and SPP may play a specific role in TNF-α-induced GTPCH expression but not in iNOS induction and may be part of the divergent sphingolipid signaling pathways previously noted to up-regulate iNOS and its cofactor BH_4, respectively (12).

Inhibition of SPHK Abrogates TNF-α-induced GTPCH Activity and Expression—To further verify that the observed effects on BH_4 and GTPCH were due to increased formation of SPP, we used the potent competitive SPHK inhibitor *N,N*-dimethylsphingosine (DMS) (39). In agreement with several previous studies (17, 28, 39–43), DMS drastically inhibited sphingosine kinase activation and SPP formation (data not shown). DMS also, in a dose-dependent manner, potently inhibited TNF-α-stimulated GTPCH activity (Fig. 5*A*). Whereas 2 μM DMS inhibited the response to TNF-α by $\sim$40%, 5 μM DMS completely abolished TNF-α-induced GTPCH activity (Fig. 5*A*). Because DMS is known to be cytotoxic for many types of cells and to induce apoptosis (44), cell viability was concomitantly determined by measurements of lactate dehydrogenase (LDH) released into the media. There were no significant differences in LDH activity between control and DMS-treated cells, indicating that the inhibitory effect of DMS was not due to cell death. RT-PCR analysis of GTPCH expression under the same conditions confirmed that DMS significantly reduced TNF-α-induced GTPCH expression (Fig. 5*B*).

The Effects of SPP on BH_4 and GTPCH Are Not Mediated via EDG-1 Receptors—The EDG-1 family of SPP receptors, which now include EDG-1, -3, -5, -6, and -8, are widely expressed in the CNS (reviewed in Refs. 21–24). Recently, it was shown that EDG-1 and EDG-5, but not EDG-3, are expressed in C6 cells and that binding of SPP to these GPCRs stimulates ERK (45, 46). It is possible that the effect on BH_4 biosynthesis of overexpression of SPHK1 and increased SPP levels could be due to either intracellular actions or possibly due to secretion and activation of cell surface receptors for SPP. In agreement with previous results (45, 46), the highest levels of EDG-5 expression were detected by RT-PCR with somewhat lower expression of EDG-8, followed by EDG-1, whereas expression of EDG-3 or EDG-6 was undetectable (data not shown). There were no obvious differences in expression of the EDG family members in parental, vector, and C6-C clone. We next examined the possibility that C6-SPHK1 cells may secrete SPP into the medium, which could then bind to and activate EDG-5 or EDG-8. We were able to readily detect secretion of [^{32}P]SPP by C6 glioma cells prelabeled to isotopic equilibrium with [^{32}P]$_i$ within 4 h, and secretion markedly increased by 24 h (Fig. 6*A*).

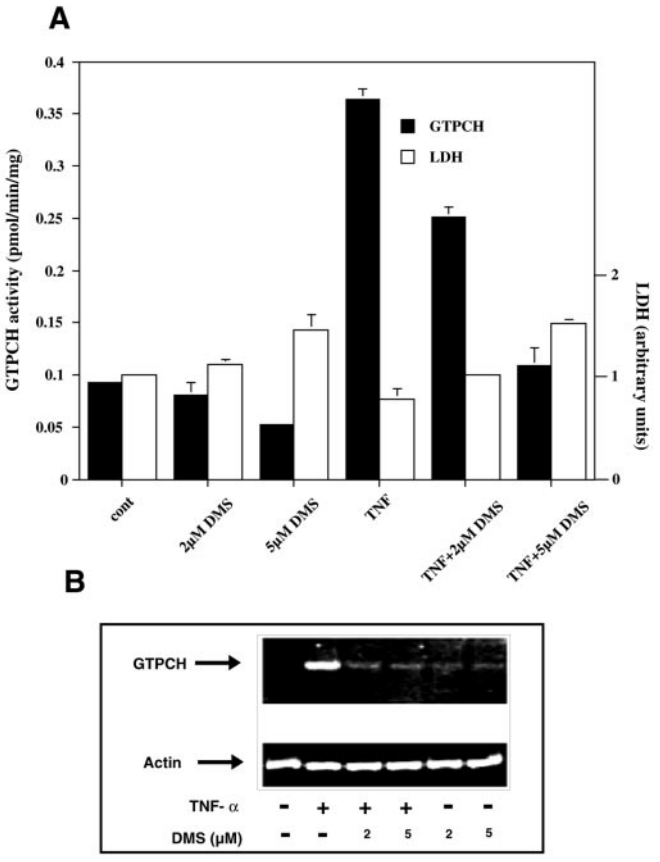

FIG. 5. **The sphingosine kinase inhibitor, *N,N*-dimethylsphingosine, inhibits TNF-α-induced GTPCH activity and expression in SPHK1 overexpressing cells.** C6-C cells expressing high levels of SPHK1 activity were pretreated for 1 h with DMS at the indicated concentrations. *A*, GTPCH activity and LDH released into the media were determined as described under "Experimental Procedures." *B*, in duplicate cultures, GTPCH and actin mRNA expression was measured by RT-PCR. Similar results were obtained in two independent experiments.

Moreover, treatment of C6-C cells with TNF-α not only stimulated sphingosine kinase (Fig. 1*B*) but also increased formation of cellular SPP and enhanced its secretion (Fig. 6*B*).

Because C6 cells express SPP receptors and can secrete SPP, it was important to determine whether the effect of SPHK1 overexpression and increased SPP levels on BH_4 synthesis was due to intracellular actions or extracellular binding to one of these receptors. Surprisingly, exogenously added SPP at low or high concentrations had no significant effects on either BH_4 levels or GTPCH activity (data not shown) and did not potentiate the effect of TNF-α (Fig. 7*A*). Moreover, dihydro-SPP, which is structurally similar to SPP and only lacks the trans 4,5-double bond and binds to and activates EDG-5 and EDG-8, also did not enhance the effect of TNF-α on BH_4 biosynthesis (Fig. 7*A*).

ERK1/2 Does Not Regulate GTPCH Activity or Expression—It has previously been shown that exogenous SPP activates EDG-1 and EDG-5 in C6 cells, which are coupled to both pertussis toxin-sensitive G_i/G_o proteins and insensitive G_q/G_{11} proteins to stimulate ERK (45) leading to induction of early growth response-1 (Egr-1), an essential transcription factor for expression of fibroblast growth factor-2 (FGF-2), a factor that influences proliferation of astroglial cells (46, 47). Although exogenous SPP had no effect on BH_4 or GTPCH (Fig. 7*A*), in agreement with previous studies, it markedly activated ERK1/2 (Fig. 7*B*), suggesting that the EDG-1 family of receptors is indeed functional in these C6 cells. Both SPP and dihy-

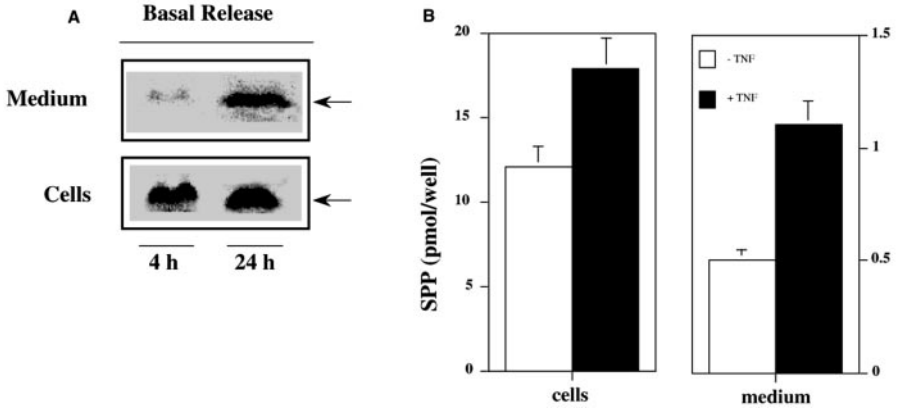

FIG. 6. **Secretion of SPP.** *A*, C6 cells grown on 6-well dishes were labeled to isotopic equilibrium with 40 μCi of [^{32}]P_i for 48 h. Cells were then washed and incubated in serum-free medium for 4 and 24 h, and [^{32}P]SPP was extracted from the cells and the media. The *arrow* indicates the location of standard SPP visualized with molybdenum blue spray. It should be noted that the total amount of [^{32}P]SPP in C6 cells was 10-fold greater than in the medium. Similar results were obtained in two independent experiments. *B*, C6-C cells were labeled to isotopic equilibrium with [^{32}]P_i for 48 h, treated without (*open bars*) or with 10 ng/ml TNF-α (*filled bars*) for 16 h, and SPP was extracted from the cells and the media. Data are expressed as picomoles per well and are means ± S.D. The level of SPP in vector-transfected cells is about 0.04 pmol/nmol phospholipid and increases by 1.28-fold after TNF-α treatment. There was no detectable SPP in the medium of the vector cells even after treatment.

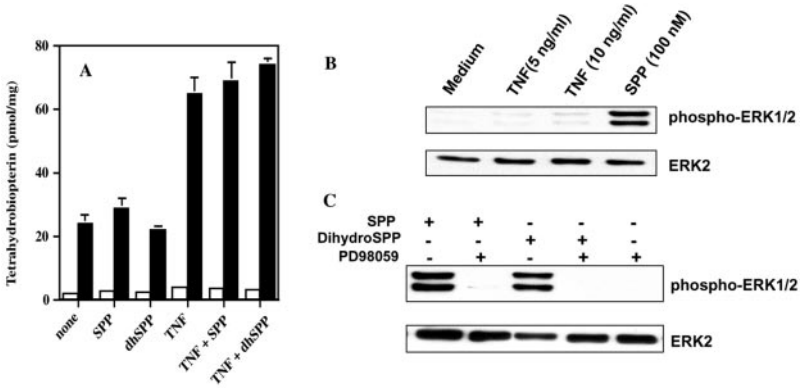

FIG. 7. **Exogenous SPP and dihydro-SPP do not affect BH_4 biosynthesis yet activate ERK1/2.** *A*, parental (*open bars*) and C6-C cells (*filled bars*) were treated for 16 h without or with TNF-α (10 ng/ml) in the absence or presence of SPP or dihydro-SPP (100 nM). Cells were harvested, and cellular BH_4 levels were measured as described under "Experimental Procedures." The data are expressed as means ± S.D. of triplicate determinations. Similar results were obtained in three independent experiments. *B*, C6-C cells were treated as in *A* with TNF-α, SPP, or dihydro-SPP for 5 min. Cytosolic fractions were prepared, and equal amounts of protein were analyzed by Western analysis using antibodies specific for phospho-ERK and then blots were stripped and reprobed with anti-ERK. *C*, where indicated, C6-C cells were preincubated with PD98059 (10 μM) for 45 min and then stimulated with SPP or dihydro-SPP (100 nM) for 5 min before measuring ERK1/2 activation as in *B*.

dro-SPP, but not TNF-α, rapidly activated ERK1/2 within 5–10 min. As expected, PD98059, an inhibitor of MEK, an upstream regulator of ERK1/2, completely prevented activation of ERK by SPP and dihydro-SPP (Fig. 7*C*).

It was of interest to determine whether ERK might be involved in regulating GTPCH and BH_4 levels. Thus, we also examined the effect of PD 98059 on GTPCH activity in C6-C cells stimulated with TNF-α. As shown in Fig. 8*A*, inhibition of Erk1/2 by PD 98059 had no effect on TNF-α-induced GTPCH activity, indicating that ERK1/2 is not involved in regulation of GTPCH in these cells. Previously, we (28) and others (48) have shown that overexpression of SPHK markedly stimulates proliferation of 3T3 fibroblasts. Similarly, stable expression of SPHK1 in C6-C cells had a dramatic stimulatory effect on proliferation of cells cultured in low serum media compared with vector cells (Fig. 8*B*). Moreover, this enhanced growth was reduced by the ERK inhibitor PD98059 (Fig. 8*C*). These results suggest that, although ERK is not important for the effect of SPHK1 on BH_4 biosynthesis, it seems to be important for the proliferative response induced by SPHK1 overexpression.

DISCUSSION

BH_4 synthesis in astroglial cells is stimulated by various proinflammatory cytokines, including TNF-α (6), which act by inducing expression of GTPCH, the rate-limiting enzyme in the *de novo* pathway for BH_4 biosynthesis. However, the mechanisms mediating these effects on GTPCH have not been fully elucidated. Here we demonstrate that stimulation of SPHK and generation of intracellular SPP by TNF-α is involved in regulation of GTPCH expression and activity. By studying several C6 clonal cell lines expressing varying levels of SPHK activity, we have been able to correlate BH_4 biosynthesis with SPHK activity and intracellular levels of SPP. Several lines of evidence implicate intracellular SPP itself in the potentiating effect of SPHK1 overexpression on TNF-α-stimulated GTPCH. First, the effect is correlated with sphingosine kinase activity and SPP production. Moreover, there were no detectable changes in any of the other phospholipids or lipid metabolites, except for a decrease in sphingosine, the precursor of SPP, in SPHK1 transfectants. Importantly, the SPHK inhibitor, DMS, blocked the potentiating effect of enforced expression of SPHK1.

Previously, we have suggested that TNF-α stimulates iNOS, which requires BH_4 for its activity, and GTPCH expression by divergent pathways. Although TNF-α stimulates iNOS expression via a ceramide-dependent pathway, TNF-α regulates GTPCH expression independently of ceramide generation. Our

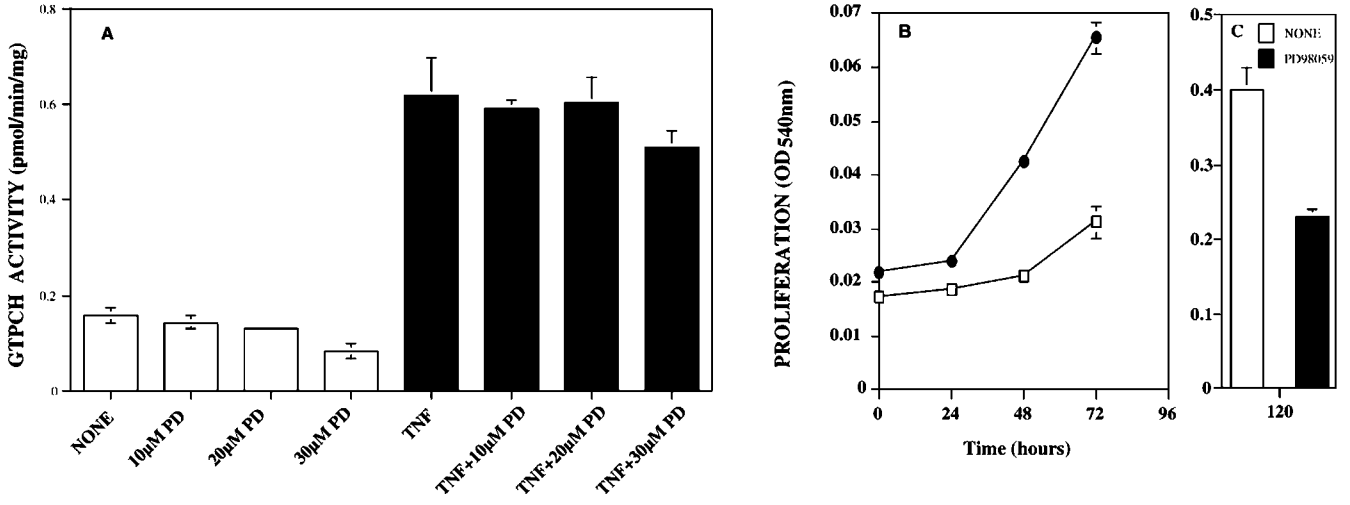

FIG. 8. **Differential effect of PD98059 on GTPCH activity and cellular proliferation in SPHK1-overexpressing C6 cells.** *A*, C6-C cells were pretreated for 45 min with PD98059 at the indicated concentrations. Cells were then stimulated without (*open bars*) or with TNF-α (10 ng/ml, *filled bars*), harvested 16 h later and GTPCH activity was determined as described under "Experimental Procedures." *B*, vector (*open symbols*) and C6-C SPHK1-transfected cells (*filled symbols*) were plated in 24-well tissue culture plates (1000 per well), cultured for the indicated days in the absence of serum, stained with crystal violet, and quantitated as described under "Experimental Procedures." Values are the means ± S.D. of six determinations. Similar results were obtained in at least two additional experiments. *C*, C6-C SPHK1-transfected cells were cultured for 5 days as described in *B* in the absence (*open bar*) or presence of 10 μM PD98059 (*filled bar*), and proliferation measured by the crystal violet assay.

findings in this study suggest that the sphingolipid metabolite SPP, but not ceramide, potentiates GTPCH mRNA expression induced by TNF-α. Conversely, TNF-α-induced SPHK activation and generation of SPP did not modulate expression of iNOS. Thus, TNF-α may induce the coordinate expression of iNOS and GTPCH via ceramide- and SPP-dependent pathways, respectively. Differential regulation of iNOS expression and levels of its cofactor BH_4 may be physiologically relevant as BH_4 has other important functions, especially in hydroxylation of the aromatic amino acids. Thus, cytokine-dependent induction of iNOS and BH_4 synthesis should be coordinately regulated only in cell types where NO production is important.

It has previously been suggested that SPP has enigmatic dual functions (25). Although it is now well established that SPP is the ligand for the EDG-1 family of GPCRs that couple to a variety of G proteins to regulate diverse biological functions (reviewed in Refs. 21–24), intracellular SPP, acting through as yet unidentified targets, regulates cell survival and calcium homeostasis (25, 49). Remarkably, in this study we found that exogenous SPP or dihydro-SPP, which bind to specific receptors of the EDG-1 family expressed by C6 cells, did not mimic the effect of overexpression of SPHK and increased intracellular SPP on GTPCH expression and activity or on BH_4 biosynthesis. However, similar to other reports (45–47), we were able to show that binding of SPP and dihydro-SPP to EDG-1 present on C6 cells markedly activated ERK. Previously, it has been shown that activation of ERK by SPP leads to induction of expression of Egr-1, one of the immediate early gene products required for expression of this essential transcriptional factor for FGF-2, an autocrine factor that stimulates proliferation of astroglial cells (46, 47). Interestingly, overexpression of SPHK1 not only potentiated TNF-α-induced BH_4 biosynthesis, but also, similar to its effect on fibroblasts (28, 48), enhanced proliferation of C6 cells. However, although the proliferative effect could be blocked by PD98059, an inhibitor of the ERK pathway, it had no effect on TNF-α-induced BH_4 biosynthesis. Thus, although some effects of SPP, *e.g.* ERK activation, are clearly mediated through interactions with EDG-1 receptors, BH_4 biosynthesis is only regulated by intracellularly generated SPP, and thus GTPCH, may be the first identified intracellular target of SPP.

In agreement with our previous results, we found that unstimulated C6 cells secreted SPP. Importantly, however, TNF-α not only stimulated SPHK and increased SPP levels, it also stimulated secretion of SPP. One of the important functions of astroglial cells is to support neuronal cells by secreting a variety of neurotrophic factors, such as FGF-2 and other peptide growth factors, and neurotransmitters (reviewed in Ref. 50). Thus, SPP may be another factor provided by glial cells *in vivo* to promote neuronal cell survival and morphology rearrangements and remodeling of the actin cytoskeleton during various stages of development. Similar to our finding, SPP-stimulated proliferation of murine striatal astrocytes, which express EDG-1 and EDG-3, was also blocked by U0126, an ERK kinase inhibitor, and it was suggested that this might have physiopathological consequences at sites of brain lesions and alterations of the blood-brain barrier (51).

It should be noted that, although C6 cells secrete SPP, which can act in an autocrine or paracrine manner through different members of the EDG-1 family present on both astroglial and neuronal cells, intracellularly generated SPP has distinct functions. Recently, we showed that SPP produced by activation of SPHK in response to platelet-derived growth factor can transactivate EDG-1, and this is a critical event in directed cell movement toward platelet-derived growth factor (52). In this study, we found that TNF-α, in a similar manner, can stimulate SPHK leading to production of SPP. SPP, in turn, can act in an autocrine or paracrine manner to regulate signaling pathways by binding to EDG-1 leading to cellular proliferation (47). Yet, in contrast, only intracellularly generated and not secreted SPP regulates BH_4 biosynthesis.

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