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Ceramide is Involved in MPP^+-induced Cytotoxicity in Human Neuroblastoma Cells

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To understand the cytotoxic mechanism of MPP^+, we examined the involvement of ceramide in MPP^+-induced cytotoxicity to human neuroblastoma SH-SY5Y cells. When MPP^+ was exposed to SH-SY5Y cells, MPP^+ induced dose-dependant cytotoxicity accompanied by an elevation of intracellular ceramide levels by 2-fold in SH-SY5Y cells. Three methods were used to test the hypothesis that the elevated intracellular ceramide is related to MPP^+-induced cytotoxicity: C_2-ceramide was directly applied to cells, sphingomyelinase (SMase) was exogenously added, and oleoylethanolamine (OE) was used to inhibit degradation of ceramide. Furthermore, inhibition of ceramide-activated protein phosphatase (CAPP), the effector of ceramide, using okadaic acid (OA) attenuated cell death but treatment of fumonisin B_1, the ceramide synthase inhibitor, did not alter the cytotoxic effect of MPP^+. Based on these, we suggest that the elevation of intracellular ceramide is one of the important mediators in MPP^+-induced cell death.

Key Words: Dopaminergic neuroblastoma, MPP^+, Sphingomyelinase, Ceramide-activated protein phosphatase

INTRODUCTION

Ingestion of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) by humans and primates leads to symptoms biochemically and behaviorally indistinguishable from those of patients with Parkinson's disease (PD). The ingested MPTP is converted to its biochemically active form 1-methyl-4-phenylpyridinium (MPP^+) by monoamine oxidase-B. The active metabolite is then taken up by dopaminergic neurons via dopamine transporter and is accumulated within the mitochondria, where it selectively inhibits mitochondrial respiratory chain leading to a depletion of ATP. Due to mitochondrial dysfunction, ATP depletion and oxidative stress were suggested to be the mechanisms of MPP^+-induced cytotoxicity (Kopin & Markey, 1988; Cleeter et al, 1992; Nakamura et al, 2000). However, the role of these cues in the MPP^+-induced cytotoxicity needs further studies (Di Monte et al, 1986; Martinovits et al, 1986; Mihatsch et al, 1991).

Sphingomyelin pathway is a ubiquitous signaling system that links external stimulation or stress to cellular responses (Kolesnick & Golde, 1994; Hannun, 1996). The pathway is initiated by hydrolysis of sphingomyelin, which is abundant in the plasma membranes of mammalian cells, by sphingomyelinase (SMase). Products of the hydrolysis, phosphatidylcholine and ceramide, are multipotent lipid second messengers, as they are generated from a series of different kinds of environmental conditions. Rather contradictory cellular responses are assigned to the role of the elevated cellular ceramide, such as differentiation (Gamard et al, 1994), proliferation (Hauser et al, 1994), and apoptosis (Obeid et al, 1993; Heller & Kronke, 1994). Details of these ceramide-induced cellular responses and functional targets are yet to be identified, however, involvement of ceramide-activated protein phosphatase (CAPP) has been proposed in these responses as contributing to neuronal apoptosis (Salinas et al, 2000). TNF-α-induced c-myc expression (Wolff et al, 1994), and ceramide's anti-proliferative effect on yeast (Nickels & Broach, 1996). A blockade of these cellular responses by okadaic acid (OA), a specific inhibitor of CAPP, strongly supports the contention that CAPP activity is tightly coupled to the elevation of intracellular ceramide levels. Recently, Brugg et al. (1996) showed that ceramide plays a role in TNF-α-induced apoptosis of dopaminergic neurons cultured from mesencephalon, the most vulnerable region of neuronal degeneration in PD.

In this study, we investigated the involvement of a novel second messenger, ceramide, in dopaminergic neuronal cytotoxicity using human neuroblastoma cell SH-SY5Y. The involvement of ceramide in MPP^+-induced cytotoxicity was determined by measuring intracellular ceramide levels. Also, enhancement of susceptibility of cells exposed to MPP^+ was examined by biochemical treatments that artificially

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ABBREVIATIONS: MPP^+, 1-methyl-4-phenylpyridinium; SMase, sphingomyelinase; OE, oleoylethanolamine; CAPP, ceramide-activated protein phosphatase; OA, okadaic acid.

cause the elevation of intracellular ceramide levels.

METHODS

Materials

MPP^+ iodide, C_2-ceramide, and ceramide (type III) were purchased from RBI (Natick, MA) and dissolved in water and DMSO, respectively. SMase, oleoylethanolamine (OE), OA, and fumonisin B_1 were from Sigma Chemical Co. (St. Louis, MO). Octyl-D-glucopyranoside and *E.coli* DAG kinase were purchased from Calbiochem, [γ-^{32}P]ATP was purchased from Amersham Pharmacia Biotech.

Cells and cell culture

SH-SY5Y cells, derived from human neuroblastoma SK-N-SH, express tyrosine hydroxylase and dopamine transporter (Ross et al, 1983; Takahashi et al, 1994). The cells were maintained in DMEM containing 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified CO_2 incubator at 37°C. The media was changed to DMEM which was supplemented with insulin and transferrin (5 μg/ml each) and left for 48 hr before exposure to drugs.

Cell viability assay

Cell viability was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT). One hundred μl of cells (approximately 10^4 cells) were plated in each well of a 96-well flat-bottom plate. After overnight, cells were maintained in defined media for 48 hr and then exposed to various drugs for specified times. At the end of treatment, 10 μl of MTT (5 mg/ml) was added to each well and incubated for another 2 hr at 37°C. The precipitated formazan was dissolved in DMSO and optical absorbance was read at 570 nm.

Measurement of intracellular ceramide

Ceramide was measured by DAG kinase assay (Dressler & Kolesnik, 1990). Following incubation with MPP^+, cells were collected by centrifugation (300×g, 10 min), washed twice with ice-cold PBS, and extracted with chloroform: methanol: HCl (100 : 100 : 1, v/v/v). Lipids in the organic phase were saved and dried using Speed Vac followed by a mild alkaline hydrolysis (0.1 N methanolic KOH for 1 hr at 37°C) to remove glycerophospholipids. Dried pellets were resuspended in a 100 μl of reaction mixture (pH 6.5) containing 150 μg of cardiolipin, 280 uM diethlyenetriaminepentaacetic acid (DTPA), 51 mM octyl-β-D-glucopyranoside, 50 mM NaCl, 51 mM imidazole, 1 mM EDTA, 12.5 mM $MgCl_2$, 2 mM DTT, 0.7% glycerol, 70 uM β-mercaptoethanol, 1 mM ATP, 10 uCi of [γ-^{32}P]ATP, and 35 μg/ml *E.coli* DAG kinase. After 30 min at room temperature, the reaction was stopped by extraction of lipids with 1 ml of chloroform: methanol: HCl (100 : 100 : 1), 170 μl of buffered saline solution (BSS, 135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, and 10 mM HEPES, pH 7.2), and 30 μl of 100 mM EDTA. The lower organic phase was dried using Speed Vac. Ceramide-1-phosphate was resolved by TLC on silica gel 60 plate using a solvent system of chloroform: methanol: acetic acid (65 : 15 : 5) and detected by autoradiography. The location of ceramide was determined by comparison with authentic ceramide (ceramide type III).

Statistical analysis

All values are expressed as mean±SEM The differences between mean values were analyzed by one-way analysis of variance followed by Tukey *post hock* test. $P<0.05$ was considered statistically significant.

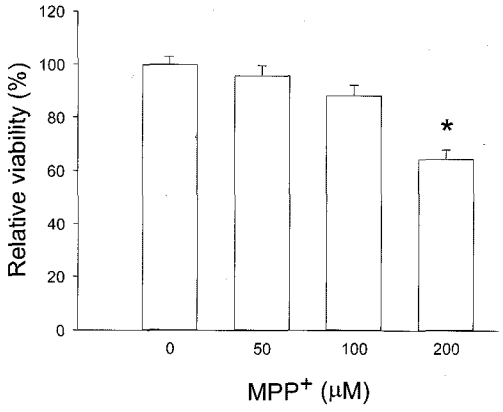

Fig. 1. Cytotoxicity of SH-SY5Y cells induced by MPP^+ treatment. The cells were maintained in serum containing DMEM medium, and then in serum-free, insulin and transferrin (5 μg/ml each) supplemented DMEM, and left for 48 hr before MPP^+ exposure. Cells were exposed to MPP^+ for 24 hr and cytotoxicity was determined at each concentration using MTT reduction assay. Viabilities are expressed as a percentage of that of untreated cells. Data are mean± SEM from 5 experiments of triplicates at each concentration. * indicates $p<0.05$, compared with no MPP^+ treatment.

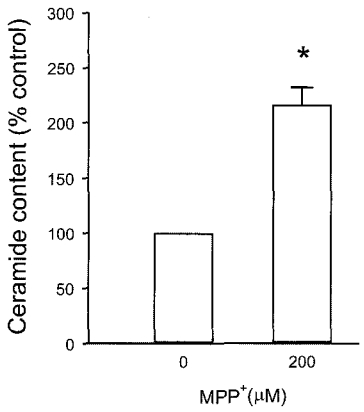

Fig. 2. Intracellular ceramide levels in SH-SY5Y cells after MPP^+ treatment. Cells were maintained as described above, exposed to 200 uM MPP^+ for 24 hr, and intracellular ceramide levels were determined. After exposure, intracellular ceramide levels in MPP^+-treated cells were approximately 220% higher than that in untreated control cells. Data are mean±SEM from 4 separate experiments. *indicates $p<0.05$, compared with no MPP^+ treatment.

RESULTS

MPP^+-induced cytotoxicity and accumulation of intracellular ceramide

As a model system of MPP^+-induced neurotoxic mechanism, we examined the viability of SH-SY5Y, human dopaminergic neuroblastoma cells, exposed to MPP^+. Cells were maintained in defined media for 48 hr before exposure to MPP^+ and cell viability was determined 24 hr after MPP^+ treatment using MTT reduction assay (Fig. 1). Cell viability decreased in a dose-dependent manner and approximately 35% of the cells died at 200 uM MPP^+ within 24 hr. Since hydrolysis of sphingomyelin has been implicated in a variety of cellular responses in several cell types including neurons (Obeid et al, 1993; Hauser et al, 1994; Heller & Kronke, 1994), and to find the mediator of MPP^+-induced cytotoxicity, intracellular ceramide levels were measured 24 hr after the addition of MPP^+. MPP^+ treatment increased the intracellular ceramide levels two-fold in SH-SY5Y cells (Fig. 2).

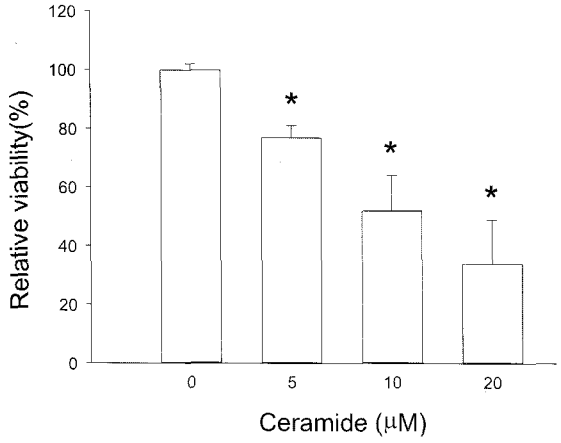

Fig. 3. Dose-dependent C_2-ceramide toxicity in SH-SY5Y cells. Exposure of cells to C_2-ceramide (5~20 uM) for 24 hr revealed a dosage-dependent cell death. Approximately 45% of the cells treated with 10 uM died within 24 hr, while about 60% of the cells died upon exposure to 20 uM ceramide. Viabilities are expressed as a percentage of that of the untreated cells. Data are mean±SEM from 5 separate experiments of triplicates. *indicates $p<0.05$, compared with no ceramide treatment.

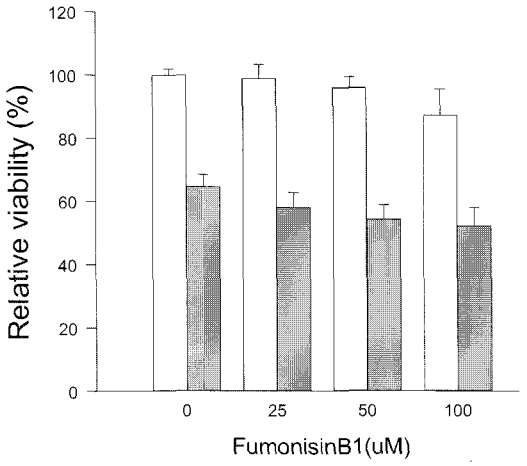

Fig. 4. Effect of inhibition of *de novo* ceramide synthesis on MPP^+-induced cytotoxicity in SH-SY5Y cells. Cells exposed to 200 uM MPP^+ with fumonisin B_1 ranging from 25 uM to 100 uM did not exhibit any significant alteration of viabilities. Blank bars represent viabilities of cells exposed to fumonisin B_1 alone and shaded bars viabilities of cells exposed to both fumonisin B_1 and MPP^+ (200 uM). Viabilities are expressed as a percentage of that of untreated cells. Data are mean±SEM from 5 separate experiments of triplicates.

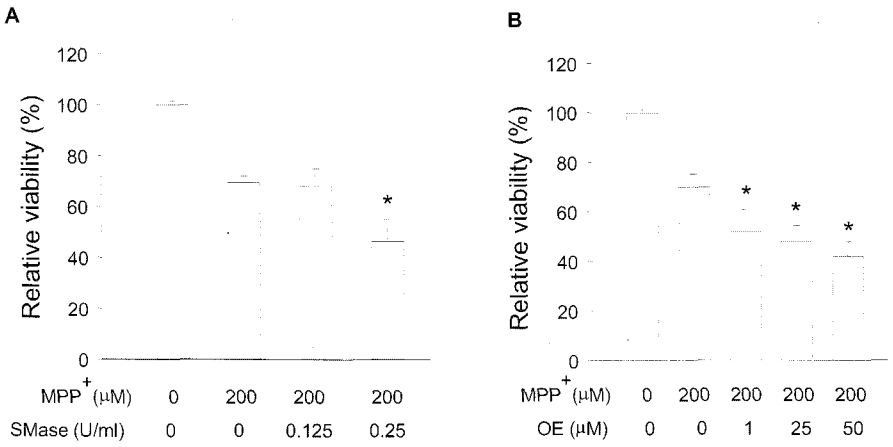

Fig. 5. Increase in susceptibility of SH-SY5Y cells to MPP^+ by forced elevation of intracellular ceramide levels. The viability of cells pretreated with 0.125 U/ml of SMase was not much different from that of the cells exposed to MPP^+ alone, however, pretreatment of 0.25 U/ml of SMase significantly increased susceptibility to MPP^+. Likewise, cells pre-exposed to OE (0~50 uM) also showed a dose-dependent enhancement of susceptibility to MPP^+. Viabilities are expressed as a percentage of that of untreated cells. Data are mean±SEM from 5 separate experiments of triplicates. *indicates $p<0.05$, compared with 200 uM MPP^+ treatment without SMase or OE respectively.

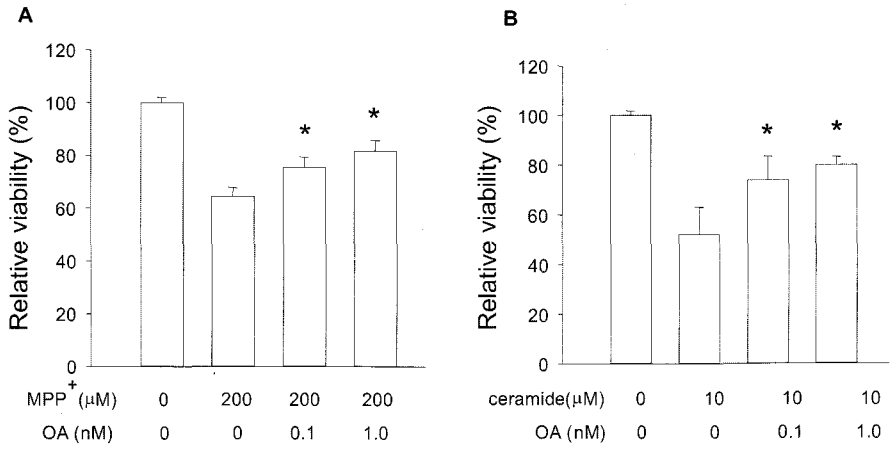

Fig. 6. Protective effects of okadaic acid (OA) on cytotoxicity induced by MPP^+ or C_2-ceramide. The activity of CAPP was inhibited by treatment of OA (1.0 nM) 30 min before exposure to 200 uM MPP^+ or 10 uM C_2-ceramide and then cell viability was determined 24h after MPP^+ or C_2-ceramide exposure. Treatment of OA significantly protected the cytotoxicity caused by MPP^+ (A) and C_2-ceramide (B). Viabilities are expressed as a percentage of that of untreated cells. Data are mean ± SEM from 5 separate experiments of triplicates. * indicates $p < 0.05$, compared with 200 uM MPP^+ or 10 uM ceramide but without OA..

Effects of C_2-ceramide and forced elevation of intracellular ceramide on MPP^+-induced cytotoxicity

To test if accumulated ceramide is responsible for the death of dopaminergic cells, we examined whether or not C_2-ceramide, the cell permeable analog of ceramide, can mimic MPP^+-induced cytotoxicity. Viability of SH-SY5Y cells exposed to various concentrations of C_2-ceramide reduced in a dose-dependent manner, with over 60% of the cells dying within 24 hr at 20 uM C_2-ceramide. Next, we checked whether MPP^+-induced cytotoxicity can be modified by changing the intracellular pathways for generation of ceramide. When cells were pretreated with ceramide synthase inhibitor, fumonisin B_1, cytotoxicity induced by MPP^+ was not significantly changed (Fig. 4). Mediation of MPP^+-induced cytotoxicity via ceramide was further examined by enhancing the hydrolysis of sphingomyelin using exogenously added SMase. Cells exposed to both MPP^+ and SMase (0.25 U/ml) exhibited a significantly lower viability than cells exposed to MPP^+ alone (Fig. 5A). Furthermore, treatment of OE, which inhibits degradation of ceramide, worsened the viability of the cells in a dose-dependent manner (Fig. 5B) suggesting that the elevation of intracellular ceramide is due to the activation of SMase and also related to MPP^+-induced cytotoxicity.

Effect of okadaic acid (OA) in MPP^+- and C_2-ceramide-induced cytotoxicity

Execution of cell death via ceramide was further examined by investigating the involvement of its effector CAPP (Kowluru & Metz, 1997). In this experiment, the activity of CAPP was inhibited by treatment of OA. Thirty minutes after OA pretreatment, SH-SY5Y cells were exposed to either MPP^+ or C_2-ceramide and cell viability was determined. Co-treatment of 1.0 nM OA and MPP^+ partially restored cell viability and reversed C_2-ceramide-induced cytotoxicity by approximately 80% (Fig. 6. A, B).

DISCUSSION

In this experiments, exposure of human neuroblastoma cells to MPP^+ showed dose-dependant cytotoxicity accompanied by an elevation of intracellular ceramide levels. Elevation of ceramide by MPP^+ is not confined to this cell line as MPP^+ also increased intracellular levels of ceramide in cerebellar granule cells (data not shown). The lethal effect of ceramide was confirmed by a direct application of cell-permeable analog C_2-ceramide, which also showed a dose-dependent cytotoxicity. Furthermore, maintaining the elevated levels of ceramide by inhibiting the natural degradation of ceramide rendered cells more susceptible to MPP^+ treatment implying that ceramide plays a pivotal role in MPP^+-induced cell death.

Consistent with our data, exogenously added SMase caused oligonucleosomal cleavage of genomic DNA in human leukemic and murine fibrosarcoma cells (Jarvis et al, 1994) and C_2-ceramide also killed Y79 retinoblastoma cells (Vento et al, 1998). Intracellular ceramide levels can be increased by activation of either ceramide synthase or SMase (Heller & Kronke, 1994; Yoshimura et al, 1998; Chan & Goldkorn, 2000; Kroesen et al, 2001). In the case of ceramide synthase activation, ceramide levels will increase without altering the levels of sphingomyelin within the membrane, and the cellular responses induced by the increased ceramide are blocked by fumonisin B_1 (Kroesen et al, 2001). However, if SMase is activated, the elevation of ceramide levels is accompanied by a reduction of sphingomyelin content in the membrane as observed in

PC12 cells exposed to hypoxia (Yoshimura et al, 1998) and in human airway epithelial cells exposed to H_2O_2 (Chan & Goldkorn, 2000). In this study, exogenously added SMase enhanced the cytotoxicity of MPP^+-treated SH-SY5Y cells, while little effect on the cytotoxicity of MPP^+ was observed when the activity of ceramide synthase was blocked by a co-treatment with fumonisin B_1. The slight but dose-dependent reduction of the viability observed with coadministration of MPP^+ with fumonisin B_1 must be due to the toxic effect of the drug (Jones et al, 2001; Sharma et al, 2001) since administration of fumonisin B_1 alone exhibited a similar degree of cytotoxicity. So, the elevation of intracellular ceramide levels responsible for cell death upon exposure to MPP^+ might be caused by a hydrolysis of sphingomyelin rather than *de novo* synthesis of ceramide.

Since hydrolysis of sphingomyelin has been implicated in a variety of cellular responses in several cell types including neurons (Obeid et al, 1993; Hauser et al, 1994; Heller & Kronke, 1994), it is very interesting how MPP^+ can increase intracellular ceramide. Activation of SMase was reported in PC12 cells exposed to hypoxia (Yoshimura et al, 1998) and in human airway epithelial cells exposed to H_2O_2 (Chan & Goldkorn, 2000). These results showed that the genareation of intracellular ceramide is closely related with oxidative stress. But it was recently reported that MPP^+-induced cell death through activation of SMase is not directly correlated with reactive oxygen species (ROS) formation in PC12 cells (Fonck & Baudry, 2001). Our previous report also supports that MPP^+ cannot activate SMase by producing ROS because the generation of ROS and the protection of cell death by antioxidants in SH-SY5Y cells after MPP^+ treatment were not shown (Lee et al, 2000). The mechanism of SMase activation in MPP^+-exposed cells is yet to be explained but one possibility is that the activation is mediated by a reduction of intracellular GSH level. Liu et al. (1998) reported that application of TNF-α to mammary carcinoma MCF7 cells resulted in a marked reduction of intracellular GSH and an enhanced hydrolysis of sphingomyelin. In addition, exogenously added GSH prevented the activation of SMase in PC12 cells under hypoxia (Obeid et al, 1993; Yoshimura et al, 1999), which suggests a relationship between a low level of intracellular GSH and SMase activation. Considering a requirement of ATP for maintaining high levels of intracellular GSH (Phelps et al, 1995; van den Dobbelsteen et al, 1996), it is more reliable that the activation of SMase in MPP^+-treated cells is primarily caused by a depletion of ATP by mitochondrial dysfunction, which results in an efflux of intracellular GSH (Fernandez-checa et al, 1988; Mithofer et al, 1992).

Mediation of cell death by ceramide was further examined by inhibiting the functional target of ceramide. CAPP is a member of the protein phosphatase 2A (PP2A) family whose activity is induced by elevated intracellular ceramide levels and is inhibited by serine/threonine phosphatase inhibitor OA (Dobrowsky et al, 1993; Kowluru & Metz, 1997). The specificity of OA was further examined in HL-60 cells where TNF-α mediated c-myc expression was inhibited by OA but similar induction by PMA was not affected by OA (Wolff et al, 1994). Retinoic acid-induced differentiation of Neuro2a cells was also hindered by OA suggesting that CAPP involvement in cellular differentiation was also affected by OA (Prinetti et al, 1997). Viewed collectively, these reports provide evidence that CAPP is the effector of ceramide whether the accumulated ceramide is inducing cellular differentiation or death. In this study we did not directly examine the inhibition of CAPP activity by OA pretreatment, but exposure of either MPP^+- or ceramide-treated cells to OA significantly reversed the cytotoxicity. So, it can be suggested that the lethal signal induced by MPP^+ or ceramide was propagated via CAPP. However, since OA was unable to completely reverse the cytotoxicity, the involvement of any other hypothetical molecular effector other than CAPP in MPP^+-induced cell death cannot be completely ruled out.

In conclusion, the present study demonstrated that MPP^+ induced the dose-dependant cytotoxicity accompanied by an elevation of intracellular ceramide levels. And maintaining the elevated levels of ceramide by inhibiting the natural degradation of ceramide or exogenously treating with SMase rendered cells more susceptible to MPP^+-induced cytotoxicity. Based on these results, it can be suggested that the elevation of intracellular ceramide is one of the important mediators in MPP^+-induced cell death.

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