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ORIGINAL PAPER

Involvement of Nitric Oxide in Maneb- and Paraquat-Induced Parkinson's Disease Phenotype in Mouse: Is There Any Link with Lipid Peroxidation?

Satya Prakash Gupta · Suman Patel · Sharawan Yadav · Anand Kumar Singh · Seema Singh · Mahendra Pratap Singh

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Abstract The study aimed to investigate the involvement of nitric oxide (NO) in maneb (MB)- and paraquat (PQ)-induced Parkinson's disease (PD) phenotype in mouse and its subsequent contribution to lipid peroxidation. Animals were treated intraperitoneally with or without MB and PQ, twice a week for 3, 6 and 9 weeks. In some sets of experiments (9 weeks treated groups), the animals were treated intraperitoneally with or without inducible nitric oxide synthase (iNOS) inhibitor-aminoguanidine, tyrosine kinase inhibitor-genistein, nuclear factor-kappa B (NF-kB) inhibitor-pyrrolidine dithiocarbamate (PDTC) or p38 mitogen activated protein kinase (MAPK) inhibitor-SB202190. Nitrite content and lipid peroxidation were measured in all treated groups along with respective controls. RNA was isolated from the striatum of control and treated mice and reverse transcribed into cDNA. RT-PCR was performed to amplify iNOS mRNA and western blot analysis was done to check its protein level. MB- and PQ-treatment induced nitrite content, expressions of iNOS mRNA and protein and lipid peroxidation as compared with respective controls. Aminoguanidine resulted in a significant attenuation of iNOS mRNA expression, nitrite content and lipid peroxidation demonstrating the involvement of nitric oxide in MB- and PQ-induced lipid peroxidation. Genistein, SB202190 and PDTC reduced the expression of iNOS mRNA, nitrite content and lipid peroxidation in MB- and PQ-treated mouse striatum. The results obtained demonstrate that nitric oxide contributes to an increase of MB- and PQ-induced lipid peroxidation in mouse striatum and tyrosine kinase, p38 MAPK and NF-kB regulate iNOS expression.

Keywords Parkinson's disease phenotype · Maneb and paraquat · Nitric oxide · Lipid peroxidation

Introduction

Parkinson's disease (PD) is characterized by an abnormal, progressive and selective degeneration of dopaminergic neurons of the nigrostriatal pathway [1–4]. Dopaminergic neurons project into the striatum, therefore, the idiopathic loss of dopaminergic neurons in the substantia nigra pars compacta region of the brain reduces the level of dopamine in the striatum [1–4]. The depletion of dopamine level in the striatum leads to the loss of control over movement and coordination [1–4]. PD is a multi-factorial disease, contributed mainly by age, genetic and environmental factors [3, 4]. Among environmental factors, herbicide and fungicide exposures have been associated with PD phenotype in animals [5–7]. Despite extensive research, the etiology and the mechanism underlying selective neuronal loss have not yet been clearly understood.

1,1′-Dimethyl-4,4′-bipyridinium (paraquat; PQ), an herbicide, induces generation of free radicals and leads to multi-organ toxicity, including neurotoxicity. PQ accepts an electron and forms PQ radical that transfers its extra electron to molecular oxygen and generates superoxide anion [8, 9]. Superoxide anion gets converted to hydrogen peroxide that in turn changes into hydroxyl radicals or is

Satya Prakash Gupta and Suman Patel have contributed equally to this work.

S. P. Gupta · S. Patel · S. Yadav · A. K. Singh · S. Singh · M. P. Singh (✉)
Indian Institute of Toxicology Research (Council of Scientific and Industrial Research), Mahatma Gandhi Marg, Post Box-80, Lucknow 226 001, UP, India
e-mail: singhmahendrapratap@rediffmail.com

directly detoxified by antioxidant enzymes [9, 10]. Hydroxyl radicals along with other free radicals readily react with polyunsaturated fatty acids to yield lipid hydroperoxides that initiate the lipid radicals chain reaction, which may cause oxidative damage to cells leading to increased membrane fluidity, permeability and loss of membrane integrity. Manganese ethylene-bis-dithiocarbamate (maneb, MB) is found to alter the function of mitochondria, therefore, expected to contribute to oxidative stress and interfere with energy metabolism [3, 11]. Systemic PQ exposure generates specific loss of dopaminergic neurons and MB augments PQ-induced neuronal damage [5, 12–14]. Co-treatment of MB and PQ selectively and synergistically targets the nigrostriatal system leading to significant reduction in motor activity, degeneration of dopaminergic neurons, neuronal toxicity, increased oxidative stress and lipid peroxidation and altered expression of toxicant responsive genes [5, 7, 14–16].

Nitric oxide (NO), an important secondary mediator of several biological functions, rapidly combines with superoxide to form peroxynitrite, a potent and versatile oxidant, which can attack a wide range of biological targets [17, 18]. Although NO plays an important role in neurotransmitter release, neurotransmitter re-uptake and regulation of gene expression, its excessive production leads to neurotoxicity [19]. NO mediates neurotoxicity by excitotoxicity, DNA damage or post-translational modification of proteins [20]. During microglial activation, an increase in oxidative stress and nitric oxide synthase (NOS)-mediated nitric oxide production are well known [21]. The involvement of tyrosine kinase pathway in iNOS-mediated nitrite oxide production is reported in a study, which has shown reduction in lipopolysaccharide and interferon-γ induced iNOS activity in presence of tyrosine kinase inhibitor [22]. The p38 mitogen activated protein kinase (MAPK) inhibitor alters nitric oxide-mediated neurodegeneration confirming the role of p38 MAPK in iNOS-mediated neuronal injury [23]. Similarly, nuclear factor kappa B (NF-kB) is also found to regulate iNOS transcriptional activity, as evidenced by the inhibition of enhanced iNOS expression by a NF-kB inhibitor, pyrrolidine dithiocarbamate (PDTC), in several experimental paradigms [24, 25].

Previously, we have reported an increased lipid peroxidation activity in the mouse striatum co-treated with MB and PQ as compared with saline treated controls [7]. The present study aimed to investigate the contribution of nitric oxide in MB- and PQ-induced lipid peroxidation and role of secondary mediators regulating this phenomenon. The study was performed with the striatum, the major site of dopamine action and motor control, as the degeneration of terminals of dopaminergic neurons initiates in the striatum [16].

Experimental Procedures

Chemicals

Bovine serum albumin (BSA), Bradford's reagent, sodium dodecyl sulfate (SDS), ethylene-diamine-tetraacetic acid (EDTA), ethylene glycol-bis (β-aminoethylether)-N, N, N′, N′-tetraacetic acid (EGTA), phenol, chloroform, MB, PQ, ethidium bromide, agarose, ethanol, sodium nitrite, sodium pyrophosphate, sodium fluoride, thiobarbituric acid (TBA), Tris (hydroxymethyl) aminomethane, Triton X-100, orthophosphoric acid (H_3PO_4), sulfanilamide, *N*-(1-naphthyl) ethylene-diamine dihydrochloride, dimethyl sulfoxide (DMSO), aminoguanidine, genistein, SB202190, PDTC, Nonidet P-40 (NP-40), protease inhibitor cocktail, etc. were procured from Sigma-Aldrich, St. Louis, USA. RT-PCR kits were purchased from MBI Fermentas, Canada. Magnesium chloride, dNTPs, *Taq* DNA polymerase, related buffers, 100 bp ladder, Western blot kits, 3,3′,5,5′-tetramethylbenzidine (TMB) and forward and reverse primers of iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were procured from Bangalore Genei Pvt. Ltd, India. Other chemicals required for this study were purchased locally from SISCO Research Laboratory (SRL), India.

Animal Treatment

Male Swiss albino mice (20–25 g) were obtained from the animal colony of Indian Institute of Toxicology Research (IITR), Lucknow. Animals were kept, maintained and sacrificed in the animal house of IITR, Lucknow. The institutional ethics committee for the use of laboratory animals approved the study. Animals were fed commercially available pellet diet along with water ad libitum. Animals were treated intraperitoneally with PQ (10 mg/kg body weight) and MB (30 mg/kg body weight) in combination for 3, 6 and 9 weeks along with saline treated controls. The animals were treated with maneb + paraquat twice a week. In some sets of experiments, the animals were treated intraperitoneally with aminoguanidine (30 mg/kg body weight), genistein (10 mg/kg body weight), SB202190 (5 mg/kg body weight) or PDTC (50 mg/kg body weight) with respective controls in 9 weeks treated animals. Inhibitors were administered to 9 weeks maneb + paraquat treated animals but not to 3 and 6 weeks treated animals, as the maximum effects on nitrite and lipid peroxidation were observed in 9 weeks treated animals. The animals were sacrificed via cervical dislocation and the striatum from two animals were pooled for biochemical analyses. TH-immunoreactivity was performed with the brain dissected after cervical dislocation and immediately kept at 4°C. A minimum of three sets of experiments for each parameter was performed.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Tri reagent was used for isolation of total RNA from mouse striatum treated with and without MB and PQ. RNA was reverse transcribed into cDNA using RT-PCR kit and oligo-dT primers at 42°C for 1 h. GAPDH and iNOS were amplified by PCR. The primers used for iNOS were: forward primer '5TGTGTTCCACCAGGAGATGT3' and reverse primer '5AGGTGAGCTGAACGAGGAG3' (Gene accession number: NM_010927), as described elsewhere [26]. The primers designed for GAPDH were: forward primer '5CTCATGACCACAGTCCATGC3' and reverse primer '5CACATTGGGGGTAGGAACAC3' (Gene accession number: NM_ 0021284), as described elsewhere [27]. The amplification was carried out as follows: 1 min at 95°C, 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, repeated for 35 cycles and 1 min at 72°C for final extension. The PCR products were visualized using ethidium bromide in agarose gels. The images were captured and densitometry was performed to quantify the band density. The values are expressed as band density ratio of iNOS/GAPDH.

Western Blot Analysis

Western blot analysis was done as described elsewhere [28] with some modifications. In brief, the 10% w/v tissue homogenate was made in lysis buffer (20 mM Tris–HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, 30 mM NaF, 30 mM sodium pyrophosphate, 0.1 % SDS, 1 % Triton X-100 and protease inhibitor cocktail) using polytron homogenizer. Protein content was measured according to Bradford's procedure [29]. The proteins (200 µg) were separated on 10% SDS–PAGE and proteins present in the gel were electroblotted onto PVDF membrane. The membrane was incubated with monoclonal antibodies for iNOS or β-actin (Sigma Aldrich, USA; 1:1000) in Tris buffered saline (TBS, pH 7.4) containing 5% non-fat dry milk, overnight at 4°C. The blot was washed thrice with TBS containing 0.2% Tween-20 to remove unbound antibodies. The blot was further incubated with rabbit anti-mouse IgG peroxidase conjugate (1:2000 dilution) for 2 h at room temperature. The blot was washed thrice with TBS and developed with TMB western blot kits (Bangalore Genei, India).

Nitrite Estimation

Nitrite was estimated in the supernatant using standard procedure [30]. In brief, supernatant of 10% w/v tissue homogenate was incubated with ammonium chloride (0.7 mM) and mixed with Griess reagent (0.1% *N*-naphthyl ethylenediamine and 1% sulfanilamide in 2.5% phosphoric acid). The tissue homogenate (10% w/v) was made in 0.7 M NH_4Cl and centrifuged at 10,000×*g* at 4°C for 10 min. The reaction mixture was incubated at 37°C for 30 min, centrifuged, and the absorbance of the supernatant was recorded at 550 nm. The nitrite content was calculated using a standard curve for sodium nitrite (10–100 µM) in terms of µmoles/ml.

Lipid Peroxidation

Lipid peroxidation was estimated using the method of Ohkawa et al. [31] with slight modifications [7]. A 10% microsomal fraction in SDS (10% w/v) was incubated for 5 min and glacial acetic acid (20% w/v) was added and further incubated for 2 min. TBA (0.8% w/v) was added in the reaction mixture and further incubated in a boiling water bath for 1 h. Following centrifugation at 10,000×*g* for 5 min at 4°C, supernatant was collected and the absorbance was recorded at 532 nm against the control blank. The values were calculated in terms of nmole malonaldehyde/mg protein.

TH-Immunoreactivity

TH-immunoreactivity and counting of TH positive neurons were performed as described previously [32] with slight modifications. Unbiased cell counting was performed, as the person who performed cell counting was not aware of the treatment paradigms. Every third serial section of each group was used for staining and counting [33]. Fixed counting dimensions were used for each tracing to count the cells bilaterally.

Statistical Analysis

Statistical analyses were performed with three or more replicates of the pooled samples. Data are expressed in means ± standard errors of means (SEM). Two-ways analysis of variance (ANOVA) with Bonferroni post-test was used for multiple comparisons (in Fig. 1). For single variable comparisons, one-way ANOVA was used (Figs. 2, 3, 4). Differences were considered statistically significant, when P values were <0.05.

Results

Expression of iNOS mRNA, Protein and Nitrite Content

An increase in the expression of iNOS mRNA and protein in mouse striatum after MB- and PQ-treatment was observed as compared with respective controls (Fig. 1a–d).

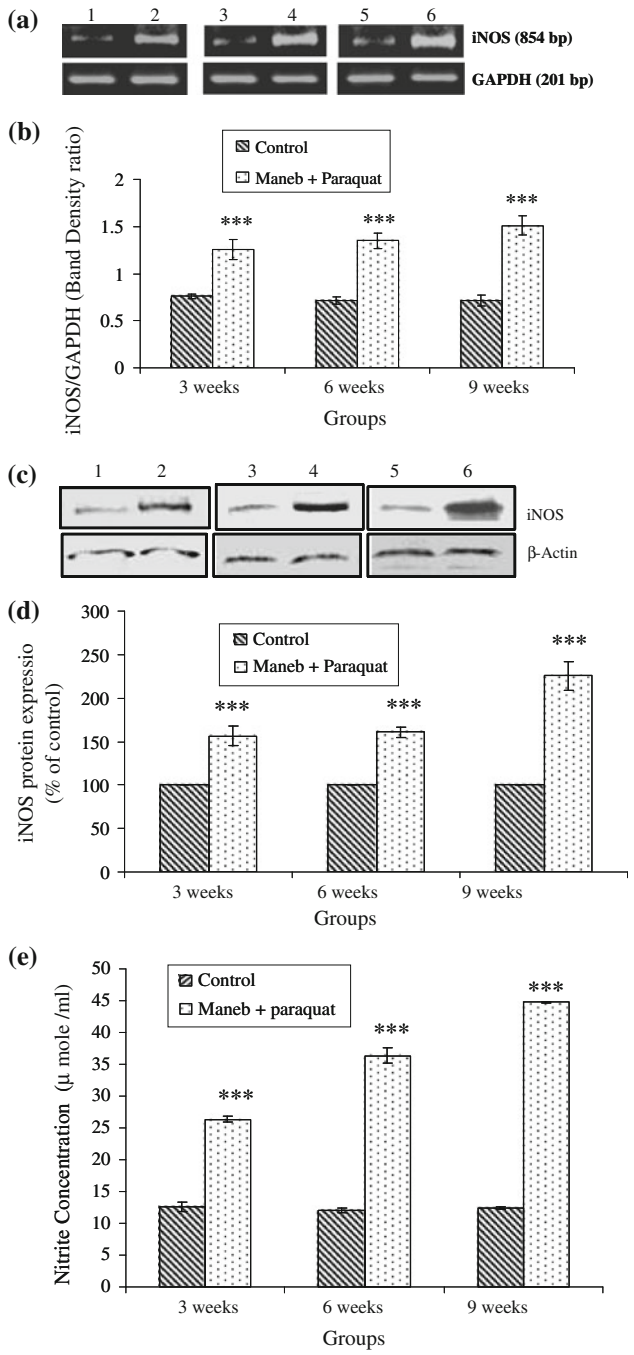

Fig. 1 RT-PCR based iNOS mRNA expression (in gel **a** and bar diagram **b**), western blot of iNOS protein (in gel **c** and bar diagram **d**) and nitrite content (**e**) in 3, 6, and 9 weeks MB- and PQ-treated mouse striatum along with respective controls. Data are expressed as means ± SEM. The iNOS protein expression is presented in terms of percentage of controls. The control values of each set of experiments were considered 100% (**d**); therefore, no standard *error bar* is shown in control. Significant changes *** ($P < 0.001$) are expressed as treated versus respective controls

Similarly, an augmentation in nitrite content was seen in MB- and PQ-induced mouse striatum as compared with respective controls (Fig. 1e).

Effect of Aminoguanidine on iNOS mRNA, Nitrite Content and Lipid Peroxidation

Aminoguanidine, a specific iNOS inhibitor, attenuated the increase of iNOS mRNA expression in MB- and PQ-treated mouse striatum (Fig. 2a, b). Aminoguanidine inhibited MB- and PQ-induced increase in nitrite content in mouse striatum (Fig. 2c). Aminoguanidine also inhibited MB- and PQ-induced increase in lipid peroxidation in mouse striatum (Fig. 3).

Effect of Inhibitors of Secondary Signaling Mediators on iNOS mRNA and Nitrite Content

Genistein, a tyrosine kinase inhibitor; SB202190, an inhibitor of p38 MAPK and PDTC, an inhibitor of NF-kB significantly reduced the increase in iNOS mRNA expression and nitrite content. None of these inhibitors abolished MB- and PQ-induced increase in iNOS mRNA and nitrite content (Fig. 2a–c).

Effect of Inhibitors of Secondary Signaling Mediators on Lipid Peroxidation

Genistein, SB202190 and PDTC significantly reduced but not completely abolished MB- and PQ-induced increase in lipid peroxidation (Fig. 3).

TH-Immunoreactivity

The animals treated with MB and PQ exhibited a significant reduction in TH-immunoreactivity as well as the number of TH-positive neurons. The animals treated with aminoguanidine exhibited partial but statistically significant restoration of TH-positive neurons. Genistein and pyrolidine dithiocarbamate treatments showed partial recovery in the number of TH-immunoreactive neurons as compared with maneb + paraquat-treated mice. Similarly, SB202190 treatment also showed partial but statistically insignificant recovery of TH-immunoreactive neurons (Fig. 4a, b).

Discussion

NO reacts with superoxide free radical and forms peroxynitrite, which is one of the most destructive molecules that mediate cell death [34–36]. Excessive secretion of inflammatory molecules during infection and cellular injury induces the expression of iNOS contributing to PD pathogenesis [19, 37, 38]. Although PQ-induced neurotoxicity is associated with redox cycling leading to formation of superoxide anion and other reactive oxygen

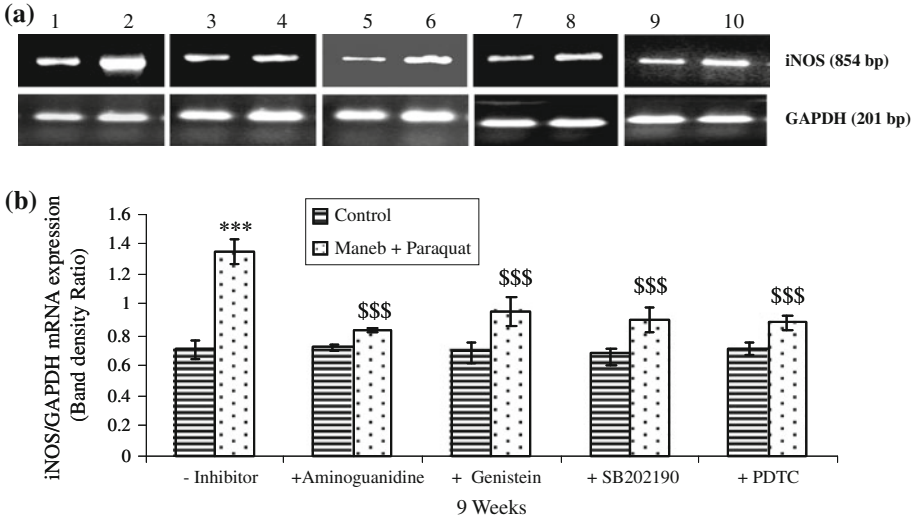

Fig. 2 Effects of aminoguanidine, genistein, SB202190 and PDTC on iNOS mRNA expression (in gel **a** and bar diagram **b**) and nitrite content (**c**) in 9 weeks MB- and PQ-treated mouse striatum and respective controls. Data are expressed as means ± SEM. Significant changes * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) are expressed as treated versus respective controls and $$ ($P < 0.01$) and $$$ ($P < 0.001$) are expressed as MB + PQ without inhibitors versus MB + PQ in presence of inhibitors

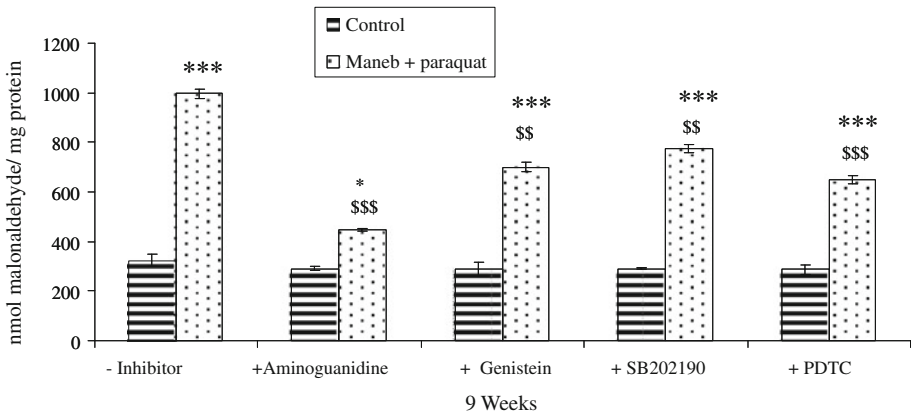

Fig. 3 Effects of aminoguanidine, genistein, SB202190 and PDTC on lipid peroxidation in control and 9 weeks MB-and PQ-treated mouse striatum. Data are expressed as means ± SEM. Significant changes * ($P < 0.05$), *** ($P < 0.001$) is expressed as treated versus control and $$ ($P < 0.01$) and $$$ ($P < 0.001$) are expressed as MB + PQ without inhibitors versus MB + PQ in presence of inhibitors

species by one-electron reduction of PQ [39, 40], the role of NO in MB- and PQ-mediated neurotoxicity in mouse striatum remains to be explored.

MB- and PQ-induced expression of iNOS mRNA, its protein and nitrite content in mouse striatum, suggests the involvement of nitric oxide in MB- and PQ-induced neurotoxicity. The elevation in iNOS mRNA expression and nitrite content was attenuated by aminoguanidine confirming the involvement of nitric oxide in MB- and PQ-induced neurotoxicity in mouse striatum The results obtained in this study are in accordance with the previous investigations showing the role of nitric oxide in neurodegeneration and iNOS inhibitors in neuroprotection [21, 41]. The involvement of iNOS in PD has also been reported using iNOS-deficient mouse [42]. RT-PCR and western blot analyses showed that the expression of iNOS

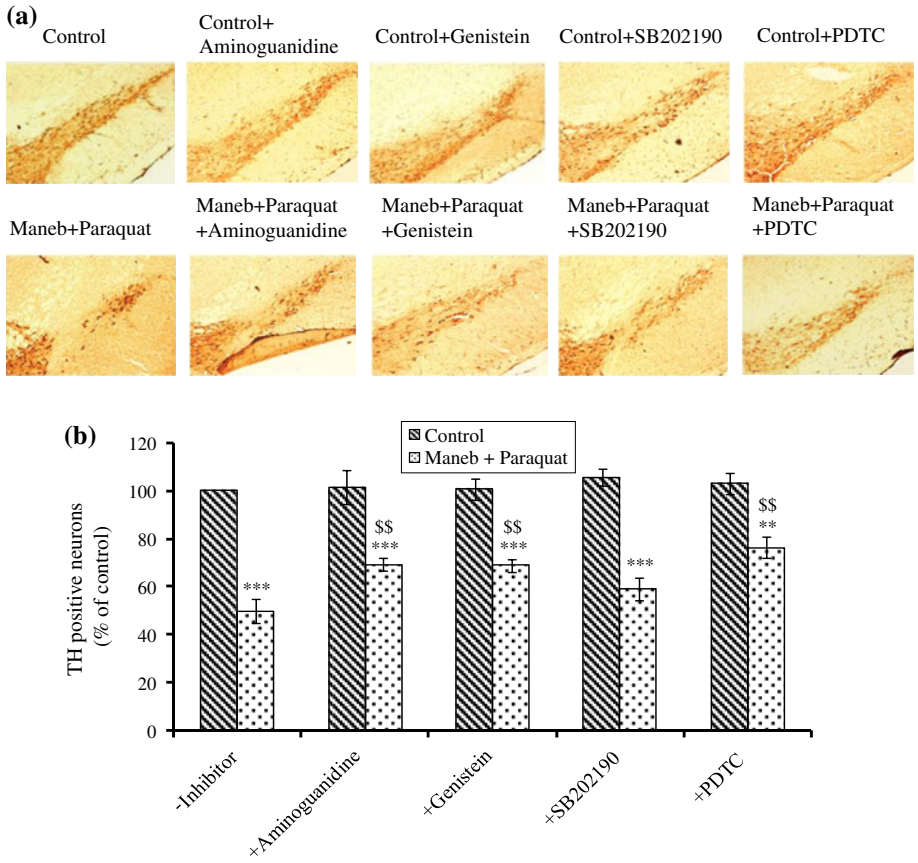

Fig. 4 TH-immunoreactivity of dopaminergic neurons in the substantia nigra pars compacta region of the brain in control and MB- and PQ-treated mouse with or without inhibitor (**a**) at 10× and number of TH positive neuronal cells (**b**). Data are expressed as means ± SEM. The control values of each set of experiments were considered 100%; therefore, no standard *error bar* is shown in control. Significant changes ** ($P < 0.01$) and *** ($P < 0.001$) are expressed as treated versus control and $$ ($P < 0.01$) are expressed as MB + PQ without inhibitor vs. MB + PQ with inhibitor

might be regulated both at the level of transcription as well as translation, however, a direct correlation between NO generation with iNOS mRNA indicated that mainly the process is regulated at the level of transcription [43].

NO ultimately converts into peroxynitrite that increases oxidative stress and neuronal cell death [44–46]. As peroxynitrite inhibits complexes I, III, IV and V of mitochondria; mitochondrial enzymes such as aconitase, creatine kinase and superoxide dismutase and mitochondrial DNA synthesis [47], it could be possible that MB- and PQ-mediated neurotoxicity occurs through induction of iNOS and consequent NO generation. We have previously demonstrated an induction in lipid peroxidation in the striatum of MB- and PQ-treated mouse [7]. In this study, we found that aminoguanidine, an inhibitor of iNOS, not only reduced iNOS mRNA expression and nitrite but also reduced MB- and PQ-induced lipid peroxidation. The inhibition of lipid peroxidation by aminoguanidine indicates that nitric oxide is involved in lipid peroxidation-mediated injury of MB- and PQ-induced PD [48]. This is in accordance with previous investigations that have shown that aminoguanidine results in reduction of oxidative stress, lipid peroxidation and possesses antioxidant property and free radical scavenging activity [49–51]. Nitric oxide and other reactive nitrogen species are known to contribute significantly in the dopaminergic neurodegeneration. This is evidenced by the abnormal nitrosylation of some key proteins, such as Parkin, in PD [52]. Although the role of nitric oxide is reported in 6-hydroxydopamine-induced PD, as 7-nitroindazole and NG-nitro-L-arginine, NOS inhibitors, improve the motor performance in treated animals, the mechanism underlying the improvement in motor behavior is not properly understood [53]. The significant restoration of TH-immunoreactivity and number of TH-positive neurons by aminoguanidine in MB- and PQ-induced PD phenotype suggests that nitric oxide plays a critical role in dopaminergic neurodegeneration. Genistein and pyrolidine dithiocarbamate, inhibitors of alternate pathways, showed only partial recovery of the TH-immunoreactive neurons in maneb + paraquat-treated mice showing the involvement of multiple molecular events including, tyrosine kinase and NF-kB in maneb- and paraquat-induced neurotoxicity.

Blocking NO and iNOS is used to understand the mechanism of iNOS-mediated biological events [54]. Genistein, a tyrosine kinase inhibitor, reduces nitric oxide-mediated injury in neuronal cells [55]. Similarly, significant reduction in iNOS expression in chemically induced dopaminergic neuronal injury is achieved by selective inhibition of p38 MAPK by SB202190 or NF-kB by PDTC [25, 56]. In this study, genistein, SB202190 and PDTC attenuated MB- and PQ-induced increase in nitrite content; iNOS mRNA and protein level and lipid peroxidation,

indicating that multiple pathways are responsible for the regulation of iNOS expression. None of these inhibitors reduced the increased lipid peroxidation completely, which indicates that some other contributory factors might be involved in the regulation of lipid peroxidation along with these pathways. Although the inhibitors of the alternate pathways reduced the nitrite content and lipid peroxidation, they did not abolish the same completely suggesting that there is a link between NO and lipid peroxidation, however, NO alone does not regulate maneb + paraquat-induced increase in lipid peroxidation. This is in accordance with previous observations that have shown the contribution of toxicant responsive genes in the augmentation of lipid peroxidation [7]. The study demonstrated that MB- and PQ-induced neurotoxicity is also regulated by NO dependent lipid peroxidation through multiple pathways.

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