Neuroprotection by misoprostol against rotenone-induced neurotoxicity in rat brain

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Objective: To investigate the effect of the prostaglandin E1 analogue misoprostol on oxidative stress and neurodegeneration caused by subcutaneous rotenone administration in rats. Methods: Rotenone was administered in a dose of 1.5 mg/kg every other day for 2 weeks. Starting from the 1st day of rotenone injection, rats were subcutaneously treated with misoprostol at doses of 10, 100 or 1000 μg/kg. Rats were evaluated for brain lipid peroxidation (malondialdehyde: MDA), reduced glutathione (GSH), nitric oxide (NO) levels, and paraoxonase-1 (PON-1) activity. The concentrations of the anti-apoptotic protein B cell/lymphoma-2 (Bcl-2) were determined in the striatum. Histopathologic examination and the expression of inducible nitric oxide synthase (iNOS) in the cerebral cortex and striatum were also performed. Results: Compared with the vehicle-treated group, rotenone caused a significant increase in brain lipid peroxidation (MDA) by 61% (P<0.05) accompanied by an increase in NO by 73.1% (P<0.05) and a decrease in GSH concentration by 29.4% (P<0.05). In addition, brain PON-1 activity significantly decreased by 63.0% (P<0.05) and striatal Bcl-2 significantly decreased by 27.9% (P<0.05) with respect to the corresponding control value. Brain sections from rotenone treated rats showed extensive dark pyknotic and apoptotic nuclei in neurons, shrunken cytoplasm and perineuronal vacuolation. Rotenone also caused pronounced expression of iNOS in the cerebral cortex and striatum. Treatment with misoprostol at doses of 100 and 1000 μg/kg resulted in decreased brain MDA (by 16.5%–23.0%) (P<0.05) and NO levels (by 37.1%–40.7%) (P<0.05) and increased GSH concentrations (by 18.8%–30.1%) (P<0.05). PON-1 activity was significantly increased by 80.0%–114.8% (P<0.05) by misoprostol at 100 and 1000 μg/kg, respectively. In addition, misoprostol treatment restored striatal Bcl-2 concentrations to its normal value. Misoprostol treatment resulted in markedly reduced brain injury and decreased iNOS expression in the cerebral cortex and striatum of rotenone intoxicated rats. Conclusions: These data suggest that misoprostol prevents the rotenone-induced neurodegeneration in rat brain by reducing brain oxidative stress.

1. Introduction

Parkinson’s disease (PD) is a progressive neurodegenerative disorder in which there is continued loss of the dopaminergic cells in the substantia nigra pars compacta of the basal ganglia of the mid-brain[1]. This results in dopamine depletion in the substantia nigra pars compacta dopaminergic neurons with its projections to the striatum and the emergence of the disease symptoms such as tremor, slowness in starting movements, muscle stiffness as well as postural instability[2-4]. The cause of this cell death in PD is not well understood but biochemical processes like free radical

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mediated oxidative damage, impaired mitochondrial complex I activity, and neuroinflammation have been uncovered in the brain of PD subjects[5-9]. PD is essentially a sporadic disorder in about 95% of cases[10] and is strongly believed to be the result of exposure to an environmental toxin in addition to a genetic component[11-13]. The role of pesticides and herbicides in the pathogenesis of PD has been inferred both from epidemiological data and animal experiments[14-18]. In the latter, injection of rotenone, a pesticide of plant origin resulted in the development of motor abnormalities, brain biochemical and pathological changes similar to those found in human PD[16-18]. Rotenone inhibits mitochondrial complex I, increases reactive oxygen metabolites and evokes oxidative stress and neuroinflammatory response in brain of injected rodents[19-23]. This provided a model for understanding the disease mechanisms and finding new therapeutic targets[17,24]. Currently, there is no treatment which could prevent or even slow the process of cell loss in PD[25]. Drugs such as L-dopa, the precursor of dopamine, irreversible selective monoamine oxidase-B inhibitors, and dopamine receptor agonists aim at replacing the midbrain dopaminergic deficit by providing dopamine or boosting dopaminergic neurotransmission in the basal ganglia[26,27]. These agents help to ease the life of individuals with PD but their efficacy declines over time due to the progressive nature of the disorder together with the emergence of side effects and motor complications eg., “on-off” phenomenon, dyskinesia and dystonia[27,28]. This illustrates the need for identifying new therapies or therapeutic targets.

Misoprostol, a gastric anti-secretory agent, has been approved by the FDA for the prevention and/or treatment of gastric ulcers[30]. The drug is a prostaglandin E1 (PGE1) analogue (15-deoxy-16-hydroxy-16-methyl PGE1) in which the 15-hydroxyl group of PGE1 is displaced to C16[31]. In the gastric mucosa, prostaglandins of the E series are endowed with antioxidant and cytoprotective properties[32,33]. Recent studies also indicated a neuroprotective action of misoprostol against experimental brain injury due to lipopolysaccharide endotoxin[34] or middle cerebral artery occlusion[35,36] in mice. The drug acts on prostaglandin E2 (PGE2) E-prostanoid (EP) EP2, EP3 and EP4 receptor subtypes[31,37]. These receptors have been shown to mediate an anti-inflammatory[38] and neuroprotective effects against amyloid beta-peptide[39] and the nigrostriatal toxin 6-hydroxydopamine in the brain[40]. In light of the above data, the present study aimed to investigate the neuroprotective potential of misoprostol in experimental PD caused by systemic rotenone injection in the rat.

Animal procedures were done following the recommendations of the Institution Ethics Committee and that of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Drugs and chemicals

Rotenone was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in dimethyl sulfoxide. Misoprostol (PGE1) was obtained from Sigma Pharmaceutical Industries (Cairo, Egypt) and dissolved in isotonic (0.9% NaCl) saline solution immediately before use. The doses selected were based on previous studies[34]. All other chemicals were of analytical grade and purchased from Sigma.

2.3. Study design

Rats were randomly divided into five equal groups, with six rats in each group. Group 1 received the vehicle (dimethyl sulfoxide) via the subcutaneous (s.c.) route. Group 2 received s.c. injection of rotenone at the dose of 1.5 mg/kg. Groups 3, 4 and 5 received s.c. rotenone at the dose of 1.5 mg/kg along with misoprostol at doses of 10, 100 or 1 000 µg/kg. Drugs or vehicle were given every other day for 2 weeks. Rats were then euthanized by decapitation for tissue collection; their brains were quickly removed out on an ice-cold plate, washed with ice-cold phosphate-buffered saline (pH 7.4), weighed, and stored at 80 °C until further biochemical studies. The brain tissues were homogenized with 0.1 mol/L phosphate buffer saline at pH 7.4 to give a final concentration of 100 g/L for the biochemical assays.

2.4. Determination of lipid peroxidation

Lipid peroxidation was determined by the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form a red colored TBA-MDA2 adduct. The absorbance was measured at 532 nm[41].

2.5. Determination of nitric oxide (NO)

The generation of NO was determined by measuring accumulation of nitrite using Greiss reagent as described previously. The concentration of nitrite was calculated using a standard curve for sodium nitrite. Absorption was measured at 540 nm[42].

2.6. Determination of reduced glutathione (GSH)

GSH was measured using the method of Ellman et al[43]. DTNB (5,5′-dithiobis (2-nitrobenzoic acid) or Ellman’s reagent is reduced by the free sulfhydryl group on GSH molecule to yield generate 5-thio-2-nitrobenzoic acid which has yellow color and can be determined by reading absorbance at 412 nm.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (180–200 g) from the National Research Centre (Cairo, Egypt) were used. Rats were housed on a 12-h light/dark cycle and standard laboratory food and tap water freely given.

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2.7. Determination of paraoxonase-1 (PON-1) activity

Arylesterase/paraoxonase activity was estimated in brain tissue with the use of phenyl acetate as a substrate. In this assay, phenyl acetate is hydrolyzed by PON-1 and the rate is which phenol is produced is measured using spectrophotometer by measuring absorbance at 270 nm. One unit of arylesterase activity is considered equivalent to 1 μmol/L of phenol formed/minute. Enzyme activity is expressed in kilo International Unit/Liter (kU/L)[44,45].

2.8. Determination of B cell/lymphoma-2 (Bcl-2)

A commercially available human Bcl-2 enzyme-linked immunosorbent assay kit from Glory Science Co, Ltd (Del Rio, TX, USA) was used.

2.9. Histopathological studies

The brain tissues were immediately fixed in 10% formalin, dehydrated in gradual ethanol (50%–100%), cleared in xylene and embedded in paraffin. Sections (4 μm) were prepared and then stained with hematoxylin and eosin (H&E) dye for photomicroscopic observations.

2.10. Immunohistochemistry for iNOS

Sections were deparaffinized and treated with 0.3% H₂O₂ in methanol at 37 °C for 30 min. Sections then were incubated overnight at 4 °C with primary anti-iNOS antibody (1:50 dilution) followed by incubation with the biotinylated secondary antibody and finally with avidin-horseradish peroxidase-conjugated biotin. The staining was visualized with 3,3′-diaminobenzidine. Sections were then counterstained with hematoxylin.

2.11. Quantitative image analysis of immunohistochemical iNOS

Leica Qwin 500 image system was used for densitometry measurement of iNOS immunoreactivity. Fifteen random fields/sections were averaged to determine means.

2.12. Statistical analysis

Data are presented as mean ± SEM. One way analysis of variance and Duncan’s multiple rang test were used for statistical evaluation of the data. SPSS software was used for this purpose. A P value less than 0.05 is considered to be statistically significant.

3. Results

3.1. Oxidative stress

Rotenone caused significant increase in brain lipid peroxidation indicated by the rise in MDA level by 61.0% (P<0.05) compared with the vehicle control value. An increase in NO by 72.1% (P<0.05) was also noted in brain of rotenone-treated rats compared to the vehicle group. On the other hand, there was significant decrease in GSH by 29.4% (P<0.05) of its control value (Table 1).

In rotenone intoxicated rats, treatment with misoprostol at doses of 100 and 1 000 μg/kg resulted in significant decrease in brain malondialdehyde level by 16.5%–23.0% (P<0.05). The content of brain NO significantly decreased by 22.0% (P<0.05), 37.1% (P<0.05) and 40.7% (P<0.05) in rats treated with 10, 100 and 1 000 μg/kg of misoprostol compared with the rotenone only group. There were also increased GSH concentrations by 18.8%–30.1% (P<0.05) by misoprostol at 100 and 1 000 μg/kg, respectively (Table 1).

3.2. PON-1 activity

A significant decrease in brain PON-1 activity by 63% (P<0.05) was observed in rotenone injected rats. PON-1 activity significantly increased by 80.0%–114.8% (P<0.05) following misoprostol treatment at 100 and 1 000 μg/kg, respectively (Table 1).

3.3. Bcl-2

In rotenone-treated rats, striatal Bcl-2 concentration was significantly decreased by 27.9% (P<0.05) of its vehicle control value. In rats treated with misoprostol, there was a dose-dependent increase in striatal Bcl-2 concentration (Table 1).

3.4. Histopathological results

The brain sections from the vehicle treated control group showed normal architecture in both cortex and striatum with neurons being round and basophilic (Figures 1A & 2A). In the rotenone group, the most consistent findings occurring in the histological tissue sections stained with H&E were those indicating severe degenerative changes such as shrunken cytoplasm and extensively dark pyknotic and apoptotic nuclei in neurons, and perineuronal vacuolation. Also, there were leukocytic infiltration, dilated blood vessels, congested capillaries with red blood cells (Figures 1B &

Table 1

| Group | MDA (nmol/g.tissue) | NO (μ mol/g.tissue) | GSH (μ mol/g.tissue) | PON1 activity (kU/L) | Bcl-2 (ng/mL) |
|-------|---------------------|---------------------|---------------------|---------------------|--------------|
| Vehicle | 23.60±0.70 | 26.00±0.61 | 6.54±0.43 | 13.50±0.85 | 3.05±0.08 |
| Rotenone | 38.00±1.51ab | 45.00±2.42bc | 4.62±0.17b | 5.00±0.61ab | 2.20±0.01a |
| Rotenone + misoprostol 10 μg/kg | 36.00±1.70a | 35.10±1.68ab | 5.16±0.32b | 5.65±0.37ab | 2.60±0.05a |
| Rotenone + misoprostol 100 μg/kg | 31.72±1.24bc | 28.30±0.95ab | 5.49±0.21b | 9.00±0.41ab | 2.83±0.04ab |
| Rotenone + misoprostol 1 000 μg/kg | 29.26±0.84bcd | 26.70±0.71bc | 6.01±0.19b | 10.74±0.68ab | 3.00±0.05ab |

Values are means ± SEM. *P<0.05 vs. vehicle. †P<0.05 vs. rotenone only. ‡P<0.05 vs. rotenone + 10 μg/kg misoprostol.
These pathological changes were ameliorated dose-dependently by treatment with misoprostol (Figures 1C–1E & 2C–2E). Sections from rats treated with 10 μg/kg misoprostol showed less histopathological changes except for pyknosis of some neurons, perivascular vacuolation, apoptotic nuclei, and acidophilic cytoplasm (Figures 1C & 2C). Moderate improvement was seen after 100 μg/kg misoprostol but with perivascular vacuolation, pyknotic, apoptotic nuclei in neurons (Figures 1D & 2D). Sections from rats treated with 1000 μg/kg misoprostol showed almost normal neuronal cells of cortex and striatum, respectively. However, few histopathological changes such as minimal vacuolation, pyknotic, apoptotic nuclei were still seen (Figures 1E & 2E).

3.5. Immunostaining for iNOS

iNOS-positive neurons were not detected in the of cortex and striatum of the vehicle control group (Figures 3A & 4A). Rotenone caused increased density of iNOS immunoreactivity (Figures 3B & 4B). In rotenone-treated rats, misoprostol administration prevented the increase of iNOS immunoreactivity of degenerating neurons in a dose-dependent manner (Figures 3C–3E & 4C–4E).
3.6. Quantitative analysis of iNOS immunoreactivity

Quantitative morphometric analysis of iNOS immunoreactivity indicated marked increase in iNOS immunoreactivity in the cerebral cortex and striatum by rotenone compared with the vehicle-treated group. Misoprostol treatment resulted in a significant and dose-dependent decrease in immunoreactivity in these areas. iNOS immunoreactivity decreased by 38.8%, 66.0%, and 88.7% in cerebral cortex and by 37.7%, 58.0%, and 79.2% in striatum by misoprostol at doses of 10, 100, and 1,000 μg/kg, respectively. Quantitative image analysis of iNOS immunoreactivity is shown in Table 2. These data clearly indicated that misoprostol suppressed iNOS expression in brain tissue.

Table 2

| Group                        | Cerebral cortex iNOS | Striatum iNOS |
|------------------------------|----------------------|---------------|
| Vehicle                      | 1.92±0.21            | 1.70±0.16     |
| Rotenone                     | 29.75±1.31           | 34.60±1.25    |
| Rotenone + misoprostol 10 μg/kg | 18.22±1.69           | 21.54±1.80    |
| Rotenone + misoprostol 100 μg/kg | 10.12±0.81          | 14.52±0.74    |
| Rotenone + misoprostol 1,000 μg/kg | 3.35±0.12           | 7.21±1.10     |

Values are means ± SEM. *p<0.05 vs. vehicle-treated group. **p<0.05 vs. rotenone control group. ***p<0.05 vs. rotenone + 10 μg/kg misoprostol. ****p<0.05 vs. rotenone + 100 μg/kg misoprostol.

4. Discussion

In this study, we have demonstrated that the administration of the synthetic prostaglandin analogue misoprostol was able to protect against rotenone-induced neurotoxicity in the rat brain. The pesticide rotenone is a mitochondrial complex-I inhibitor that is widely used in rodents to model human PD.[17]. The toxicant has been shown to cause nigrostriatal damage, alpha-synuclein-like intracellular deposits, dopamine depletion and decreased tyrosine hydroxylase immunoreactivity in the substantia nigra and striatum of treated rats and mice.[16,21,46,47]. In this study, rotenone administered via the subcutaneous route caused marked neuronal damage in the form of neuronal apoptosis and necrosis, and focal gliosis. The administration of rotenone was associated with markedly elevated brain oxidative stress. There was increased lipid peroxidation (MDA) along with decreased level of the antioxidant and free radical scavenging molecule GSH. These results agree with previous studies indicating increased generation of reactive oxygen metabolites by the toxicant.[18,21,46,48]. Rats treated with s.c. rotenone exhibited increased MDA and decreased GSH concentrations in different brain regions.[18,21,46,48-50]. Rotenone caused increased superoxide and NO in organotypic striatal slice cultures[48] and increased intracellular reactive oxygen metabolites in SH-SY5Y human neuroblastoma cell line[51]. Rotenone inhibits the mitochondrial complex I and causes increased O2•− generation resulting in damage to complexes I and II and mitochondrial dysfunction[52]. Moreover, microglia activation by the pesticide results in increased superoxide anion production via NADPH oxidase[59] or hypochlorous acid (HOCl) via myeloperoxidase[53]. The neurotoxic effects of rotenone are largely ascribed to its ability to cause oxidative mediated cell damage. This is because the antioxidant vitamin E (α-tocopherol) could prevent the rotenone effects such as protein oxidation and the decrease in tyrosine hydroxylase protein in midbrain organotypic slices[20]. In mice with orally administered rotenone, the synthetic antioxidant lipoylcarnitine prevented the increased generation of reactive oxygen metabolites in forebrain and midbrain regions and improved motor performance[54].

Our results in addition indicate markedly increased brain nitric acid content by rotenone. Moreover, immunohistochemical studies indicated that iNOS which was very rarely detected in neurons in the vehicle treated rats showed strong expression after rotenone treatment. These data are in line with other studies showing increased NO and 3-nitrotyrosine and prominent iNOS expression in different brain regions in rodents administered rotenone.[21,55,56]. The increased generation of NO from iNOS during inflammatory or toxic conditions by activated astrocytes or microglia contributes to neuronal death. This is caused by the inhibition of cellular respiration and the fall in cellular ATP due to cytochrome-c oxidase inhibition and decreased activity of mitochondrial complexes (I and II).[57,58]. When present in high concentrations, NO reacts with the superoxide anion or molecular oxygen to form reactive nitrogen intermediates such as nitrogen dioxide (•NO2), dinitrogen trioxide (N2O3) or peroxynitrite radical (ONOO−) capable of amine and thiol nitrosation.
Rotenone induces apoptotic neuronal death [52,67-70]. Studies with developing to PD after exposure to insecticides [14,65]. The decrease indicating that decreased activity might increase the susceptibility for been a focus of much interest because of the epidemiological data been shown to reduce the increase in MDA and NO and to increase the brain of rotenone intoxicated rats which is consistent with PON-1 activity in the brain of rotenone intoxicated rats which is consistent with our previous observations following rotenone injection in rodents. PON-1 is a calcium-dependent esterase and plays an important role in detoxifying some organophosphate insecticides such as parathion, diazinon and chlorpyrifos [62]. It has also been shown to exhibit an anti-inflammatory and antioxidative actions [63,64]. The enzyme has been a focus of much interest because of the epidemiological data indicating that decreased activity might increase the susceptibility for developing to PD after exposure to insecticides [14-65]. The decrease in the enzyme activity after rotenone injection might be due to direct inhibition by the toxicant or is caused by the increased oxidative burden. The latter has been shown to result in inactivation of PON-1 [66].

Rotenone induces apoptotic neuronal death [52,67-70]. Studies with SH-SY5Y human dopaminergic cells in vitro indicated that rotenone causes neuronal death via apoptosis. This involves activation of Bad, a member of the proapoptotic BH3-only subfamily of BCL-2 proteins, caspase-9, c-Jun N-terminal protein kinase and the p38 mitogen pathways [68,69]. Rotenone also induced apoptosis in human promyelocytic leukaemia cell line (HL-60 cells) and in rat ventral mesencephalic dopaminergic neurons along with an increase in reactive oxygen metabolites, cytochrome c release and caspase-3 activation [52,67]. Protection was conferred by antioxidants or by overexpression of manganese superoxide dismutase. This suggested the involvement of increased reactive oxygen metabolites in the rotenone-induced apoptosis [52]. In this study, there was a marked decrease in concentration of the antiapoptotic protein Bcl-2 in the striatum of rotenone intoxicated rats, which is in accordance with earlier observations [46]. The antiapoptotic protein Bcl-2 is a member of the Bcl-2 family of proteins [71], Bcl-2 also exhibits an antioxidative action [72].

Our results clearly indicated a neuroprotective action for the PGE1 analogue misoprostol against rotenone neurotoxicity. The drug attenuated neuronal apoptosis and necrosis in the cerebral cortex and striatum and restored the level of the antiapoptotic protein Bcl-2 in the striatum. Misoprostol also reduced the oxidative stress and the expression of the inducible form of nitric oxide synthase in the brain of rotenone-treated rats. Misoprostol might thus protect against the neurotoxic action of rotenone by reducing the brain level of reactive oxygen and nitrogen species. The drug has also been shown to reduce the increase in MDA and NO and to increase GSH concentration and PON-1 activity in the brain of mice treated with lipopolysaccharide endotoxin [34]. Misoprostol activates PGE2 EP receptor subtypes EP2 and EP3 [31]. High expression of the PGE2 EP2 receptor occurs in the cerebral cortex, hippocampus and striatum [73] and mediates an anti-inflammatory and neuroprotective effect in the brain [36,38-40,73]. In-vitro studies show EP2 receptor activation was associated with decreased neuronal death due to glutamate toxicity or oxygen glucose deprivation [73]. In neuronal cultures, the EP2 agonist butaprost, and the EP4/EP3 agonist 1-hydroxy-PGE1 protected against neurotoxicity by amyloid beta-peptide. Stimulation of EP4/EP3 receptors was found to increase cAMP signaling and alleviate the increase in reactive oxygen metabolites [39]. In mice with focal brain ischaemia, genetic deletion of the EP2 receptor increased the extent of cerebral infarction in mice [73]. In contrast, treatment with misoprostol was shown to protect against experimental cerebral ischaemia in mice via the PGE2 EP2 and/or EP4 receptor stimulation [35,36].

In summary, the present study indicates that the synthetic PGE1 analogue and the antiulcer agent misoprostol exerted neuroprotective effects against the rotenone neurotoxicity in the rat brain. The drug shows an antioxidant and antiapoptotic actions and decreased the expression of iNOS in both the cerebral cortex and striatum. These observations suggest that misoprostol could have a place in the treatment of PD.

Conflicts of interest statement

The authors declare that they have no conflicts of interest.

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