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ORIGINAL ARTICLE

The mechanism of action of MPTP-induced neuroinflammation and its modulation by melatonin in rat astrocytoma cells, C6

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Abstract

The 1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine (MPTP) induces reactive astrogliosis, the cellular manifestation of neuroinflammation, in various models of Parkinson's disease (PD), but its mechanism of action on astrocytes is not understood. The effect of melatonin on MPTP-induced neuroinflammation in astrocytes is also not known. The present study demonstrated that MPTP treatment of rat astrocytoma cells, C6 for 24 h significantly increased nitrative and oxidative stress and intracellular calcium (Ca^{2++}) level. MPTP also activated phosphorylated p38 mitogen activated protein kinase (P-p38 MAPK) and up-regulated expressions of inflammatory proteins. Moreover, MPTP modulated mRNA expressions of pro-inflammatory cytokine genes via activating nuclear factor kappa-B (NF-kB) translocation. Treatment of melatonin with MPTP reversed all these MPTP-induced changes. Study with deprenyl demonstrates that MPTP is inducing neuroinflammation in astrocytoma cells. The present findings elucidated the molecular mechanism of MPTP-induced neuroinflammation and its modulation by melatonin in astrocytoma cells (C6).

Keywords: *Neuroinflammation, Parkinson's disease, rat astrocytoma cells (C6), NF-kB activation, MPTP, melatonin*

Abbreviations: *MPTP, 1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine; ROS, reactive oxygen species; RNS, reactive nitrogen species; TNF-α, tumour necrosis factor-α; NF-kB, nuclear factor kappa-B; COX-2, cyclooxygenase-2; GFAP, glial fibrillary acidic protein; CHOP, C/EBP, homologous protein 10; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DCF-DA, dichlorofluorescein diacetate; iNOS, inducible nitric oxide synthase; MDA, melondialdehyde; IL-1α, interleukin-1α; IL-1β, interleukin-1β; IL-6, interleukin-6; Mela, melatonin; P-p38 MAPK, Phosphorylated p38 mitogen activated protein kinase; Dpl, (–)-deprenyl; MAO'B, Monoamine oxidase B; NO, nitrite.*

Introduction

The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes selective dopaminergic neurodegeneration and has widely been used in the various models of Parkinson's disease (PD) [1]. MPTP in primates produces neurological changes comparable to those observed in Parkinson's disease pathophysiology [2,3]. MPTP-induced neurotoxicity in the brain is associated with the oxidative stress and inflammatory mediators [4,5]. MPTP is converted in to MPP^+ by the monoamine-oxidase B (MAO'B), present in the glial cells and most abundant in astrocytes [6]. MPP^+ formed is then selectively taken up by the dopaminergic neurons causing their demise [1]. During this conversion of MPTP to MPP^+ the fate of astrocytes response is largely unknown. Particularly, the actions of MPTP and MPP^+ in astrocytes activation associated to the inflammatory mediators production remain in the ambiguity. In the brain, MPTP induces reactive gliosis, the cellular manifestation of neuroinflammation, which is a common response of the brain to neuronal injury [7]. Reactive gliosis is characterized by activation of either microglia or astroglia, both able to respond to injury and to participate in

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pathological events [5,8]. Activated astrocytes and microglia contribute significantly to neuronal degeneration because they produce a broad array of neurotoxic molecules, including pro-inflammatory cytokines, chemokines, prostaglandins and a large amount of reactive oxygen and nitrogen species [4,9]. The major intracellular events that may be associated to the glial cell activation are the induction of p38 mitogen activated protein kinase (p38 MAPK) cascade and elevated expressions of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and C/EBP, homologous protein 10 (CHOP) [10,11]. In addition to p38 kinase, nuclear factor kappa-B (NF-kB) may also be activated, which in turn regulates the expressions of many genes including pro-inflammatory cytokines; in particular of tumour necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), interleukin-1α (IL-1α) and interleukin-6 (IL-6) [12]. These intracellular events of neuroinflammation in astrocytes are still poorly understood in response to MPTP and in PD pathophysiology. Deprenyl is a MAO'B inhibitor and clinically used drug against PD [13,14], but its mechanism of action associated to the neuroinflammatory response is not properly known and needs to be elucidated in more detail. Although accumulating evidence suggests that astroglial activation may be the key response for MPTP-induced dopaminergic neurodegeneration [15], the exact mechanism of action in deleterious events ultimately leading to dopaminergic neuronal cell death remains elusive and is of great importance towards development of novel therapeutics.

Melatonin (*N*-acetyl-5-methoxytryptamine) is an indolamine mostly produced in the pineal gland during the dark phase [16]. Melatonin, being a good antioxidant [17,18], has shown its protective and anti-inflammatory effect in numerous neurological disorders including PD [19,20]. However, several reports insist on the neuroprotective ability of the melatonin in brain-related disorders [21,22], but the effect of melatonin in the cascade of events associated to the neuroinflammatory signal transduction pathway particularly in astrocytes is still less clearly explored.

Therefore, the present study was undertaken primarily to investigate the mechanism of action of MPTP on a neuroinflammatory cascade of events associated to the nitrative and oxidative stress, intracellular Ca^{2++} ion level, mRNA expression profile of pro-inflammatory cytokine genes, expression profile of GFAP, COX-2, P-p38, iNOS, CHOP proteins and NF-kB translocation in rat astrocytoma C6 cells. Secondarily, we have investigated the action of melatonin on these MPTP-induced neuroinflammatory changes in C6 cells. Moreover, to understand the fate of MPTP and MPP^+ in triggering these inflammatory events, the effect of deprenyl was investigated in MPTP-induced C6 cells.

Materials and methods

Materials

Primary antibodies, rabbit polyclonal anti-COX-2, anti-NF-kB (p-65), anti-iNOS, anti-CHOP, anti-P-p38, anti-β-actin and secondary goat anti-rabbit HRP-conjugated were purchased from Santa Cruz Biotechnology (CA, USA). Secondary goat anti-rabbit fluorescent-conjugated (Alexa-fluor-546) antibody was purchased from Invitrogen (CA, USA). RT-PCR (5-prime two steps) kit was purchased from Eppendorf (Chennai, India) and primers specific for TNF-α, GAPDH, IL-1α, IL-1β and IL-6 were purchased from Metabion International AG (Hamburg, Germany). Rabbit polyclonal anti-GFAP and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). MPTP.HCl (RBI), -(–) Deprenyl. HCl (RBI) and melatonin (Sigma-Aldrich) were dissolved in sterile water and subsequent dilutions were made in medium as per the required concentrations.

Cell culture and treatment

Rat astrocytoma cell line (C6) was obtained from National Centre for Cell Sciences (Pune, India) and maintained in a CDRI tissue culture facility. C6 cells were cultured in DMEM nutrient mixture medium supplemented with 10% heat-inactivated foetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. C6 cells were incubated with MPTP in the absence or presence of melatonin and melatonin alone for 24 h. Measurements for nitrite, ROS generation, MDA formation, intracellular glutathione (GSH), Ca^{2++} ion level, NF-κB translocation were done. Measurement for mRNA expressions of pro-inflammatory genes (TNF-α, IL-1α, IL-1β and IL-6) and protein expressions of COX-2, GFAP, iNOS, CHOP and P-p38 MAPK were also done in respective groups.

Cell viability assay by MTT

MTT assay was used for cell viability [23]. C6 cells (10 000/well) were seeded in 96-well plates. After the treatment period, MTT salt (20 µl/well containing 100 µl of cell suspension; 5 mg/ml in PBS) was added. Colour was read at 530 nm, using a multi-well microplate reader (Biotech-Atlanta, USA).

Nitrite estimation by Griess reagent

Nitrite estimations in culture supernatant from different groups was done by the Griess reagent [24]. C6 cells were seeded at the cell density of 1×10^4 cells/well in 96-well plates. After the treatment period an equal amount of culture supernatant (100 µl) from treated groups were mixed with an equal amount

(100 µl) of Griess reagent (1% p-amino-benzene sulphonamide, 0.01% napthylethylenediamide in 2.5 % phosphoric acid) and an incubation period of 20 min was given in the dark and absorbance was read at 570 nm. Nitrite release was expressed as percentage increase from basal.

Measurement of reactive oxygen species generation

Measurement of intracellular reactive oxygen species (ROS) was accomplished by fluorescence dye DCF-DA. C6 cells were seeded at the cell density of 1×10^4 cells/well in the 96-well plates. After treatment period medium was aspirated and DCF-DA 100 µl/well (10 µM final concentrations) in phenol red free HBSS buffer was added to the plate. A minimum incubation period of 30 min in the dark at 37°C was given to the cells in the CO_2 incubator. Fluorescence was measured at wavelength excitation = 485 nm and emission at = 530 nm, by microplate fluorescence reader (using Cary Eclipse software, VARIAN Optical Spectroscopy Instruments, Mulgrave, Australia). ROS generation was expressed as percentage increase from basal.

Melondialdehyde estimation

Melondialdehyde (MDA) estimation in C6 cells was done according to the method of Colado et al. [25]. C6 cells were seeded at the cell density of 4×10^5 cells/well in 6-well plates and were left for 24 h for the proper attachment. After the treatment, cells were scraped in 100 µl of the sodium phosphate buffer pH 7.0. Cells were lysed by sonication (a pulse of 30 s for each) and then centrifuged at 10 000 g for 5 min. Pellet was discarded and supernatant was collected. In supernatant (100 µl), 60 µl of TCA (trichloroacitic acid) and 30 µl of 5N HCl was added. After 5 min of incubation, 30 µl of TBA (Thiobarbituric acid) in 1 N NaOH was added and then samples were heated at 90°C. After heating, samples were again centrifuged at 10 000 rpm for 10 min. Supernatants were then transferred to a micro-plate and absorbance was read at 532 nm. MDA level was expressed as percentage increase in basal.

Glutathione estimation

Reduced glutathione (GSH) was measured by the method of Anderson and Greenwald [26]. C6 cells were seeded at the cell density of 4×10^5 cells/well in 6-well plates. After the incubation period cells were scraped in 100 µl of PBS (ice cold). After lysis (by ultrasonication) cell lysate was centrifuged at 10 000 g for 5 min at 4°C. Cell supernatants were then deproteinized by adding pre-cooled 10% trichloroacetic acid (100 µl) with an incubation period of 1 h at 4°C. Samples were further re-centrifuged at 5000 g for 5 min. Supernatants were collected and pellets were discarded. Supernatants (75 µl) were mixed with 25 µl of the distilled water + 100 µl of buffer (0.25M of Tris base + 20 mM EDTA) + 50 µl of DTNB (0.1%). After 10 min of incubation absorbance was measured at 412 nm. Results were expressed as relative GSH level.

Estimation of intracellular calcium (Ca^{++}) ion by FURA-2AM

Intracellular calcium level was measured by fluorescence probe FURA-2 AM following the method of Grynkiewicz et al. [27]. C6 cells were seeded at the cell density of 100 000 cells/ml in 6-well plates. After treatment for 24 h, medium was aspirated and cells were then incubated with HBSS buffer (137 mM NaCl, 5.4 mM KCl, 0.49 mM $MgCl_2$, 0.44 mM KH_2PO4, 0.64 mM Na_2HPO_4, 3 mM $NaHCO_3$, 5.5 mM glucose,1.26 mM $CaCl_2$, 20 mM HEPES) for 1 h at 37°C in an incubator containing 5 µM FURA-2AM as a final concentration. HBSS buffer was removed and replaced with the fresh HBSS buffer. Fluorescence was read using a spectrofluoremeter (using Cary Eclipse software, VARIAN optical spectroscopy instruments, Australia) at dual wave length ex = 340/380 nm and em = 510 nm. Calcium level was calculated according to Grynkiewicz et al. [27]. Calcium influx was expressed as percentage increase in basal.

Immunocytochemistry for NF-kB translocation

C6 cells (2×10^5/well) were seeded in 6-well plates containing cover slips at their bottom and treatment was given after 24 h. Cells were treated with 0.05% H_2O_2 in methanol for 1 h at room temperature in the dark with mild agitation to make them permeable. Cells were treated with blocking buffer (0.02% BSA + 0.002% triton-X 100) for 30 min at room temperature. Cells were then treated with the primary antibody of NF-kB (p65 sub-unit), 1:100 dilutions for 1 h with mild agitation. Cells were again treated with secondary fluorescent-conjugated antibody at 1:200 dilutions in blocking buffer for 1 h with mild agitation. Cells were washed with PBS at each step. Images were captured by an upright fluorescence microscope at 100× magnification.

Western blotting for COX-2, iNOS, CHOP, GFAP and P-p38 expressional analysis

Cell lysates of glial cells from different treatment groups were prepared in 200 µl of the lysis buffer, containing 100 mM NaCl, 50 mM Tris-HCL, 1 mM EDTA, 1 µg/ml aprotenin, 100 µg/ml phenylmethylsulphonyle fluoride (PMSF), 10 µg/ml pepstatin for non-phosphorylated

proteins and for phosphorylated proteins 2 mM NaF, 2mM Na_3Vo_4, 1% triton-X 100 were additionally added. Cell lysate was then centrifuged at 10 000 g for 5 min at 4°C. Protein estimation in supernatants were done by Lowry et al.'s [28] method. Samples were mixed with the 3× loading buffer containing 100 mM Tris-HCl (pH = 6.8), 200 mM Dithiothritol (DTT), 4% sodiumdodecyl sulphate (SDS), 0.2% bromophenol blue, 20% glycerol and were boiled for 5 min at 100°C. Proteins (50–100 µg) were separated on 8–15% SDS-PAGE, transferred to PVDF membrane. Membranes were then blocked by blocking buffer (5% non-fat dry milk, 10 mM Tris pH = 7.5, 100 mM NaCl, 0.1% tween-20) overnight at 4°C and washed with wash buffer. Membranes were treated with primary anti-COX-2, anti-iNOS, anti-CHOP, anti-GFAP or anti-P-p38 antibodies at 1:1000 dilutions at room temperature for a period of 2 h. After washing, membranes were again treated with HRP-conjugated secondary antibodies in a 1:2000 dilution for 1 h at room temperature. Blots were then developed by the ECL (Enhanced chemiluminescence) system provided by Amersham Biosciences (Uppsasa, Sweden). Densitometry analyses of bands were accomplished by the Alpha Image gel documentation system (Alpha Innotech, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) for transcriptional analysis of TNF-α, IL-1β, IL-1α, IL-6 genes

RNA was isolated by tri-reagent (Sigma-Aldrich). RNA (2 µg) was quantified spectrophotometrically and was reverse transcribed using oligo-(dT) primers by kit 5 prime (manual master script kit and manual master script RT-PCR system) according to the manufacturer's protocol (eppendorff). An equal amount of cDNA was subjected to subsequent PCR analysis in a total volume of 50 µl containing 0.5 µM of primers specific for TNF-α, IL-1β, IL-1α, IL-6 and GAPDH (glyceraldehyde phosphate dehydrogenase). Detail of primers is given in Table I. PCR was performed in the following conditions: (1) 5 min at 94°C; (2) 45 s at 94°C, 45 s at 60°C for TNF-α or 68°C for GAPDH or 58°C for IL-1β or 65°C for IL-6 and IL-1α, 45 s at 72°C for 35 cycles to all except GAPDH (30 cycles); and (3) 10 min at 70°C. Controls included RNA subjected to the RT–PCR procedure without addition of reverse transcriptase and PCR performed in the absence of cDNA, which always yielded negative results.

Statistical analysis

All data are expressed as means ± SEM and are representative of an average of at least three separate experiments. Statistical analysis was done by one-way analysis of variance (ANOVA), followed by Newman-Keuls post-hoc test. GraphPad prism (version-3) was used to perform statistical tests. The value $p < 0.05$ was considered statistically significant.

Results

Dose-dependent study of MPTP on the cell viability of C6 cells

Prior to assessing the mechanism of neuroinflammation, we have determined the cell viability of C6 cells in response to MPTP concentrations in a 24-h incubation period. To accomplish this, C6 cells were incubated with various concentrations of MPTP (312 µM, 625 µM, 1250 µM, 2500 µM 5000 µM and 10 000 µM) for a period of 24 h and cell viability was measured by MTT assay. As demonstrated in Table II, MPTP concentrations up to 2500 µM did not affect the cell viability of the C6 cells, showing non-cytotoxic concentrations. MPTP concentrations 5000 µM and 10 000 µM caused significant reduction in the cell viability of C6 cells showing cytotoxic concentrations. We used non-cytotoxic concentrations of MPTP below 2500 µM in our subsequent mechanistic study.

Dose-dependent study of MPTP on the ROS generation and nitrite release by C6 cells

To study the mechanism of neuroinflammation, dose-dependent study on ROS generation and nitrite

Table I. Details of PCR conditions used.

Probe	Cycles	Orientation	Sequence	Length (bp)	T-an (°C)
GAPDH	30	Forward	5'-TGAAGGTCGGTGTGAACGGATTTGGC-3'	983	68
		Reverse	5'-CATGTAGGCCATGAGGTCCACCAC-3'		
TNF-α	35	Forward	5'-TTCTGTCTACTGAACTTCGGGTGATCGGTCC-3'	111	58
		Reverse	5'-TATGAGATAGCAAATCGGCTGACGGTGTGGG-3'		
IL-1β	35	Forward	5'-CACCTCTCAAGCAGAGCACAG-3'	79	58
		Reverse	5'-GGGTTCCATGGTGAAGTCAAC-3'		
IL-1α	35	Forward	5'-AAGACAAGCCTGTGTTGCTGAAGG-3	85	65
		Reverse	5'-TCCCAGAAGAAAATGAGGTCGGTC-3		
IL-6	35	Forward	5'-TCCTACCCCAACTTCCAATGCTC-3'	79	65
		Reverse	5'-TTGGATGGTCTTGGTCCTTAGCC-3'		

Table II. Effect of MPTP (μM) on the cell viability of rat astrocytoma cells, C6.

Groups	Control	MPTP312	MPTP625	MPTP1250	MPTP2500	MPTP5000	MPTP10000
Mean ± SEM	100 ± 0	92.63 ± 3.43	90.83 ± 2.08	88.77 ± 4.75	87.18 ± 4.80	71.58 ± 8.84**	7.68 ± 0.95***

**$p < 0.01$ and ***$p < 0.001$ significant with control.

release was conducted in a 24-h incubation period because both are major characteristics of glial cells response in the Parkinson's disease pathophysiology [29–32]. To ascertain this, C6 cells were incubated with non-cytotoxic concentrations of MPTP (50 μM, 100 μM, 200 μM, 400 μM and 800 μM) dose dependently and ROS and nitrite release were measured after 24 h of incubation [33–35]. MPTP caused dose-dependent ROS generation and nitrite release in 24 h (Table III). MPTP (400 μM) caused significant ROS generation (~200%) and nitrite release. We used 400 μM of MPTP concentration in our subsequent experiments to study molecular mechanism of action.

Effect of melatonin on MPTP-induced nitrite release, ROS generation, MDA formation, GSH level and intracellular calcium ion level in C6 cells

To explore the effect of melatonin on MPTP-induced nitrative and oxidative stress, we measured the nitrite release and ROS generation in C6 cells. As shown in Figures 1A and B, MPTP (400 μM) significantly increased nitrite release and ROS generation by C6 cells which were significantly inhibited by the melatonin (50 μM, 100 μM and 200 μM) in a dose-dependent manner. Further, we also measured MPTP-induced MDA formation and GSH level in C6 cells after a 24-h incubation period. As shown in Figures 1C and D, MPTP (400 μM) significantly increased MDA formation and a significant decrease in GSH level. MPTP-induced MDA formation was significantly attenuated with an increase in GSH level by the melatonin (50–200 μM) dose-dependently. However, melatonin (200 μM), *per se*, did not show any significant difference on these parameters. Further, we have also estimated intracellular calcium ion level after 24 h of incubation with MPTP and melatonin. In this study, MPTP (400 μM) significantly up-regulated intracellular Ca^{2++} ion level which was down-regulated by melatonin in a dose-dependent manner (Figure 2).

Influence of melatonin on MPTP-induced mRNA expressions of pro-inflammatory cytokine genes in C6 cells

Further, we also measured the mRNA expression profile of inflammatory cytokine genes (IL1α, IL-1β, TNF-α and IL-6), because they have widely been involved in the initiation and progression of PD [36]. MPTP (400 μM) significantly down-regulated IL-1α expression with significant up-regulation in IL-1β (Figure 3A). Melatonin (200 μM) significantly up-regulated MPTP-induced deficit in IL-1α expression with significant down-regulation in IL-1β expression. However, melatonin (200 μM), *per se*, did not show any change in the IL-1α expression but significantly increased IL-1β expression. As illustrated in Figure 3B, MPTP (400 μM) significantly down-regulated IL-6 expression with significant up-regulation in TNF-α expression. Melatonin (200 μM) significantly up-regulated MPTP-induced decrease in IL-6 expression. Melatonin (100 μM, 200 μM) also significantly down-regulated MPTP-induced TNF-α expression. However, melatonin (200 μM), *per se*, did not show any significant effect on IL-6 and TNF-α mRNA expressions.

Influence of melatonin on MPTP-induced cyclooxigenase-2 (COX-2) and glial fibrillary acidic protein (GFAP) expressions in C6 cells

We also assessed the effect of MPTP on COX-2 and GFAP protein expressions and their modulation by melatonin. MPTP (400 μM) significantly up-regulated COX-2 protein expression (Figure 4). Melatonin (200 μM) significantly down-regulated MPTP-induced COX-2 expression. Melatonin (200 μM), *per se*, also significantly down-regulated COX-2 expression. However, MPTP alone did not show significant change in GFAP protein expression (Figure 4). Melatonin alone and in combination with MPTP significantly down-regulated GFAP protein expression.

Table III. Dose-dependent study of MPTP (μM) on ROS generation and nitrite release in rat astrocytoma cells, C6.

		Control	MPTP50	MPTP100	MPTP200	MPTP400	MPTP800
ROS	*M* ± SEM	100 ± 0	143.1 ± 4.6**	149 ± 5.1**	167.4 ± 5.0***	206.7 ± 7.54***	181.5 ± 10.49
Nitrite	*M* ± SEM	100 ± 0	565.2 ± 63.17*	1014 ± 139***	1150 ± 122.6***	1783 ± 190.1***	1159 ± 160.7***

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ significant with control.

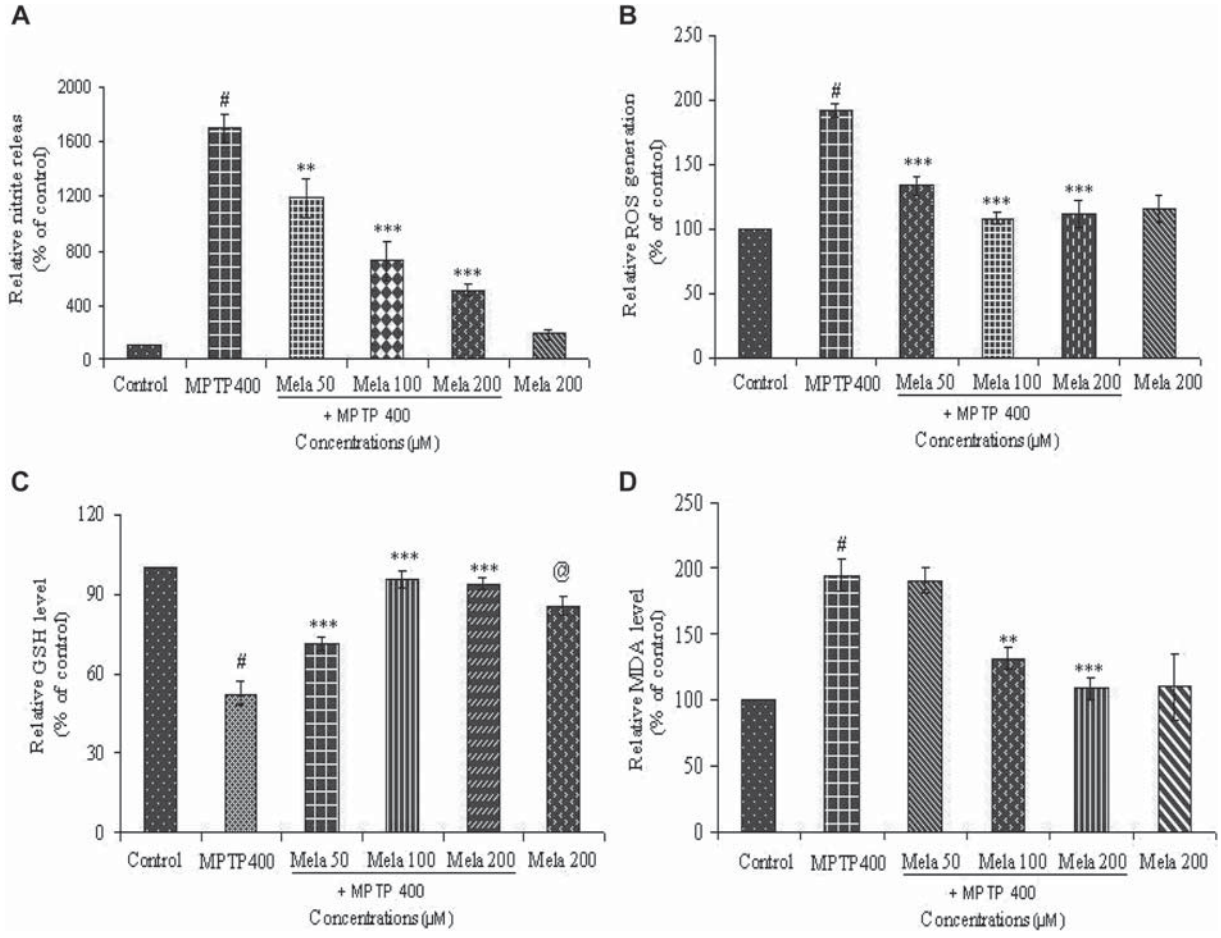

Figure 1. Effect of melatonin on MPTP-induced nitrite release, ROS generation, MDA formation and GSH level. Histograms show relative change expressed as Mean ± SEM of three independent experiments ($n = 3$). (A) $^{\#}p < 0.001$ significant with control, $^{**}p < 0.01$ and $^{***}p < 0.001$ significant with MPTP-treated group. (B) $^{\#}p < 0.001$ significant with control, $^{***}p < 0.001$ significant with MPTP treated group. (C) $^{\#}p < 0.001$ significant with control, $^{**}p < 0.01$ and $^{***}p < 0.001$ significant with MPTP-treated group. (D0 $^{\#}p < 0.001$ significant with control, $^{***}p < 0.001$ significant with MPTP-treated group.

Effect of melatonin on MPTP-induced nuclear factor kappa B (NF-kB) translocation, P-p38 MAPK expression, iNOS and CHOP expressions in C6 cells

To explore the mechanisms underlying the protective effect of melatonin against MPTP, measurements for NF-kB translocation and P-p38 kinase expression were done. Treatment of C6 cells with MPTP (400 µM) caused NF-κB (p65) translocation, from cytosol to nucleus which was inhibited by melatonin (Figure 5A). Further, as illustrated in Figure 5A, MPTP (400 µM) significantly up-regulated the expression of phosphorylated form of p38 kinase (P-p38), which was down-regulated by the melatonin in a dose-dependent manner. Melatonin (200 µM), *per se*, also significantly down-regulated P-p38 kinase expression. To unravel the further downstream targets of melatonin and MPTP actions, we measured CHOP and iNOS expressions by western blotting [11]. Treatment of C6 cells with MPTP (400 µM) significantly increased iNOS and CHOP expressions which were attenuated by melatonin in a dose-dependent manner (Figure 5B).

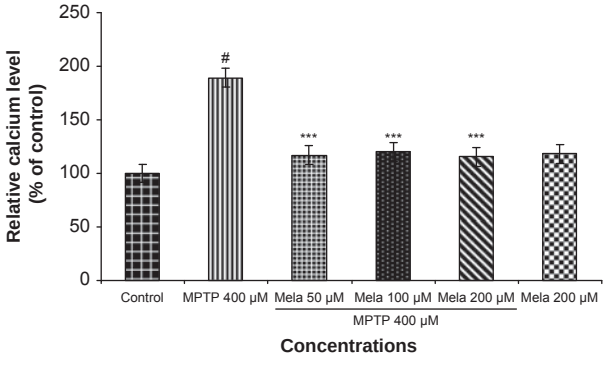

Figure 2. Effect of melatonin on MPTP-induced Ca^{2++} ion level in C6 cells. Histograms show relative calcium level expressed as Mean ± SEM of three independent experiments ($n = 3$). $^{\#}p < 0.001$ significant with control, $^{***}p < 0.001$ significant with MPTP-treated group.

Effect of deprenyl on MPTP-induced nitrite release, ROS generation, COX-2 and GFAP expressions in C6 cells

To determine the fate of MPTP and MPP^{+} in astrocytoma cells, we also studied the effect of deprenyl,

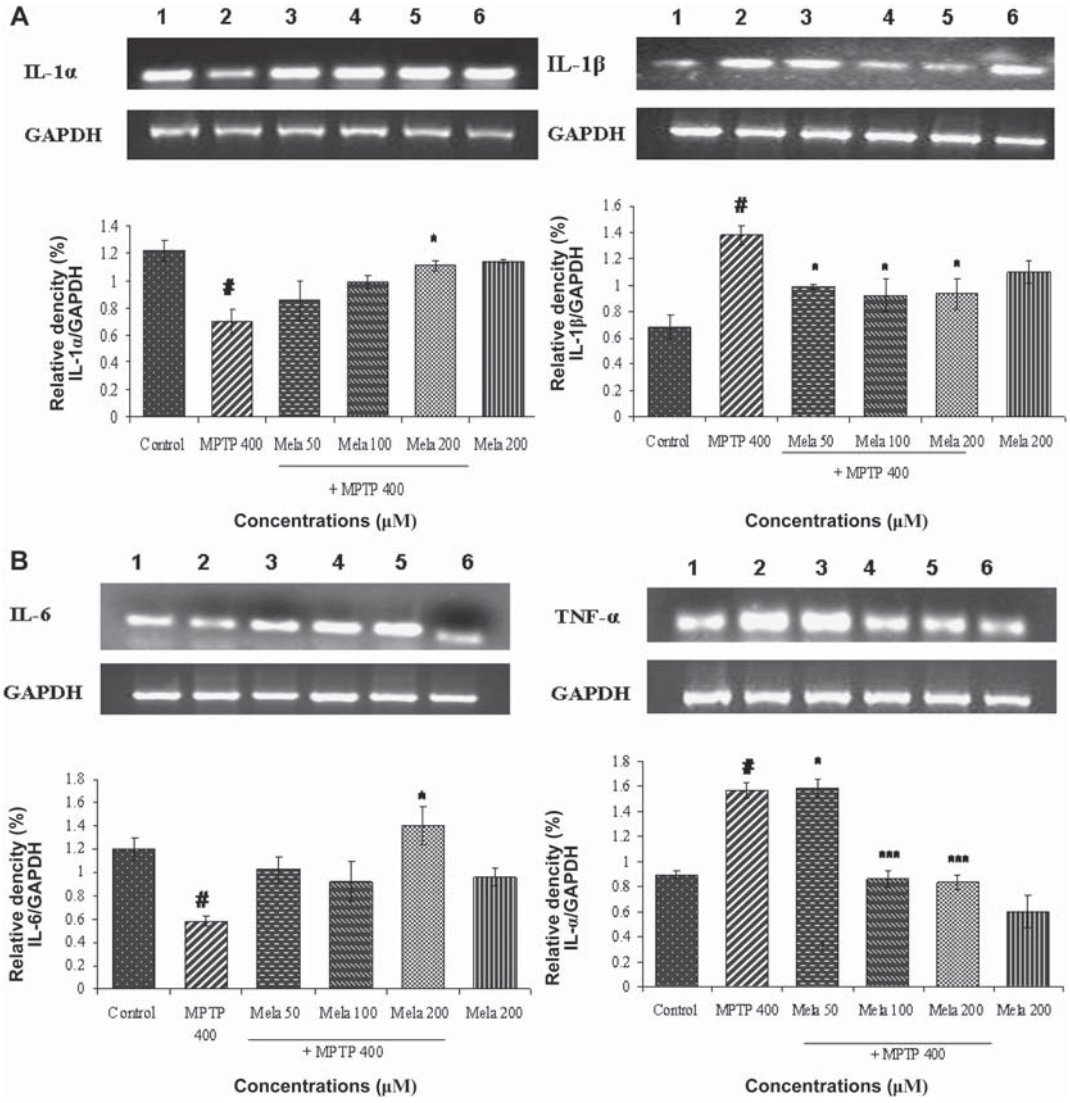

Figure 3. Effect of melatonin on MPTP-induced mRNA expressions of pro-inflammatory cytokine genes (IL-1α, IL-1β, IL-6 and TNF-α). Lane-1 = control, Lane-2 = MPTP 400 μM, Lane-3 = MPTP + Mela 50 μM, Lane-4 = MPTP + Mela 100 μM, Lane-5 = MPTP + Mela 200 μM, Lane-6 = Mela 200 μM. Histograms represent relative density (%) of IL-1α, IL-1β, IL-6 and of TNF-α DNA expressed as Mean ± SEM of three independent experiments. GAPDH was taken as internal control. (A) In IL-1α expression (left-panel), $^{\#}p < 0.01$ significant with control, $^{*}p < 0.05$ significant with MPTP-treated group. In IL-1β expression (right-panel), $^{\#}p < 0.01$ significant with control, $^{*}p < 0.05$ significant with MPTP-treated group. (B) In IL-6 expression (left-panel), $^{\#}p < 0.05$ significant with control, $^{*}p < 0.01$ significant with MPTP-treated group. In TNF-α (right-panel) expression, $^{\#}p < 0.001$ significant with control, $^{***}p < 0.001$ significant with MPTP-treated group.

a monoamine oxidase'B (MAO'B) inhibitor, on the MPTP-induced nitrite release, ROS generation, COX-2 and GFAP expressions [14]. To accomplish this, C6 cells were incubated with MPTP (400 μM) alone and with MPTP (400 μM) plus deprenyl (1 μM, 10 μM, 100 μM) and deprenyl alone (100 μM) for 24 h. MPTP-induced nitrite release was not significantly inhibited by the deprenyl (Figure 6A). Further, MPTP-induced elevated ROS generation was significantly decreased by the deprenyl (1 μM, 10 μM and 100 μM) (Figure 6B). However, deprenyl (100 μM), *per se*, did not show significant difference on ROS generation. Deprenyl (100 μM), significantly potentiate MPTP-induced COX-2 expression without significant effects *per se* (Figure 6C). MPTP did not significantly affect GFAP expression (Figure 6D). Treatment of deprenyl (100 μM) with MPTP down-regulated GFAP expression. Deprenyl (100 μM), *per se*, also down-regulated GFAP expression.

Discussion

The present findings showed the mechanism of action of MPTP and its modulation by melatonin on neuroinflammatory cascade of events in rat astrocytoma cells, C6. After the assessment of cytotoxicity of MPTP on C6 cells, we determined the MPTP concentrations able to generate the peak

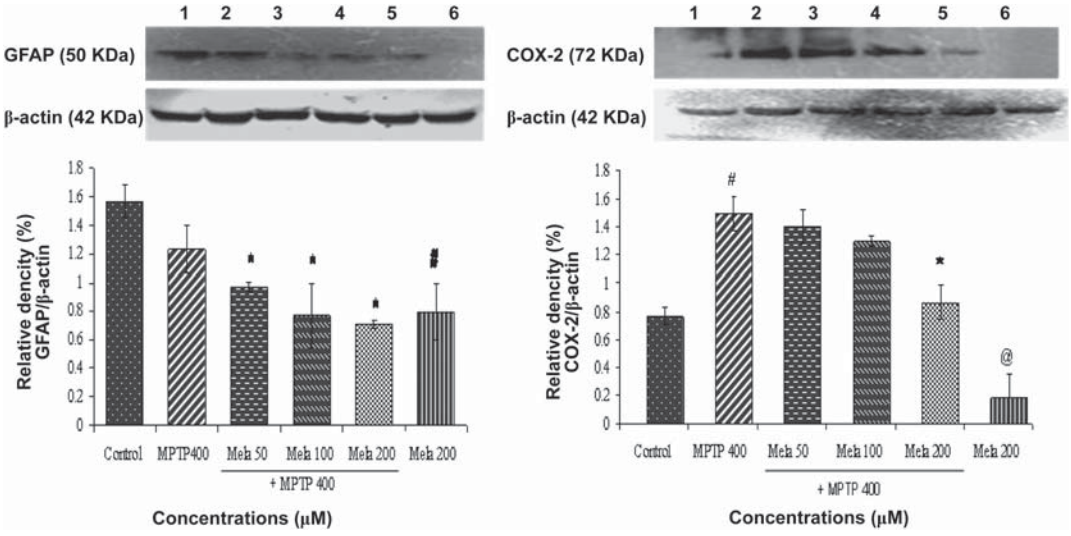

Figure 4. Effect of melatonin on MPTP-induced expressions of GFAP and COX-2 proteins. Histograms represent relative density of protein (%). Beta-actin was taken as an internal control. In GFAP expression pattern (left panel), $^{*}p < 0.05$ significant with MPTP-treated group, $^{\#}p < 0.05$ significant with the control group. In COX-2 expression pattern (right panel) $^{\#}p < 0.01$ significant with the control group, $^{*}p < 0.05$ significant with MPTP-treated group, @ < significant with the control group.

neuroinflammatory response. As determined by the dose-dependent study, MPTP 400 μM generated the peak neuroinflammatory response on nitrite release and ROS generation (see Table III) and was selected to study further molecular mechanisms [37]. In addition to the multiple factors such as cytokines, fatty acid metabolites produced from activated glial cells, ROS and nitrite may be the key mediators of glia-facilitated MPTP neurotoxicity [32,38,39]. Dopaminergic neurons in the nigra are known to be particularly vulnerable to oxidative stress, presumably due to their lower antioxidant capacity, increased accumulation of ion and oxidation-prone dopamine (DA) and possible defects in mitochondria [40,41]. Previous studies have described the involvement of reactive oxygen and nitrogen species in the pathophysiology of PD, but the effect of MPTP and melatonin on astroglia facilitated oxidative and nitrative stress was not known. Our results demonstrated that MPTP has significantly up-regulated nitrite release, ROS generation, MDA formation and has reduced intracellular GSH level; this depicts that astroglial oxidative and nitrative stress may be a more potential pathological event causing degeneration of neighbouring dopaminergic neurons. Melatonin significantly down-regulated the MPTP-induced oxidative and nitrative stress in this study, indicating that antioxidant effect is responsible of neuroprotection by melatonin in Parkinson's disease pathophysiology [42].

In addition to oxidative and nitrative stress, Ca^{2++} ion release in astrocytes is an important event and involved in the regulation of number of mechanisms including release of S100B and other neurotoxins [43,44]. However, earlier studies have reported alteration in the calcium ion homeostasis in PD [45], but the state of intracellular Ca^{2++} ion level in astrocytes in response to MPTP and melatonin was not demonstrated. This study showed that MPTP elevated Ca^{2++} ion level, which was down-regulated by the melatonin and possibly subsequent Ca^{2++} ion-mediated release of neurotoxins.

Polymorphism studies of IL-1α gene have revealed its important role in the PD pathophysiology [46]. IL-1α has also been shown to modulate glial cell proliferation and thereby guidance and trophic factors for growth of new fibres of neurons, in response to brain injury [47]. The state of expression of IL-1α gene in PD and in response to MPTP and melatonin associated to the astroglia was not explored. We observed that MPTP significantly down-regulated IL1-α expression which correlate to the reduced glial cell proliferation and lack of neurotrophic factor for dopaminergic neurons. Melatonin has up-regulated the IL-1α expression, thus showing its mechanism of protective effect. Reports from PD patients describe the conflicting information associated to the interleukin-6 functions [48]. Muller et al. [49] have also described that IL-6 level inversely correlates with the severity of PD. IL-6 is also important for the trophic factors for neurons. We observed that IL-6 expression was significantly down-regulated by MPTP, which supports the similar results in the PD pathophysiology. Elevated levels of IL-1β and TNF-α are well documented in the serum levels from the PD patients and in the animal models [50]. In the present work, we also showed that MPTP significantly up-regulated TNF-α and IL-1β cytokine genes expressions which may emphasize astroglial cells as a major culprit in PD pathophysiology because TNF-α and

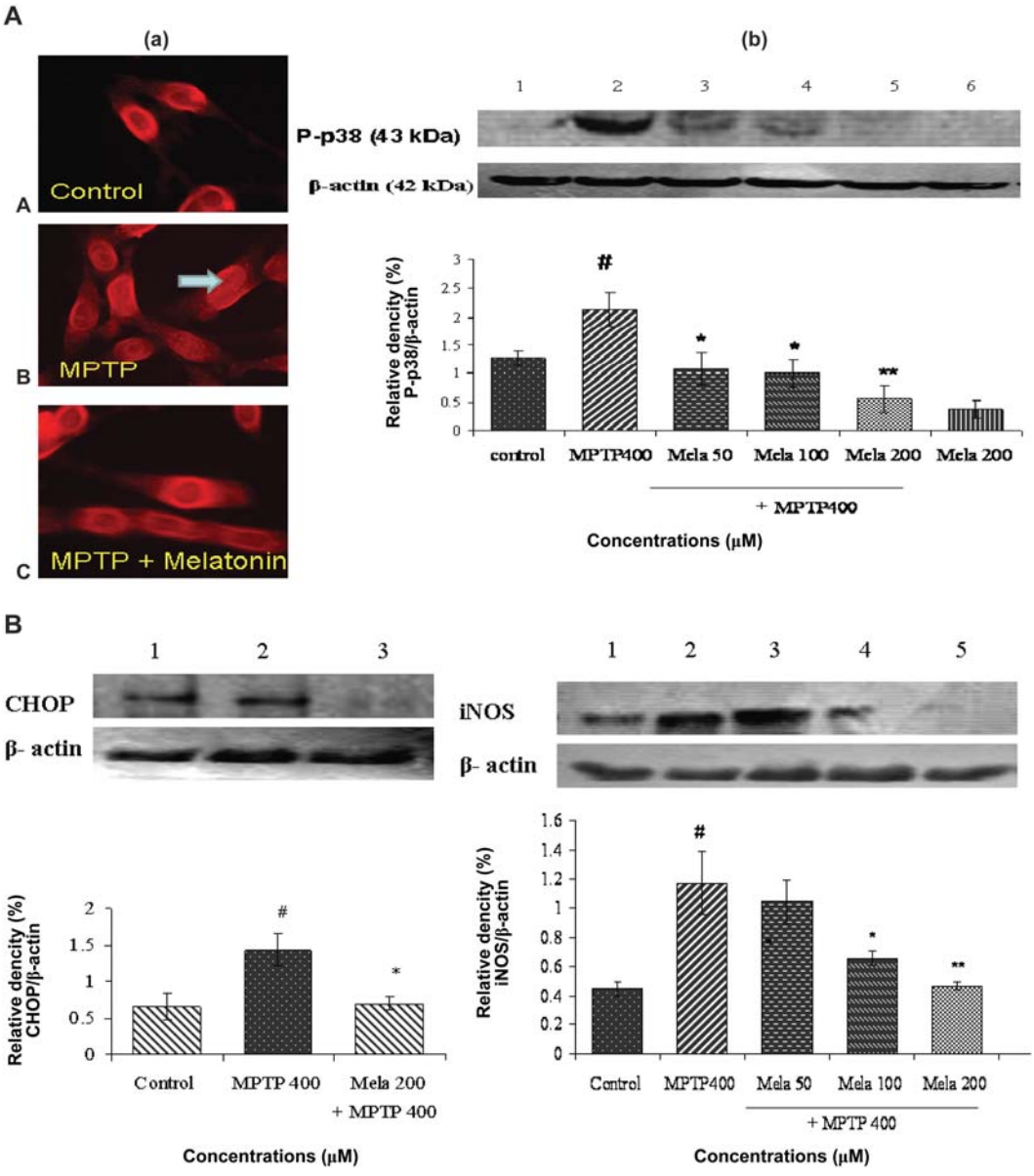

Figure 5. Effect of melatonin on MPTP-induced NF-kB translocation and expression of P-p38, CHOP and iNOS proteins. Histograms represent relative density of protein (%). Beta-actin was taken as an internal control. (A) (a) A-control, B-MPTP 400 μM, C-MPTP 400 μM + Mela 200 μM. (b) $^{\#}p < 0.05$ significant with control, $^{*}p < 0.05$ and $^{**}p < 0.01$ significant with MPTP-treated group. (B) In CHOP expression pattern (left panel), $^{\#}p < 0.05$ significant with the control group, $^{*}p < 0.05$ significant with MPTP-treated group. In iNOS expression pattern (right panel), $^{\#}p < 0.05$ significant with control group, $^{*}p < 0.05$ and $^{**}p < 0.01$ significant with MPTP-treated group.

IL-1β have previously been thought to cause dopaminergic neurodegeneration [51,52]. Melatonin has down-regulated MPTP-induced expression of TNF-α and IL-1β mRNA levels depicting its regulatory mechanism behind its neuroprotective effects [53].

Recently it has been reported that activation of p38 MAPK in the substantia nigra leads to nuclear translocation of NF-kB in MPTP-treated mice, but the astrocytes-specific regulation of NF-kB translocation and p38 activation remain elusive [10]. Importantly, melatonin also showed its potent NF-kB inhibiting activity in cell culture models [54,55], but its astrocytes-specific action was not known. In the present study, MPTP significantly caused NF-kB translocation and increased P-p38 expression revealing the intracellular mechanism of action in the release of inflammatory mediators by C6 cells. Melatonin has significantly inhibited MPTP-induced NF-kB translocation and P-p38 activation, which reveals that the inhibition of NF-kB and p38 activation may be involved in the astrocytes-facilitated neuroprotective action of melatonin in PD pathophysiology. Activation of CHOP transcription factor, a downstream target of P-p38 MAPK activation (Figure 7) was unknown in response to MPTP and melatonin with respect to astrocytes [56]. However, a previous study

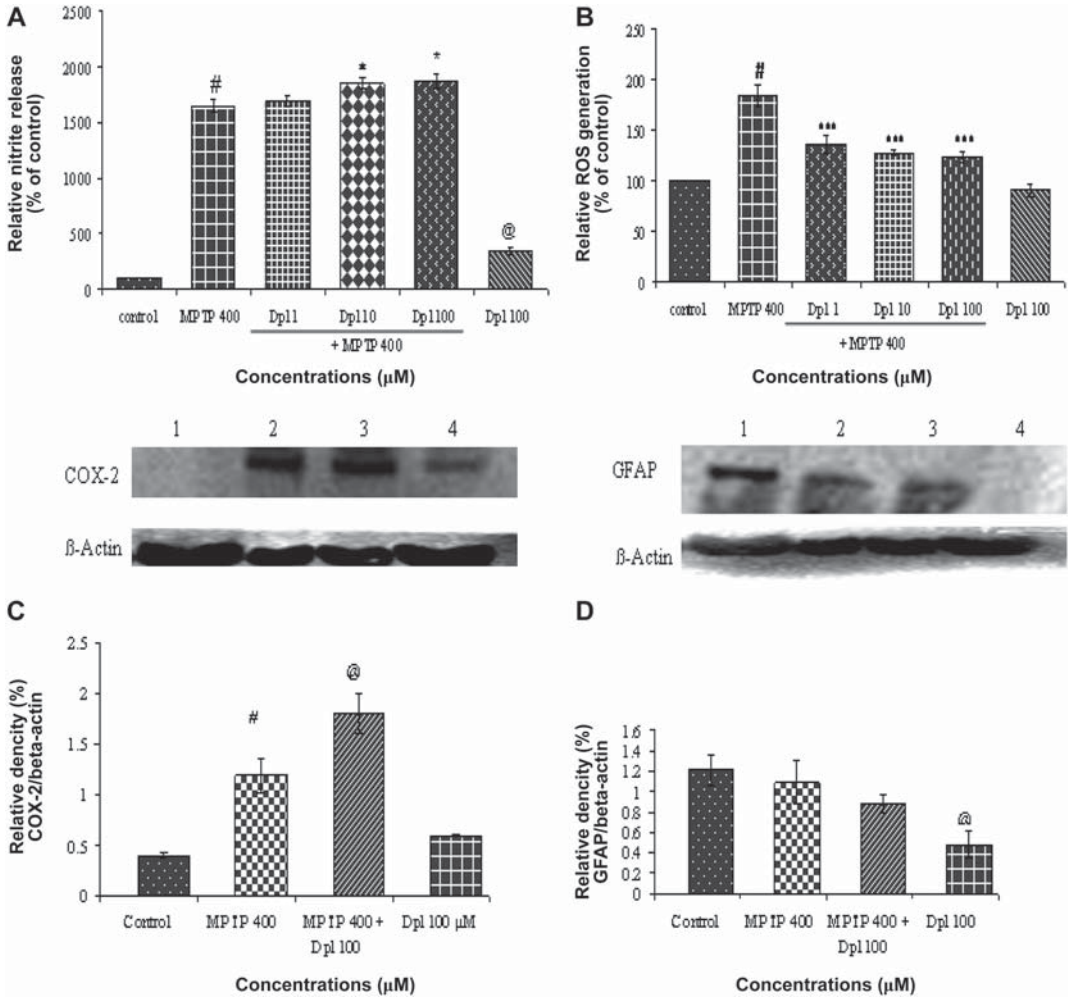

Figure 6. Effect of deprenyl on MPTP-induced nitrite release, ROS generation, expressions of GFAP and COX-2 in C6 cells. Beta-actin was taken as an internal control. (A) $^{\#}p < 0.001$ significant with control, $^{*}p < 0.05$ and significant with MPTP-treated group, @ $p < 0.001$ significant with control. (B) $^{\#}p < 0.001$ significant with control, $^{***}p < 0.001$ significant with MPTP-treated group. (C) In COX-2 expression pattern (left panel), $^{\#}p < 0.01$ significant with the control group, @ $p < 0.05$ significant with MPTP-treated group. (D) In GFAP expression pattern (right panel) @ $p < 0.05$ significant with control group.

has described that CHOP up-regulation is involved in the apoptotic events in the substantia nigra of animal model of PD [57], but its astroglial mediated expression was still unclear with special aspects of regulation of inflammatory mediators release. Our results demonstrated that MPTP has up-regulated CHOP expression which was subsequently down-regulated by the melatonin emphasized CHOP, a mere target for melatonin in astrocytes in PD pathophysiology.

Previous study has described that up-regulation of iNOS has been instrumental in the dopaminergic neurodegeneration [30]. There are also reports that melatonin down-regulated the iNOS induction in various models [54,58]. However, these studies showed iNOS inhibiting activity of melatonin, but the exact role of melatonin on MPTP-induced astroglial cells was not clearly understood [59]. We demonstrated that melatonin significantly down-regulated the MPTP-induced iNOS expression and subsequent nitrite release, insisting that neuroprotection by melatonin may be mediated by the inhibition of nitrite release by astroglial cells in PD.

Several reports have described that there is a vital role of COX-2 enzyme in dopaminergic neurodegeration [60]. COX-2 up-regulation is thought to mediate neuronal damage, presumably by producing an excessive amount of harmful prostanoids and free radicals [61]. MPTP elevated COX-2 expression in animal models of PD [58]. Melatonin also showed down-regulation in COX-2 expression in MPTP-induced models of PD [62]. However, these reports described the reciprocal expression of COX-2 in response to MPTP and melatonin, but their cell-specific actions with special respect to astroglia were largely unknown. In the present study, MPTP significantly up-regulated the COX-2 expression, which may represent prostaglandins-mediated neurodegeneration. Melatonin significantly down-regulated MPTP-induced COX-2 expression, thus inhibiting the prostaglandins synthesis and subsequent neuroprotection.

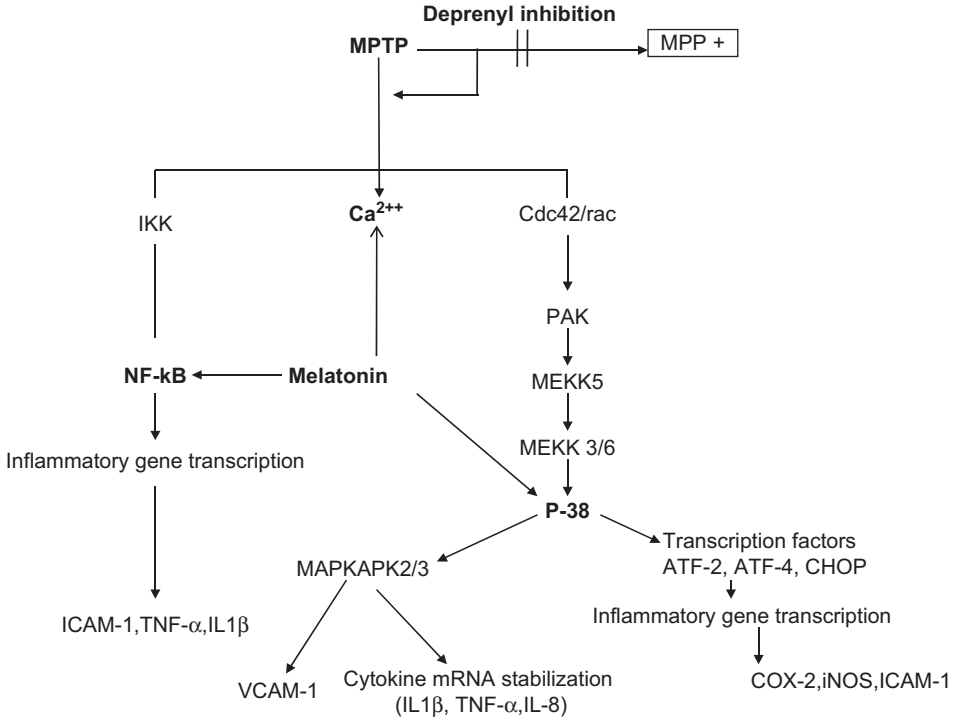

Figure 7. Diagrammatic representation of mechanism of action of melatonin on MPTP-induced neuroinflammation in rat astrocytoma cells, C6. MPTP enters in C6 cells and triggers calcium overload. Subsequently, it activates NF-kB and p-p38 kinase which in turn regulate inflammatory genes. Melatonin may mitigate this inflammation by normalizing calcium overload and inhibiting NF-kB and P-p38 kinase activation.

Reactive astrogliosis, the cellular manifestation of neuroinflammation, is characterized by formation of glial cell soma and increased level of GFAP expression [33]. Our study hereby demonstrates that MPTP does not influence the GFAP expression, but melatonin has significantly down-regulated the MPTP-induced GFAP expression. Hu et al. [63] showed that amyloid-beta peptide did not influence the GFAP expression, which supports the present findings. The results indicate that the MPTP could not initiate the GFAP expression, which may be the limiting factor in the MPTP-induced dopaminergic neurodegeneration in rats. Melatonin could be effective enough in attenuating GFAP expression, which may be one of the mechanisms of its neuroprotective functions.

An interesting question arises from the fact that MPTP itself or MPP^+ is responsible for triggering this neuroinflammatory response inside astroglial cells [1]. Studies related with MAO-B inhibition showed deprenyl as a protective agent in various models of PD, postulating that it could happen by blocking the MPP^+ formation but multiple mechanisms may exist for this protective effects [6]. Importantly, Thiffault et al. [64] have described that deprenyl has no effect on MPTP-induced dopaminergic neurodegeneration. The present study demonstrated deprenyl caused significant up-regulation of COX-2 expression, which revealed that MPTP may alone be responsible for neuroinflammatory response rather than MPP^+. Moreover, deprenyl also failed to reduce MPTP-induced NO release, suggesting that MPTP is responsible for iNOS-mediated NO release rather than MPP^+. It again becomes important to know how deprenyl has shown its protective effect in various models of PD [13,14]. Furthermore, Muralikrishnan et al. [65] have reported that the protective effect of deprenyl against MPTP was because of antioxidant mechanisms rather that its inhibitory actions on formation of MPP^+ from MPTP. Our results demonstrated that deprenyl could be able to reduce ROS generation, which emphasizes its predominant antioxidant mechanisms. In addition to ROS, deprenyl alone also down-regulated GFAP expression, which may indicate its protective role, possibly by reducing astrogliosis (astroglial proliferation).

In conclusion, the present study demonstrates that MPTP activates astrocytes cells, resulting in a sustained NF-kB and CHOP activation and the release of potentially neurotoxic molecules including ROS and nitrite. The pro-inflammatory cytokine genes expression profile could focus astroglial-mediated neurodegeneration. Further, GFAP expression pattern explored the understanding of reactive astrogliosis. The modulatory effect of melatonin on these MPTP-induced neuroinflammatory mediators elucidated its mechanism of protective effect. Study with deprenyl revealed that MPTP rather than MPP^+ is responsible for initiating this inflammatory response in astrocytoma cells, C6.

Collectively, our findings elucidated the molecular mechanism of MPTP-induced neuroinflammation and its modulation by melatonin in C6 cells, which contributes in basic understanding of astroglial-mediated neuroinflammation in PD pathophysiology.

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