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Critical Role for Sphingosine Kinase-1 in Regulating Survival of Neuroblastoma Cells Exposed to Amyloid-β Peptide

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ABSTRACT

We examined the role of sphingosine kinase-1 (SphK1), a critical regulator of the ceramide/sphingosine 1-phosphate (S1P) biostat, in the regulation of death and survival of SH-SY5Y neuroblastoma cells in response to amyloid β (Aβ) peptide (25-35). Upon incubation with Aβ, SH-SY5Y cells displayed a marked down-regulation of SphK1 activity coupled with an increase in the ceramide/S1P ratio followed by cell death. This mechanism was redox-sensitive; *N*-acetylcysteine totally abrogated the down-regulation of SphK1 activity and strongly inhibited Aβ-induced cell death. SphK1 overexpression impaired the cytotoxicity of Aβ, whereas SphK1 silencing by RNA interference mimicked Aβ-induced cell death, thereby establishing a critical role for SphK1. We further demonstrated that SphK1 could mediate the well established cytoprotective action of insulin-like growth factor (IGF-I) against Aβ toxicity. A dominant-negative form of SphK1 or its pharmacological inhibition not only abrogated IGF-I-triggered stimulation of SphK1 but also hampered IGF-I protective effect. Similarly to IGF-I, the neuroprotective action of TGF-β1 was also dependent on SphK1 activity; activation of SphK1 as well as cell survival were impeded by a dominant-negative form of SphK1. Taken together, these results provide the first illustration of SphK1 role as a critical regulator of death and survival of Aβ-treated cells.

The sphingolipid metabolites ceramide and sphingosine 1-phosphate (S1P) have received much attention in the last decade as key regulators of cell death and survival (Hannun and Obeid, 2002; Spiegel and Milstien, 2003). Ceramide mediates a wide array of stress signals leading to growth arrest or cell death, whereas S1P exerts prosurvival capabilities by antagonizing ceramide effects. The opposing directions of ceramide- and S1P-mediated signaling gave birth to the concept of a ceramide/S1P biostat and the assumption that the balance between these two sphingolipids could determine whether a cell survives or dies (Cuvillier et al., 1996). The intracellular balance between ceramide and S1P is strongly regulated by sphingosine kinase-1 (SphK1), the enzyme that phosphorylates sphingosine (the catabolite of ceramide) to form S1P. When overexpressed, SphK1 promotes cell survival in response to stresses that increase ceramide content (Olivera et al., 1999; Edsall et al., 2001; Nava et al., 2002; Pchejetski et al., 2005; Bonhoure et al., 2006; Pchejetski et al., 2007) by shifting the ceramide/S1P biostat toward S1P. On the contrary, SphK1 down-regulation is associated with an accumulation of ceramide and has been correlated with cell death induced by anticancer treatments (Nava et al., 2000; Taha et al., 2004; Pchejetski et al., 2005; Bonhoure et al., 2006).

The amyloid-β peptide (Aβ), the main constituent of amyloid plaques, is believed to play a causative role in the neurodegenerative process occurring in Alzheimer's disease (Roher et al., 1993; Selkoe, 2001). Although Aβ-mediated neuronal cell death demonstrates biochemical characteristics of apoptosis, the molecular mechanism underlying Aβ toxicity remains largely undefined. It is noteworthy that in-

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ABBREVIATIONS: S1P, sphingosine 1-phosphate; SphK1, sphingosine kinase-1; Aβ, amyloid-β; IGF-I, insulin-like growth factor I; TGF-β, transforming growth factor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; TLC, thin-layer chromatography; NAC, *N*-acetylcysteine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; siRNA, small interfering RNA; n-SMase, neutral sphingomyelinase; CA074-Me, [L-3-*trans*-(propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester.

creased levels of ceramide have been found in the brain of patients with Alzheimer's disease (Han et al., 2002; Cutler et al., 2004; Satoi et al., 2005), thereby implying that ceramide accumulation could contribute to Alzheimer's disease pathogenesis. In addition, Aβ toxicity was recently shown to be linked with ceramide generation in both cell culture models (Ayasolla et al., 2004; Cutler et al., 2004; Jana and Pahan, 2004; Lee et al., 2004; Yang et al., 2004; Zeng et al., 2005) and an animal model (Alessenko et al., 2004).

Survival of neurons is dependent on extracellular signals from neurotrophic factors and related factors with trophic activity. Compelling data have revealed the potential involvement of insulin-like growth factor I (IGF-I) in Alzheimer's disease pathophysiology. Low serum levels of IGF-I is correlated with premature brain amyloidosis, and IGF-I has been found to regulate Aβ clearance from the brain (Carro et al., 2002; Carro and Torres-Aleman, 2004). Furthermore, IGF-I prevents Aβ-induced neuronal cell death (Doré et al., 1997; Niikura et al., 2001) including in SH-SY5Y cells (Wei et al., 2002). The TGF-β1 peptide growth factor also protects neurons from a variety of insults (Flanders et al., 1998) including Aβ (Chao et al., 1994; Prehn et al., 1996; Ren and Flanders, 1996; Ren et al., 1997). Levels of TGF-β1 are decreased in human Alzheimer's disease serum (De Servi et al., 2002) and TGF-β receptor expression reduced (Tesseur et al., 2006). Via genetic manipulation of TGF-β1 signaling, it has been demonstrated that reduction of TGF-β1 signaling in neurons of transgenic mice caused age-dependent neurodegeneration and promoted Alzheimer's disease-like pathologic conditions in a mouse model for Alzheimer's disease (Tesseur et al., 2006).

Herein, we report that Aβ treatment of SH-SY5Y cells triggered a strong inhibition of SphK1 activity coupled with an elevation of the ceramide/S1P biostat, in a redox-sensitive fashion. Knocking-down SphK1 by an RNA interference strategy mimicked the effects of Aβ, whereas its overexpression rendered cells resistant to Aβ. We further established that SphK1 could transduce the prosurvival action of both IGF-I and TGF-β1. Overall, this study strongly suggests that SphK1 could play a critical role in the regulation of Aβ-induced neuronal cell death and the neuroprotective effect of IGF-I and TGF-β1.

Materials and Methods

Cell Lines. SH-SY5Y cells (DSMZ, Braunschweig, Germany) were cultured in DMEM containing 10% fetal bovine serum (FBS) under a humidified atmosphere of 5% CO_2 at 37°C. Retinoic acid was used to induce neuronal differentiation (Fig. 1A) similar to primary cell cultures (Uberti et al., 1997; Datki et al., 2003), which was controlled by immunohistochemistry with MAP2 (Sigma-Aldrich) and anti-tau Alz50 (gift from Dr. Peter Davies) antibodies. FLAG epitope-tagged wild-type human SphK1 (hSphK1) cDNA and hSphK1 containing the G82D mutation, subcloned into pcDNA3* vector (Pitson et al., 2000), were used for stable transfection in SH-SY5Y cells. Mass pools of stable transfectants were selected in growth medium containing 0.4 mg/ml G418. Empty vector-, wild-type

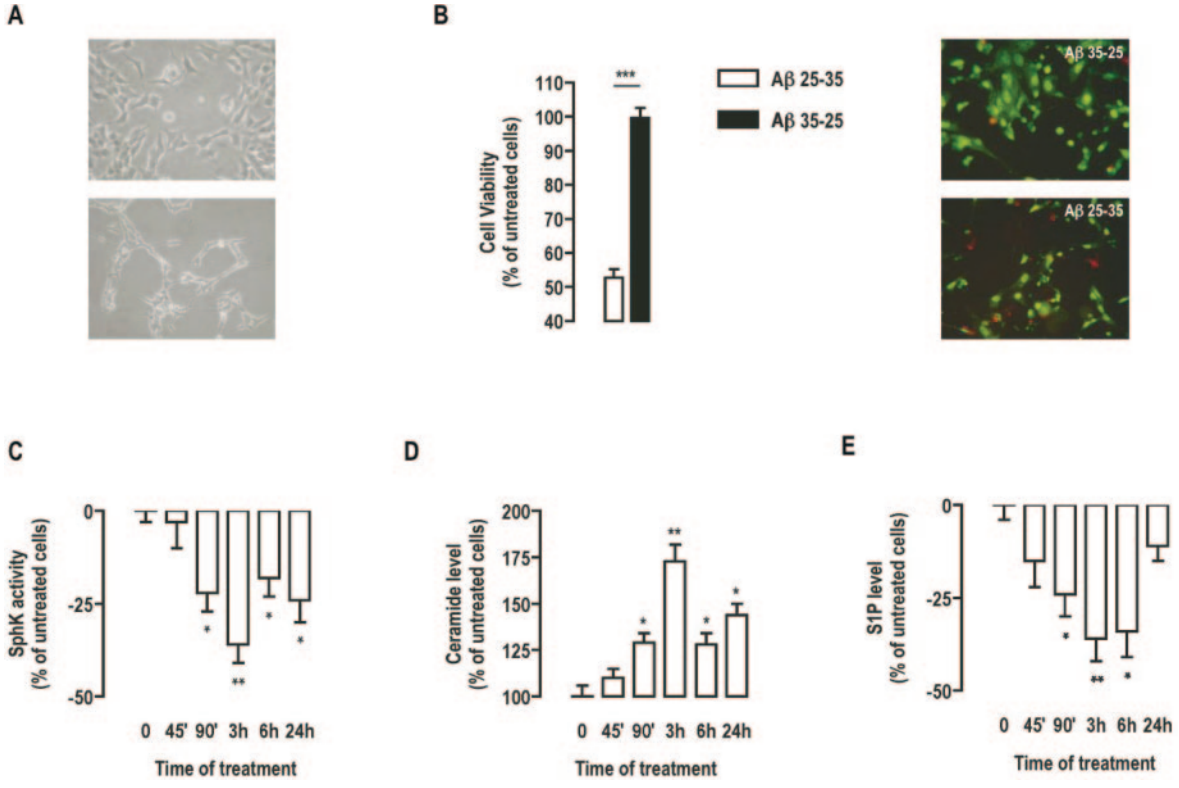

Fig. 1. Cell death induced by Aβ 25-35 peptide was associated with alteration in sphingolipid metabolism as a result of SphK1 inhibition. A, representative phase-contrast images of SH-SY5Y before (top) and after (bottom) differentiation into neuron-like cells with retinoic acid (10 μM, 5 days). B, SH-SY5Y cells were treated with 10 μM Aβ peptides for 24 h and cell viability was assessed by the MTT assay. Columns, mean of more than ten experiments; bars, S.E. Inset, representative images of cells treated for 24 h with Aβ peptide and stained with Syto13-propidium iodide. Cells were incubated with 10 μM Aβ 25-35 peptide for the indicated times, then tested for SphK1 activity (C), ceramide (D), and S1P (E) levels. Basal SphK1 activity was 31.1 ± 2.3 pmol/mg/min. Basal ceramide and S1P contents were 2650 ± 110 pmol/mg of protein and 22.6 ± 1.7 pmol/μg of protein, respectively. Columns, mean of six experiments performed in triplicate; bars, S.E. The two-tailed P values between the means are as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.1$.

hSphK1-, and hSphK1^{G82D}-transfected SH-SY5Y cells were designated SH-SY5Y/Neo, SH-SY5Y/SphK1, and SH-SY5Y/SphK1-DN, respectively. All experiments were conducted in the presence of 2% FBS.

Materials. Medium, serum, and antibiotics were obtained from Invitrogen (Carlsbad, CA). Aβ peptides (25-35) and (35-25) were from Bachem (Weil am Rhein, Germany) and freshly solubilized in water before treatment. Thioflavine T fluorescent binding and Congo Red spectroscopic assay indicated a high β-sheets content characteristic of amyloid aggregates (data not shown). *Escherichia coli* diacylglycerol kinase, human IGF-I, human TGF-β1, CA074-Me, and sphingosine kinase inhibitor (2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole; CAS number 312636-16-1) inhibitor were from Calbiochem (San Diego, CA). [γ-^{32}P]ATP (3000 mCi/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA), and silica gel 60 high-performance TLC (Partisil LK6D) plates were from Whatman (Maidstone, UK). *N*-Acetylcysteine (NAC), hydrogen peroxide (H_2O_2), and retinoic acid were from Sigma-Aldrich (St. Louis, MO).

Cell Viability and Staining of Apoptotic Nuclei. Cell viability was measured using the MTT dye reduction assay as described previously (Cuvillier and Levade, 2001). Apoptosis was visually assessed by double-staining cells with Syto 13 (1 μM) and propidium iodide (6 μg/ml) for 5 min at 37°C (Pchejetski et al., 2005). The cells were then examined with a Leica fluorescent microscope and apoptotic cells distinguished by condensed, fragmented nuclear regions.

Western Blot Analysis. Western blotting was carried out as reported previously (Cuvillier et al., 1999) Mouse anti-FLAG (Sigma) and mouse anti-β-actin (Sigma) were used as primary antibodies. Proteins were visualized by ECL detection system (Pierce, Madison, WI) using anti-mouse horseradish peroxidase-conjugated IgG (Bio-Rad Laboratories, Hercules, CA).

SphKs Assay and Mass Measurements of Sphingolipids. SphK1 activity was determined in the presence of sphingosine prepared with Triton X-100 (final concentration 0.25%) whereas for assaying SphK2 activity, sphingosine was prepared in bovine serum albumin (final concentration, 4 mg/ml) and 1 M KCl, a condition in which SphK2 is optimal and SphK2 is inhibited (Maceyka et al., 2005). Ceramide and S1P levels were measured and normalized to protein content (Edsall et al., 2000).

RNA Interference. Transient interference was achieved by double-stranded SphK1-specific siRNA 5′-GGGCAAGGCCUUGCAGCUCd(TT)-3′ and 5′-GAGCUGCAAGGCCUUGCCCd(TT)-3′ or scrambled siRNA (Qiagen, Valencia, CA) using OligofectAMINE reagent (Invitrogen) as reported previously (Pchejetski et al., 2005). In brief, 75 pM siRNA was complexed with a 1:125 final dilution of OligofectAMINE (Invitrogen) reagent and applied to 1×10^5 cells in a final volume of 250 μl of Opti-MEM (Invitrogen) without FBS or antibiotics. After incubation for 4 h at 37°C under 5% CO_2, 150 μl of DMEM with 30% FBS was added. Twenty hours later the medium was changed to 1 ml of DMEM with 10% FBS. Cell viability assays and SphK1 activity determinations were performed daily.

Statistical Analysis. The statistical significance of differences between the means of two groups was evaluated by unpaired Student's *t* test. All statistical tests were two-sided, and the level of significance was set at $P < 0.1$. Calculations were performed using Instat (GraphPad Software, San Diego, CA).

Results

The Aβ 25-35 Peptide-Induced Cell Death Was Associated with Down-Regulation of Sphingosine Kinase-1 Activity. As reported previously (Li et al., 1996; Luetjens et al., 2001; Olivieri et al., 2001; Wei et al., 2002; Pettifer et al., 2004; Arias et al., 2005), Aβ 25-35 peptide induced a strong loss of cell viability in SH-SY5Y cells with an approximate EC_{50} of 10 μM at 24 h (Fig. 1B). In contrast, the 10 μM reverse Aβ 35-25 peptide treatment was not toxic to SH-SY5Y cells (Fig. 1B), thus confirming the specificity to the observed toxic effects of Aβ 25-35 peptide. Syto 13/propidium iodide staining revealed that the loss of cell viability observed in Aβ 25-35-treated cells could be attributed to apoptosis (Fig. 1B, inset), as confirmed by flow cytometry analysis with Annexin V-propidium iodide (data not shown). Previous studies have recently suggested that Aβ-induced cell death could be mediated by the proapoptotic sphingolipid metabolite ceramide (Jana and Pahan, 2004; Lee et al., 2004; Yang et al., 2004), we thus sought to determine whether not only ceramide but also other bioactive sphingolipids could be implicated in Aβ-induced toxicity. As already reported (Jana and Pahan, 2004; Lee et al., 2004; Yang et al., 2004), Aβ 25-35 treatment resulted in ceramide increase that was detectable as early as 45 min of incubation with a peak at around 3 h (Fig. 1D). It is noteworthy that SphK1 activity was strongly inhibited as soon as 45 min, with a decrease of more than 30% within 3 h of treatment (Fig. 1C). The SphK1 inhibition was paralleled by a marked decrease in S1P content (Fig. 1E). It is noteworthy that there were no changes in the levels of ceramide and S1P in SH-SY5Y cells incubated with reverse Aβ 35-25 (data not shown), suggesting that down-regulation of the ceramide/S1P biostat was seen only in cells undergoing apoptosis. Treatment with Aβ 25-35 did not significantly alter activity of the other sphingosine kinase isoform (SphK2; 6.3 ± 1.9 pmol/mg/min), the optimal basal activity of which (6.8 ± 1.2 pmol/mg/min) was approximately 5-fold lower than that of optimal SphK1 (31.1 ± 2.3 pmol/mg/min).

Ceramide generation during Aβ-induced cell death has been shown to involve changes in the cellular redox state and/or glutathione metabolism that controls neutral sphingomyelinase activation (Jana and Pahan, 2004; Lee et al., 2004; Yang et al., 2004). Therefore, we sought to determine whether the glutathione precursor NAC could also affect SphK1 activity. NAC totally hampered Aβ 25-35-induced SphK1 down-regulation (Fig. 2A) and prevented cytoxicity (Olivieri et al., 2001), thus implying a redox-sensitive mechanism for Aβ 25-35-mediated inhibition of SphK1. After having established that Aβ 25-35-induced SphK1 inhibition was inhibited by antioxidant NAC, it was of interest to determine whether addition of exogenous H_2O_2 could mimic the effect of Aβ 25-35 on SphK1 activity. As shown in Fig. 2B, addition of H_2O_2 led to a strong inhibition of SphK1. It is noteworthy that pretreatment with NAC could fully prevent SphK1 inhibition as well as cytotoxicity (Fig. 2B).

Sphingosine Kinase-1 Overexpression Inhibits Aβ 25-35 Peptide-Induced Cell Death. Because an inhibition of SphK1 is observed during Aβ 25-35 peptide-induced cell death, transfection of SH-SY5Y with this enzyme might render these cells resistant to Aβ toxicity. Transfection efficiency was verified by Western blotting with FLAG antibody (Fig. 3A). The SphK1 activity of SH-SY5Y overexpressing SphK1 (Fig. 3B) was increased to ~950 pmol/mg of protein/min (i.e., ~30-fold higher compared with that of empty vector-transfected cells). This increase of SphK1 activity led to a shift in the sphingolipid balance and, notably, in the ceramide-to-S1P ratio. The enforced expression of SphK1 in SH-SY5Y diminished the level of total intracellular ceramide in resting cells by ~25%. The basal S1P level was increased by ~50% (Fig. 3B, inset and right). The role of SphK1 inhibition in cell death induced by Aβ 25-35 peptide was confirmed by cell viability assays, which showed that SphK1-overexpressing SH-SY5Y were ~40% more resistant to Aβ 25-35 peptide

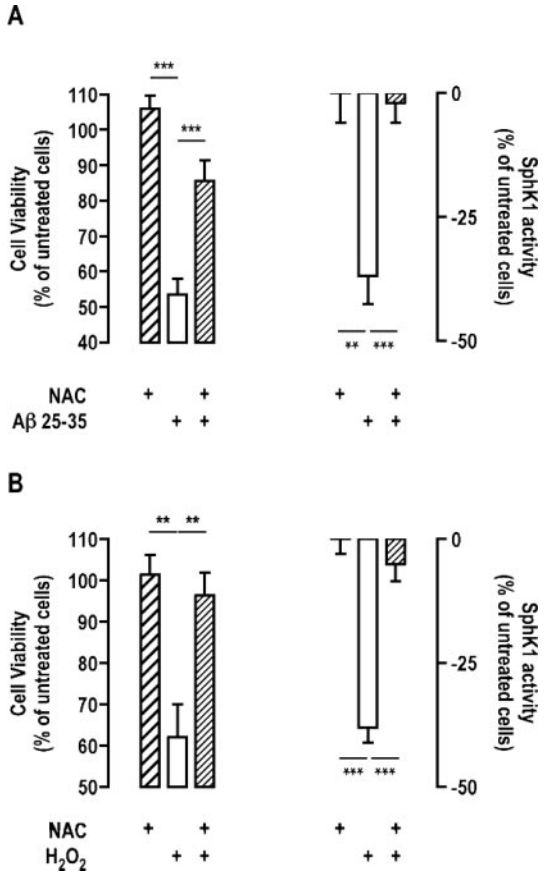

Fig. 2. SphK1 inhibition by Aβ 25-35 peptide is redox-sensitive. A, SH-SY5Y cells were preincubated for 2 h with 5 mM NAC and then treated with 10 μM Aβ 25-35 peptide for 3 h (SphK1 assay) or 24 h (MTT assay). B, SH-SY5Y cells were preincubated for 1 h with 5 mM NAC and then treated with 10 μM H_2O_2 for 60 min (SphK1 assay) or 24 h (MTT assay). Columns, mean of six experiments for MTT and SphK1 assays; bars, S.E. The two-tailed *P* values between the means are as follows: ***, $P < 0.001$; **, $P < 0.01$.

than SH-SY5Y transfected with empty vector (Fig. 3C). The cytoprotective effect of SphK1 overexpression was illustrated by a significant decrease of the ceramide-to-S1P ratio (Fig. 3D). In addition, SphK1 overexpression could block the loss of cell viability of SH-SY5Y cells treated with H_2O_2 (cell viability = 59.5 ± 3.9% in SH-SY5Y/Neo versus 85.5 ± 3.6% in SH-SY5Y SphK1, $P < 0.001$).

The Manipulation of the Ceramide/Sphingosine 1-Phosphate Rheostat by Sphingosine Kinase-1 Silencing Promotes Cell Death. To establish proof of concept that SphK1 down-regulation has a critical effectiveness on the cytotoxicity of Aβ 25-35, we examined the effects of siRNA targeted against SphK1 (Pchejetski et al., 2005; Bonhoure et al., 2006; Pchejetski et al., 2007) on SphK1 activity and SH-SY5Y viability. SphK1 activity was strongly decreased compared with scrambled siRNA (Fig. 4A). This was further illustrated by reduction in S1P content (Fig. 4A, inset). Western blot analysis revealed a significant down-regulation of the SphK1 protein in SH-SY5Y/SphK1 cells after siRNA treatment for 72 h (Fig. 4B). The decrease in SphK1 activity was accompanied by an increase in the ceramide to S1P ratio (Fig. 4C) and a substantial loss of cell viability (Fig. 4D). These results clearly indicate that SphK1 is required for cell survival and that the lowering of SphK1 may be crucial to the execution of cell death as recently reported in breast (Taha et al., 2006) and prostate adenocarcinoma (Pchejetski et al., 2005), leukemia cells (Taha et al., 2004; Bonhoure et al., 2006), as well as in cardiomyocytes (Pchejetski et al., 2007).

Sphingosine Kinase-1 Mediates the Prosurvival Effects of IGF-I. The capability of IGF-I to protect from Aβ toxicity is well established (Doré et al., 1997; Wei et al., 2002). In agreement with previous reports (Wei et al., 2002), incubation of SH-SY5Y cells with 75 ng/ml IGF-I had a pronounced cytoprotective impact toward Aβ 25-35 peptide (Fig. 5A). Because SphK1 overexpression enhanced survival of SH-SY5Y in response to Aβ 25-35 peptide (Fig. 3C), it was of interest to determine whether SphK1 could be involved in a

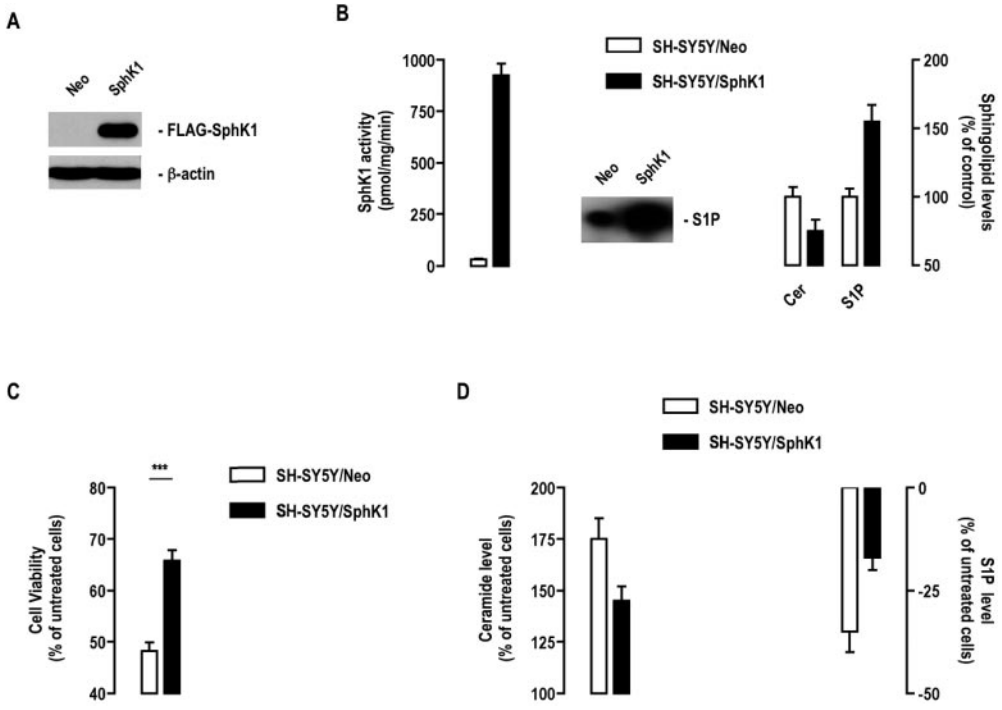

Fig. 3. SphK1 enforced expression protects against Aβ 25-35 peptide-induced cell death in SH-SY5Y cells. A, SphK1 expression in SH-SY5Y cells was analyzed by Western blotting using an anti-FLAG antibody. B, basal SphK1 activity and content of ceramide and S1P were measured in SH-SY5Y/Neo- and SphK1-overexpressing cells. Inset, corresponding S1P content produced in vitro by SphK1 in SH-SY5Y/Neo and SphK1 overexpressing as shown by TLC. C, cell viability was assessed after 24-h Aβ 25-35 peptide treatment of SH-SY5Y overexpressing or not SphK1, and expressed as a percentage of respective untreated cells, respectively. D, sphingolipid levels were estimated after 3 h of incubation with Aβ 25-35 peptide. Columns, mean of more than ten experiments performed in triplicate; bars, SE. The two-tailed *P* values between the means are as follow: ***, $P < 0.001$.

survival pathway such as the IGF-I one. Figure 5B shows that IGF-I markedly and rapidly up-regulated SphK1 activity (maximum within 60 min of incubation). This increase of SphK1 activity translated into a shift in the sphingolipid balance toward S1P: the level of ceramide was decreased, whereas S1P level was strongly increased (Fig. 5B, inset). To further evaluate the contribution of SphK1 in the cytoprotective effect of IGF-I, we generated SH-SY5Y cells overexpressing SphK1^{G82D}, a catalytically inactive form of SphK1. In contrast to SH-SY5Y/SphK1 cells (Fig. 3B), overexpression of SphK1^{G82D} produced no detectable increase in basal SphK1 activity compared with empty vector-transfected cells (data not shown). Although this mutation did not have any effect on basal SphK1 activity, it did noticeably prevent its stimulation by IGF-I (Fig. 6A), indicating that the mutated SphK1 could function as a dominant-negative (Pitson et al., 2000; Bonhoure et al., 2006). We next evaluated the prosurvival impact IGF-I in SH-SY5Y/SphK1^{G82D} cells when challenged with Aβ 25-35 peptide. Only a partial resistance to Aβ was found (Fig. 6B, left), hence suggesting that SphK1 was to a large extent accounting for the cytoprotective effect of IGF-I (Fig. 6, B, left, versus C). Furthermore, the role of SphK1 was also examined by performing experiments with 2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole, known as the most selective SphK1 pharmacological inhibitor available (French et al., 2003). Figure 6C shows that this SphK1 inhibitor could significantly block the cytoprotective effect of IGF-I in a dose-dependent fashion, in a similar manner to the SphK1-DN cell line model (Fig. 6B). We also investigated whether siRNA targeted against SphK1 could have an impact on the cytoprotective effect of IGF-I against Aβ. The prosurvival effect of IGF-I was significantly reduced in SphK1 siRNA-treated SH-SY5Y cells with respect to scrambled siRNA-treated cells (Fig. 6C). As a whole, our results suggest that IGF-I protected against Aβ-induced cell death by markedly up-regulating SphK1.

The TGF-β1 Protective Effect Against Aβ Peptide Cytotoxicity Involves Sphingosine Kinase-1 Activation. A number of studies have established that peptide

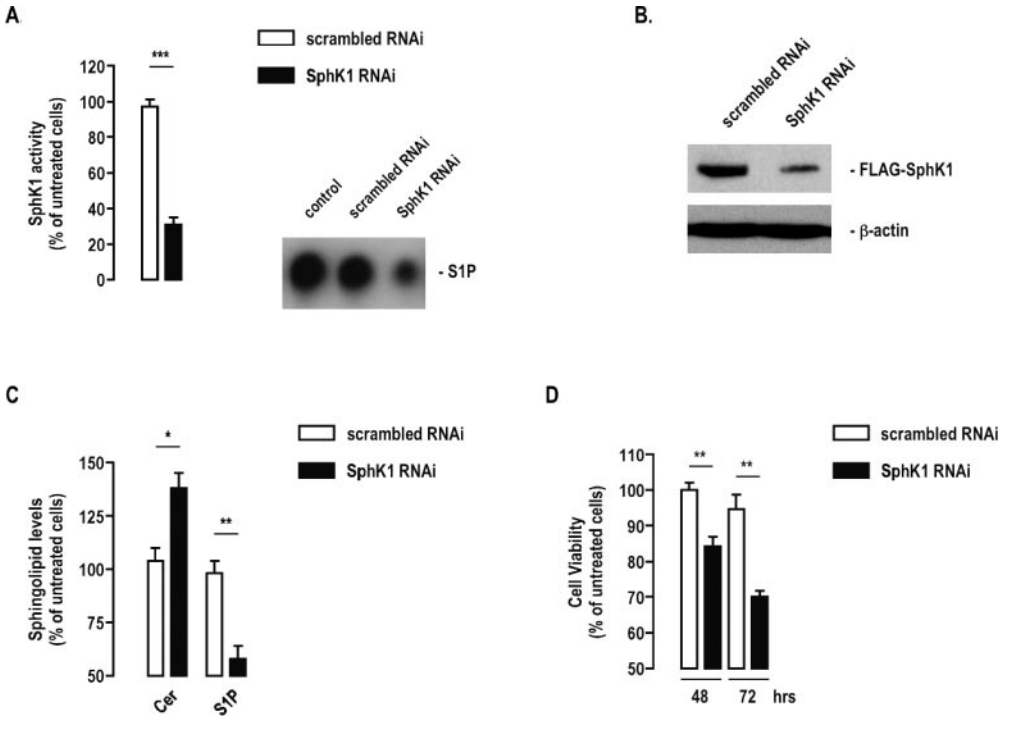

Fig. 4. SphK1-targeted inhibition by RNA interference induces cell death in SH-SY5Y cells. A, SphK1 activity in SH-SY5Y cells was assessed after a 72-h treatment with scrambled or hSphK1 siRNA (20 nM). Inset, S1P content of control, scrambled, or hSphK1 RNA interference-treated SH-SH5Y cells as shown by TLC. Columns, average of three independent assessments of SphK1 activity; bars, S.E. B, SphK1 expression in SH-SY5Y/SphK1 cells treated for 72 h with scrambled or hSphK1 siRNA was analyzed by Western blotting using an anti-FLAG antibody. C, sphingolipid levels were measured after 72 h of incubation with scrambled or hSphK1 siRNA in SH-SY5Y cells. D, cell viability was determined by MTT after treatment of SH-SY5Y cells with scrambled or hSphK1 siRNA for the indicated times. Columns, average of three independent experiments expressed as percentage of untreated cells; bars, S.E. The two-tailed *P* values between the means are as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.1$.

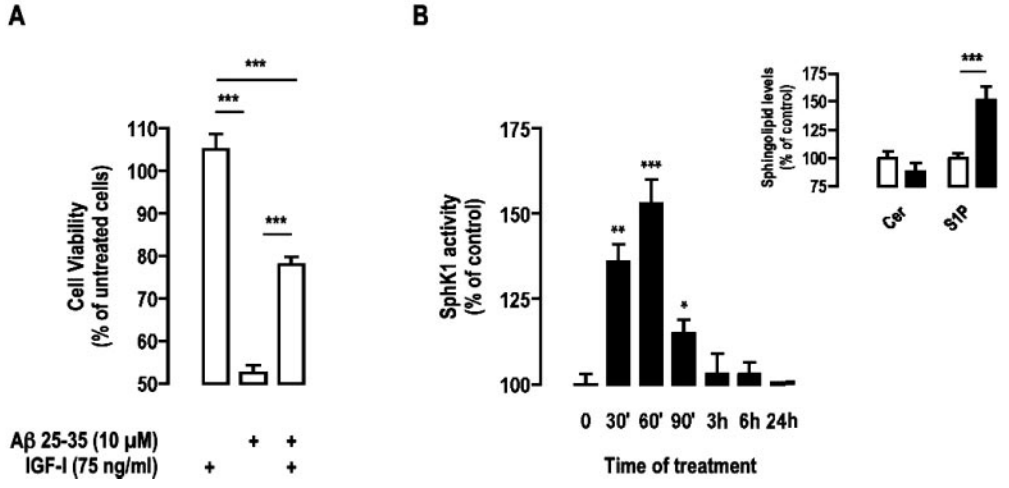

Fig. 5. SphK1 transmits the cytoprotective effects of IGF-I. A, SH-SY5Y cells were treated for 24 h with the indicated drugs, and cell viability was assessed by MTT. Columns, mean of 10 experiments performed in triplicate. B, cells were incubated with 75 ng/ml IGF-I for the indicated times, then tested for SphK1 activity. Inset, ceramide and S1P levels were determined in SH-SY5Y incubated with 75 ng/ml IGF-I for 1 h. Columns, mean of six and three experiments performed in triplicate for B and C, respectively. Bars, S.E. The two-tailed *P* values between the means are as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.1$.

growth factor, TGF-β1 can protect against the damaging effects of Aβ in human fetal brain cell cultures (Chao et al., 1994), in primary hippocampal neurons (Prehn et al., 1996; Ren and Flanders, 1996), in differentiated human teratocarcinoma cells (Ren et al., 1997), and in neuroblastoma cell line models (Ren and Flanders, 1996). Pretreatment of SH-SY5Y/Neo cells with 10 ng/ml TGF-β1 led to a pronounced cytoprotective effects toward Aβ 25-35 peptide (Fig. 7A) in agreement with previous studies (Ren and Flanders, 1996). Similar to IGF-I, we found a weaker protection from Aβ 25-35 peptide in SH-SY5Y/SphK1^{G82D} cells coincubated with TGF-β1 (Fig. 7A), and a slightly higher protection in SH-SY5Y cells overexpressing SphK1 (Fig. 7A), implying that SphK1 was a key regulator for the cytoprotective effect of TGF-β1. It has been reported that TGF-β1 could increase SphK1 activity in dermal fibroblasts (Yamanaka et al., 2004). We therefore asked whether SphK1 activity could be stimulated in our cell system in response to TGF-β1. As shown in Fig. 6B, SphK1 was found to be rapidly activated after exposure of SH-SY5Y/Neo to TGF-β1. It is noteworthy that the dominant-negative SH-SY5Y/SphK1^{G82D} cells, in contrast to SH-SY5Y/Neo, did not display SphK1 activation upon TGF-β1 treatment (Fig. 7B, right). The increase of SphK1 activity in SH-SY5Y/Neo cells led to a shift in the sphingolipid balance toward S1P: the level of ceramide was slightly decreased, whereas the S1P level was increased by almost 50% (Fig. 7B). As anticipated, there were no significant changes in the levels of ceramide and S1P in the dominant-negative SH-SY5Y/SphK1^{G82D} cells after treatment with TGF-β1 (Fig. 7C).

Discussion

The major finding of the present study relates to the critical role of SphK1 isoform in regulating cell survival against Aβ-induced toxicity. SphK1 is a key enzyme in the sphingo-

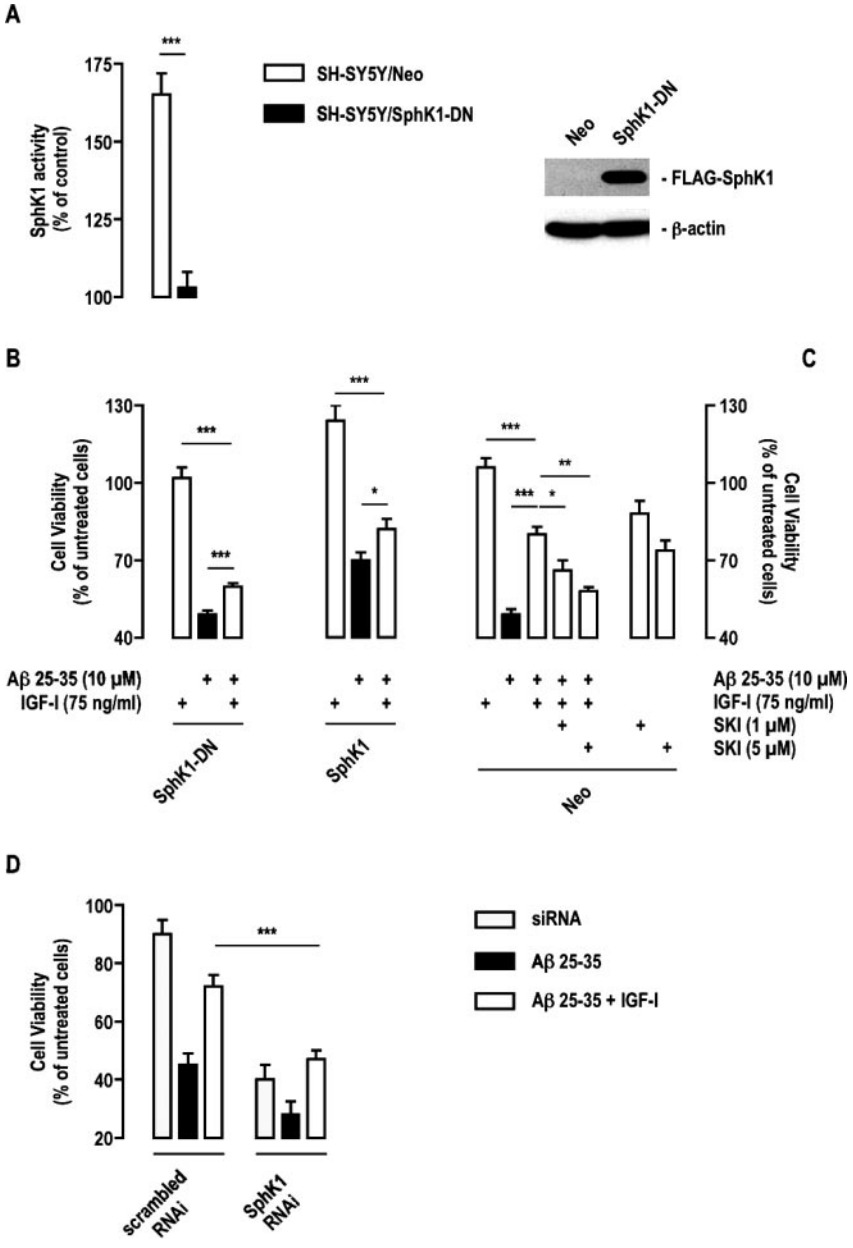

Fig. 6. Blockade of SphK1 activity impedes IGF-I-induced prosurvival effects. A, Neo and SphK1-DN overexpressing SH-SY5Y cells were incubated with 75 ng/ml IGF-I for 60 min, then tested for SphK1 activity. Inset, expression of SphK1 in SH-SY5Y/Neo and SphK1-DN cells was analyzed by Western blotting using an anti-FLAG antibody. Columns, mean of three experiments performed in triplicate. Bars, S.E. B, SphK1-DN and SphK1 overexpressing SH-SY5Y cells were treated with 10 μM Aβ peptides for 24 h in the presence or absence of 75 ng/ml IGF-I, and cell viability was quantified by MTT assay. Columns, mean of ten experiments performed in triplicate. Bars, S.E. C, cell viability was assessed in SH-SY5Y/Neo cells were incubated as indicated with the following compounds: 10 μM Aβ 25-35 peptide, 75 ng/ml IGF-I, and 1 and 5 μM SphK inhibitor SKI. Columns, mean of 10 experiments performed in triplicate. Bars, S.E. D, cell viability was determined in SH-SY5Y cells preincubated for 72 h in presence of scrambled or SphK1 RNA interference then treated for an additional 24 h without or with 10 μM Aβ peptides and/or 75 ng/ml IGF-I. Columns, mean of three to five experiments performed in triplicate. Bars, S.E. The two-tailed P values between the means are as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.1$.

lipid metabolism because it serves the dual function of regulating ceramide and S1P level, two sphingolipids with opposite function on cell survival (Cuvillier et al., 1996).

Although the mechanism by which Aβ-peptides induce neuronal loss is poorly understood, a wealth of reports support the notion that ceramide could be a mediator of Aβ-induced toxicity. Ceramide generation by a n-SMase-mediated sphingomyelin degradation in response to Aβ treatment was indeed described both in vitro and in vivo (Alessenko et al., 2004; Ayasolla et al., 2004; Cutler et al., 2004; Jana and Pahan, 2004; Lee et al., 2004; Yang et al., 2004; Zeng et al., 2005). The generation of ceramide during Aβ-induced neuronal cell death is instrumental as for its blockade by n-SMase antisense oligonucleotides, pharmacological n-SMase inhibitors or antioxidants such as the glutathione (GSH) precursor NAC, diphenyl iodonium can hinder the cytotoxic effect of Aβ-peptides (Jana and Pahan, 2004; Lee et al., 2004; Yang et al., 2004). It has been hypothesized that the decrease in GSH level observed after Aβ exposure (Müller et al., 1997; Pereira et al., 1999) could activate n-SMase, therefore leading to ceramide production (Lee et al., 2004). In this study, we establish that not only is the proapoptotic ceramide produced during Aβ-induced neuronal cell death, but also the levels of the prosurvival S1P are diminished as a result of SphK1 down-regulation, thus tilting the ceramide/S1P biostat toward ceramide, as previously observed in tumor cells after anticancer treatments (Nava et al., 2000; Taha et al., 2004; Pchejetski et al., 2005; Bonhoure et al., 2006). There was up to a 3-fold increase in the ceramide/S1P ratio at 3-h incubation time (calculated from the relative amounts of ceramide and S1P levels shown in Fig. 1, D and E, respectively; 1.75:0.65).

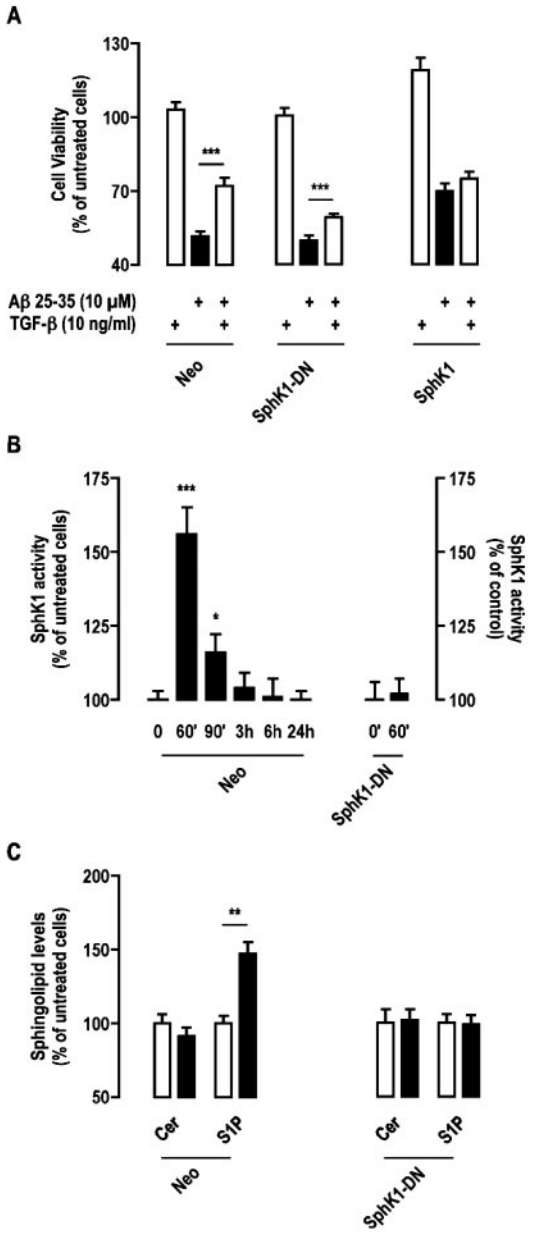

Fig. 7. TGF-β1 cytoprotective effect involves the SphK1/S1P pathway. A, Neo, SphK1, and SphK1-DN overexpressing SH-SY5Y cells were incubated in the presence of 10 ng/ml TGF-β1 or vehicle for 24 h, then treated with 10 μM Aβ peptides for 24 h, and cell viability was quantified by MTT assay. Columns, mean of 10 experiments performed in triplicate. Bars, S.E. B, Neo and SphK1-DN overexpressing SH-SY5Y cells were incubated with 10 ng/ml TGF-β1 for the indicated times, then tested for SphK1 activity. C, ceramide and S1P levels were determined in SH-SY5Y/Neo and SphK1-DN overexpressing cells incubated with 10 ng/ml TGF-β1 for 1 h. Columns, mean of three experiments performed in triplicate. Bars, S.E. The two-tailed P values between the means are as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.1$.

As a proof of the strategic role for SphK1 in regulating Aβ-induced neuronal cell death, our study showed that SphK1 enforced expression markedly inhibited the cytotoxicity of the Aβ peptide. These results are in line with previous reports establishing that SphK1 overexpression can offer protection against proapoptotic stimuli, including serum withdrawal, short-chain ceramides, or anticancer drugs (Olivera et al., 1999; Edsall et al., 2001; Nava et al., 2002; Pchejetski et al., 2005; Bonhoure et al., 2006). Enhanced SphK1 activity is known to reduce ceramide levels by driving ceramide metabolism toward the generation of S1P, which blocks the apoptotic machinery (Pchejetski et al., 2005, 2007; Bonhoure et al., 2006). We further confirmed the specific role of SphK1 by showing that siRNA against SphK1 induced a strong loss of cell viability, thus implying that SphK1 was required for survival of SH-SY5Y cells and that its inhibition was a key feature in apoptosis. Loss of SphK1 has been recently shown to activate the intrinsic pathway of apoptosis through enhanced oligomerization of Bax in the mitochon-

Oxidative stress—which is suggested to play a central role in Aβ-induced toxicity and Alzheimer's disease—seemed to be instrumental for mediating SphK1 down-regulation by Aβ peptide because the GSH precursor NAC could impede SphK1 inhibition. Moreover, direct addition of H_2O_2 also triggered SphK1 inhibition that could be blocked by NAC, and H_2O_2-induced cell death was overcome by SphK1 overexpression. Such a role for oxidative stress in controlling SphK1 activity has been recently reported in cardiomyocytes after ischemia/reperfusion (Pchejetski et al., 2007). With respect to the significance of SphK1 inhibition during Aβ-induced cell death, one can anticipate it as a means to make sure that the ceramide produced—in response to Aβ—will not give rise to augmented prosurvival S1P. It should be noted that SphK1 inhibition could be seen after treatment of leukemic cells with C_2-ceramide (Bonhoure et al., 2006), a cell-permeable analog of natural ceramide, which is known to be metabolized to produce natural long-chain ceramides when added to the cells (Abe et al., 1996). It is noteworthy that C_2-ceramide-induced cell death in SH-SY5Y cells can be blocked by SphK1 overexpression (data not shown).

drial membrane, resulting in cytochrome *c* release and downstream caspase activation (Taha et al., 2006).

Last, our studies not only demonstrated that SphK1 inhibition was required for Aβ-toxicity in SH-SY5Y cells but also established for the first time that SphK1 was a major transducer of two important growth factors, IGF-I and TGF-β1, whose neuroprotective effects against Aβ are well recognized (Flanders et al., 1998; Carro and Torres-Aleman, 2004; Tesseur et al., 2006). Both IGF-I and TGF-β1 triggered a rapid stimulation of SphK1 activity, tipping the ceramide/S1P balance toward S1P, which in turn could protect SH-SY5Y cells from Aβ-toxicity. The activation of SphK1 was essential for the action of IGF-I and TGF-β1 because a dominant-negative form of SphK1 or its pharmacological inhibition or knocking-down by RNA interference strategy could markedly impede their cytoprotective effect against Aβ peptide.

As a whole, this report shows for the first time the implication of SphK1 in the regulation of death and survival of Aβ-treated neuronal cells, highlighting the notion that the ceramide/S1P biostat could be a regulator of life and death of neurons. The capability of SphK1 to promote neuronal survival suggests that analogs of S1P or stimulators of SphK1 activity might provide a strategy toward forestalling the symptoms of Alzheimer's disease.

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