Published in final edited form as:

Neurochem Res. 2010 September ; 35(9): 1413–1421. doi:10.1007/s11064-010-0200-9.

The Protective Role of D-Glucose Against 1-Methyl-4-Phenylpyridinium Ion (MPP⁺): Induced Mitochondrial Dysfunction in C6 Astroglial Cells

Ramesh B. Badisa, Selina F. Darling-Reed, and Karam F. A. Soliman

College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, Tallahassee, FL 32307, USA

Karam F. A. Soliman: karam.soliman@fam.u.edu

Abstract

Impaired mitochondrial function in glial and neuronal cells in the substantia nigra is one of the most likely causes of Parkinson's disease. In this study, we investigated the protective role of glucose on early key events associated with MPP⁺-induced changes in rat C6 astroglial cells. Studies were carried out to examine alterations in mitochondrial respiratory status, membrane potential, glutathione levels, and cell cycle phase inhibition at 48 h in 2 and 10 mM glucose in media. The results obtained suggest that MPP⁺ caused significant cell death in 2 mM glucose with LC₅₀ 0.14 ± 0.005 mM, while 10 mM glucose showed highly significant protection against MPP⁺ toxicity with LC₅₀ 0.835 ± 0.03 mM. This protection was not observed with cocaine, demonstrating its compound specificity. MPP⁺ in 2 mM glucose decreased significantly mitochondrial respiration, membrane potential and glutathione levels in a dose dependent manner, while 10 mM glucose significantly restored them. MPP⁺ in 2 mM glucose arrested the cells at G0/G1 and G2/M phases, demonstrating its dual inhibitory effects. However, in 10 mM glucose, MPP⁺ caused G0/G1 arrest only. In summary, the results suggest that loss of cell viability in 2 mM glucose group with MPP⁺ treatments was due to mitochondrial dysfunction caused by multilevel mechanism, involving significant decrease in mitochondrial respiration, membrane potential, glutathione levels, and dual arrest of cell phases, while 10 mM glucose rescued astroglial cells from MPP⁺ toxicity by significant maintenance of these factors.

Keywords

D-glucose; Rat astroglial cells; MPP⁺; Mitochondrial respiration; Membrane potential

Introduction

One of the neuropathological hallmarks of Parkinson's disease (PD) is the selective and progressive degeneration of the dopaminergic neurons in the substantia nigra of the brain [1]. Despite several years of extensive research, the exact molecular events in PD are not yet totally understood. Even though many theories are proposed for the causes of PD, none is proven. There are several drugs available to alleviate the symptoms of this disease, but unfortunately, there is no cure available. Post-mortem examinations of PD patients suggest that the impaired mitochondrial function in the neurons of substantia nigra is the most likely cause of the disease, possibly due to oxidative stress [2] or inflammation [3,4]. Since

mitochondria are the main organelles producing reactive oxygen species (ROS), they are highly vulnerable for oxidative damage, leading to their dysfunction and inhibition of ATP synthesis. The mitochondrial impairment could also lead to an unusual glucose oxidation, which is observed in certain central nervous system associated diseases namely, schizophrenia and Alzheimer's disease [5].

The environmental neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was shown to produce several biochemical, pathological and clinical features of PD in several animal models by inhibiting complex I of the electron transport chain (ETC.) in the mitochondria. It was demonstrated that MPTP itself was not toxic to any brain cells. Upon the in vivo administration of MPTP, which is a lipophilic, it crosses the blood brain barrier with ease. Monoamine oxidase type B (MOAB) converts MPTP into 1-methyl-4-phenyl-1,2-dihydropyridinium ion (MPDP⁺) [6,7], which is spontaneously oxidized to toxic metabolite MPP⁺ (1-methyl-4-phenylpyridinium). Interestingly, this enzyme (MOAB) is absent in dopaminergic neurons [8] but exists mainly in glial cells and serotonergic neurons [9,10]. Since astroglial cells are densely localized in substantia nigra par compacta [11], it is obvious that MPTP is converted first into the toxic MPP⁺ metabolite in these cells, causing damages to the mitochondria. It was demonstrated that the release of MPP⁺ into the extracellular spaces due to lysis of glial cells enables accumulation of these ions in nearby dopaminergic neurons via the plasma membrane dopamine transporters. MPP⁺ has been shown to inhibit complex I of mitochondrial ETC. selectively in these cells [12,13].

Although the toxic nature of MPP⁺ against various cell cultures was well-demonstrated earlier [14–16], the mechanism of MPP⁺ toxicity was not investigated thoroughly so far in glial cells. Since glial cells represent a significant portion of central nervous system in maintaining the health and viability of neurons, the effect of MPP⁺ and the significant role of glucose against MPP⁺ toxicity would help in understanding MPP⁺ mechanism of toxicity. Glucose is the preferred energy source in the CNS and its availability can influence several vital cellular processes.

In the present investigation, we have employed rat C6 astroglial (glioma) cell line to examine the role of MPP⁺ on the onset of early events associated with PD, such as changes in mitochondrial respiratory status, mitochondrial membrane potential and cellular glutathione levels in 2 and 10 mM glucose. In addition, rate of cell proliferation and compound specific protection by high level of glucose were also determined. Finally, cell cycle analysis was performed to assess for the role of glucose on MPP⁺ effect on the different phase of cell cycle. In these studies, 2 mM glucose in the culture media represents the physiological concentration in the extra cellular fluid of brain in wake up animals [17], and thus may bring a step closer to in vivo situation. On the other hand, 10 mM glucose represents lower range hyperglycemia [18] under in vivo conditions and may help for better speculation of the early key events associated with PD.

Experimental Procedure

Materials

Supplies include RPMI 1640 medium, Dulbecco's Modified Eagle Medium (DMEM) fetal bovine serum (FBS), penicillin/streptomycin, amphotericin B, phosphate-buffered saline (PBS), and L-glutamine were purchased from Media Tech, Inc., (Herndon, VA, US). Crystal violet, D-glucose, 5,5-dithiobis-2-nitrobenzoic acid (DTNB), EDTA, L-glutaraldehyde, trypan blue, MPP⁺, rhodamine—123, nicotinamide adenosine dinucleotide phosphate (NADPH) and 5-sulfosalicylic acid were supplied by Sigma Chemical Company (St. Louis, MO, US). CellTiter 96 Aqueous One Solution Reagent kit was purchased from Promega (Madison, WI, US).

C6 Astroglial Cell Culture

The central nervous system derived rat C6 astroglial (glioma) cell line was purchased from American Type Culture Collection (Rockville, MD, US) and maintained as an adherent monolayer culture in RPMI 1640 1X medium (Cat. No. 10-040-CV, Media Tech, Inc., Herndon, VA, US) with phenol red, containing 11.1 mM glucose, 2 mM L-glutamine, and supplemented with 10% (v/v) FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. Cells were grown in a humidified atmosphere containing 5% CO₂ in air at 37 °C in an incubator and sub-cultured twice a week. For cytotoxic studies, the culture was harvested by treating with 0.05% EDTA in PBS for 2 min or less. Cell counts and cell viability were assessed immediately by using 0.4% trypan blue stain on a haemocytometer under a light microscope. Dye stained cells (blue) were counted as dead cells, while dye excluded cells were counted as viable cells. The actual cell numbers were determined by multiplying diluted times compared with initial cell numbers. When cell viability exceeded more than 90%, cells were diluted in complete RPMI 1640 medium and seeded in culture plates or dishes for the experiments.

Preparation of Experimental Medium

The experimental medium was prepared with DMEM powder (Cat. No. 90-113-PB) in distilled water, supplemented with phenol red (5 µg/ml or 0.5% v/v), sodium bicarbonate (44 mM), 2 or 10 mM D-glucose, 2 mM L-glutamine, 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. The medium was filtered and used in the experimental studies.

Cell Doubling Period

Cells were plated in 96-well microtiter plates at a starting density of 5×10^3 cells per well in DMEM containing 2 or 10 mM glucose supplemented with 10% FBS. The number of cells at various time points was evaluated by crystal violet as described earlier [19]. The culture plates were read at 540 nm in a plate reader (Bio-Tek Instruments Inc, Wincoski, VT). The doubling period in hours was determined as reported earlier [20] from the average absorbance values ($n = 24$) of cells growing at exponential phase.

Treatments with MPP⁺

The cells were seeded at a starting density of 2×10^4 cells per well in a total volume of 196 µl of complete RPMI 1640 growth medium with 10% FBS. The cells were allowed to adhere overnight in the incubator. Then the medium was replaced completely with DMEM with phenol red containing 10% FBS either with 2 or 10 mM D-glucose. Stocks and working stocks of MPP⁺ were prepared always fresh in PBS and used in the studies. The cells (typically 60–70% confluent) were treated with MPP⁺ at different concentrations in a final volume of 4 µl under sterile conditions. MPP⁺ was added in increasing concentrations (0.1, 0.2 and 0.3 mM). In all studies, cells in the medium alone or cells in the medium containing equal volume of PBS served as controls. Controls and the treated samples were always present in the same culture plates. These plates were incubated for 48 h continuously without further renewal of growth medium in a 5% CO₂ in air at 37 °C with the plates capped in the normal fashion. All studies were repeated at least twice ($n = 16$). At the end of incubation, the cytotoxicity of MPP⁺ was evaluated by dye uptake assay using crystal violet [19]. The plates were read at 540 nm in a plate reader.

Treatments with Cocaine

The role of glucose concentration on cocaine- induced toxicity on glial cell was performed in the presence of 2 or 10 mM glucose in complete DMEM. Cocaine was tested at six different concentrations (2–7 mM) for 24 h as per earlier report [20]. These studies were

repeated twice ($n = 12$). Cytotoxicity was evaluated by dye uptake assay using crystal violet [19] and the plates were read at 540 nm in a plate reader.

Effect of MPP⁺ on Mitochondrial Respiratory Activity

Mitochondrial respiratory activity was measured according to Denizot and Lang [21]. In brief, glial cells were exposed to various concentrations of MPP⁺ (0.1, 0.2 and 0.3 mM) in 2 or 10 mM glucose for 48 h. Three hours prior to end of incubation, 10 μ l of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was added per well. The titer plates were read in a plate reader at 490 nm. These studies were repeated at least twice ($n = 6$).

Assessment of Mitochondrial Membrane Potential

MPP⁺ treatments were performed at 0.1, 0.2 and 0.3 mM in 2 or 10 mM glucose for 48 h. In brief, at the end of incubation, cells were fixed with 100 μ l of 0.25% aqueous glutaraldehyde, containing rhodamine—123 to yield a final concentration of 1 μ M for 30 min at room temperature as described earlier [20]. The plates were read with excitation filter set at 485 nm and emission filter at 538 nm on a microplate Fluorometer model 7620, Version 5.02, Cambridge Technology, Inc., (Watertown, MA, US). These studies were repeated at least twice ($n = 8$).

Estimation of Cellular Glutathione Levels

Total cellular glutathione was assayed according to Smith et al. [22]. In brief, after treating with MPP⁺ at 0.1, 0.2 and 0.3 mM in 2 or 10 mM glucose for 48 h, the cells were deproteinized with 2% 5-sulfosalicylic acid (10 μ l/well) for 30 min at 37 °C, followed by addition of 90 μ l of reaction mixture containing 0.416 mM sodium EDTA, 0.416 mM NADPH, 0.835 mM DTNB and 0.083 mM sodium phosphate buffer, pH 7.5, and 0.216 units of glutathione reductase. The plates were incubated at 37°C for 30 min. The absorbance was measured at 412 nm on a plate reader. These studies were repeated at least twice ($n = 12$).

Cell Cycle Progression

Treatments with MPP⁺ (0.1, 0.15 and 0.25 mM) were performed for 48 h. Then the cells were collected and fixed in 95% ice-cold ethanol for at least 24 h at 4°C. The next day, the tubes were centrifuged at 2,600 rpm for 7 min and fixed in 100 μ l chilled ethanol. Cells were stained and analyzed as described elsewhere [23]. The proportion of cells in each stage was performed within 2 h by using FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). In each sample, 10,000 individual events from the gated subpopulation were analyzed separately. Cell Quest Software was used for acquisition and analysis of the data, and the percentage of cells in each phase was determined by using ModFit 3.0 Software (Verity Software House, Topsham, ME, US). The studies were repeated at least twice.

Statistical Analysis

In this study, “ n ” represents the final number of wells per each treatment. The absorbance values were converted to percentage with respect to the control (100%) and the experimental results were presented as mean $\pm$ SEM (standard error mean) in the plots. The data were analyzed for significance by one-way analysis of variance (ANOVA) and then compared by Bonferroni's multiple comparison tests, using GraphPad Prism Software, Version 3.00 (San Diego, CA, US). The test values of $P < 0.05$ and $P < 0.01$ were considered significant and highly significant, respectively. The LC₅₀ or EC₅₀ values, representing the millimolar concentration of MPP⁺ needed to show 50% response on glial cells was determined from the graphs where both curves crossed [24].

Results

Excess Glucose does not Increase Cell Proliferation Rate

The data on cell growth studies for 2 and 10 mM glucose groups (Fig. 1) was used to determine the cell doubling periods. It was found that the difference in the absorbance values between 2 and 10 mM glucose groups shown in this figure at any time point was insignificant ($P > 0.05$). In comparison to zero hour control (100%), the number of cells in 2 mM glucose was increased by 178, 386, and 832% after 24, 48, and 72 h, respectively (Fig. 1). This increase was almost the same as those cells in 10 mM glucose (178, 384, and 858% at 24, 48, and 72 h, respectively). These growth rates clearly suggest that cells in both glucose groups proliferated linearly with time ($P < 0.001$) with an initial lag of about 24 h after seeding of the cells (Fig. 1). The average doubling period ($\pm$ SEM) of cells in 2 and 10 mM glucose groups was 22.53 ± 0.9 and 22.63 ± 0.39 h, respectively. The data clearly suggest that fivefold difference in the glucose concentration in the medium had no significant role on the rate of cell proliferation.

Excess Glucose Specifically Protects Glial Cells Against MPP⁺ Toxicity

Our subsequent studies were focused on MPP⁺ toxicity to glial cells, and the protection by glucose. For this purpose, the MPP⁺ concentrations (0.1, 0.2 and 0.3 mM) were selected based on non-linear regression analysis method (Fig. 2a). The correlation coefficient, R^2 , indicating the linearity was 0.9944. Ideally, an R^2 value of 1.0 indicates that all data points are on the straight line, while zero R^2 value indicates lack of such linearity. The R^2 value obtained from our data clearly indicates that there was more than 99% linear relationship between MPP⁺ concentration and the cell viability. It was observed that MPP⁺ caused a progressive and statistically significant ($P < 0.01$) cell death in 2 mM glucose in comparison to control after 48 h with an average LC₅₀ of 0.14 ± 0.005 mM. This value was much lower than a previous report [0.5 mM, 25] presumably due to differences in glucose concentrations or incubation periods or serum concentrations in media or other factors, but was closer to another study [160 μ M, 26]. Interestingly, 10 mM glucose significantly ($P < 0.01$) increased the percentage of cell viability at all MPP⁺ treatments in comparison to the treatments in 2 mM glucose (Fig. 2b). The average LC₅₀ in 10 mM glucose was 0.835 ± 0.03 mM MPP⁺. This increase of about sixfold higher value clearly demonstrates the protective role of glucose on cells.

We next designed an experiment to determine if 10 mM glucose protection is limited to MPP⁺-induced toxicity alone or extended to any other toxic compounds. For this purpose, studies were performed with cocaine, a widely abused drug that causes toxicity to various cells. Six different concentrations of cocaine were tested (2–7 mM) on glial cells for 24 h in the presence of 2 or 10 mM glucose in DMEM with 10% FBS. The selection of cocaine concentrations in this study was based on our previous report [20]. The results (Fig. 2c) revealed that 10 mM glucose did not protect the cells significantly at any cocaine concentrations ($P > 0.05$). Instead, a similar significant dose dependent cytotoxicity was observed in both 2 and 10 mM glucose groups ($P < 0.01$) at all concentrations of cocaine. The LC₅₀ of cocaine in both 2 and 10 mM glucose was 4.4 ± 0.02 mM and 4.6 ± 0.02 mM, respectively. These values were significantly closer to earlier 24 h studies with cocaine 4.3 mM, [20,27]. Since no protection was observed with 10 mM glucose at 24 h exposure, further increase in the incubation period to 48 h would only enhance cocaine toxicity to cells. Thus, studies were not repeated with cocaine. From these results, it is obvious that 10 mM glucose protection is compound specific.

MPP⁺ Decreases Mitochondrial Respiratory Function

Exposure of glial cells to MPP⁺ at increasing concentrations (0.1, 0.2 and 0.3 mM) for 48 h caused significant ($P<0.01$) decrease in mitochondrial respiration in 2 mM glucose group (Fig. 3). This was evidenced in terms of progressive decrease in the amount of formazan produced in MPP⁺ treated cells in 2 mM glucose. The average EC₅₀ of MPP⁺, where 50% loss of mitochondrial respiratory activity in 2 mM glucose observed was found to be 0.174 ± 0.01 mM. Interestingly, the amount of formazan produced in MPP⁺ treated cells in 10 mM glucose group was significantly ($P<0.01$) higher in comparison to 2 mM glucose group. These observations reflect the increased state of mitochondrial respiratory activity of glial cells in 10 mM glucose. The average EC₅₀ of MPP⁺ in 10 mM glucose was found to be 0.299 ± 0.06 mM. The results clearly suggest the ability of 10 mM glucose for restoring the respiratory status in MPP⁺ treated cells.

MPP⁺ Decreases Mitochondrial Membrane Potential

Glial cells were treated with MPP⁺ at 0.1, 0.2 and 0.3 mM for 48 h in the presence of 2 or 10 mM glucose in DMEM containing 10% FBS and then stained with 1 μ M rhodamine 123. It is clear from Fig. 4 that MPP⁺ decreased significantly ($P<0.05$) the mitochondrial membrane potential of glial cells in 2 mM glucose in a dose dependent manner, which is consistent from earlier reports [28]. In 2 mM glucose group, the average EC₅₀ of MPP⁺ was found to be 0.26 ± 0.02 mM. In comparison to MPP⁺ treatments in 2 mM glucose, a dose dependent significant ($P<0.05$) increase in mitochondrial membrane potential was observed (Fig. 4) in 10 mM groups. The average EC₅₀ in the case of 10 mM glucose was found to be 0.428 ± 0.02 mM. The increased membrane potential by excess glucose was consistent with earlier studies [25,29] albeit no quantified effective dose values were reported.

MPP⁺ Decreases Total Cellular Glutathione Levels

It was observed that MPP⁺ treatments at 0.1, 0.2 and 0.3 mM for 48 h decreased the total glutathione levels significantly ($P<0.05$) in cells of 2 mM glucose group (Fig. 5) with the EC₅₀ of 0.36 mM. On the other hand, the total glutathione levels in 10 mM glucose group restored significantly ($P<0.05$) at all MPP⁺ treatments in comparison to MPP⁺ treatments in 2 mM glucose group. The EC₅₀ in this case was >1 mM. Since we used glutathione reductase in the assay, the total glutathione measurements in our study represent both oxidized (GSSG) and reduced form (GSH) of glutathione. The data on MPP⁺ treatments in 10 mM glucose again confirms the protective role of 10 mM glucose.

Dual Cell Cycle Arrest by MPP⁺

The effect of MPP⁺ at 0.1, 0.15 and 0.25 mM in 2 and 10 mM glucose was studied on different phases of glial cell cycle for 48 h and analyzed by flow cytometry. It was observed that in 2 mM glucose, MPP⁺ caused a dual cell cycle inhibition. For instance, MPP⁺ treatment in the presence of 2 mM glucose caused a dose dependent cell cycle arrest both at G0/G1 and G2/M phases (Fig. 6a). These phase arrests were significant ($P<0.05$) at 0.15 and 0.25 mM MPP⁺. In 10 mM glucose, MPP⁺ caused significant arrest ($P<0.05$) only at G0/G1 phase, but no G2/M phase arrest was observed (Fig. 6b). In 2 and 10 mM glucose, the number of cells in S-phase was decreased significantly. While the dual inhibition by MPP⁺ in 2 mM glucose was not reported earlier, its G0/G1 inhibition in 10 mM glucose was consistent with previous studies [30]. The results clearly show the dual inhibitory nature of MPP⁺ in glial cells in 2 mM glucose.

Discussion

The high sensitivity of cells to MPP⁺ in low levels of glucose (1 or 2 mM) was in agreement with previous reports, where low glucose was shown to potentiate MPP⁺ induced toxicity [31,32]. In our study, the decreased cell viability due to MPP⁺ treatments in 2 mM glucose group does not appear to be an additive affect of both MPP⁺ toxicity and insufficient availability of glucose to cells. If insufficiency of glucose was the cause, then cells in both 2 and 10 mM glucose control groups would not have shown similar absorbance values as a function of the time (Fig. 1). Since cells in 2 or 10 mM glucose exhibited almost the same absorbance values at any given time point up to 72 h in our growth curve studies (Fig. 1), it is clear that the cells in 2 mM glucose were as much viable as those in 10 mM glucose group prior to MPP⁺ treatments, and thus the observed cell death during treatments was solely attributed to MPP⁺ toxicity.

Based on the cell doubling results, we used 48 h incubation, where cells were proliferating actively, as an end point in all our studies. Consistent with earlier reports on different cell lines [13,25,29]; we also observed that 10 mM glucose significantly protected the glial cells against MPP⁺ toxicity (Fig. 2b). In our study, this protection was reflected in terms of having about 6 times higher LC₅₀ value in 10 mM glucose (0.835 ± 0.03 mM) in comparison to 2 mM glucose (0.14 ± 0.005 mM).

Various studies demonstrated that MPP⁺ inhibits NADH-ubiquinone oxidoreductase (complex I) of ETC. in the mitochondria [13,33]. The inhibition at complex I results in very low or no ATP production in the mitochondria, depending on the potency of inhibition by the compounds, such as MPP⁺ [34]. In addition, inhibition at complex I may also generate more ROS [35], which affects the rate of mitochondrial respiration and its membrane potential. Because of several disadvantages associated with Clark oxygen electrode measurements [36–39], we preferred measuring the mitochondrial respiration by evaluating succinate dehydrogenase activity directly in cells as per the established method [21]. Since this enzyme is located on the inner membrane of mitochondria, measuring its activity may provide direct evidence on the general respiratory status of mitochondria in terms of their ability to reduce the tetrazolium compound of MTS to formazan in cells. We observed that the amount of formazan produced due to MPP⁺ treatment in 2 mM glucose was significantly lower than the control (Fig. 3). This indicates that MPP⁺ treatment significantly inhibited the mitochondrial respiration in 2 mM glucose group.

Under such circumstances, we next sought to know the state of mitochondrial membrane potential of MPP⁺ treated cells in 2 mM glucose group. For this purpose, we used rhodamine —123 fluorescent dye. Since this dye is selectively taken up by mitochondria [40], the amount of rhodamine —123 present in the mitochondria is directly proportional to its membrane potential. We observed that MPP⁺ treatment resulted significant loss of mitochondrial membrane potential in 2 mM glucose group (Fig. 4). It was further observed that MPP⁺ treatment decreased the total glutathione content significantly in 2 mM glucose cells, while 10 mM glucose significantly restored and almost reached (94%) to the level of control at 0.3 mM MPP⁺ (Fig. 5). While the decreased glutathione level was known to be associated with neurodegeneration, this is the first report to observe the glucose dependent regulation of glutathione levels by MPP⁺.

Significant decrease and restoration of mitochondrial respiratory status, its membrane potential and glutathione levels in MPP⁺ treated glial cells in 2 and 10 mM glucose respectively may indicate that MPP⁺ inhibition at complex I of ETC. was reversible type. This speculation was consistent with earlier reports [41,42], where reversal of MPP⁺ inhibition was shown in different cell systems. The mechanism of restoration of the

mitochondrial membrane potential by 10 mM glucose was not the objective of this investigation. It may be possible that high glucose in our studies relieved the MPP⁺ inhibition at complex I site, which resulted in increased cell viability. Further studies, however, are required to corroborate this speculation.

The data obtained from cell cycle study indicate that high level of glucose was associated with the lack of G2/M cell arrest, indicating the increase in cell mitosis and confirm the cell viability data (Fig. 2b). The obtained results indicate that glucose protection against MPP⁺ and the lack of G2/M cell cycle arrest might be related to the mitochondrial protection offered by glucose. Cell protection by 10 mM glucose observed in this study seems to be compound specific based on the observation that 10 mM glucose did not offer similar protection against cocaine treated glial cells (Fig. 2c). This was evidenced by exhibiting closer LC₅₀ values of cocaine in both 2 mM glucose (4.4 mM) and 10 mM glucose (4.6 mM). These values were also found closer to the LC₅₀ values of earlier studies [4.3 mM, 20,27]. Recent studies from our group indicated that cocaine interaction decreases the mitochondrial membrane potential in glial cells [20]. This observation reminds a similar trend of decrease in mitochondrial membrane potential by MPP⁺ in glial cells observed in the present study. The ability of 10 mM glucose to protect glial cells against MPP⁺ toxicity (Fig. 2b) and its inability to protect cocaine treated cells under the same conditions (Fig. 2c) may indicate that the mode of action of both MPP⁺ and cocaine at mitochondria was different in glial cells. This speculation is further supported from the results of our previous cell cycle analyses with cocaine [20]. For instance, our earlier studies with cocaine for 48 h under similar conditions as of the present study, resulted significant glial cell cycle arrest at G2/M phase. On the other hand, MPP⁺ treatment for the same period of time in the present study led to significant G0/G1 and G2/M arrests in 2 mM glucose group and G0/G1 arrest in 10 mM glucose cells (Fig. 6a, b). These observations clearly suggest that even though both MPP⁺ and cocaine interact with mitochondria and decrease membrane potential, their mode of action for cytotoxicity were different. Based on the differences in LC₅₀ values or EC₅₀ values or cell cycle arrest of MPP⁺ and cocaine, it appears that the MPP⁺ toxicity is specific rather than non-specific.

The data obtained in this investigation demonstrate the role of glucose in MPP⁺ induced changes on glial cell viability. While earlier reports [25] concluded that the increased glial cell viability in 10 mM glucose with MPP⁺ treatment was exclusively due to anaerobic glycolysis, here, based on the results of our additional studies, we found that the increased cell viability in 10 mM glucose with MPP⁺ treatments was not associated with a single mechanism but multi level mechanisms such as significant restoration of mitochondrial respiratory activity, its membrane potential, and increased total glutathione content. One of our findings was consistent with earlier studies on PC12 cells [29], where glucose protection against MPP⁺ toxicity was clearly demonstrated due to maintenance of mitochondrial membrane potential.

The data also indicate that MPTP toxicity to neurons might be related more to glial cells damage rather than the ability of glial cells to metabolize MPTP and release of the toxic metabolite, MPP⁺. Damaged and injured glial cells can produce toxic cytokines [43] to neurons and play important role in the inflammatory process in nervous system [43]. Inflammation may be associated with the neuropathology of PD as evidenced by the excessive glial activation and increased levels of the pro-inflammatory cytokines, tumor necrosis factor-alpha and interleukin-1beta in the substantia nigra of patients with PD [44]. Since anti-inflammatory drugs and microglial activation inhibitors decrease susceptibility to PD, it is likely that their use may emerge as a therapy in PD [44].

It was concluded that from this study that high levels of glucose were protective against MPP⁺-induced changes on glial cell viability, alterations in mitochondrial general respiratory status, mitochondrial membrane potential, total glutathione levels and dual cell cycle phase arrest. The data also indicate that MPTP toxicity to neurons is more likely related to glial cell activation rather than the ability of glial cells to release the toxin MPP⁺.

Acknowledgments

The authors acknowledge the critical reading of the manuscript and the valuable suggestions of Dr. Sandra Suther. This study was supported by a grant obtained from the National Institutes of Health, Division of Research Resources, Research Centers in Minority Institution (RCMI) G12 RR 03020.

References

1. Langston JW, Langston EB, Irwin I. MPTP-induced parkinsonism in human and non-human primates: clinical and experimental aspects. *Acta Neurol Scand* 1984;100(Suppl):49–54.
2. Hunot F, Boissiere F, Faucheux B. Nitric oxide synthase and neuronal vulnerability in Parkinson's disease. *Neuroscience* 1996;72:355–363. [PubMed: 8737406]
3. McGeer PL, Itagaki S, Boyes BE, McGeer EG. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. *Neurology* 1988;38:1285–1291. [PubMed: 3399080]
4. Hunot B, Brugg B, Ricard D. Nuclear translocation of NF-kappa B is increased in dopaminergic neurons of patients with Parkinson' disease. *Proc Natl Acad Sci USA* 1997;94:7531–7536. [PubMed: 9207126]
5. Blass JP. Glucose/mitochondria in neurological conditions. *Int Rev Neurobiol* 2002;51:325–376. [PubMed: 12420364]
6. Heikkila RE, Manzino L, Duvoisin RC, Cabbat FS. Protection against the dopaminergic neurotoxicity of 1-methyl-4- phenyl-1, 2, 3, 6-tetrahydropyridine by monoamine oxidase inhibitors. *Nature* 1984;311:467–469. [PubMed: 6332989]
7. Markey SP, Johannessen JN, Chiueh CC, Burns RS, Herkenham MA. Intraneuronal generation of a pyridinium metabolite may cause drug-induced parkinsonism. *Nature* 1984;311:464–467. [PubMed: 6332988]
8. Westlund KN, Denney RM, Kochersperger LM, Rose RM, Abell CW. Distinct monoamine oxidase A and B populations in primate brain. *Science* 1985;230:181–183. [PubMed: 3875898]
9. Takada M, Li ZK, Hattori T. Astroglial ablation prevents MPTP-induced nigrostriatal neuronal death. *Brain Res* 1990;509:55–61. [PubMed: 2306638]
10. Di Monte DA. Mitochondrial DNA and Parkinson's disease. *Neurology* 1991;41(suppl 2):38–42. [PubMed: 1904141]
11. Forno LS, Delanney LE, Irwin I, Di Monte D, Langston JW. Astrocytes and Parkinson's disease. *Prog Brain Res* 1992;94:429–436. [PubMed: 1287728]
12. Singer TP, Ramsay RR. Mechanism of the neurotoxicity of MPTP: an update. *FEBS Lett* 1990;274:1–8. [PubMed: 2253761]
13. Mazzio E, Soliman KFA. The role of glycolysis and gluconeogenesis in the cytoprotection of neuroblastoma cells against 1-methyl-3-phenyl-pyridinium ion toxicity. *Neurotoxicology* 2003;24:137–147. [PubMed: 12564389]
14. Itano Y, Kitamura Y, Nomura Y. Biphasic effects of MPP⁺, a possible parkinsonism inducer, on dopamine content and tyrosine hydroxylase mRNA expression in PC12 cells. *Neurochem Int* 1995;26:165–171. [PubMed: 7599536]
15. Sheehan JP, Palmer PE, Helm GA, Tuttle JB. MPP⁺ induced apoptotic cell death in SH-SY5Y neuroblastoma cells: an electron microscope study. *J Neurosci Res* 1997;48:226–237. [PubMed: 9160245]
16. De Girolamo LA, Hargreaves AJ, Billett EE. Protection from MPTP-induced neurotoxicity in differentiating mouse N2a neuroblastoma cells. *J Neurochem* 2001;76:650–660. [PubMed: 11158235]

17. Lund-Andersen H. Transport of glucose from blood brain to brain. *Physiol Rev* 1979;59:305–352. [PubMed: 375257]
18. McGowan TA, Dunn SR, Falkner B, Sharma K. Stimulation of urinary TGF- β and isoprostanes in response to hyperglycemia in humans. *Clin J Am Soc Nephrol* 2006;1:263–268. [PubMed: 17699215]
19. Badisa RB, Tzakou O, Couladis M, Pilarinou E. Cytotoxic activities of some Greek Labiatae herbs. *Phytother Res* 2003;17:472–476. [PubMed: 12748981]
20. Badisa RB, Darling-Reed SF, Goodman CB. Cocaine induces alterations in mitochondrial membrane potential and dual cell cycle arrest in rat C6 astroglial cells. *Neurochem Res* 2010;35(2):288–297. [PubMed: 19757036]
21. Denizot R, Lang R. Rapid colorimetric assay for cell growth and survival. *J Immunol Methods* 1986;89:271–277. [PubMed: 3486233]
22. Smith IK, Vierheller TL, Thorne CA. Assay of glutathione reductase in crude tissue homogenates using 5, 5'-dithiobis(2-nitrobenzoic acid). *Anal Biochem* 1988;175:408–413. [PubMed: 3239770]
23. Badisa RB, Darling-Reed SF, Joseph P, Cooperwood JS, Latinwo LM, Goodman CB. Selective cytotoxic activities of two novel synthetic drugs on human breast carcinoma MCF-7 cells. *Anticancer Res* 2009;29:2993–2996. [PubMed: 19661306]
24. Ipsen, J.; Feigl, P. Bancroft's introduction to biostatistics. Harper & Row; New York: 1970. p. 455
25. Williams ZR, Goodman CB, Soliman KFA. Anaerobic glycolysis protection against 1-methyl-4-phenyl pyridinium (MPP⁺) toxicity in C6 glial cells. *Neurochem Res* 2007;32:1071–1080. [PubMed: 17401669]
26. Rollema H, Skolnik M, D'Engelbronner J, Igarashi K, Usuki E, Castagnoli N. MPP (+)-like neurotoxicity of a pyridinium metabolite derived from haloperidol: in vivo microdialysis and in vitro mitochondrial studies. *J Pharmacol Exp Ther* 1994;268:380–387. [PubMed: 8301579]
27. Cunha-Oliveira T, Rego AC, Cardoso SM, Borges F, Swerdlow RH, Macedo T, de Oliveira CR. Mitochondrial dysfunction and caspase activation in rat cortical neurons treated with cocaine or amphetamine. *Brain Res* 2006;1089:44–54. [PubMed: 16638611]
28. Lambert CE, Bondy SC. Effects of MPTP, MPP⁺ and paraquat on mitochondrial potential and oxidative stress. *Life Sci* 1989;44:1277–1284. [PubMed: 2785626]
29. Chalmers-Redman RME, MacLean Fraser AD, Carlile GW, Pong A, Tatton WG. Glucose protection from MPP⁺—induced apoptosis depends on mitochondrial membrane potential and ATP synthase. *Biochem Biophys Res Commun* 1999;257:440–447. [PubMed: 10198232]
30. Bai J, Nakamura H, Ueda S, Kwon YW, Tanaka T, Ban S, Yodoi J. Proteasome-dependent degradation of cyclin D1 in 1-methyl-4-phenylpyridinium ion (MPP⁺)-induced cell cycle arrest. *J Biol Chem* 2004;279:38710–38714. [PubMed: 15247282]
31. Basma AN, Heikkila RE, Saporito MS, Philbert M, Geller HM, Nicklas WJ. 1-methyl-4-(2'-ethylphenyl)-1, 2, 3, 6-tetrahydropyridine-induced toxicity in PC12 cells is enhanced by preventing glycolysis. *J Neurochem* 1992;58:1052–1059. [PubMed: 1737983]
32. Chan P, Langston JW, Irwin I, Delanney LE, Di Monte DA. 2-deoxyglucose enhances 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine- induced ATP loss in the mouse brain. *J Neurochem* 1993;61:610–616. [PubMed: 8336145]
33. Mizuno Y, Saitoh T, Sone N. Inhibition of mitochondrial NADH-ubiquinone oxidoreductase activity by 1-methylphenylpyridinium ion. *Biochem Biophys Res Commun* 1987;143:294–299. [PubMed: 3103619]
34. Scotcher KP, Irwin I, Delanney LE, Langston JW, Di Monte D. Effects of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine and 1-methyl-4-phenylpyridinium ion on ATP levels of mouse synaptosomes. *J Neurochem* 1990;54:1295–1301. [PubMed: 2313288]
35. Lotharius J, Dugan LL, O'Malley KL. Distinct mechanisms underlie neurotoxin-mediated cell death in cultured dopaminergic neurons. *J Neurosci* 1999;19:1284–1293. [PubMed: 9952406]
36. Amao Y, Miyashita T, Okura I. Novel optical oxygen sensing material: platinum octaethylporphyrin immobilized in a copolymer film of isobutyl methacrylate and tetrafluoropropyl methacrylate. *React Funct Polym* 2001;47:40–54.

37. Mamchaoui K, Saumon G. A method for measuring the oxygen consumption of intact cell monolayers. *Am J Physiol Lung C* 2000;278:L858–L863.
38. Starlinder H, Lubber DW. Methodical studies on the polarographic measurement of respiration and critical oxygen pressure in mitochondria and isolated cells with membrane-covered platinum electrodes. *Pflugers Archiv* 1972;337:19–28. [PubMed: 4674010]
39. Vanderkooi JM, Erecinska M, Silver IA. Oxygen in mammalian tissue: methods of measurement and affinities of various reactions. *Am J Physiol* 1991;260:1131–1150.
40. Johnson LV, Walsh ML, Chen LB. Localization of mitochondria in living cells with Rhodamine-123. *Proc Natl Acad Sci USA* 1980;77:990–994. [PubMed: 6965798]
41. Ramsay RE, Kowal AT, Johnson MK, Salach JI, Singer TP. The inhibition site of MPP⁺, the neurotoxic bioactivation product of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine is near the Q-binding site of NADH dehydrogenase. *Arch Biochem Biophys* 1987;259:645–649. [PubMed: 2827583]
42. Di Monte DA, Wu EY, Delaney LE, Irwin I, Langston JW. Toxicity of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine in primary cultures of mouse astrocytes. *J Pharmacol Exp Ther* 1992;261:44–49. [PubMed: 1560384]
43. Elsis N, Darling-Reed S, Lee EY, Oriaku ET, Soliman KF. Ibuprofen and apigenin induce apoptosis and cell cycle arrest in activated microglial. *Neuroscience Lett* 2005;375:91–96.
44. Long-Smith CM, Sullivan AM, Nolan YM. The influence of microglia on the pathogenesis of Parkinson's disease. *Prog Neurobiol* 2009;89:277–287. [PubMed: 19686799]

Fig. 1.

Growth curves of glial cells. Cells with starting density of 5×10^3 per well in 96-well plates ($n = 4$, and each value is the average absorbance of 6 wells, so final n at each time point = 24) with complete DMEM containing 2 or 10 mM glucose and 10% FBS were incubated for designated time points. The cells were stained with crystal violet and the absorbance at 540 nm was read in a plate reader. The absorbance values at any time point were so close to each other that error bars were not seen clearly on the plot. * Significant when compared to the respective zero hour controls ($P < 0.001$). # There was no significant difference between the 2 and 10 mM glucose ($P > 0.05$) groups

Fig. 2.

a Concentration-effect curve. Glial cells were treated with MPP⁺ (0.1, 0.2 and 0.3 mM) in 2 mM glucose for 48 h. The absorbance for viability was measured, and a graph was plotted between log of MPP⁺ concentrations and the mean absorbance values by non-linear regression analysis method. **b** Effect of MPP⁺ on glial cell viability in 2 and 10 mM glucose after 48 h. * MPP⁺ treatments had significant effect when compared to the respective controls. # MPP⁺ treatments had significant effect on cell viability when 2 and 10 mM glucose groups were compared. Values represent the average of three independent studies ($P < 0.001$, $n = 16$). **c** Lack of 10 mM glucose protection in glial cells treated with cocaine after 24 h. * Treatments had significant effects when compared to the respective controls ($P < 0.05$, $n = 12$). # There was no significant difference between 2 and 10 mM glucose groups ($P > 0.05$, $n = 12$)

Fig. 3.

Effect of MPP⁺ on mitochondrial respiratory status (MRS) in glial cells. Cells were grown in DMEM with 2 or 10 mM glucose containing 10% FBS, and were treated with various concentrations of MPP⁺ for 48 h. Values represent the average of two independent studies ($n = 6$). * MPP⁺ had significant effect on MRS in comparison to the control containing 2 or 10 mM glucose ($P < 0.05$, $n = 6$ One-way ANOVA, Bonferroni's multiple comparison test). # Significant difference between MPP⁺ treatment groups with 2 and 10 mM glucose ($P < 0.001$, $n = 6$, One-way ANOVA, Bonferroni's multiple comparison test)

Fig. 4. Effect of MPP⁺ on mitochondrial membrane potential in glial cells. Cells were grown in DMEM with 2 or 10 mM glucose containing 10% FBS, and were treated with various concentrations of MPP⁺ for 48 h. Values represent the average of two independent studies ($n = 8$). * Significant effect of MPP⁺ in comparison to control groups containing 2 or 10 mM glucose ($P < 0.05$, $n = 8$, One-way ANOVA, Bonferroni's multiple comparison test). # Significant difference between MPP⁺ treatment groups with 2 and 10 mM glucose in media ($P < 0.001$, $n = 8$, One-way ANOVA, Bonferroni's multiple comparison test)

Fig. 5.

Effect of MPP⁺ on GSH levels in glial cells. Cells were grown in DMEM with 2 or 10 mM glucose containing 10% FBS, and were treated with various concentrations of MPP⁺ for 48 h. Values represent the average of two independent studies ($n = 12$). * Significant in comparison to control containing 2 or 10 mM glucose in media ($P < 0.05$, $n = 12$ One-way ANOVA, Bonferroni's multiple comparison test). # Significant difference between MPP⁺ treatment groups with 2 and 10 mM glucose in media ($P < 0.001$, $n = 12$, One-way ANOVA, Bonferroni's multiple comparison test)

Fig. 6.

Effect of MPP⁺ on glial cell cycle. Cells were grown in 100 × 15 mm culture dishes in DMEM with 2 mM (a) or 10 mM (b) glucose containing 10% FBS, and were treated with various concentrations of MPP⁺ for 48 h. Cells were stained by propidium iodide staining solution for 1 h in dark and analyzed by flow cytometry. Data were presented as mean ± SEM ($n = 2$, * $P < 0.05$, significant in comparison to control, One-way ANOVA, Bonferroni's multiple comparison test)