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The key role of sphingosine kinases in the molecular mechanism of neuronal cell survival and death in an experimental model of Parkinson's disease

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Abstract

Sphingosine kinases (Sphk1/2 EC 2.7.1.91) are responsible for synthesis of sphingosine-1-phosphate (S1P) and for regulation of the bioactive sphingolipids homeostasis. Sphingosine-1-phosphate can act as a potent messenger in an autocrine/paracrine manner through five specific G protein-coupled receptors (GPCR) S1P1-5. This sphingolipid is involved in the mechanism of transcription, mitochondrial function, neuronal viability and degeneration. Until now the involvement of Sphk1/2 and sphingolipid alterations in Parkinson's disease (PD) remains unknown. Recent studies have indicated the role of sphingolipids in the regulation of alpha-synuclein (ASN) in the PD brain. Our latest data demonstrated significant inhibition of Sphk1 gene expression and activity in an in vitro PD model, induced by 1-methyl-4-phenylpyridinium (MPP+). The aim of this study was to investigate the role of Sphks inhibition in ASN secretion and in the molecular mechanism of neuronal death in the PD model. Our study was carried out using neuronal dopaminergic SH-SY5Y control cells, transfected with the human gene for ASN or with an empty vector. These cells were treated with MPP+ (1-3 mM), which represents an experimental PD model, or with the Sphks inhibitor (1-5 μM SKI II) for 3-24 h. Our data indicated that MPP+ (3 mM) induced significant alterations of Sphks and S1P lyase (SPL) gene expression. Reduced activity of Sphk1 and Sphk2 in the cytosolic fraction and in the crude nuclear fraction, respectively, was observed. Sphks inhibition evoked enhancement of ASN secretion, suppression of PI3K/Akt phosphorylation and activation of gene expression for the pro-apoptotic Bcl-2 proteins Bax and BH3-only protein Harakiri. Moreover, a lower level of cytochrome c in the mitochondrial fraction and caspase-dependent degradation of DNA-bound enzyme poly(ADP-ribose) polymerase (PARP-1) were observed. The caspase inhibitor (20 μM Z-VAD-FMK) significantly enhanced neuronal cell viability in MPP+ oxidative stress. However, exogenous S1P (1 μM) exerted a more efficient neuroprotective effect as compared to Z-VAD-FMK. In summary, these data indicated that Sphk1 inhibition plays an important role in caspase-dependent apoptotic neuronal death in an experimental PD model.

Key words: *sphingosine kinases, dopaminergic neurons, apoptosis, PD model.*

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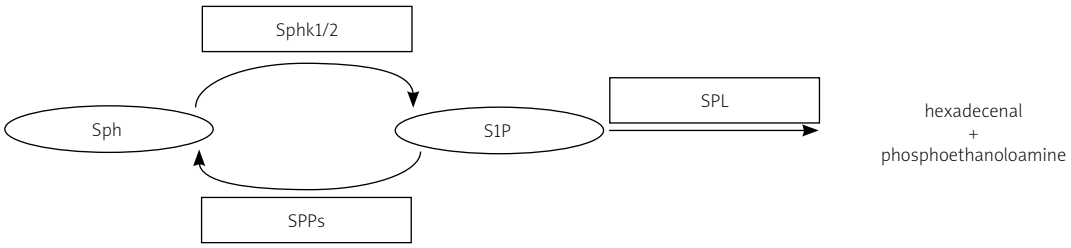
Introduction

Sphingosine kinases (Sphk1 and Sphk2) catalyse ATP-dependent phosphorylation of sphingosine (Sph) to sphingosine 1-phosphate (S1P). Sphks are crucial enzymes in maintaining a balance between the lipid signalling molecules, such as ceramide/sphingosine, and S1P [36,37,50,53]. Sphk1 and Sphk2 share overall homology and synthesize S1P, which depending on intracellular localisations of Sphks isoforms plays different roles within the cell [36,50,55]. Sphk2 is located mainly in the nucleus, although it is also present in the other cell compartments (mitochondria, endoplasmic reticulum). The S1P pool synthesized by this isoform is demonstrated to exert an anti-proliferative and pro-apoptotic effect [22,29]. S1P synthesized by Sphk2 enhances histone H3 acetylation and is involved in regulation of genes encoding the cyclin-dependent kinase inhibitor p21 and c-fos [19]. The latest data reported by Takasugi *et al*. [52] indicated that the S1P pool synthesized by Sphk2 regulates β-amyloid (Aβ) precursor protein (APP) cleaving enzyme 1 (BACE1) activity and the Aβ level. Sphk1 resides predominantly in the cytosol and is translocated to the plasma membrane followed by ERK1/2-mediated phosphorylation [39,45,47]. Sphk1 is well known to synthesize the S1P pool, which exerts pro-survival effects within the cells. Sphk1 can be stimulated in response to neuronal growth factor and cytokines [13,32,46,58]. The S1P pool synthesized by Sphk1 can promote cell survival, proliferation and migration, and can also regulate differentiation, neurogenesis, angiogenesis, carcinogenesis and inflammation [16,33,50]. The level of intracellular S1P is regulated not only by Sphks but also by enzymes involved in its degradation, that means by S1P lyase (SPL) and by specific S1P phosphatase (SPP1) and also by less-specific SPP2 and other phosphatases (Fig. 1) [36,37]. S1P formed at the plasma membrane can be easily exported from the cell and can act in an autocrine or paracrine fashion through five specific G protein-coupled receptors (S1P1-S1P5), located in sphingolipid microdomains of cell membranes [49]. It is known that sphingolipids play a very important role in Alzheimer's disease (AD). However, there are only a few data about the involvement of sphingolipid metabolism in the pathogenesis/pathomechanism of Parkinson's disease (PD). Brugg *et al.* [28] reported that dopaminergic neurons in PD undergo apoptosis through a ceramide-dependent mechanism [5]. Recent observations indicated that heterozygous mutations in glucocerebrosidase (GBA), which catalyses the breakdown of glucosylceramide to ceramide and glucose, predispose to PD [28]. A recent study by Abbott *et al.* [1] showed an altered level of ceramide and ceramide synthase-1 gene expression in PD anterior cingulate cortex, containing Lewy body pathology. The main component of a Lewy body is α-synuclein (ASN), a small presynaptic protein that easily changes conformation under different types of stress [54,56]. Overexpression of ASN or its mutation plays a crucial role in PD pathogenesis/pathomechanism [40,56]. The ASN mutation is responsible for the familiar form of PD. ASN can be secreted from the cells and can act not only intra- but also extracellularly and can transmit protein pathology from cell to cell [56]. ASN is most toxic in the form of oligomers [20,56]. It was observed that ASN binds with high affinity to glycolipoprotein microdomains termed lipid rafts, which are enriched in sphingolipids [15]. The relationship between sphingolipids and ASN in neurodegeneration remains poorly understood. A study by Gómez-Santos *et al.* (2002) showed that MPP+ increases ASN expression in SH-SY5Y



Sph – sphingosine, Sphk1/2 – sphingosine kinases 1/2, SPL – S1P lyase, SPPs – S1P phosphatases, S1P – sphingosine 1-phosphate

Fig. 1. Schematic representation of sphingosine-1-phosphate metabolic pathway.

cells and suggested that ASN could play an important role in this cellular model of PD [18]. Our latest data demonstrated that MPP+ and Sphks inhibition enhanced the free radical level and down-regulated Sphk1 expression and activity in the SH-SY5Y. Moreover, we observed that S1P protected against MPP+ evoked oxidative stress and alteration of gene expression for Sphk1 and for pro-apoptotic proteins (Bax, Hrk/DP5). S1P significantly enhanced cell viability mainly by activation of S1P1 receptor mediated signalling [41]. In our previous and in this study we used human dopaminergic SH-SY5Y cells, because they express tyrosine hydroxylase (HT), dopamine β-hydroxylase (DBH) [38] and the dopamine transporter (DAT) [24,51,59] and have been widely used to gain a better understanding of the molecular processes and pathomechanism of PD. In the present study we investigated gene expression for Sphk1/2 and for enzymes involved in S1P degradation (SPL and SPP). Moreover, we analyzed the role of Sphks activity inhibition in ASN secretion and in the molecular mechanism of neuronal cell death in oxidative stress induced by MPP+.

Material and methods

Cell culture

The studies were carried out using the human neuroblastoma cell line SH-SY5Y, control (non-transfected) and transfected with the human gene for alpha-synuclein (ASN), SH-SY5Y-T, or with an empty vector, SH-SY5Y-V (a kind gift from Prof. Nakamura, Kobe University Graduate School of Medicine, Japan; control SH-SY5Y cells were a kind gift from Prof. Anne Eckert, Neurobiology Laboratory for Brain Aging and Mental Health, Psychiatric University Clinics, University of Basel). The cells were cultured in MEM/F-12 Ham Nutrient Mixtures (1 : 1) supplemented with 15% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin and 2 mM glutamine. The cells were maintained at 37°C in a humidified incubator containing 5% CO_2. For the experiments, SH-SY5Y control cells were sub-cultured into collagen-coated 100 mm^2 dishes. Prior to treatment, the medium was changed to low-serum medium (2% FBS). SH-SY5Y cells transfected with the gene for ASN and with the empty vector were sub-cultured into 6-well clusters at 4×10^4 cells/well and differentiated with 10 μM all-trans-retinoic acid (ATRA) for 5 days. Prior to treatment, the medium was changed to a serum-free medium with 10 μM ATRA.

Cell treatment protocols

SH-SY5Y control cells (non-transfected) were treated with 3 mM MPP+ (Sigma-Aldrich), with the Sphk1 inhibitor (SKI II 5 μM, Sigma-Aldrich) or with staurosporine (100 nM, Sigma-Aldrich) for 3-24 h. In some experiments the cells were pre-incubated with 1 μM S1P (Enzo Life Sciences) or with the caspase inhibitor (Z-VAD-FMK 20 μM, R&D Systems) added to the culture medium 1-2 h before MPP+, SKI II treatment or staurosporine treatment. SH-SY5Y-T or SH-SY5Y-V were treated with 1 mM MPP+ or with 1 μM SKI II for 24 h.

Assay of alpha-synuclein precipitation and immunochemical detection

The extracellular medium from SH-SY5Y treated cells and transfected with the ASN gene or with the empty vector was collected and rapidly frozen in liquid nitrogen for 0.5 h. After thawing at 95°C, 50 μg of BSA as a carrier protein was added. Protein precipitation was carried out by the addition of 100% TCA and incubating for 0.5 h at room temperature (RT). This mixture was then centrifuged at 13 000 × g for 4 min. Then the pellet was washed twice with 1.5 ml and 0.2 ml of cold acetone and centrifuged at 13 000 × g for 4 min. The pellet was dried at 95°C for 10 min to remove acetone, then resuspended in 25 μl of PBS and incubated at RT for 1 h. The pellet was denatured with 0.55 M Tris-based sample buffer (pH = 6.8) for 10 min. 40 μg of proteins were loaded into 15% polyacrylamide denaturing gels and subjected to electrophoresis (applied constant voltage, 100 V). After protein separation, each gel was placed on a nitrocellulose membrane (Amersham Biosciences) in a transfer buffer and the proteins were transferred to the membrane by applying 50 V for 2 h. After the transfer was completed the ASN level was detected by immunochemical reaction (Western blot) analysis. In order to block non-specific binding, the membrane was incubated at RT for 1 h in 5% dried skimmed milk and 0.3% Tween 20 in phosphate-buffered saline (PBS), pH 7.4. Incubation with primary antibody (anti-α synuclein 1 : 1000 dilution, Sigma-Aldrich) in PBS with 0.1% Tween 20 and 1% bovine serum albumin (BSA) was performed overnight at 4°C. Then the blots were washed three times for 15 min at RT in PBS with 0.1% Tween 20.

The membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody diluted 1 : 800 (Sigma-Aldrich) at RT for 1 h. Then the blots were washed as described above and bound antibodies were visualised by enhanced chemiluminescence (Thermo Scientific). Incubation with the anti-β-actin antibody additionally confirmed equal protein loading.

Cell viability analysis

Mitochondrial function and cellular viability were evaluated by using 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After 24 h incubation with appropriate compounds, MTT (2.5 mg/ml) was added to all of the wells. The cells were incubated at 37°C for 2 h. Then the cells were lysed in DMSO and spectrophotometric measurement at 595 nm was performed.

Preparation of the subcellular fraction

Preparation of the subcellular fraction was performed as described previously by Cieślik *et al*. (2013) [8]. Cells were washed and scraped into ice-cold PBS and were pelleted at 1000 rpm for 3 min at 4°C. The pellet was resuspended in hypotonic buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1.5 mM $MgCl_2$, 10 mM KCl and protease inhibitor cocktail). The homogenate was prepared using a 26-gauge needle (10 passes). Then it was pelleted at 500 g for 10 min at 4°C. The pellet (P1, the crude nuclear fraction) was resuspended in 25 mM Tris, pH 7.4 with protease inhibitors and was used for Western blot analysis. The supernatant (S1) was centrifuged at 15 000 g for 15 min at 4°C to obtain a cytosolic (S2) and crude mitochondrial fraction (P2).

Measurement of Sphk1 and Sphk2 activity

Sphk activity assay was performed according to a previous report [52]. The pellet, P1 (the crude nuclear fraction), or supernatant, S1 (cytosolic together with the crude mitochondrial fraction), was resuspended in lysis buffer and used for enzymatic reaction. A total of 100 μg of protein of P1, S1 and NBD-Sphingosine (10 μM; Avanti Polar Lipids) was mixed in the reaction buffer (50 mM HEPES, pH 7.4, 15 mM $MgCl_2$, 0.5 mM KCl, 10% glycerol, and 2 mM ATP) and incubated in a final volume of 100 μl for 30 min at 30°C. The reactions were stopped by the addition of an equal amount of 1 M potassium phosphate, pH 8.5, followed by the addition of 500 μl of chloroform/methanol (2 : 1), and then centrifuged at 15 000 rpm for 1 min. The reactant NBD-S1P (but not the substrate NBD-Sphingosine) was collected into the alkaline aqueous phase. After the aqueous phase was combined with an equal amount of dimethylformamide, the fluorescence value was measured (λex = 485 nm, λem = 538 nm).

Determination of PARP-1 and cytochrome c protein level

After protein measurement according to Lowry, the homogenate of SH-SY5Y cells and the mitochondrial fraction were mixed with 5 × Laemmli sample buffer and denatured for 5 min at 95°C. A total of 40 μg of protein was loaded per lane on 10% acrylamide gels and examined by SDS-PAGE. The proteins were transferred onto PVDF membranes at 100 V. The membranes were incubated in 5% dry milk in TBS with Tween 20 (TBS-T) for 1 h and exposed overnight to specific antibodies: anti-cytochrome c (1 : 750, from Santa Cruz Biotechnology), anti-Actin (1 : 4000, from MP Biomedicals), anti-PARP-1 (1 : 500, from Sigma-Aldrich), anti-phospho-Akt (Ser473) and anti-Akt (1 : 750, Cell Signaling). After treatment for 1 h with horseradish-peroxidase-coupled secondary antibodies (anti-rabbit from Amersham Biosciences or anti-mouse from GE Healthcare), the protein bands were detected by ECL reagent (Thermo Scientific). Then the membranes were treated with stripping buffer (50 mM glycine, pH 2.5, 1% SDS) for further blots.

Analysis of the mRNA level using real-time PCR

RNA was isolated using a TRI reagent from Sigma-Aldrich. The isolated RNA was dissolved in RNase-free water (Ambion). The amount and purity of the RNA were determined using spectrophotometric measurement at 260 and 280 nm. The OD260/OD280 ratio of the RNA samples ranged from 1.8 to 2.0. Isolated RNA (5 μg) was treated with DNase and then used in RT-PCR. Reverse transcription was performed by using a High Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol (Applied Biosystems). Quantitative PCR was performed by using pre-developed TaqMan Gene Expression Assays (Applied Biosystems): actb Hs99999903_m1,

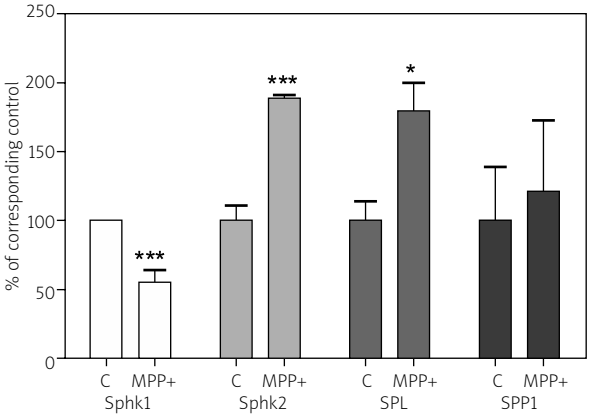


Fig. 2. Sphk1/2, SPL and SPP1 gene expression in MPP+-treated SH-SY5Y cells. SH-SY5Y cells were treated with MPP+ (3 mM) for 12 h. The mRNA level for Sphk1/2, SPL and SPP1 was normalized against β-actin (Actb). The relative level of mRNA was calculated by the ΔΔCt method. Data represent the mean value ± S.E.M from three independent experiments with three replications. Student's *t*-test was used. **p* < 0.05, ****p* < 0.001 – vs. control (non-treated) SH-SY5Y cells

bax Hs00180269_m1, hrk Hs02621354_s1, sphk1 Hs01116530_g1, sphk2 Hs01016543_g1, sgpl1 Hs00187407_m1, sgpp1 Hs00229266_m1 on an ABI PRISM 7500 apparatus according to the manufacturer's instructions. Actb was selected and used in all of the studies as a reference gene. The level of mRNA was calculated by the ΔΔCt method.

Statistical analysis

The results were expressed as mean values ± S.E.M. Differences between means were analysed using Student's *t*-test or one-way ANOVA followed by the Newman-Keuls *post hoc* test. Values of $p < 0.05$ were considered statistically significant.

Results

Human dopaminergic cells (SH-SY5Y) were exposed to 3 mM MPP+ for 24 h. Significant changes in the gene expression level for enzymes involved in the synthesis and degradation of S1P in MPP+-treated SH-SY5Y cells took place. It was observed that MPP+ reduced gene expression for Sphk1 and up-regulated Sphk2. These changes were accompanied by signif-

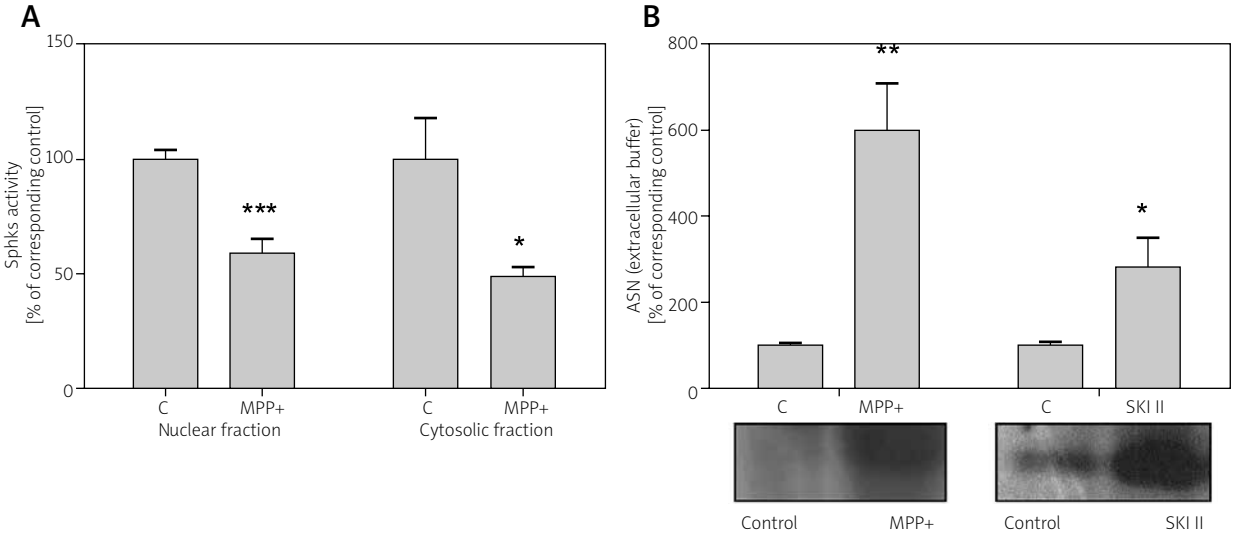


Fig. 3. Sphk1/2 activity in MPP+-treated SH-SY5Y cells and the role of Sphks inhibition and MPP+ in ASN release from the SH-SY5Y-T cells. **A)** SH-SY5Y cells were treated with MPP+ (3 mM) for 24 h. Sphks activity was measured in the cytosolic and crude nuclear fraction. Data represent the mean value ± S.E.M from three independent experiments with two replications. Student's *t*-test was used; **p* < 0.05, ****p* < 0.001 – vs. control (non-treated) SH-SY5Y cells. **B)** SH-SY5Y-T cells were treated with MPP+ (1 mM) and SKI II (1 μM) for 24 h. Control represents non-treated SH-SY5Y-T cells. The immunochemical level of ASN in the extracellular buffer was detected. Data represent the mean value ± S.E.M from three independent experiments. Student's *t*-test was used. **p* < 0.05, ***p* < 0.01 – vs. control (non-treated) SH-SY5Y-T cells.

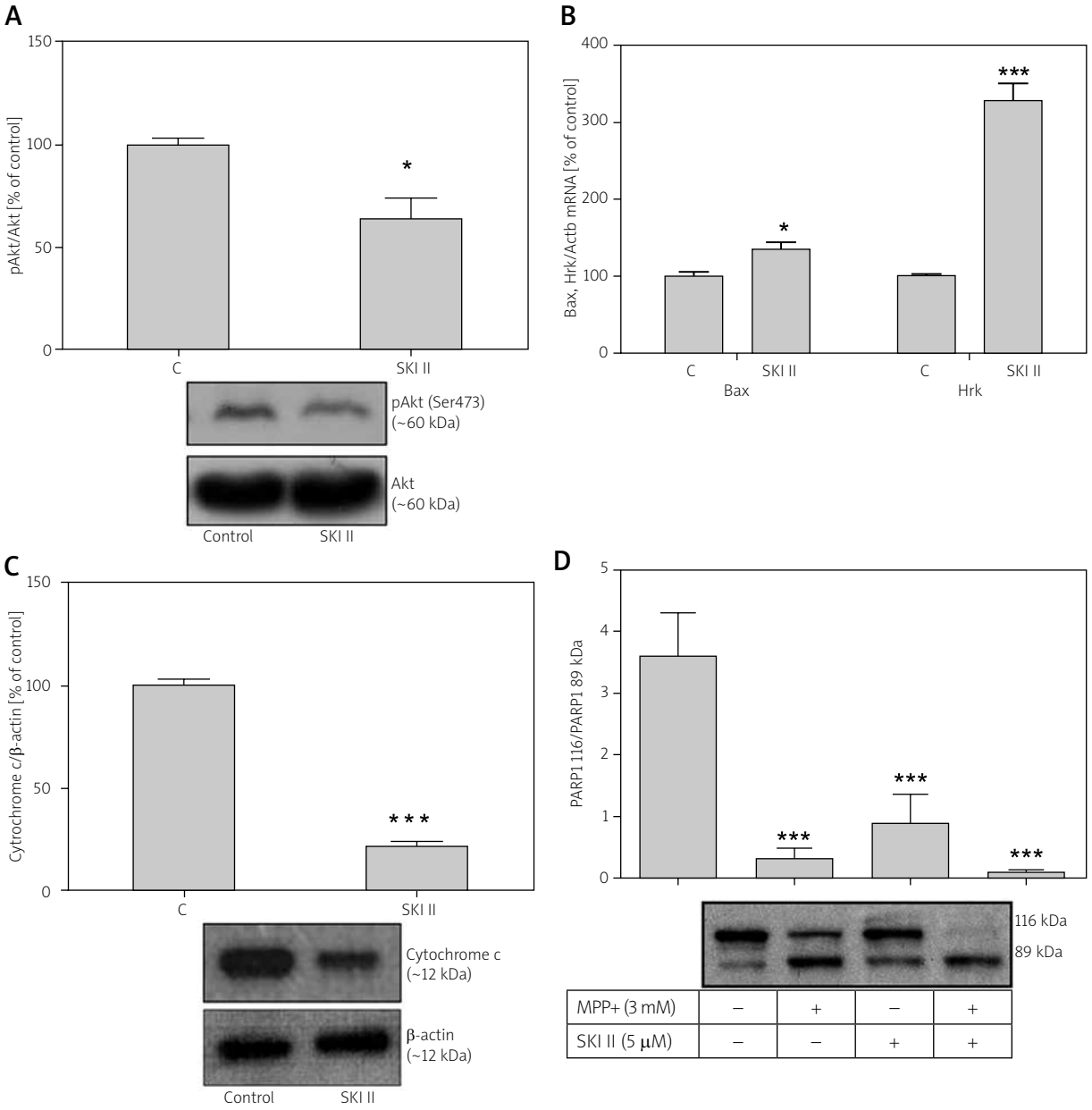
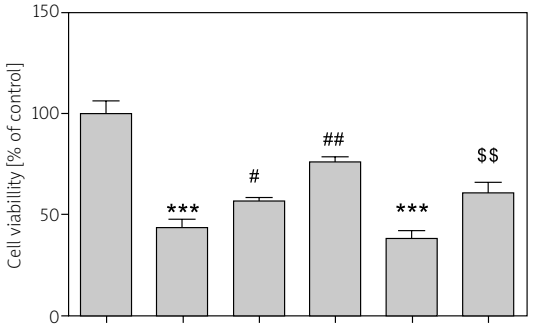


Fig. 4. Effect of Sphks inhibition (using SKI II) on Akt phosphorylation, Bax and Hrk gene expression, level of cytochrome c in the mitochondrial fraction and PARP1 degradation. **A)** SH-SY5Y cells were treated with SKI II (5 μM) for 24 h. Immunochemical detection of pAkt(Ser473)/Akt in cell homogenate was measured. Data represent the mean value ± S.E.M from four independent experiments. Student's *t*-test was used. **p* < 0.05 – vs. control (non-treated) SH-SY5Y cells. **B)** The mRNA level for Bax and Hrk was normalized against β-actin (Actb). The relative level of mRNA was calculated by the ΔΔCt method. Data represent the mean value ± S.E.M from three independent experiments with three replications. Student's *t*-test was used. **p* < 0.05, ****p* < 0.001 – vs. control (non-treated) SH-SY5Y cells. **C)** Immunochemical detection of cytochrome c/β-actin in the mitochondrial fraction was measured. Data represent the mean value ± S.E.M from four independent experiments. Student's *t*-test was used. ****p* < 0.001 – vs. control (non-treated) SH-SY5Y cells. **D)** SH-SY5Y cells were treated with 3 mM MPP+, 5 μM SKI II and with both compounds together for 24 h. The immunochemical detection of PARP1 116 kDa/PARP1 89 kDa in cell homogenate was measured. Data represent the mean value ± S.E.M from four independent experiments. One-way ANOVA followed by the Newman-Keuls *post-hoc* test was used. ****p* < 0.001 – vs. control (non-treated) SH-SY5Y cells.



MPP + 3 mM	–	+	+	+	–	–
Z-VAD-FMK 20 μM	–	–	+	–	–	+
S1P 1 μM	–	–	–	+	–	–
Staurospo-rine 100 nM	–	–	–	–	+	+

Fig. 5. Effect of caspase inhibitor and S1P on MPP+-treated SH-SY5Y cell viability. SH-SY5Y cells were treated with caspase inhibitor (20 μM Z-VAD-FMK) for 2 h or with 1 μM S1P for 1 h and then exposed to 3 mM MPP+ or 100 nM staurosporine for 24 h. Cell viability was determined using MTT assay. Data represent the mean value ± S.E.M from four independent experiments with 4-6 replications. One-way ANOVA followed by the Newman-Keuls post-hoc test was used. $^{***}p < 0.001$ vs. control (non-treated) SH-SY5Y cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. MPP+-treated cells; $^{\$\$} < 0.01$ – vs. staurosporine-treated cells.

icant enhancement of mRNA for SPL, but there was no alteration of S1P phosphatase (SPP1) gene expression (Fig. 2). In these stress conditions, Sphk1 activity in the cytosolic fraction and Sphk2 in the nuclear fraction were measured. Spectrofluorometric analysis indicated that the activity of both enzymes was reduced in MPP+-treated cells (Fig. 3A). Subsequently, we examined the effect of Sphks inhibitor (SKI II 1 μM) and MPP+ (1 mM) (at a concentration that did not change cell viability) on ASN secretion from ASN-transfected cells (SH-SY5Y-T and SH-SY5Y-V). Our data indicated that these compounds significantly enhanced ASN release into the extracellular space (Fig. 3B). Then the role of Sphks inhibition in other molecular events involved in neuronal death, evoked by MPP+, was analyzed. Here we found that the Sphks inhibitor (5 μM SKI II) caused significant suppression of PI3K/Akt phosphorylation/activity (Fig. 4A) and an increase in gene expression for the pro-apoptotic Bcl-2 proteins Bax and Hrk (Fig. 4B). Moreover, Sphks inhibition by 5 μM SKI II significantly reduced the level of cytochrome c in the mitochondrial fraction from the SH-SY5Y cells (Fig. 4C). It was also demonstrated that Sphks inhibition led to degradation of nuclear enzyme poly(ADP-ribose) polymerase PARP1, the preferential substrate for caspase 3 (Fig. 4D). A similar effect of PARP1 degradation was evoked by MPP+. Inhibitor of caspases (20 μM Z-VAD-FMK) enhanced cell viability in MPP+ evoked oxidative stress. However, 1 μM S1P exerts a more efficient neuroprotective effect (Fig. 5).

Discussion

In this study we analysed the role of Sphks inhibition on ASN secretion and in the molecular mechanism of dopaminergic neuronal death in oxidative stress evoked by MPP+. We found altered gene expression and activity of both Sphk1 and Sphk2. The activity of both kinases was significantly decreased in MPP+-evoked stress. We demonstrated that Sphks inhibition evoked death signalling by stimulation of ASN secretion and several molecular events, including inhibition of PI3K/Akt, activation of pro-apoptotic protein expression, release of cytochrome c from the mitochondria and caspase-dependent apoptosis. PI3K/Akt participates in Bax phosphorylation and in stabilization of Bad/14-3-3 complexes [12,42,48]. These events protect against translocation of the pro-apoptotic proteins from the cytosol to the mitochondria and reduce cell death. Several studies have found that S1P, synthesized by Sphk1, is responsible for promoting pro-survival processes through PI3K/Akt [27,31,35]. Here we demonstrated that inhibition of Sphks/S1P formation by SKI II reduced PI3K/Akt phosphorylation on Serine 473 and reduced its activity. Subsequently, these events may reduce BAD phosphorylation. In our experiments we observed lower BAD phosphorylation, but the results were statistically insignificant (data not shown). In this study we found that inhibition of Sphks induced significant up-regulation of Bax and Hrk. Both of these pro-apoptotic proteins are engaged in alteration of mitochondrial membrane potential/permeability and cytochrome c release. Lower content of cytochrome c in mitochondria was observed in our study. It was previously demonstrated that S1P regulates cell survival/death through Bcl-2 proteins [3,4,9,26,43]. Exogenous S1P (1 μM)

enhanced the expression of anti-apoptotic Bcl-2 protein and cell survival in oxidative stress evoked by ceramide [11]. Recently, we demonstrated that S1P (1 μM) reduced the Bax and Hrk mRNA level in MPP+-treated SH-SY5Y cells [41]. In this study we observed that S1P (1 μM) exerted a more efficient neuroprotective effect than the caspase inhibitor. There are no data showing Sphk1/S1P alteration in the PD human brain, as opposed to several post-mortem studies on AD brains demonstrating disturbances in sphingolipid metabolism, including changes in the expression of Sphk1/2, SPL and SPP [6,21,23]. It was shown that Sphk1 and Sphk2 activity declined concomitantly with progression of AD pathology in the hippocampus and in temporal grey matter. Subsequently, the S1P/sphingosine ratio decreased in parallel with an increase in neuropathological changes as described in Braak stages in AD [10]. Recent data demonstrated ceramide and lipid rafts alterations in PD [7,14,15]. It was found that reduced glucocerebrosidase and, in consequence, accumulation of glucosylceramide are associated with an increased ASN level in sporadic PD [34]. The data of Martinez *et al*. [30], Czubowicz and Strosznajder [11] demonstrated that ceramide dose-dependently reduced the viability of primary dopaminergic neurons and neuroblastoma cells respectively. Moreover, the data of Martinez *et al*. [30] showed that the inhibitors of sphingomyelinases protected dopaminergic neurons against ceramide formation, endoplasmic stress, caspase 3 activation and decrease in Akt phosphorylation. Our data suggest that inhibition of Sphks could be responsible for the transient enhancement of sphingosine and for the accumulation of ceramide. The latest study by Murphy *et al*. [34] indicated a significant relationship between lipids and ASN in PD. Our results demonstrated that inhibition of Sphks leads to ASN secretion from dopaminergic neuronal cells. Sphks inhibition and S1P have no effect on ASN gene expression and protein level (data not shown). It was found that MPP+ also evoked ASN release. A previous study suggested that ASN might be a key player in oxidative stress and in MPP+-induced mitochondrial dysfunction of SH-SY5Y cells [57]. Oxidative/nitrosative stress, evoked by different experimental conditions, leads to ASN release from the brain synaptosomal fraction [2]. It seems that in our experimental conditions, the enhanced free radical level and oxidative stress could also be involved in ASN release. Extracellular ASN may be responsible for propagation of pathology from cell to cell. ASN oligomerisation causes oxidative stress and leads to mitochondrial failure and cell death. A rapidly growing amount of evidence indicates that extracellular ASN in oligomeric form contributes to PD pathology [25,56]. In summary, our data indicate that Sphk1 inhibition plays an important role in ASN release and in caspase-dependent apoptotic dopaminergic neuronal death in the experimental PD model.

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Disclosure

Authors report no conflict of interest.

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