

Guanosine exerts neuroprotective effects by reversing mitochondrial dysfunction in a cellular model of Parkinson's disease

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Received April 19, 2014; Accepted August 7, 2014

DOI: 10.3892/ijmm.2014.1904

Abstract. The mitochondria are the most important cytoplasmic organelles in determining cell survival and death. Mitochondrial dysfunction leads to a wide range of disorders, including neurodegenerative diseases. The central events in the mitochondrial-dependent cell death pathway are the activation of the mitochondrial permeability transition pore (mPTP) and the disruption of mitochondrial membrane potential, which cause the release of apoptogenic molecules and finally lead to cell death. This is thought to be at least partly responsible for the loss of dopaminergic neurons in Parkinson's disease (PD); thus, the attenuation of mitochondrial dysfunction may contribute to alleviating the severity and progression of this disease. Guanosine is a pleiotropic molecule affecting multiple cellular processes, including cellular growth, differentiation and survival. Its protective effects on the central nervous system and on several cell types by inhibiting apoptosis have been shown in a number of pathological conditions. This study aimed to analyze the ability of guanosine to protect neuronal PC12 cells from the toxicity induced by 1-methyl-4-phenylpyridinium (MPP⁺), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which mediates selective damage to dopaminergic neurons and causes irreversible Parkinson-like symptoms in humans and primates. Our results demonstrated that the apoptosis of PC12 cells induced by MPP⁺ was significantly prevented by pre-treatment for 3 h with guanosine. In addition, guanosine attenuated the MPP⁺-induced collapse of mitochondrial transmembrane potential and prevented the

subsequent activation of caspase-3, thereby protecting dopaminergic neurons against mitochondrial stress-induced damage.

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the gradually progressive and selective loss of dopaminergic neurons in the substantia nigra (1). The progressive loss of dopaminergic neurons is a complex process, and multiple pathological events are involved in this process (2-6). While the underlying mechanisms of nigrostriatal dopaminergic neuron degeneration are not yet completely understood, accumulating evidence indicates that mitochondrial dysfunction may be a central event in neurodegenerative diseases (7-9). The mitochondria are multifunctional organelles that are important for living cells. Mitochondrial dysfunction has a multitude of consequences for cells, including apoptosis (10). The activation of the mitochondrial permeability transition pore (mPTP) and the collapse of the mitochondrial membrane potential may be major contributors to mitochondrial-dependent cell death and at least partly responsible for the pathogenesis of PD and several other neurodegenerative disorders (7-9).

PC12 cells treated with 1-methyl-4-phenylpyridinium (MPP⁺) provide a reliable *in vitro* model for investigating the pathogenesis of PD. MPP⁺ is an active metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is known to selectively kill dopaminergic neurons and cause irreversible Parkinson-like symptoms in humans and primates (11-13). MPTP is a lipophilic molecule that can rapidly cross the blood-brain barrier; it is subsequently oxidized in the brain to its toxic metabolite, MPP⁺, by type B monoamine oxidase (14). The neurotoxic action of MPP⁺ is related to the activation of the mPTP and the collapse of mitochondrial membrane potential through oxidative damage, which together initiate the downstream apoptotic pathway, including the release of cytochrome c and the activation of caspases, finally leading to neuronal cell death (7-9). Damage to the mitochondria is considered as an initial and irreversible step towards apoptosis; thus, mitochondrial-targeted therapeutic strategies may be a promising treatment for PD. Guanosine, a non-adenine-based purine, is an intercellular signaling molecule affecting multiple cellular processes, including cellular growth, differentiation and survival (15-17). In multiple cell types, it exerts protective

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Abbreviations: PD, Parkinson's disease; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP⁺, 1-methyl-4-phenylpyridinium; mPTP, mitochondrial permeability transition pore; DCF, 2',7'-dichlorodihydrofluorescein; Bcl-2, B-cell lymphoma 2; ROS, reactive oxygen species

Key words: Parkinson's disease, mitochondrial dysfunction, apoptosis, guanosine, neuroprotection, 1-methyl-4-phenylpyridinium

effects against apoptosis induced by a number of agents, such as staurosporine (18), β -amyloid (19) and MPTP (20). The neuroprotective effects of guanosine in the central nervous system have also been recognized (15). The present study was designed to investigate the effects of guanosine on MPP⁺-induced apoptosis in PC12 cells and the underlying mechanisms for these actions. Our results demonstrated that guanosine effectively prevented MPP⁺-induced PC12 cell apoptosis by stabilizing the mitochondrial membrane potential and attenuating the subsequent activation of caspases. In addition, guanosine inhibited the production of reactive oxygen species (ROS) and increased the expression levels of glutathione (GSH), further supporting the protective role of guanosine in oxidative conditions. Overall, these findings indicate the protective role of guanosine in mitochondrial stress-induced dopaminergic neuronal damage, thus providing potential effective strategies for the treatment of PD.

Materials and methods

Drugs and chemicals. All reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

PC12 cell cultures. The PC12 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 4.00 mM L-glutamine, 100 U/ml of penicillin and 100 μ g/ml of streptomycin (Gibco, Grand Island, NY, USA). The cultures were maintained in a humidified 5% CO₂ atmosphere at 37°C. The culture medium was changed every 3-4 days and the cells were seeded at a density of 30,000 cells/cm².

Cell viability assay. Cell viability was measured using the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a mitochondrial dye which is converted into a blue formazan product by mitochondrial dehydrogenases in metabolically active cells. The PC12 cells were plated at a density of 30,000 cells/cm² in 96-well plates and incubated for 24 h. To assess the neuroprotective effects of guanosine on MPP⁺-induced toxicity in PC12 cells, the cells were pre-treated with various concentrations of guanosine (0.01-1,000 μ M) for 3 h and were then exposed to 500 μ M MPP⁺ for 72 h, under optimal conditions for the assessment of the neuroprotective effects as previously described (21). MTT (5 mg/ml) solution was added to the wells and the cells were incubated for 4 h. Subsequently, the culture medium was removed, and dimethyl sulfoxide was added to each well to solubilize the formazan into a colored solution. The absorbance of colored solution was measured at 570 nm using a microplate reader (Epoch; BioTek, Winooski, VT, USA). The results were expressed as the percentage of the absorbance of the control culture wells. Based on these results, we used guanosine at a dose of 10 μ M in all the subsequent experiments.

Nuclear staining assay. The morphological signs of apoptosis induced by MPP⁺ were detected using acridine orange (AO)/ethidium bromide (EB) staining of the PC12 cells. The cells were plated in 6-well plates at a density of 30,000 cells/cm² and were incubated in DMEM medium at 37°C. After 3 h of pre-

treatment with guanosine (10 μ M), MPP⁺ (500 μ M) was added to the medium for 72 h. The cells were washed and resuspended in phosphate-buffered saline (PBS) and AO/EB was added at a final concentration of 1 μ g/ml. Subsequently, the number of apoptotic cells was randomly counted under a fluorescence microscope (IX71; Olympus, Tokyo, Japan). Viable cells with intact structures stained with AO only showed bright green nuclear staining; the early apoptotic cells were bright green and later apoptotic cells were red-orange with condensed chromatin. The number of apoptotic cells is expressed as a percentage of the total cells counted.

Measurement of apoptosis in cells. Apoptosis was assessed by measuring DNA fragmentation with single-stranded DNA (ssDNA) apoptosis enzyme-linked immunosorbent assay (ELISA) kits (Chemicon International, Temecula, CA, USA) according to the manufacturer's instructions. The cells plated at a concentration of 30,000 cells/cm² were cultured for 24 h, followed by treatment with 10 μ M guanosine prior to the addition of 500 μ M MPP⁺ for 3 h. The cells were washed 3 times with PBS and formamide was then added which selectively denatures DNA in apoptotic cells. Anti-ssDNA monoclonal antibody and peroxidase-conjugated secondary antibody were then added to the cells; the ssDNA was then measured at 450 nm using a microplate reader (Epoch; BioTek).

Measurement of mitochondrial transmembrane potential. Mitochondrial membrane potential is a key indicator of mitochondrial function and cell death or injury, which can be detected using the mitochondrial dye, 3,3-dihexyloxycarbocyanine iodide [DiOC₆(3)]. This dye is a lipophilic fluorescent stain and becomes highly fluorescent when incorporated into membranes. The cells at a concentration of 30,000 cells/cm² were cultured in 24-well plates for 24 h, followed by treatment with 10 μ M guanosine prior to the addition of 500 μ M MPP⁺ for 3 h. Following 72 h of incubation, 1 ml of serum-free culture medium containing DiOC₆(3) was added to each well with the final concentration of 1 μ M, and the cells were cultured in a humidified incubator for 15 min. The cells were collected and centrifuged at 1,000 x g for 5 min, and the cell pellets were resuspended in PBS containing 0.5 mM EDTA. The intensity of DiOC₆(3) fluorescence was recorded using a flow cytometer (Becton-Dickinson, San Diego, CA, USA).

Western blot analysis. Following treatment, the PC12 cells were collected and lysed with cell lysis solution containing 4% sodium dodecyl sulfate (SDS), 2 mM EDTA and 50 mM Tris-HCl, pH 6.8. Equal amounts of protein were loaded onto a 12% SDS-polyacrylamide gel. Following electrophoretic separation, the polyacrylamide gels were transferred onto PVDF transfer membranes (Amersham Biosciences, Uppsala, Sweden). The membranes were incubated in Tris-buffered saline/Tween-20 (TBST) supplemented with 5% fat-free milk for 1 h to block non-specific binding. The blots were incubated using rabbit anti-Bax, anti-B-cell lymphoma 2 (Bcl-2) antibodies. Horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies were used as the secondary antibodies.

Measurement of ROS production. Intracellular ROS produced during the inhibition of mitochondrial complex I was detected

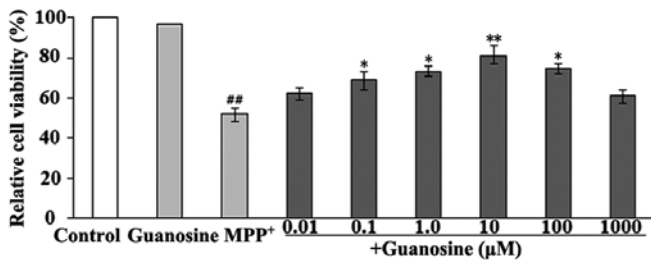


Figure 1. Effect of guanosine on 1-methyl-4-phenylpyridinium (MPP⁺)-induced toxicity in PC12 cells. PC12 cells were pre-treated with guanosine (0.01-1,000 µM) for 3 h, and then exposed to 500 µM MPP⁺ for 72 h. Cell viability was determined by MTT assay as described in the Materials and methods. Data are presented as the means ± standard error of the mean (SEM) of 3 independent experiments performed in sixuplicate. ##p<0.01 vs. control and *p<0.05 or **p<0.01 compared to treatment with MPP⁺.

using 2'-7'-dichlorofluorescein diacetate (DCFH-DA). This is a non-fluorescent cell-permeating compound that can easily diffuse into cells and be converted into dichlorofluorescein (DCFH) by intracellular esterase. DCFH is then trapped within the cell and oxidized into fluorescent dichlorofluorescein (DCF) by intracellular ROS. Following treatment, the cells were incubated in BSA-free DMEM with DCFH-DA at a final concentration of 20 µM for 30 min at 37°C. Thereafter, 10,000 cells of each group were analyzed by flow cytometry using the FL1 flow cytometer detection channels. The excitation wavelength was 485 nm and the reading was performed at 530 nm.

Measurement of GSH levels. GSH levels were measured using GSH reductase, as previously described (22). Briefly, following centrifugation and washing with PBS, the cells were dissolved with 2% 5-sulfosalicylic acid and incubated in 100 µl of the reaction mixture containing 20 mM sodium EDTA, 600 µM nicotinamide adenine dinucleotide phosphate (NADPH), 12 mM 5,5'-dithiobis(2-nitrobenzoic acid) and 105 mM NaH₂PO₄. GSH reductase was added to each well, and the cells were cultured in a humidified incubator for 10 min. Absorbance was measured at 450 nm, and the calibration curve was performed with standard GSH solutions. The results are expressed as percentages of the control condition.

Evaluation of caspase-3 activity. Caspase-3 activity was measured using an ApoAlert caspase-3 assay kit according to the manufacturer's instructions. Briefly, the cells were lysed and centrifuged at 1,000 x g for 10 min, then the supernatant was added to the reaction mixture containing dithiothreitol and caspase-3 substrate (N-acetyl-Asp-Glu-Val-Asp p-nitroanilide). The cells were incubated for 1 h at 37°C, and the absorbance of the chromophore p-nitroanilide produced was measured at 450 nm. The standard curves were obtained from the absorbance of p-nitroanilide standard reagent diluted with cell lysis buffer. One unit of the enzyme was defined as the activity producing 1 nmol of p-nitroanilide.

Statistical analysis. Data are expressed as the means ± standard error of the mean (SEM). Statistical analysis was performed by one-way analysis of variance, followed by Dunnett's

multiple-comparisons test. Differences between mean values were considered statistically different at p<0.05.

Results

Guanosine reduces the MPP⁺-induced loss of cell viability. The ability of guanosine to reverse the cytotoxicity to PC12 cells induced by MPP⁺ was investigated using MTT, which is a mitochondrial dye and can be converted into a blue formazan product by mitochondrial dehydrogenases; therefore, it can partially detect the levels of metabolically active cells. The measurements revealed a significant decrease in the viability of the PC12 cells following exposure to 500 µM MPP⁺ for 72 h; however, the cells treated with guanosine alone did not show a decrease in cell viability. Pre-treatment with 10 µM guanosine significantly decreased the MPP⁺-induced cytotoxicity (Fig. 1).

Guanosine attenuates MPP⁺-induced apoptosis in PC12 cells. To determine whether guanosine prevents MPP⁺-induced apoptosis in PC12 cells, AO/EB and DNA fragmentation assays were performed. Apoptosis is a process of programmed cell death characterized by a series of distinct nuclear morphological changes. These changes can be detected by AO/EB staining. This assay identified 3 types of cells under a fluorescence microscope: live cells (green), early apoptotic cells (bright green with condensed chromatin) and later apoptotic cells (red-orange with condensed chromatin). The administration of guanosine alone did not induce changes in the number of apoptotic cells, while the administration of MPP⁺ significantly increased the number of apoptotic cells compared to the control group (p<0.01). Pre-treatment with 10 µM guanosine significantly decreased the number of apoptotic cells induced by exposure to MPP⁺ (p<0.01; Fig. 2), indicating that guanosine plays an anti-apoptotic role. To clarify the neuroprotective role of guanosine on MPP⁺-induced toxicity in PC12 cells, DNA fragmentation, a marker of late apoptosis, was further investigated by ssDNA assay. The results revealed that the increase in DNA fragmentation induced by exposure to MPP⁺ was markedly attenuated by pre-treatment with guanosine (Fig. 3), supporting the protective role of guanosine in conditions of oxidative stress.

Guanosine modulates Bax and Bcl-2 protein expression. Bax and Bcl-2 are key members of the Bcl-2 family of proteins that contribute to the opening of mPTP, leading to the induction of apoptosis. To investigate the changes in Bax and Bcl-2 protein expression levels, western blot analysis was performed on the untreated cells and the cells treated with 500 µM MPP⁺ alone or 500 µM MPP⁺ in the presence of 10 µM of guanosine. The administration of MPP⁺ significantly increased the levels of Bax expression and decreased Bcl-2 expression. These changes were markedly reversed by pre-treatment with guanosine. Treatment with guanosine alone did not induce changes in the expression levels of these proteins (Fig. 4), thus further demonstrating the protective role of guanosine in mitochondrial-stress induced cell damage.

Guanosine prevents the MPP⁺-induced collapse of mitochondrial transmembrane potential. Mitochondrial membrane potential maintenance is essential for living cells, and its collapse is a key event in the activation of the mitochondrial-dependent

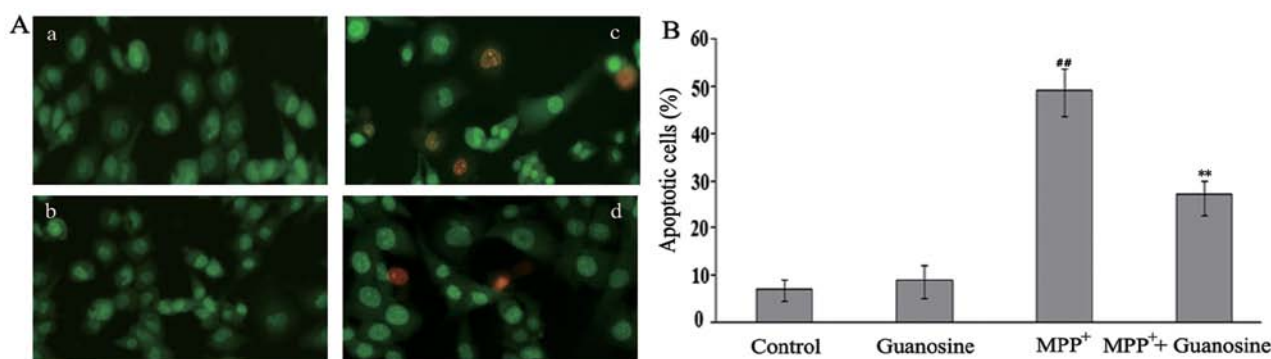


Figure 2. (A) Effects of guanosine on 1-methyl-4-phenylpyridinium (MPP⁺)-induced neuronal cell apoptosis were analyzed by acridine orange (AO)/ethidium bromide (EB) assay. (a) Control; (b) treatment with 10 μ M guanosine; (c) treatment with 500 μ M MPP⁺; (d) treatment with 10 μ M guanosine plus 500 μ M MPP⁺. (B) Histograms show percentages of values in total cells. Data are presented as the means \pm standard error of the mean (SEM). $n=3$. ^{**} $p<0.01$ vs. control and ^{**} $p<0.01$ compared to treatment with MPP⁺.

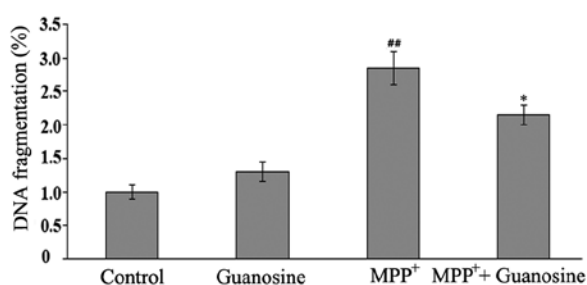


Figure 3. Effects of guanosine on 1-methyl-4-phenylpyridinium (MPP⁺)-induced apoptosis of PC12 cells was determined by single-stranded DNA assay. The histograms show percentages of values in total cells. Data are presented as the means \pm standard error of the mean (SEM). $n=3$. ^{**} $p<0.01$ vs. control and ^{*} $p<0.01$ compared to treatment with MPP⁺.

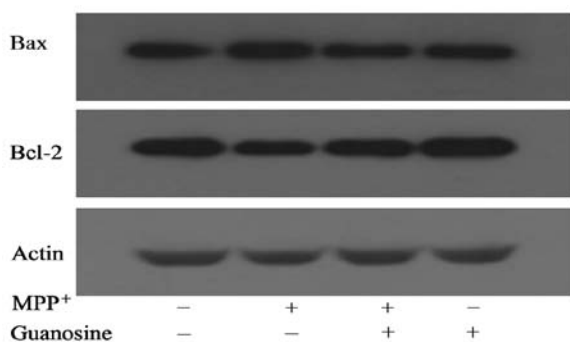


Figure 4. Effects of guanosine on the levels of Bax and Bcl-2 protein expression in 1-methyl-4-phenylpyridinium (MPP⁺)-exposed PC12 cells. PC12 cells were either untreated, or treated with 500 μ M MPP⁺ alone or 500 μ M MPP⁺ in the presence of 10 μ M guanosine. The protein expression levels of Bax and Bcl-2 were determined by western blot analysis, and actin levels were measured as a loading control.

pathway. The collapse of mitochondrial transmembrane potential was assessed by measuring the response to the mitochondrial dye, DiOC₆(3), which is converted into a highly green fluorescent dye following incorporation into mitochondrial membranes, thereby allowing the qualitative assessment of mitochondrial membrane potential. The administration of MPP⁺ in comparison with the control cells induced a significant decrease in fluorescence intensity, indicating the increasing

percentage of the cells with collapse of mitochondrial membrane potential. The results also revealed a marked reduction in the number of cells with the collapse of mitochondrial membrane potential, when guanosine was administered prior to exposure to MPP⁺; no significant change was observed following treatment with guanosine alone (Fig. 5). These results suggest that the mitochondrial dysfunction induced by MPP⁺ can be partly restored by the administration of guanosine.

Guanosine inhibits the MPP⁺-induced production of ROS. The levels of ROS production were evaluated by flow cytometry with DCFH-DA. DCFH-DA is a stable compound that can easily diffuse into cells, where it is converted into DCFH by intracellular esterase. DCFH is then trapped within cells and oxidized to highly fluorescent DCF by intracellular ROS; thus, the intensity of fluorescence produced by DCF may reflect an intracellular oxidative state.

The administration of guanosine alone, compared with the control group, did not elicit changes in the levels of DCFH oxidation. The administration of MPP⁺ induced a significant increase in DCFH oxidation in the PC12 cells, which was markedly reversed by pre-treatment with guanosine (Fig. 6A), thus indicating that guanosine may play an antioxidant role.

Guanosine reverses the MPP⁺-induced reduction in GSH levels. GSH protein is a major non-enzymatic antioxidant that plays a crucial role in protecting neurons from oxidative damage in the central nervous system (23). To assess the protective role of guanosine in MPP⁺-induced oxidative damage, the levels of GSH were measured in the PC12 cells. The administration of MPP⁺ in comparison with the control cells induced a significant decrease in GSH levels; this effect was markedly reversed by pre-treatment with guanosine. The administration of guanosine alone did not elicit any changes in the levels of GSH (Fig. 6B).

Guanosine reduces caspase-3 activity. Caspase-3 is an effector caspase that cleaves a wide range of signal transduction proteins in the apoptotic process (24). To determine whether guanosine protects neuronal PC12 cells against MPP⁺-induced cell death, the activity of caspase-3 was measured by ELISA with an ApoAlert caspase-3 assay kit. The PC12 cells exposed

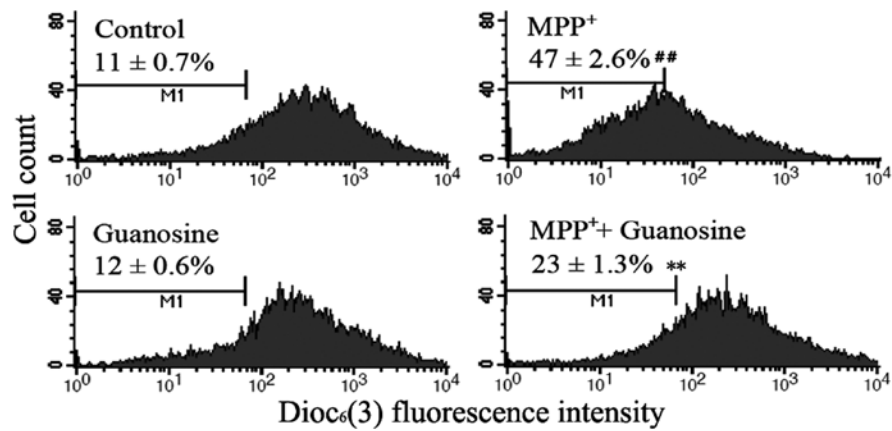


Figure 5. Mitochondrial transmembrane potential was determined by flow cytometry with the mitochondrial dye, 3,3-dihexyloxacarbocyanine iodide [DiOC₆(3)]. The x-axis shows the log scale of fluorescence intensity and the y-axis represents the cell count. Data are presented as the means ± standard error of the mean (SEM) of 3 independent experiments. ##p<0.01 vs. control and **p<0.01 compared to treatment with 1-methyl-4-phenylpyridinium (MPP⁺).

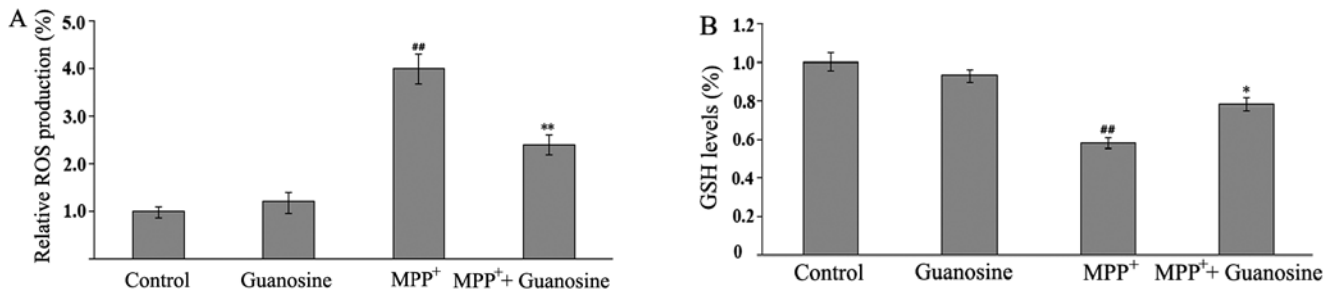


Figure 6. Effects of guanosine on 1-methyl-4-phenylpyridinium (MPP⁺)-induced reactive oxygen species (ROS) and glutathione (GSH) production. PC12 cells were pre-treated with guanosine for 3 h and then either exposed or not to MPP⁺ for 72 h. The production of ROS in the cells was measured by flow cytometry with DCFH-DA, and GSH levels were measured by enzyme-linked immunosorbent assay (ELISA). The graphs display the relative levels of (A) ROS production and (B) GSH production compared to the production in the control. Data are presented as the means ± standard error of the mean (SEM). n=3. ##p<0.01 vs. control and *p<0.05 or **p<0.01 compared to treatment with MPP⁺.

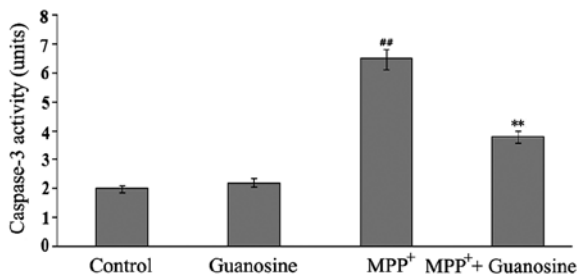


Figure 7. Effects of guanosine on the 1-methyl-4-phenylpyridinium (MPP⁺)-induced caspase-3 activity in PC12 cells. The cells were either untreated, or treated with 500 μM MPP⁺ alone or 500 μM MPP⁺ in the presence of 10 μM guanosine. Caspase-3 activity was determined by enzyme-linked immunosorbent assay (ELISA). Data are expressed as units for caspase-3 activity, and presented as the means ± standard error of the mean (SEM). n=3. ##p<0.01 vs. control and **p<0.01 compared to treatment with MPP⁺.

to 500 μM MPP⁺ showed a significant increase in caspase-3 activity; however, treatment with guanosine alone did not induce any changes in caspase-3 activity. Pre-treatment with guanosine markedly inhibited the MPP⁺-induced the increment in caspase-3 activity (Fig. 7), illustrating the protective role of guanosine against MPP⁺-induced toxicity in PC12 cells.

Discussion

The non-adenine-based purine, guanosine, is a multifaceted intercellular signaling molecule affecting multiple cellular processes, including cellular growth, differentiation and survival (15). Its protective roles have been reported in previous studies. It protects several cell types against apoptosis induced by a number of agents, such as staurosporine, β-amyloid and MPP⁺ through its interactions with several steps of the biochemical and cellular cascade (18,19). The protective role of guanosine has also been reported in neurodegenerative diseases (20). The present study demonstrates that guanosine exerts protective effects against apoptotic cell death elicited by MPP⁺ by alleviating mitochondrial dysfunction, inhibiting the activation of caspase-3 and, subsequently, attenuating cytotoxic cell damage in a reliable cellular model of PD.

PD is a common neurodegenerative disease clinically characterized by rigidity, resting tremor, bradykinesia and postural instability caused by the degeneration and death of dopaminergic neurons in the pars compacta of the substantia nigra (25). Although the cellular and molecular events underlying the loss of dopaminergic neurons remain unclear, accumulating evidence indicates that mitochondrial dysfunction may be a central event in the pathogenesis of PD (2,8). The

mitochondria are the most important cytoplasmic organelles responsible for the life and death of cells (26). The maintenance of membrane potential and the low-conductance of the mPTP are the major properties of mitochondria in living cells, and any changes related to these properties are considered to be critical factors associated with mitochondrial dysfunction and cell death in neurodegenerative diseases (7,8). Multiple proteins are involved in intrinsic apoptotic events associated with mitochondrial dysfunction. The Bcl-2 family of proteins are recognized as key messengers for delivering the apoptotic signal to the mitochondria in response to various insults (27). Pro-apoptotic Bax and anti-apoptotic Bcl-2 are key members of the Bcl-2 family in apoptosis mediated by mitochondrial stress. Under pathogenic conditions, Bax is upregulated and translocates from the cytoplasm to the mitochondria. Once located in the mitochondrial membrane, this protein causes mitochondrial membrane disruption by sequestering Bcl-2 and oligomerizing within the mitochondrial membrane, leading to the opening of the mPTP, the collapse of mitochondrial membrane potential and the subsequent release of pro-apoptotic molecules into the cytoplasm (28,29). Compared to Bax, Bcl-2 is a key protein that preserves mitochondrial integrity, thereby preventing stress-induced mitochondrial damage in cells (30). Bcl-2 proteins are crucial effectors in the opening of the mPTP and the collapse of mitochondrial potential, thus determining the induction of downstream events in the mitochondrial-dependent cell death pathway, including the release of pro-apoptotic molecules and the activation of caspases (28).

Our results revealed that treatment with MPP⁺ induced the adverse expression levels of two Bcl-2 proteins and the disruption of the mitochondrial membrane potential, supporting the involvement of mitochondrial dysfunction in dopaminergic neuronal degeneration. These changes were reversed by the administration of guanosine prior to exposure to MPP⁺, demonstrating the protective role of guanosine in mitochondrial-stress induced cell damage, which was partly mediated through the regulation of the expression of proteins involved in the mitochondrial stage of the apoptotic cascade. However, the underlying mechanism responsible for this effect of guanosine is unclear.

A number of studies have indicated that the anti-apoptotic effects of guanosine are mediated by modulating the phosphatidylinositol 3-kinase (PI3K)/Akt/protein kinase B (PKB) and the mitogen-activated protein kinase (MAPK) cell survival pathways (18,19,31,32). PI3K is an upstream signal of glycogen synthase kinase 3 (GSK-3) that plays a central role in the mitochondrial-dependent cell death pathway through the regulation of anti-apoptotic and pro-apoptotic Bcl-2 family proteins, including Bcl-2 and Bax (33-35). GSK-3 β can directly phosphorylate Bax on serine 163, which results in the activation of Bax, and, subsequently, in its translocation from the cytoplasm to the mitochondria. The inhibition of GSK-3 β suppresses the levels of Bax expression, but increases Bcl-2 expression, thereby promoting cell survival by alleviating the mitochondrial dysfunction under multiple pathological conditions (34,36,37). Thus, the neuroprotective effects of guanosine may be mediated through the activation of PI3K, which inactivates the downstream signal protein, GSK-3 β , leading to the attenuation of the opening of the mPTP through the regulation of Bcl-2 family proteins. This hypothesis is

supported by our results that guanosine reversed the collapse of mitochondrial membrane potential, the downstream event of the opening of the mPTP in mitochondrial-mediated cell death. The opening of the mPTP causes the collapse of mitochondrial membrane potential and, subsequently, the release of apoptotic proteins from the mitochondria into the cytoplasm. Perhaps the most intriguing pro-apoptotic protein that is released is cytochrome *c*, which triggers the activation of the caspase cascade (28). Caspase-3 is a key effector in the mitochondrial-stress-induced apoptotic pathway, and its activation leads to the irreversible process toward apoptosis (38,39). Our results also demonstrated that guanosine, when administered to MPP⁺-treated neuronal PC12 cells, effectively prevented the collapse of mitochondrial potential and inhibited caspase-3 activity, further supporting its protective role in mitochondrial stress-induced neuronal cell damage.

Oxidative stress is another pathological event associated with cell death mechanisms in PD. Studies using postmortem samples of PD have demonstrated that oxidative markers, including soluble protein carbonyl modifications, lipid peroxidation and DNA oxidative damage are selectively observed in the dopaminergic neurons in the pars compacta of the substantia nigra, indicating the correlation of oxidative damage with striatal dopaminergic neurodegeneration (4,5,40). The inhibition of mitochondrial complex I with MPP⁺ and rotenone, well-established inducers of Parkinson-like symptoms in humans and primates, can lead to an increase in ROS production and selective dopaminergic neuronal loss in the substantia nigra, supporting the involvement of oxidative stress in the pathogenesis of PD (41). ROS are mainly produced as by-products of oxidative phosphorylation in the mitochondria, and many mitochondrial proteins, which possess iron-sulfur clusters for oxidation-reduction reactions and lack protective histones, are particularly vulnerable to ROS attack (42). Generally, cells develop complex antioxidant systems to scavenge ROS. The GSH protein is recognized as the major non-enzymatic antioxidant in the central nervous system (23). A number of studies have indicated that increased levels of GSH exert protective effects in various neurodegenerative diseases, such as Alzheimer's disease and PD (22,43,44). The reduction of GSH levels contributes to the dysfunction of the mitochondria and increases the sensitivity of neurons to toxic insults (45). The neurotoxin, MPP⁺, is an inhibitor of the mitochondrial respiratory chain and an inducer of ROS in the mitochondria. Our results revealed that pre-treatment with guanosine reduced the MPP⁺-induced increase in the production of ROS, the crucial contributors to mitochondrial dysfunction through oxidative damage and the opening of the mPTP. Moreover, guanosine alleviated the decreased levels of GSH induced by the administration of MPP⁺, reinforcing its protective role in oxidative conditions and its role as a potential neuroprotectant in mitochondrial-mediated neurodegenerative diseases.

In conclusion, this study clearly demonstrates that the neuroprotective effects of guanosine promote dopaminergic neuronal survival by alleviating mitochondrial dysfunction in a cellular model of PD. These neuroprotective effects are partly mediated through the stabilization of mitochondrial membrane potential via the modulation of the expression levels of intrinsic apoptotic proteins involved in the mitochondrial apoptotic pathway. Further studies are required to fully eluci-

date the mechanisms responsible for the protective effects of guanosine in neurodegenerative diseases, which may promote the development of potentially effective treatments for neurodegenerative diseases by targeting mitochondria-mediated neuronal damage.

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