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Impairment of Nrf2- and Nitroergic-Mediated Gastrointestinal Motility in an MPTP Mouse Model of Parkinson's Disease

C. Sampath¹, R. Kalpana¹, T. Anshah², C. Charlton², A. Hale³, K. M. Channon⁴, S. Srinivasan^{5,6}, P. R. Gangula¹

¹Department of ODS and Research, School of Dentistry, Meharry Medical College, 1005 Dr. D.B. Todd Jr. Blvd, Nashville, TN 37208, USA

²Department of Cancer Biology Physiology Pharmacology and Neuroscience, School of Medicine, Meharry Medical College, Nashville, TN, USA

³Department of Oncology, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford, UK

⁴Oxford Heart Centre, John Radcliffe Hospital, University of Oxford, Oxford, UK

⁵Division of Digestive Diseases, Department of Medicine, Emory University, Atlanta, GA, USA

⁶Atlanta VA Health Care System, Decatur, Atlanta, GA, USA

Abstract

Background—Gastrointestinal (GI) motility dysfunction is the most common non-motor symptom of Parkinson's disease (PD). Studies have indicated that GI motility functions are impaired before the onset of PD.

Aims—To investigate the underlying mechanism of PD-induced GI dysmotility in MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine)-induced animal model.

Methods—C57BL/6 mice were administered with or without a selective dopamine neurotoxin, MPTP, to induce parkinsonian symptoms. In addition to in vivo studies, in vitro experiments were also conducted in colon specimens using 1-methyl-4-phenylpyridinium (MPP⁺), a metabolic product of MPTP. Gastric emptying, colon motility, nitroergic relaxation, and western blot experiments were performed as reported.

Results—MPTP-induced PD mice showed decreased expression of nuclear factor erythroid 2-related factor (Nrf2) and its target phase II genes in gastric and colon neuromuscular tissues. Decreased levels of tetrahydrobiopterin (BH₄, a critical cofactor for nNOS dimerization) associated with uncoupling of nNOS in gastric and colon tissues exposed to MPTP. Impaired enteric nitroergic system led to delayed gastric emptying and slower colonic motility compared to

P. R. Gangula pgangula@mmc.edu.

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the control mice. In vitro results in colon specimens confirm that activation of Nrf2 restored MPP⁺-induced suppression of alpha-synuclein, tyrosine hydroxylase (TH), Nrf2, and heme oxygenase-1. In vitro exposure to L-NAME [N(w)-nitro-l-arginine methyl ester], a NOS synthase inhibitor, reduced protein expression of TH in colon tissue homogenates.

Conclusions—Loss of Nrf2/BH₄/nNOS expression in PD impairs antioxidant gene expression, which deregulates NO synthesis, thereby contributing to the development of GI dysmotility and constipation. Nitric oxide appears to be important to maintain dopamine synthesis in the colon.

Keywords

Gastrointestinal motility; Parkinson's disease; Nrf2; Antioxidants; Tetrahydrobiopterin; Nitric oxide synthase; Tyrosine hydroxylase; α -Synuclein

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by motor impairment that occurs due to loss of dopaminergic neurons in the *substantia nigra* pars compacta (SNpc), formation of Lewy bodies (LB), oxidative stress, and chronic low-grade inflammation, followed by non-motor symptoms. Recent studies have shown that during the initial stages of PD, the extrinsic and intrinsic innervation of the gastrointestinal (GI) tract, the dorsal motor nucleus of the vagus (DMV), and the enteric nervous system (ENS) were first affected before *substantia nigra* [1]. Thus, non-motor symptoms, the most familiar of which is GI dysfunction, occur earlier than the motor symptoms of PD [2, 3]. GI dysfunction characterized by faster or easy satiety, weight loss from delayed gastric emptying (gastroparesis), and impaired colonic transit contributes directly to the pathogenesis of PD and complicates the disease's clinical management [4, 5].

GI dysfunction is observed in almost all PD patients during progression of the illness and impairs gastric or colonic motility [6]. GI motility is controlled by the complex neural network of intrinsic enteric nervous system (ENS). ENS consists of a deep myenteric and superficial submucosal plexus acting as the "second brain," providing a link between the stomach and the brain through the vagus nerve [7, 8]. It has been reported that the aggregation of the protein, namely alpha-synuclein (α -synuclein or α -S) found in LB and Lewy neurites (LN), spreads from neuron to neuron within the gut wall, reaches the vagal projections, and enters the central nervous system, thereby leading to manifestation of motor symptoms [1, 9]. Previous studies in GI biopsies of PD patients have shown that aggregated α -S is accumulated prior to the onset of motor symptoms. Furthermore, these studies demonstrate that α -S may spread also from the GI system to the brain [10–12].

Results from our laboratory and others have shown that GI motility dysfunction occurs due to diminished neuronal nitric oxide synthase (nNOS) activity, which catalyzes the formation of nitric oxide (NO), initiating smooth muscle relaxation [13, 14]. In turn, nNOS activity is regulated by tetrahydrobiopterin (BH₄), a cofactor for nNOS dimerization and enzyme activity that couples electron flow to NO generation [15]. BH₄ is synthesized from guanosine 5'-triphosphate (GTP) de novo by the rate-limiting enzyme GTP cyclohydrolase 1 (GCH-1) or from a salvage pathway via dihydrofolate reductase (DHFR) using arginine as

a substrate [16]. Although previous studies have demonstrated that loss of dopaminergic neurons and/or impaired nitrergic relaxation could lead to GI dysfunction in MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine)-induced PD rodent models, the mechanism of nitrergic-mediated GI motility has not been fully investigated [4].

NOS uncoupling results in superoxide generation. Oxidative stress plays an important role in PD patients who exhibit increased levels of oxidized lipids, decreased levels of reduced glutathione (GSH), mutations of proteins, and DNA [17, 18]. Mechanisms to handle misfolded proteins more effectively within the cell include modulating endogenous cellular stress response pathways that upregulate the protein degradation machinery or sequester the misfolded proteins into inclusion bodies [19]. Nuclear factor erythroid 2-related factor (Nrf2) coordinates a whole program of gene expression that counters stress at multiple levels [20]. In response to electrophilic and reactive oxygen species-producing agents, Nrf2 binds to the antioxidant responsive element and regulates the expression of detoxification genes and oxidative stress-inducible enzymes [21–23]. However, a growing body of evidence indicates that Nrf2 regulates a much broader gene expression response, including genes involved in protein homeostasis, such as chaperones and proteasome subunits, and thus, it is considered as a potential target to deal with damaged proteins [24–26]. However, the protective role of Nrf2 in enteric neurons is yet to be determined.

Due to the lack of proper treatment available for GI dysmotility among PD patients, it is essential to discover new therapies that allow us to improve not only motor symptoms, but also non-motor symptoms, including cognitive impairment and dysfunction of the autonomic nervous system, and thereby modulate disease progression. PD may be initiated and/or precipitated by environmental or endogenous toxins by a mechanism similar to that of MPTP in genetically predisposed individuals [27]. The MPTP model has been widely accepted as a PD model that shows parkinsonogenic effects [28, 29]. In addition, 1-methyl-4-phenylpyridinium (MPP⁺), the active metabolite of MPTP, is shown to deplete dopaminergic neurons and induce oxidative stress [30]. In addition, a previous study from our group has shown that administration of MPTP to adult C57BL/J6 either short term or over several days leads to destruction of substantia nigra, dopaminergic neurons, and depletion of striatal dopamine [31]. The above studies suggest that exposure of the brain to toxic environmental agents in adult life may be an important causative factor for the development of PD. Hence, the current study aimed to evaluate the underlying mechanisms involved in MPTP-induced GI dysmotility in vivo. In addition, we also have investigated whether in vitro activation of Nrf2 restored MPP⁺-induced suppression of PD and antioxidant markers in the colon.

Materials and Methods

Experimental Design

Animals—C57BL/6J male mice (at least 10 weeks old) were obtained from Jackson Laboratories (The Jackson Laboratory, Bar Harbor, ME). The animals were maintained in the Meharry Medical College (MMC, Nashville, TN) Animal Care Facility under controlled temperature, humidity, and light–dark cycle (12:12 h), with free access to a purified diet (Purina LabDiet, St. Louis, MO) and water. The Institutional Animal Care and Use Committee at MMC approved all experiments in this study, in accordance with

recommendations of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Mice were divided into two groups: The control group ($n = 6$) received PBS (vehicle) only and the PD-induced group ($n = 6$) received MPTP. To develop the MPTP-PD model, the adult mice were injected with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, Sigma Chemical, St. Louis, MO), 30 mg/kg body weight; intraperitoneally/day/7 days [31]. Animals were euthanized 7 days after last vehicle or MPTP treatment. Our previous publications using this model and time regimen for MPTP treatment have been shown to develop parkinsonism [31].

Solid Gastric Emptying (GE)

Solid GE studies were performed according to Gangula et al. [32]. Briefly, after fasting overnight (providing water), known amounts of food were fed to the animals for 3 h. At the end of 3 h, the remaining food was weighed to establish the amount of food intake. Animals were fasted for 2 h without food and water and then killed. Gastric tissue was collected, and the weight of the whole stomach and the empty stomach weight were noted after removing the food contents. The rate of GE was calculated according to the following equation: GE (% in 2 h) = $(1 - \text{gastric content/food intake}) \times 100$.

Colonic Transit Time

Colon motility experiments were performed using the bead expulsion method according to Anitha et al. [33]. A 3-mm glass bead was placed 2 cm proximal to the anal opening using a plastic Pasteur pipette lightly lubricated with lubricating jelly. Distal colonic transit time was assessed by measuring the amount of time between bead placement and expulsion of the bead. The bead expulsion test was performed in the last week of the study.

Gastric and Colon Organ Bath Studies

Electric field stimulation (EFS)-induced NANC relaxation was studied in circular gastric and colon neuromuscular strips [34]. Gastric muscle strips were obtained from the antrum region of the stomach. The colon was dissected into three parts—proximal, middle, and distal—and was used for organ bath studies. Strips were exposed to atropine, phentolamine, and propranolol (10 μmol , Sigma-Aldrich, St. Louis, MO) in bath solution for 1 h to block cholinergic and adrenergic responses. 5-Hydroxytryptamine (100 μmol , Sigma-Aldrich, St. Louis, MO) pre-contracted strips were exposed to EFS (90 V, 2 Hz, 1-ms pulse for the duration of 1 min) to elicit NANC relaxation. A relaxation response elicited by low-frequency (2 Hz) stimulus under NANC conditions, as used in this study, was demonstrated as predominantly nitrenergic (nNOS) in origin, and this was further confirmed using the NOS inhibitor L-NAME (100 μmol , Sigma-Aldrich, St. Louis, MO) [35]. At the end of the experiment, each muscle strip was blotted dry with filter paper and weighed. Comparisons between groups were made by measuring the area under the curve (AUC/mg of tissue) of the EFS-induced relaxation (AUC_R) for 1 min and the baseline for 1 min (AUC_B), according to the formula “ $(\text{AUC}_R - \text{AUC}_B)/\text{weight of tissue (mg)} = \text{AUC/mg of tissue}$.”

nNOS α Dimerization in Gastric and Colon Neuromuscular Tissue

Gastric and colon neuromuscular tissue specimens were homogenized using radio-immunoprecipitation assay buffer as reported earlier [36]. Levels of the nNOS α monomer and dimer were quantified by western blot (WB) via LT-PAGE in gastric antrum homogenates as described previously [16]. LT-SDS (sodium dodecyl sulfate)-PAGE was performed on ice. The LT process was used to identify nNOS dimers and monomers in the native state, as LT is known to prevent monomerization of nNOS dimers. For the LT process, 30 μ g of protein in the standard Laemmli buffer at 4 °C was used for SDS-PAGE. The mixture was incubated at 0 °C for 30 min before LT loading onto a 6% gel. All gels and buffers were pre-equilibrated to 4 °C prior to electrophoresis, and the buffer tank was placed in an ice bath during electrophoresis to maintain the gel temperature below 15 °C. A polyclonal antibody specific to nNOS α (ab229785, 1: 500, Abcam, Cambridge, MA) and anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (31,430, 1:10,000, Thermo Fisher Scientific, Waltham, MA) were used as the primary and secondary antibodies, respectively.

WB Analysis

Tissue lysates containing 30 μ g proteins were separated by SDS-PAGE, and the proteins were transferred to a nitrocellulose membrane. The membrane was immunoblotted with polyclonal Nrf2 (sc13032, 1:1000), HO-1 (sc1369601:1000), glutamate-cysteine ligase modifier subunit (GCSm, sc22754, 1:1000), GCH-1 (sc376483, 1:1000), and DHFR (sc33184, 1:1000) as primary antibodies, which were purchased from Santa Cruz (Santa Cruz Biotechnology, Dallas, TX), and anti-rabbit IgG conjugated with horseradish peroxidase (31,430, 1:10,000, Thermo Fisher Scientific, Waltham, MA) as a secondary antibody. Primary nNOS α (1:500, ab229785) antibody, α -synuclein (α -S, ab1903, 1:1000), and tyrosine hydroxylase (TH, ab112, 1:200) were purchased from Abcam (Abcam, Cambridge, MA). P-Glycogen synthase kinase-3beta (p-GSK-3 β , 9331s, 1:1000) was from Cell Signaling (Cell Signaling Technology, Danvers, MA). Binding of antibodies to the blots was detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ) following the manufacturer's instructions. Stripped blots were re-probed with β -actin-specific polyclonal antibodies (sc8432, 1:1000, Santa Cruz Biotechnology, Dallas, TX) to enable normalization of signals between samples. Band intensities were analyzed using Bio-Rad Gel Doc (Bio-Rad, Hercules, CA).

Measurement of Gastric Biopterin Levels

Biopterin levels were determined from gastric tissues obtained from controls and PD-induced mice by high-performance liquid chromatography (HPLC) using electrochemical and fluorescent detection methods as described [37]. Following homogenization and centrifugation of the gastric tissues (15 min at 13,000 \times g at 4 °C), the samples were transferred to new, cooled microtubes and precipitated with an equal volume of a solution of cold phosphoric acid (1 M, Sigma Chemical, St. Louis, MO), trichloroacetic acid (2 M, Sigma Chemical, St. Louis, MO), and dithioerythritol (1 mM, Sigma Chemical, St. Louis, MO). The samples were vigorously mixed and centrifuged again for 15 min at 13,000 \times g at 4 °C. The supernatants were injected onto an isocratic HPLC system and quantified using

sequential electrochemical (Coulochem III, ESA Inc., Mundelein, IL) and fluorescence (Jasco, Easton, MD) detection. HPLC separation was performed using a 250-mm ACE C-18 column (Hichrom, Berkshire, UK) and mobile phase comprised of sodium acetate (50 mM), citric acid (5 mM), EDTA (48 μ M), and dithioerythritol (160 μ M) (pH 5.2) (all ultrapure electrochemical HPLC grade, Sigma Chemical, St. Louis, MO) at a flow rate of 1.3 mL/min. Background currents of + 500 μ A and – 50 μ A were used for the detection of BH₄ on electrochemical cells E1 and E2, respectively. Biopterin and BH₂ were measured using a Jasco FP2020 fluorescence detector. Quantification of BH₄, BH₂, and biopterin was done by comparison with authentic external standards and normalized to sample protein content [37].

In Vitro Nitrite Measurement in Gastric and Colon Specimens

In vitro nitrite experiments were performed as described previously by Gangula et al. [16]. Briefly, animals from both groups were killed by CO₂ asphyxiation, and the whole dissected stomach was transferred to chilled oxygenated Krebs bicarbonate solution of the following composition (in mmol): 118.0 NaCl; 4.7 KCl; 25.0 NaHCO₃; 1.5 CaCl₂; 1.2 MgSO₄; 1.2 KH₂PO₄; and 11.5 glucose (pH 7.4). All these chemicals were purchased from Sigma Chemical (St. Louis, MO). Gastric and colon neuromuscular tissues were harvested and cut into mucosa-free strips and were cultured for 24 h (37 °C, 5% CO₂ in 500 μ l of phenol red-free DMEM (Thermo Fisher Scientific, Waltham, MA) supplemented with NB27 (2%, Thermo Fisher Scientific, Waltham, MA) and antibiotics (1%, Thermo Fisher Scientific, Waltham, MA). After incubation, DMEM (500 μ l) was collected and stored at – 80 °C for analysis of nitrite released in medium during incubation period. Nitrite released in the medium was analyzed as total nitrite (metabolic byproduct of NO) according to the manufacturer protocol supplied with a commercially available kit (EMD Chemicals, Gibbstown, NJ).

In Vitro Organ Culture

Colon neuromuscular strips were organ cultured with MPP⁺ with or without Nrf2 activators such as CNM (100 μ M, Sigma Chemical, St. Louis, MO), CUR (100 μ M, Sigma Chemical, St. Louis, MO), and GSK3 β inhibitor (SB216763, 30 μ M, Tocris, Minneapolis, MN) at 37 °C and 5% CO₂ in a humidified chamber [38]. In a separate set of experiment, colon strips were cultured with NOS inhibitor L-NAME (100 μ M, Sigma-Aldrich, St. Louis, MO). Full-thickness colon tissues were maintained in DMEM/F12 (Gibco, Waltham, MA) medium supplemented with antibiotics (penicillin/streptomycin, Gibco, Waltham, MA) for 48 h, respectively. WB methods were used to determine changes in targeted proteins.

Statistical Analysis

Data were presented as the mean \pm standard error (SE). Statistical comparisons between groups were made by the Student's *t* test or Tukey's test after one-way analysis of variance using the GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). A *p*-value of less than 0.05 was considered statistically significant.

Results

Our previous study has shown that treatment with MPTP for 7 days produces parkinsonism in a mouse model [31]. The current study focused on whether MPTP-induced PD alters GI motility, and if so, investigated the possible mechanisms involved.

PD Induction Results in Delayed Gastric Emptying (GE) and Reduced Intestinal Transit Time

The data depicted in Fig. 1a show that solid GE was significantly ($p < 0.05$) reduced in PD-induced mice compared with the control group. Colonic motility, as measured by the bead latency test, was also reduced ($p < 0.05$) in PD-induced mice, where time to expel the bead was longer than the time in the control group (Fig. 1b).

Impairment of Gastric and Colon Nitroergic Relaxation in PD-Induced Mice

To demonstrate whether delayed GE and reduced colon motility are due to altered nitroergic relaxation, we performed a series of in vitro organ bath experiments to assess electric field stimulation-induced non-adrenergic non-cholinergic (NANC) relaxation at 2 Hz in gastric and colon tissues (proximal, middle, and distal) obtained from control, and PD-induced mice. As shown in Fig. 2, the gastric tissue (a) and colon tissue (b) nitroergic relaxation was reduced significantly ($p < 0.05$) in the PD-induced mice.

PD Induction Decreases Nrf2 and Phase II Enzyme Protein Levels

The protein levels of Nrf2 in gastric (Fig. 3a) and all regions of colon (Figs. 3b–d) neuromuscular tissue were significantly ($p < 0.05$) decreased following PD induction. As shown in Fig. 4, loss of Nrf2 protein expression resulted in the suppression of oxidant detoxifying systems (GcSm) in gastric (Fig. 4a) and all regions of intestinal (Fig. 4b–d) neuromuscular strips.

PD-Induced Impairment of Gastric Neuronal Nitric Oxide Synthase alpha (nNOS α) Protein Expression and nNOS α Dimerization

The functional isoform of nNOS (nNOS α) protein expression in gastric (Fig. 5a) and all regions of colon (Fig. 5b–d) neuromuscular tissues was significantly decreased ($p < 0.05$) following PD induction. Next, dimerization (an indirect measurement of nNOS enzyme activity) studies were performed by low-temperature (LT)-polyacrylamide gel electrophoresis (PAGE) gel to establish whether the decreased nNOS α was the result of altered nNOS α dimer levels in PD-induced mice. As depicted in Fig. 6, a significant decrease in the dimer/monomer ratio of gastric (Fig. 6a) and colon (Fig. 6b–d) nNOS α was noticed in PD-induced mice compared with that of the control group.

PD Induction Decreased Gastric and Colon Dihydrofolate Reductase (DHFR) Protein Expression and Biopterin Levels

Several cofactors are known to be important for nNOS activity, including tetrahydrobiopterin (BH₄). The biosynthesis of bioactive BH₄ is dependent on the tissue availability of GCH-1 (de novo) and DHFR (salvage). Our data presented in Figs. 7 and 8

show that induction of PD reduced the protein expression of GCH-1 and DHFR in both gastric neuromuscular tissues. However, DHFR does not show any changes in colon neuromuscular tissues. Next, we quantified the levels of BH₄ and its oxidized metabolites in control and MPTP-induced PD mice gastric tissues. Our data demonstrated that MPTP treatment significantly reduced gastric BH₄ (reduced and active form that promotes nNOS enzyme activity) availability, while oxidized biopterins BH₂ and B (7,8-dihydrobiopterin and biopterin, respectively) were elevated (Fig. 9).

As PD induction reduced protein expression and dimerization of nNOS α , we examined whether nitrite (end product of NO) levels are altered in PD-induced GI specimens. As shown in Fig. 10, nitrite levels were significantly ($p < 0.05$) reduced in PD-induced gastric (A) and colon (B) specimens compared with control groups.

In Vitro Effects of Nrf2 Activators and GSK3 β Inhibitor in the Colon Exposed to MPP⁺, a Metabolic Product of MPTP

Our data presented in Fig. 11a show that in vitro treatment with MPP⁺ significantly ($p < 0.05$) elevated protein expression of alpha-synuclein (α -synuclein or α -S) in colon tissues. Pre-treatment with Nrf2 activators, cinnamaldehyde (CNM, 100 μ M), curcumin (CUR, 100 μ M), or glycogen synthase kinase 3 beta (GSK3 β) inhibitor (30 μ M, SB216763) attenuated MPP⁺-induced elevation of α -S (Fig. 11a). In addition, activation of Nrf2 or inhibition of GSK3 β restored MPP⁺-induced reduction of tyrosine hydroxylase (TH, Fig. 11b), Nrf2, and heme oxygenase-1 (HO-1) protein expressions in colon tissues (Fig. 11c–d). In a separate set of experiment colon tissues exposed to NOS inhibitor L-NAME (100 μ M) tyrosine hydroxylase protein expression was reduced (Fig. 11e).

Discussion

Approximately 30% of PD patients have reported GI dysfunction at all stages of PD. Clinical studies and animal models of PD have shown disturbances in the ENS involving nitrergic nerves [39–41]. Earlier studies have demonstrated that delayed GE, impaired colonic contractility, and stool frequency observed in a rodent model of PD are due to an altered ENS [39]. The results from this study support the previous findings that PD induction led to delayed GE and impaired colon motility. In addition, our study further shows an impairment of nitrergic relaxation due to reduced dimerization of nNOS α and BH₄ availability in PD mice. Our data in gastric and colon neuromuscular tissue indicate that Nrf2 and antioxidant protein expression was reduced in PD-induced mice. In addition, in vitro activation of Nrf2 or inhibition of GSK-3 β restored MPP⁺-induced alteration of α -S, TH, Nrf2, and HO-1 protein expression in colon specimens. Collectively, the above findings suggest that an impairment of the Nrf2/BH₄/nNOS mechanism could lead to alterations in α -S, TH, delayed GE, and colon dysmotility normally seen in PD patients.

Previous studies have shown that PD induction with MPTP treatment leads to increased oxidative stress in the ileum [9]. Other study reported that loss of Nrf2 increases MPTP sensitivity and affects behavioral, neurochemical, and transcriptional changes in Nrf2 knockout mice, revealing the importance of Nrf2 [42]. A growing body of evidence suggests that Nrf2 has emerged as a pharmacological target for the treatment of PD [43]. In addition,

Nrf2 activation showed neuroprotective effects in MPTP mice, and these were associated with a reduction in oxidative damage and neuroinflammation in astrocytes [44, 45]. Nrf2 protects against oxidative stress by activating the antioxidant enzymes, such as GSH synthesis enzymes (GCSs, GCSm), critical for the protection of cells. Loss of Nrf2 exacerbates neuronal degeneration, whereas Nrf2 expression protects against neurodegeneration in genetic and toxin models of PD [21, 39–41]. Likewise, activation of Nrf2 may modify: (1) proteasome and autophagy, (2) oxidative stress (by increasing the expression of phase II enzymes), and (3) neuroinflammation (enhancing antiinflammatory markers) in neurodegenerative brain tissue homogenates [21, 45–49]. Postmortem brain samples from PD patients showed that Nrf2-mediated protein expression of NQO1 and p62 was partly reduced in LB [50]. Previous *in vitro* and *in vivo* studies have reported that Nrf2 mitigates neuronal toxicity of rotenone and MPP⁺ [51]. Skibinski et al. demonstrated that activation of Nrf2 blocks degeneration caused by α -S in neurons in the brain [26]. Earlier studies from our laboratory show that loss of Nrf2 led to reduced nitric (nNOS)-mediated gastric motility and delayed GE in Nrf2 knockout mice, suggesting that Nrf2 plays an important role in GI motility function [14]. Recently, we also have reported that *in vivo* activation of Nrf2 attenuates delayed gastric emptying and improves nitric relaxation in high-fat-induced diabetic mice [52]. Our current study demonstrates that PD induction reduced Nrf2 and antioxidant protein expression in gastric and colon tissues. Overall, the above studies suggest that activation of Nrf2 pathway helps GI functions and also protects brain against oxidative damage during the onset of PD.

Glycogen synthase kinase-3 β (GSK-3 β) dysregulation played a significant role in the pathogenesis of PD [53]. Further, inhibition of GSK-3 β restored PD symptoms [53]; one of the pathways that regulate Nrf2 is GSK3 [54]. Previous studies have shown that both CNM and CUR activate Nrf2 via AKT/JNK and Keap-1 pathways, respectively [55]. Our *in vitro* results show the activation of Nrf2 or the inhibition of GSK-3 β restored MPP⁺-induced suppression of Nrf2 and HO-1. In addition, our data demonstrate that activation of Nrf2 by CNM, CUR, and GSK-3 β inhibitor blocks α -S aggregation in colon tissues.

In the ENS, nNOS (nitric neuron)-derived NO is an important neurotransmitter regulator of motility, vascular tone, blood supply, mucosal secretion, and barrier function [42, 56]. nNOS is shown to be expressed in the ventral tegmental area and SNpc of the brain [57]. The activity of the constitutive NOS isoforms is regulated primarily by calcium fluxes, mediated by calmodulin, and by the availability of BH₄. The current study demonstrates that reduced BH₄ synthesis is associated with delayed GE, altered colon transit, reduced nitric relaxation (nNOS function), and nNOS α dimerization in PD-induced mice. Limited reports are available on possible alterations of gastric nitric pathways in the onset of PD [58, 59]. Among multiple pathogenic mechanisms observed in PD, two of the major neuropathological hallmarks are: (1) the loss of dopaminergic neurons from the SNpc and the presence of α -S-containing LB in the surviving neurons, (2) signals from nNOS-NO may influence glutamatergic and dopaminergic neurotransmission, which plays a key role in PD [60, 61]. Our studies further demonstrate that treatment with L-NAME reduced the protein expression of TH in colon. Collectively, the above data suggest that loss of Nrf2–nNOS–NO signaling in the GI system may diminish dopaminergic neurons and thus lead to progression of PD.

Previous studies have demonstrated that low BH₄ levels impair the production of NO and lead to increased superoxide radical production due to nNOS uncoupling [62]. BH₄ deficiency is associated with both Alzheimer's disease and PD, implying a contribution to these neurodegenerative diseases [63, 64]. Mainly, two pathways synthesize BH₄: de novo and salvage. GCH-1 is the rate-limiting enzyme in the de novo synthetic pathway of BH₄. Pyruvoyl-tetrahydropterin synthase and sepiapterin reductase are two additional enzymes required for the final production of BH₄ [65]. In the salvage pathway, BH₄ that is oxidized to BH₂ or B may be recycled back to BH₄ by the activity of DHFR [65]. This conversion is particularly crucial, as elevated levels of BH₂ may compete with BH₄ to bind to nNOS and thus lead to uncoupling of enzyme and superoxide production. In this study, we have observed reduced levels of BH₄ and nNOS activity, which in turn affects homeostasis of NO production in the PD-induced mouse model. Levels of BH₄ in the brain have been shown to be decreased in PD patients and in the MPP⁺ model of PD, although the subsequent mechanisms have not been investigated fully [66, 67]. In a clinical study, it has been shown that BH₄ treatment for 6 months improved memory and brain activation with neural changes in working memory performance at the 6-month time point [68]. Administration of MPTP in mice did not change BH₄ levels in the brain but has significantly decreased tyrosine hydroxylase and dopamine concentrations [69]. Our data show that both GCH-1 and BH₄ levels are reduced in the GI specimens. In contrast, DHFR levels are reduced only in the stomach but not in the colon. In addition, previous studies have shown that GCH-1 is regulated by Nrf2 [70]. NO generated from nNOS is known to induce GI smooth muscle relaxation via its downstream signaling pathway, cyclic GMP (cGMP)–protein kinase G (PKG)–phosphodiesterase type 5 (PDE5) [71]. Based on numerous experimental studies, West et al. have suggested that NO-cGMP be recommended as one of the potential therapeutic agents to protect against PD [72]. Collectively, the above studies suggest that an impairment of the Nrf2–BH₄–nNOS–NO–cGMP pathway could lead to aggregation of α-S in ENS, GI dysfunction, and subsequent development of PD [73].

In vitro studies using neuroblastoma cells have shown the protective role of sepiapterin (elevates intracellular BH₄ via salvage pathway) against MPP⁺-induced increases in proteasome degradation, oxidative stress, and dopaminergic neuronal degradation [74]. Previous studies from our laboratory demonstrated that in vivo supplementation of BH₄/sepiapterin attenuated delayed GE and nNOS function in the onset of diabetes [34, 35]. Nrf2 activators, such as sulforaphane, CDDO-methyl ester (also known as bardoxolone methyl), and DMF, have proven efficacy in normalizing oxidative stress [75]. Our current findings show that in vitro activation of Nrf2 or inhibition of GSK-3β was effective in normalizing dopamine synthesis by enhancing the expression of TH protein in colon tissues after MPP⁺ treatment. Additional studies are warranted to investigate the mechanistic role of these pathways in both GI motility and parkinsonism.

In summary, MPTP is a selective dopaminergic neurotoxin which produces a physiological impairment of ENS inhibitory function causing behavioral symptoms of slow GI motility. Our results in MPTP-induced PD mice show significant alterations in the gastric and colon Nrf2/phase II pathway, reduced gastric and intestinal BH₄ biosynthesis enzymes, and nNOS expression and dimerization, leading to an impaired nNOS function (Fig. 12). The changes observed among Nrf2–BH₄–nNOS signaling may result in gastroparesis and an altered

intestinal transit time, leading to constipation. Finally, our studies suggest that activation of Nrf2 or inhibition of GSK-3 β attenuated MPP⁺-induced alterations in the colon α -S, TH, Nrf2, and HO-1 protein expression in vitro. We suggest that understanding molecular interactions between Nrf2, nNOS, NO-cGMP, dopamine synthesis, and aggregation of α -S in both the GI system and brain is critical for the advancement of proper interventions against the development of PD.

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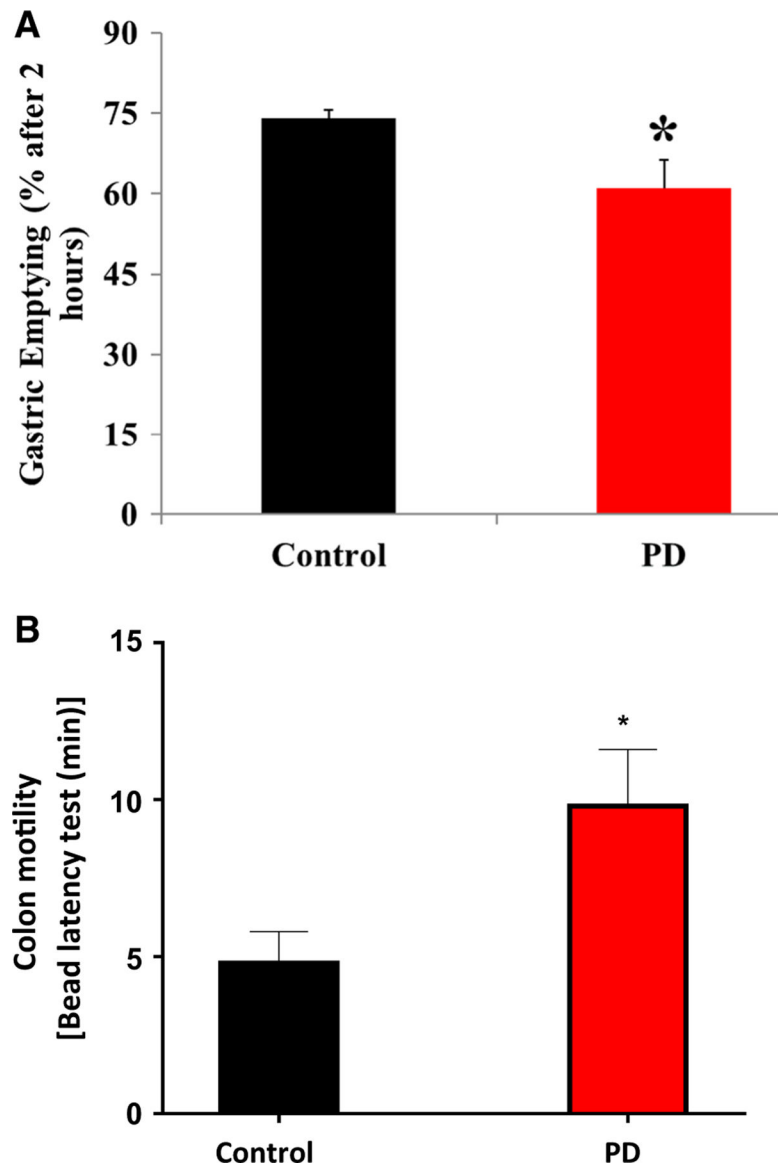


Fig. 1. Effect of MPTP-induced PD on solid GE and colon motility. **a** PD-induced mice showed delayed solid GE compared with control group and **b** bead expulsion time was longer in the PD-induced mice showing colon motility dysfunction. The values are mean \pm SE ($n = 4$) animals. Statistical significance was determined by Student's *t* test. * $p < 0.05$ compared with control group animals. *PD* Parkinson's disease

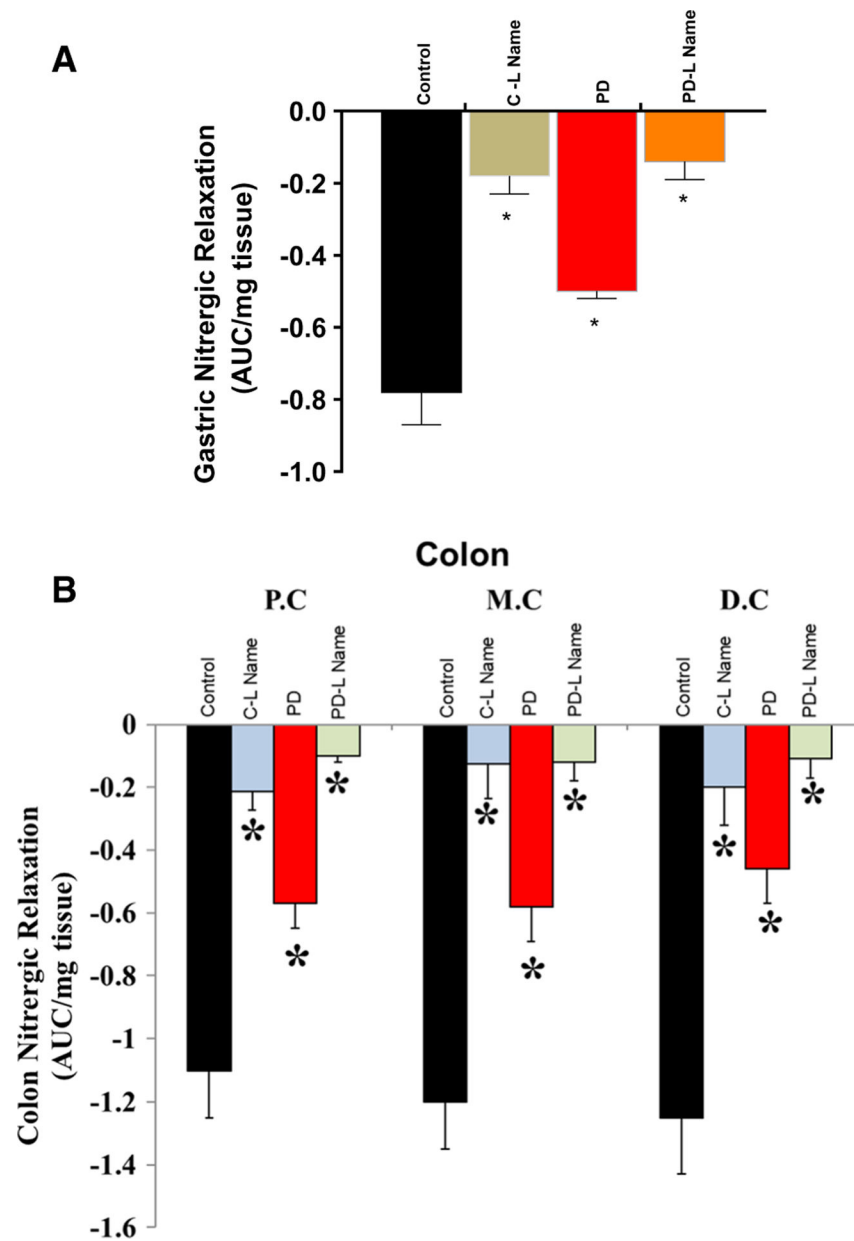


Fig. 2. Effect of MPTP on nitregic relaxation at 2 Hz in gastric and colon tissues. Nitregic relaxation was reduced in PD-induced mice when compared with control group animals. **a** Nitregic relaxation in gastric antrum tissues, **b** intestinal nitregic relaxation. Values are mean \pm SE ($n = 4$). Statistical significance was determined by Student's t test. $*p < 0.05$ compared with wild-type animals. *C* Control, *Cntrl* control, *PD* Parkinson's disease, *P.C* proximal colon, *M.C* mid colon, *D.C* distal colon

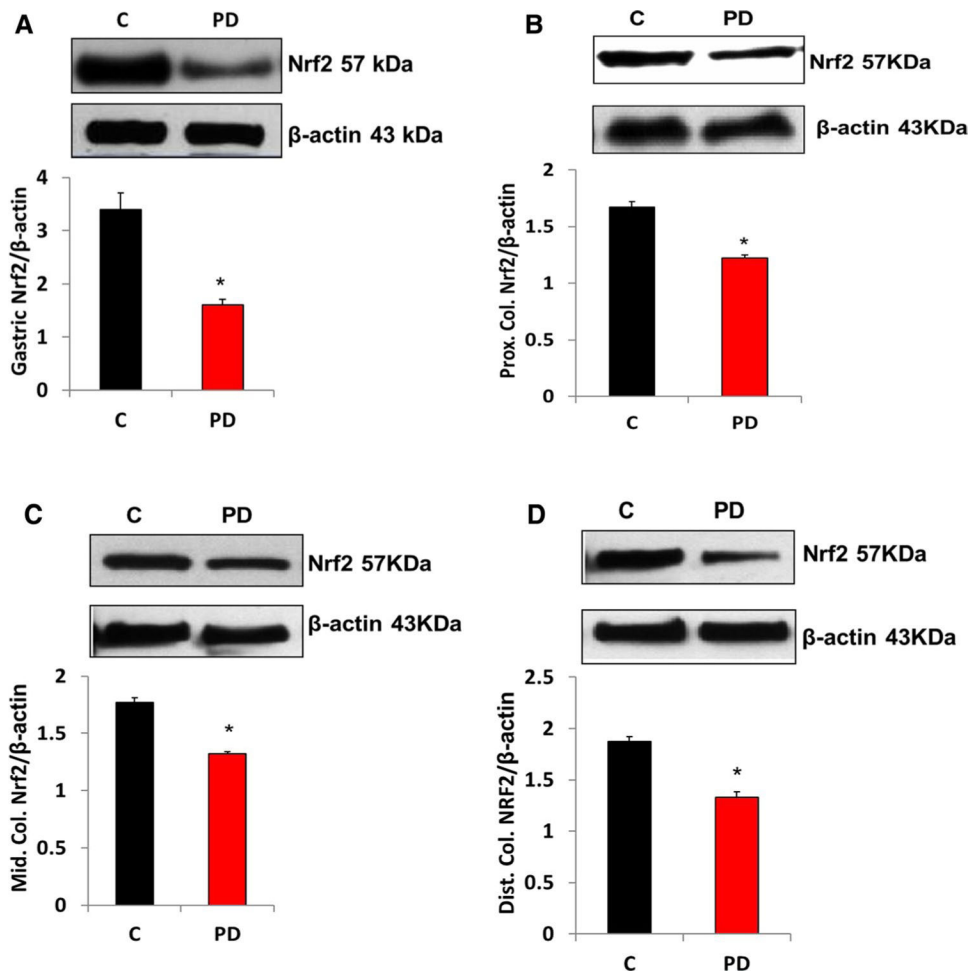


Fig. 3. Effect of MPTP-induced PD on Nrf2 protein expression in gastric and colon tissues. Representative immunoblot and densitometric analysis data for Nrf2 protein. **a** A significant decrease in Nrf2 was noticed in PD-induced mice compared with the control group. **b–d** A significant reduction in Nrf2 protein expression was noticed in all parts of colon in MPTP-treated mice. * $p < 0.05$ compared with control group animals ($n = 4$). *C* Control, *PD* Parkinson's disease

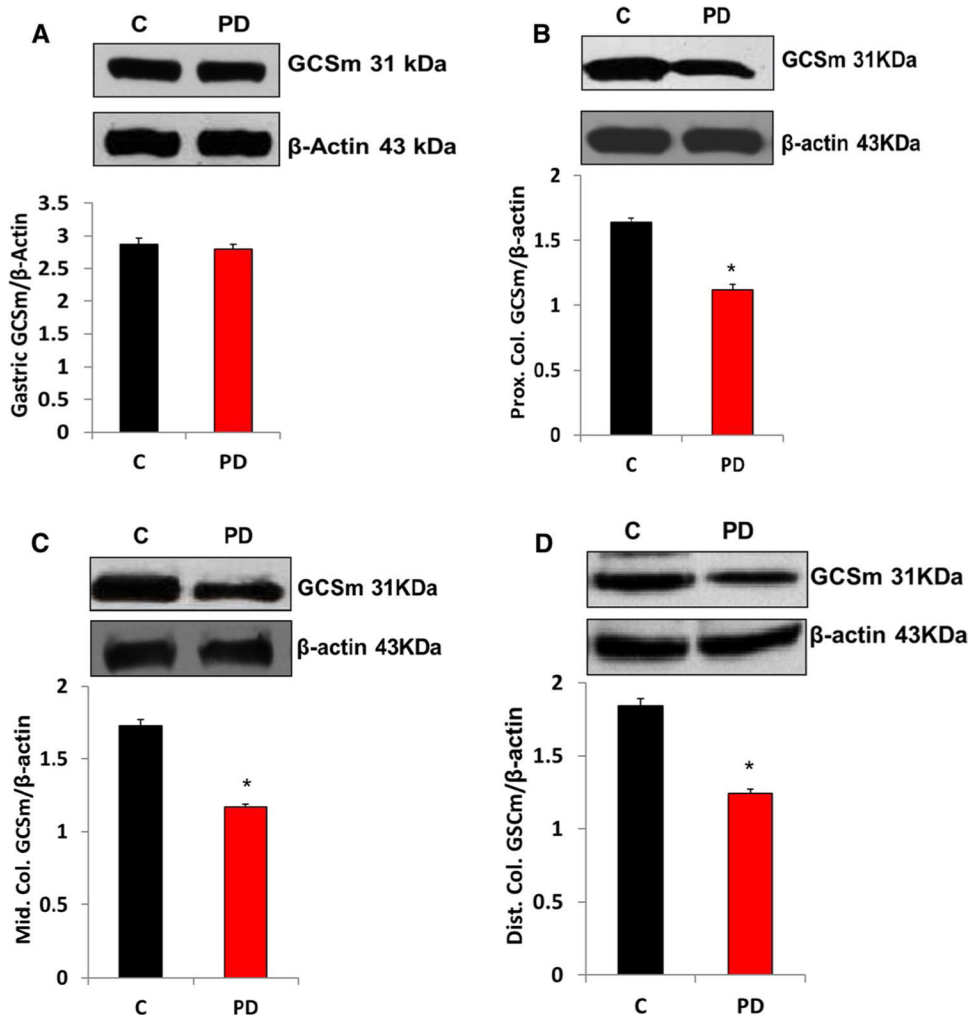


Fig. 4. Changes in protein expression of GCSm in MPTP-induced PD mice gastric and colon specimens. A significant decrease in GCSm was noticed in PD-induced mice compared with the control group. **a** Representative immunoblot and densitometric analysis in gastric antrum tissue, **b** representative immunoblot and densitometric analysis in proximal colon, **c** representative immunoblot and densitometric analysis in middle colon, and **d** representative immunoblot and densitometric analysis in distal colon. * $p < 0.05$ compared with control group animals ($n = 4$). *C* Control, *PD* Parkinson's disease

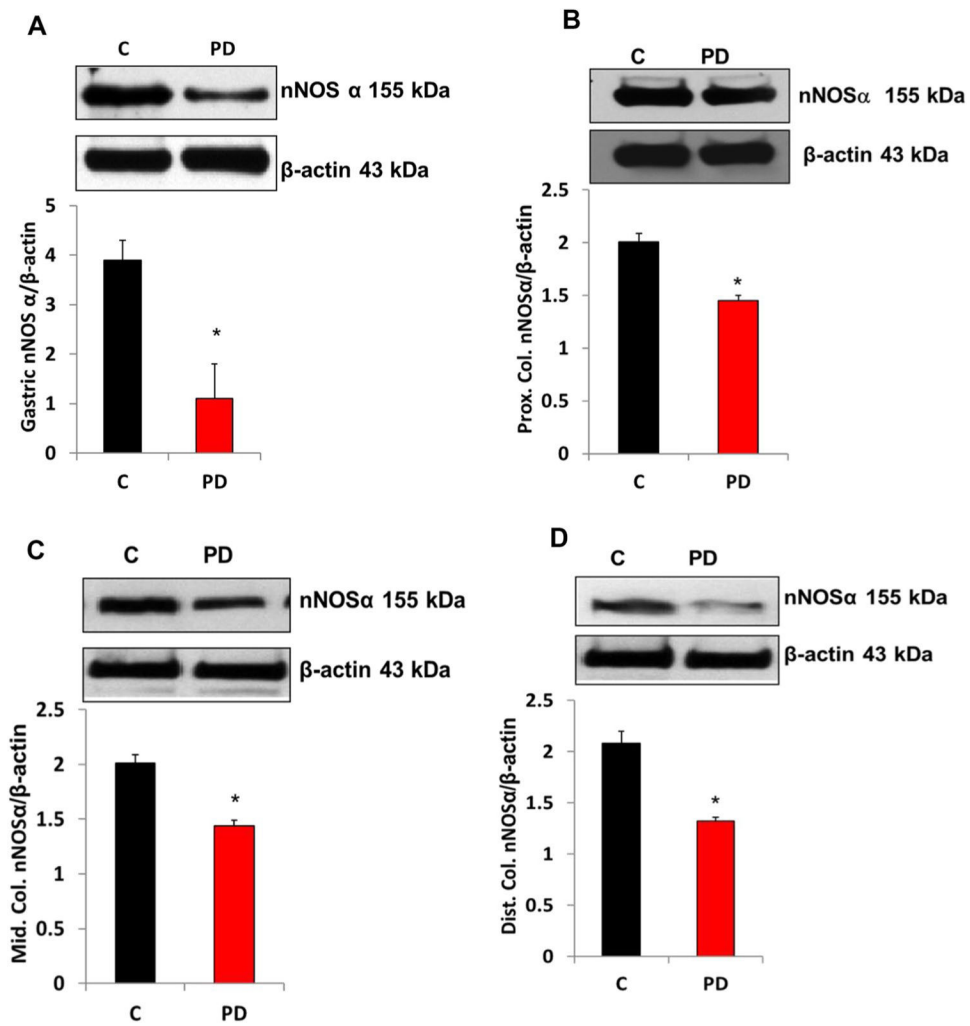


Fig. 5. Changes in protein expression of nNOS α in gastric and colon tissues of control and in MPTP-induced PD mice. nNOS α expression was reduced significantly in PD-induced mice. **a** Representative immunoblot and densitometric analysis in gastric antrum tissue, **b** representative immunoblot and densitometric analysis in proximal colon, **c** representative immunoblot and densitometric analysis in middle colon, and **d** representative immunoblot and densitometric analysis in distal colon. The values are mean \pm SE of four samples in each group. Statistical significance was determined by Student's *t* test. * $p < 0.05$ compared with wild-type animals. *C* Control, *PD* Parkinson's disease

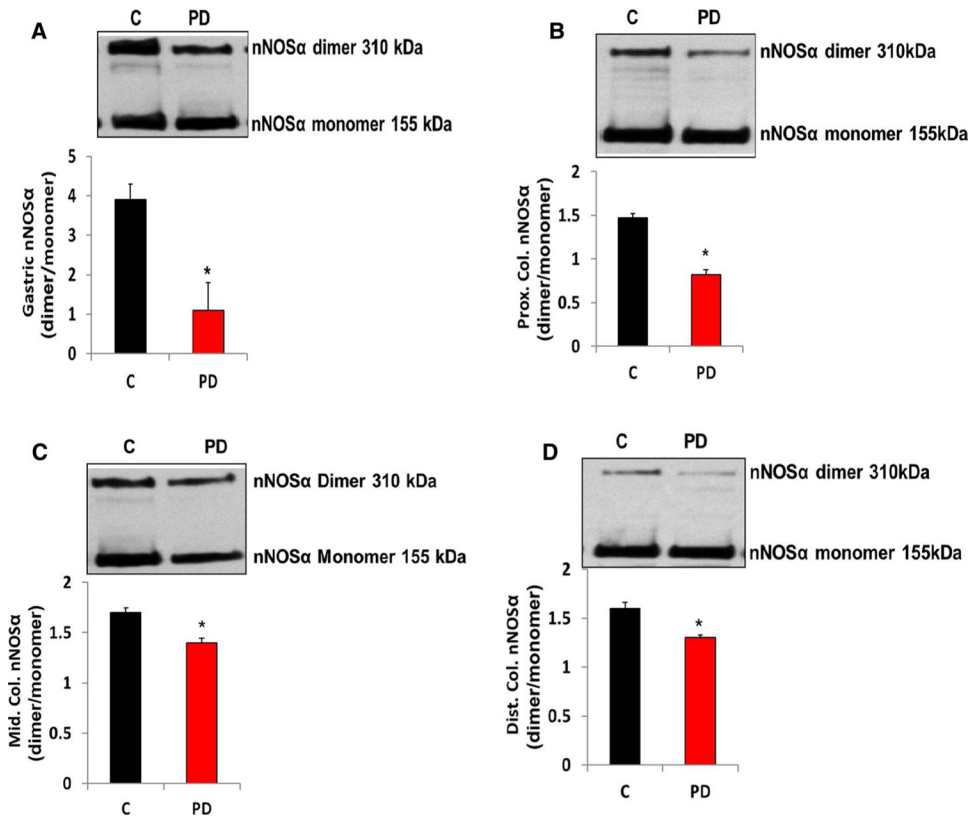


Fig. 6.

Changes in protein expression of nNOS α dimerization in gastric and colon tissues of control and in MPTP-induced PD mice. **a** nNOS α dimerization in gastric antrum tissue, **b** representative immunoblot and densitometric analysis in proximal colon, **c** nNOS α dimerization in middle colon, and **d** nNOS α dimerization in distal colon. The values are mean \pm SE of four samples in each group. Statistical significance was determined by Student's *t* test. **p* < 0.05 compared with wild-type animals. *C* Control, *PD* Parkinson's disease

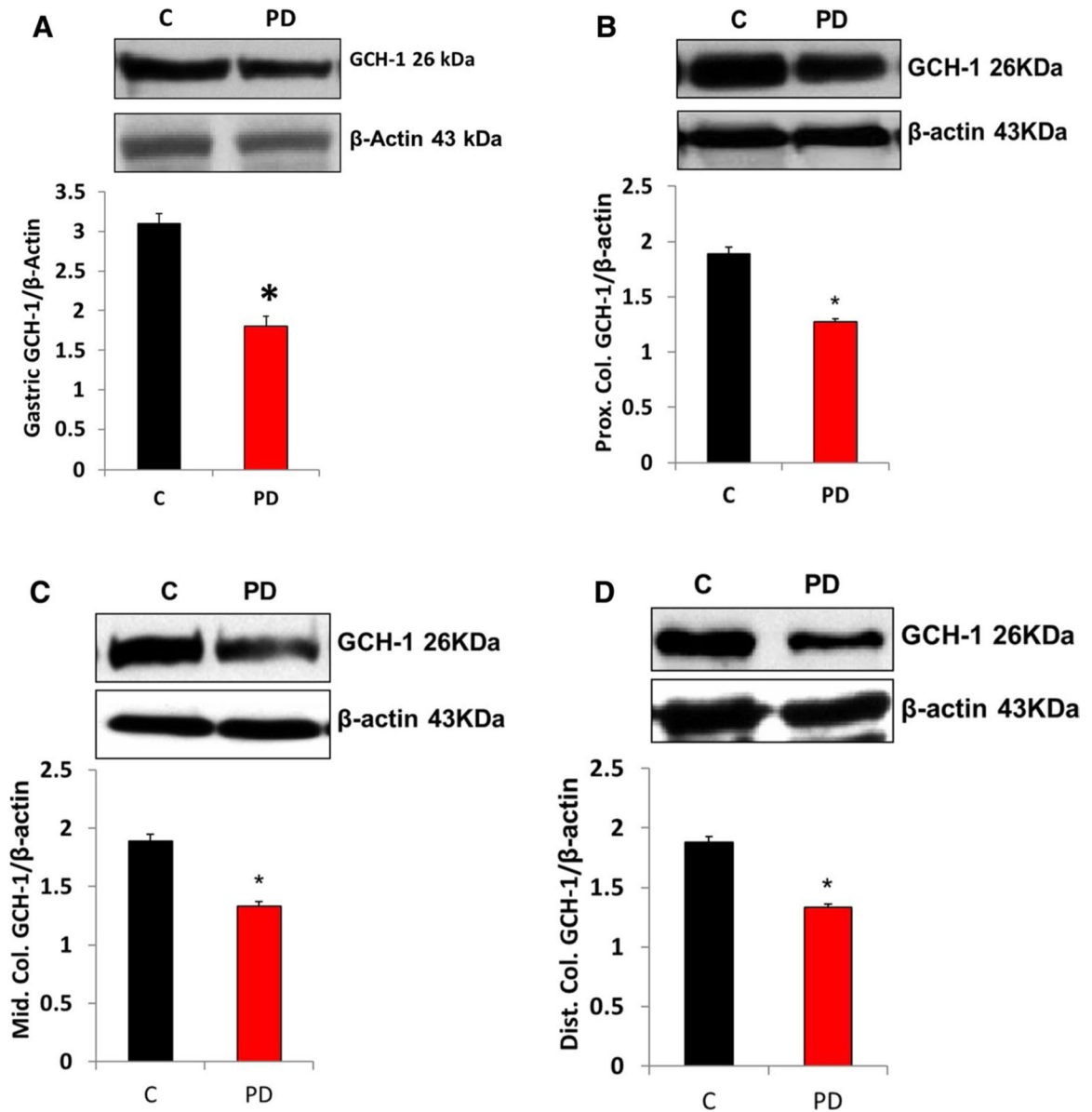


Fig. 7.

Changes in protein expression of GCH-1 in PD mice gastric and colon tissues. **a** Representative immunoblot and densitometric analysis in gastric antrum tissue, **b** representative immunoblot and densitometric analysis in proximal colon, **c** representative immunoblot and densitometric analysis in middle colon, and **d** representative immunoblot and densitometric analysis in distal colon. * $p < 0.05$ compared with control ($n = 4$). *C* Control, *PD* Parkinson's disease

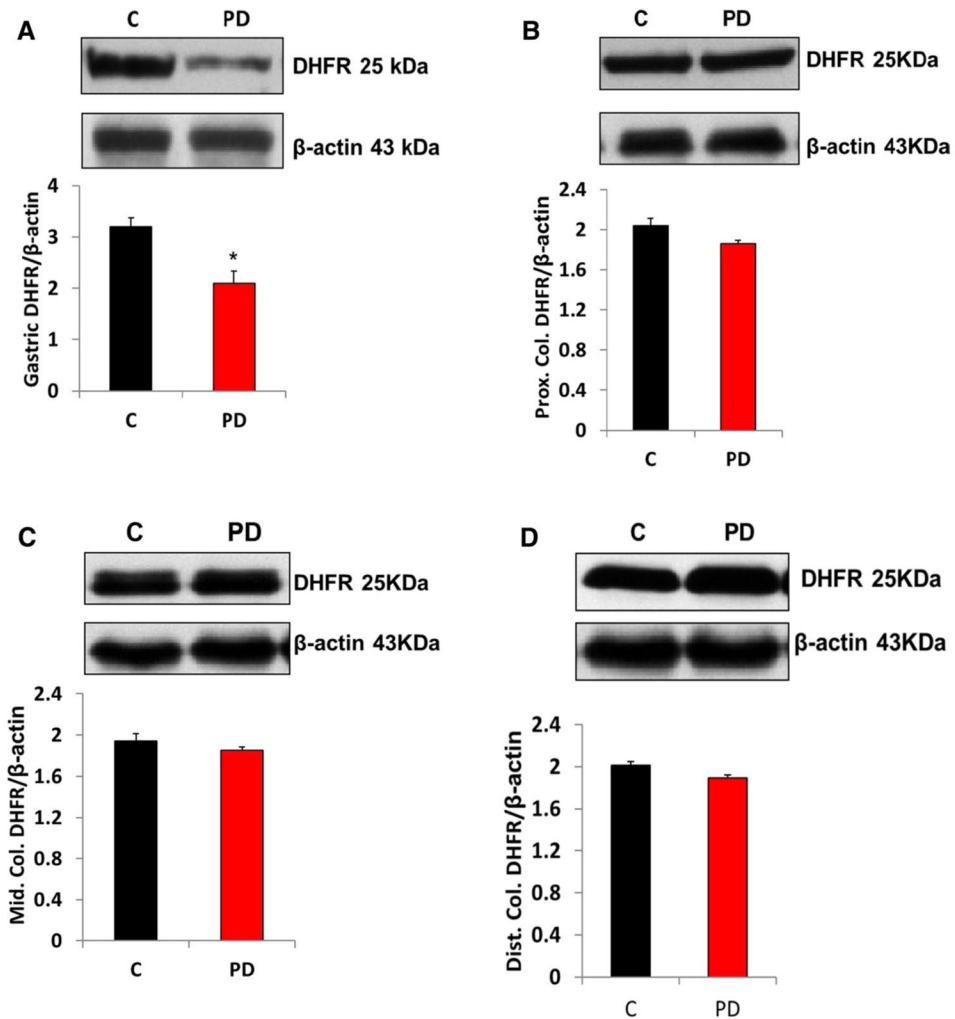


Fig. 8. DHFR protein expression in MPTP-induced PD in gastric and colon tissues. **a** Representative immunoblot and densitometric analysis of DHFR in gastric antrum tissue, **b** representative immunoblot and densitometric analysis of DHFR in proximal colon, **c** representative immunoblot and densitometric analysis of DHFR in middle colon, and **d** representative immunoblot and densitometric analysis of DHFR in distal colon. * $p < 0.05$ compared with control ($n = 4$). *C* Control, *PD* Parkinson's disease

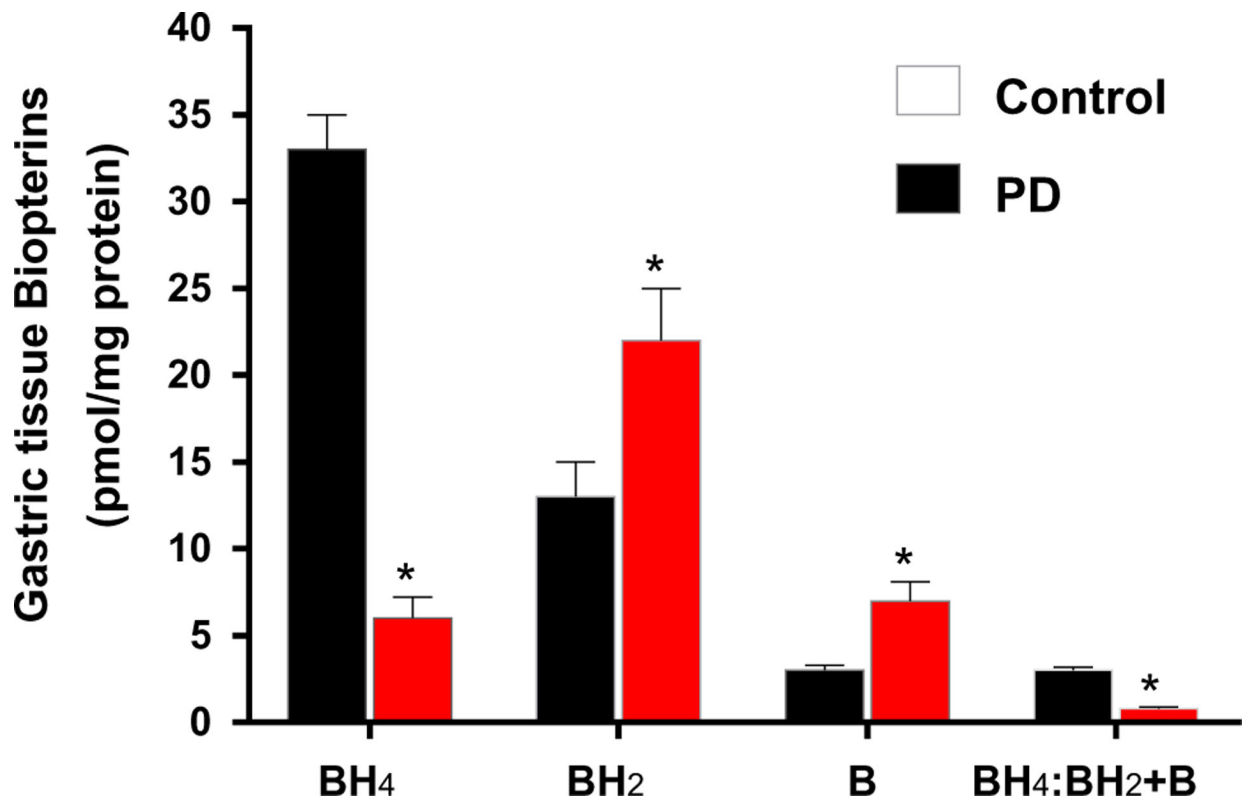


Fig. 9. Effect of MPTP treatment in reduced (BH₄, biologically active) and oxidized (BH₂ and B, biologically inactive) biopterins in gastric muscular tissues. * $p < 0.05$ vs. controls. $p < 0.05$ vs. PD-induced group. C Control, BH₄ tetrahydrobiopterin, BH₂ oxidized form dihydrobiopterin, B biopterin

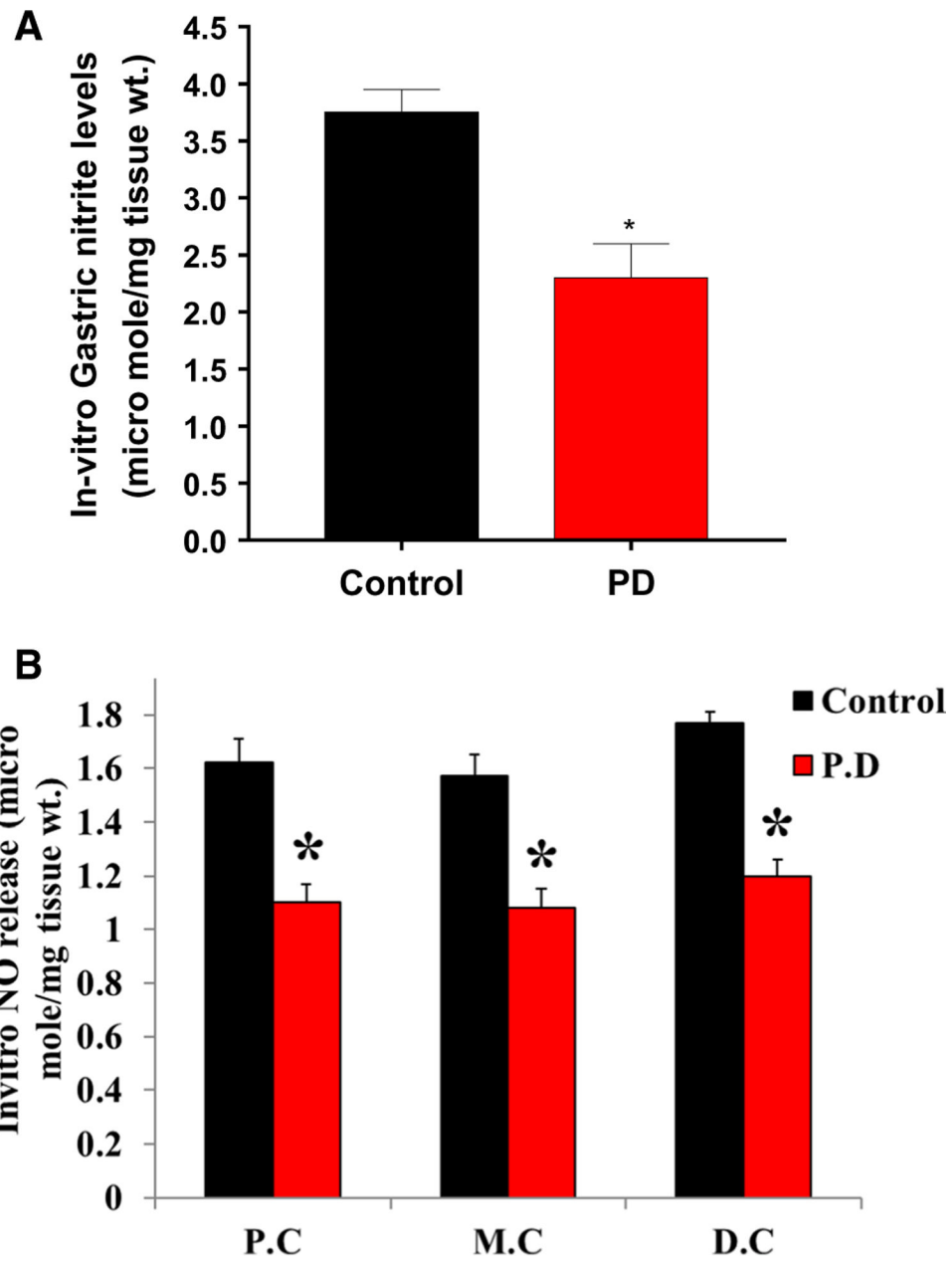


Fig. 10. Effect of MPTP treatment in gastric and colon neuromuscular nitrite levels. Nitrite levels at 48 h were measured using the NO assay kit (ELISA). Values are mean \pm SE ($n = 4$). Statistical significance was determined by Student's *t* test. * $p < 0.05$ compared with wild-type animals. *C* Control, *PD* Parkinson's disease, *PC* proximal colon, *M.C* mid colon, *D.C* distal colon

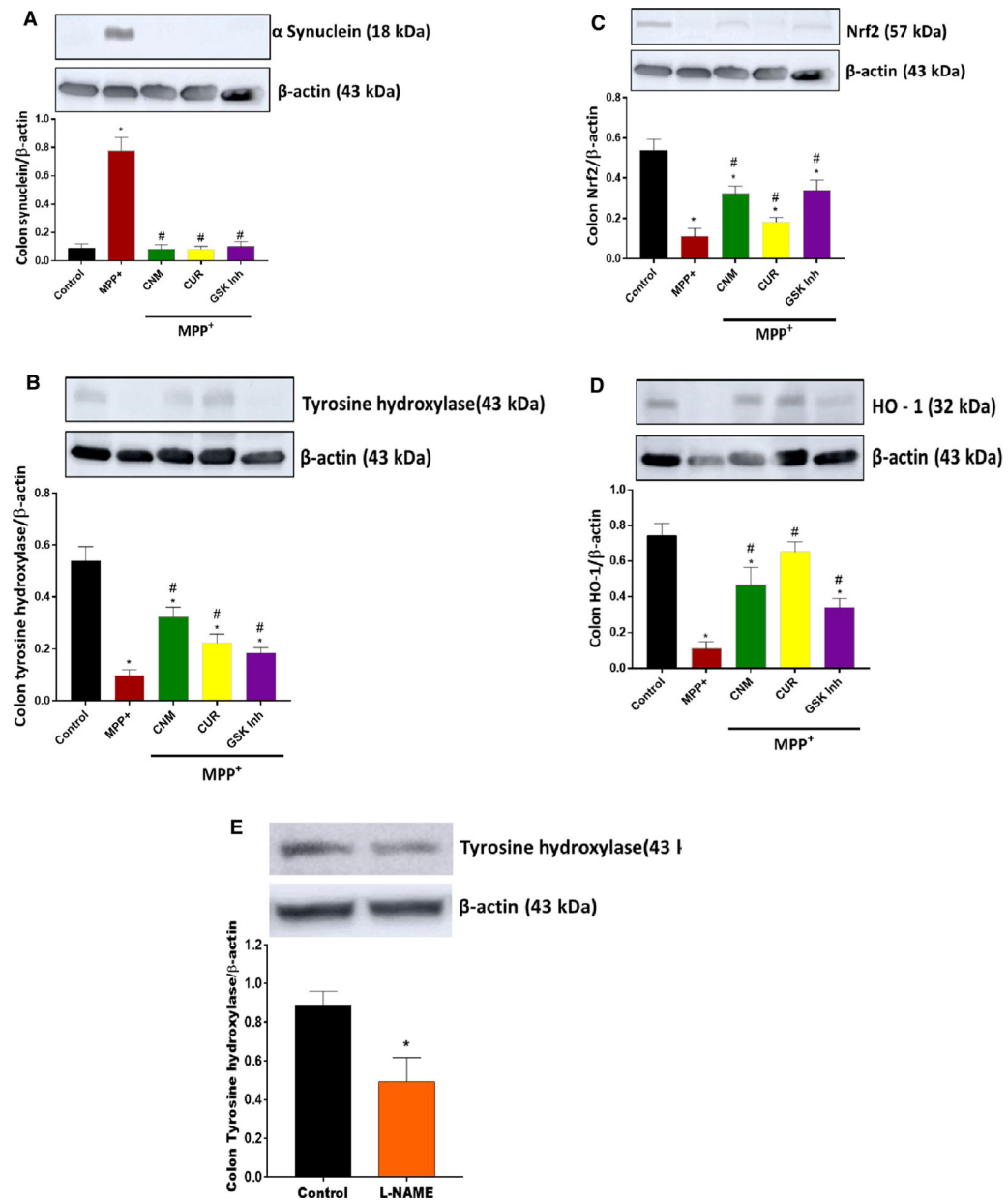


Fig. 11. Effects of Nrf2 activators cinnamaldehyde (CNM, 100 μ M), curcumin (CUR, 100 μ M), or GSK3 β inhibitor (SB216763, 30 μ M) on MPP⁺-induced alterations of α -synuclein, tyrosine hydroxylase, Nrf2, and HO-1. Effect of L-NAME (100 μ M) on tyrosine hydroxylase protein expression in colon tissues. Colon specimens were incubated for 48 h and WB experiments were performed for targeted proteins. **a** Representative immunoblot and densitometric analysis of α -synuclein in colon tissue, **b** representative immunoblot and densitometric analysis of tyrosine hydroxylase in colon tissue, **c** representative immunoblot and densitometric analysis of Nrf2 in colon tissue, **d** representative immunoblot and densitometric analysis of HO-1 in colon tissue, and **e** representative immunoblot and densitometric analysis of tyrosine hydroxylase in colon tissue. * $p < 0.05$ compared with

control ($n = 4$), # $p < 0.05$ compared to MPP^+ . *C* Control, *PD* Parkinson's disease, MPP^+ (1-methyl-4-phenylpyridinium), $MPP^+ + CNM$ (cinnamaldehyde), $MPP^+ + CUR$ (curcumin), $MPP^+ + GSK-3\beta$ inhibitor (SB216763)

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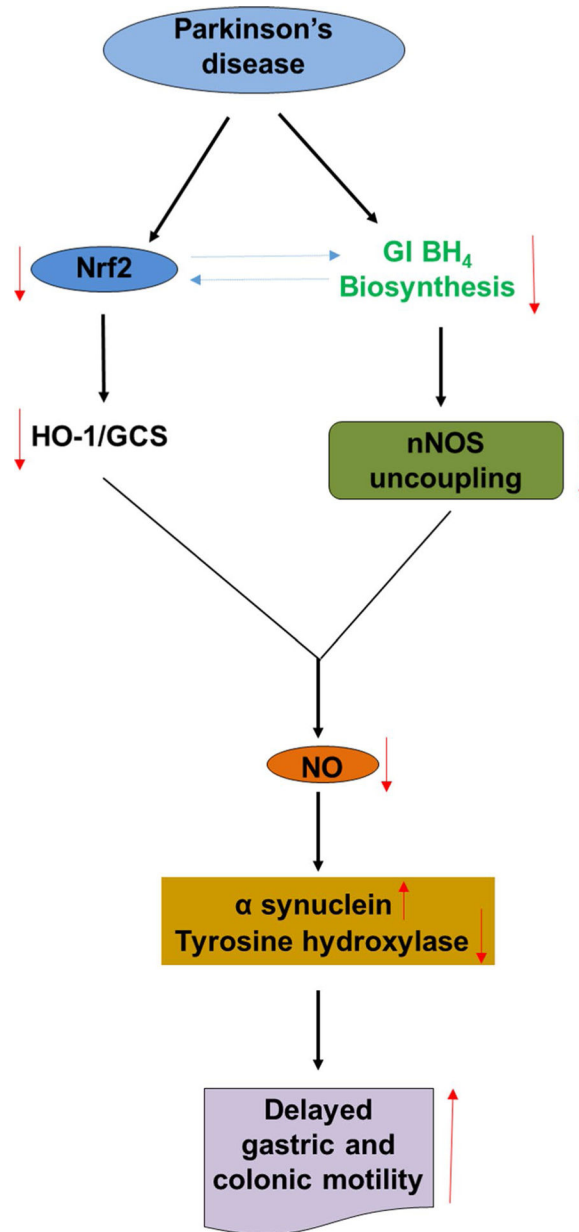


Fig. 12. Schematic diagram of proposed BH₄/Nrf2/nNOS mediated gastrointestinal motility in MPTP-induced PD mice. The solid lanes indicate the sequential events occur through protein–protein interactions that connect the gene promoters to execute transcription of target genes. The dotted connections indicate possible interactions between the proteins. The reversible arrows indicate possible cross talk between the proteins. ↑ and ↓ arrows indicate increase and decrease levels, respectively