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The neuroprotective effect of erythropoietin on experimental Parkinson model in rats

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ABSTRACT

Dopaminergic neuronal loss in Parkinson's disease (PD) results from oxidative stress, neuroinflammation and excitotoxicity. Because erythropoietin (EPO) has been shown to have antioxidant, anti-inflammatory and neuroprotective effects in many previous studies, present study was designed to evaluate the effect of EPO on rotenone-induced dopaminergic neuronal loss. The rats in which PD was induced by stereotaxical infusion of rotenone showed increased MDA and TNF-alpha levels and decreased HVA levels. On the other hand, EPO treatment resulted in markedly decreased MDA and TNF-alpha levels and increased HVA levels. EPO treatment in rotenone-infusion group resulted in improvement of striatal neurodegeneration and a significant increase in decreased total number of neurons and immunohistochemical TH positive neurons. Results of the present study demonstrate the neuroprotective, anti-inflammatory and antioxidant effects of EPO in a rotenone-induced neurodegenerative animal model.

1. Introduction

Parkinson's disease (PD) is a chronic and progressive disease leading to clinical findings results from the lack of dopamine due to the neuroinflammation and neurodegeneration. Parkinson syndrome is characterized by four cardinal symptoms including bradikinesia, resting tremor, rigidity and postural instability and has primary or secondary origin. Many reasons can cause secondary Parkinson syndrome; they are called as symptomatic Parkinsonisms. Primary Parkinson syndrome may be familial or sporadic. In primary Parkinson syndrome (Parkinson disease), although it is commonly considered that neuronal loss is primarily seen in substantia nigra, pathological process is considered to be present initially in caudal brain stem structures, olfactory bulbus and even in enteric system (Braak et al., 2003, 2006; Parkkinen et al., 2009). The most affected dopaminergic neurons are those in the ventrolateral of substantia nigra pars compacta, while ventral tegmental area is relatively intact (Esposito et al., 2007). Striatum is the most commonly affected area from the neurotoxicity, hypoxia and metabolic disturbances, because dopaminergic neurons are abundant in this area. The first clinical signs usually occur after loss of about 60% of the neurons in substantia nigra pars compacta (Poewe and Wenning, 1998).

Many previous studies have suggested that the major pathophysiological mechanisms in PD are mitochondrial dysfunction, oxidative stress, excitotoxicity and inflammation (Przedborski, 2007). Furthermore, the Lewy bodies, in which the main protein is alpha-sinuclein, has been also suggested to have a role in PD pathophysiology. Oligomeric alpha-sinuclein is considered to generate holes in the cell membrane, increasing the vulnerability of the cell to the toxicity (Lashuel et al., 2002).

One of the action mechanism of the rotenone used in the 1-methyl-4-phenyl-1, 2, 3,6-tetrahydropyridine (MPTP) induced experimental PD model is inhibition of complex I (Fukae et al., 2007). Mitochondrial dysfunction resulting from the oxidative stress – a major pathophysiological mechanism in PD – leads to the lack of complex I, rendering the dopaminergic neurons to become vulnerable.

Erythropoietin (EPO) is a 30.5 kDa protein and is produced by liver hepatocytes during the fetal period and mainly by renal fibroblast-like cells after the birth (Jelkmann, 2004). Even less, it is also produced by neurons, glial cells and cerebral endothelial cells (Velly et al., 2010). The neuroprotective effect of EPO is considered to results from of antioxidant, antiapoptotic, angiogenic and anti-inflammatory effects of the hormone (Ehrenreich et al., 2004). Although antioxidant effect of the EPO depends on increased levels of superoxide dismutase and catalase enzymes, many previous studies have demonstrated that EPO did not result in a significant

Abbreviations: EPO, erythropoietin; MPTP, 1-methyl-4-phenyl-1, 2, 3,6-tetrahydropyridine; MDA, malondialdehyde; HVA, homovalinic acid.

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change in serum malondialdehyde (MDA) levels (Çavdar et al., 1997; Djordjević et al., 1993; Luciak et al., 1991). The free radicals generated in many cases including ischemia and metabolic disturbances are considered to induce proinflammatory cytokines such as IL-1, IL-6 and tumor necrosis factor-alpha (TNF-α), which mediate EPO release in CNS neurons (Nagai et al., 2001). EPO also exert neuroprotective effect by decreasing the tissue damaging molecules such as reactive oxygen derivatives and glutamate and by modulating the inflammatory process. In addition, some previous studies have suggested EPO to play a role in synaptic plasticity by leading to the stimulation or inhibition of neurotransmitters (Genc et al., 2004; Kumral et al., 2005). In the light of all these data, the present study investigated the antioxidant, neuroprotective and anti-inflammatory effects of EPO on the basis of laboratory and clinical findings in an experimental neurodegenerative PD model induced by rotenone in rats.

2. Materials and methods

2.1. Animals

Twenty-one adult male Sprague Dawley rats, weighing 200–210 g, were used in the study. Animals were housed in cages and maintained under standard conditions with 12-h light/dark cycles at room temperature (22 ± 2 °C). They were fed by standard pellet diet and tap water *ad libitum* throughout the study. The Institutional Animal Care and Ethical Committee of the University of Gaziosmanpasa approved the study protocol. All chemicals were obtained from Sigma-Aldrich Inc. unless otherwise noted.

2.2. Experimental protocol

Animals were randomly divided into two groups: stereotaxical rotenone infusion group ($n = 14$) and stereotaxical DMSO infusion group (vehicle, $n = 7$). Rats were deeply anesthetized by intraperitoneal (i.p.) injection of the mixture of ketamine hydrochloride (80 mg/kg, Alfamine®, Ege Vet, Alfasan International B.V. Holland) and xylazine hydrochloride (4 mg/kg, Alfazyne®, Ege Vet, Alfasan International B.V. Holland), and placed in a stereotaxic frame. Rotenone (3 μg/μl in DMSO, $n = 14$) was infused into the left substantia nigra pars compacta (SNc, AP: 5.0 mm, L: 2.0 mm, DV: 8 mm) and ventral tegmental area (VTA, AP: 5.0 mm; L: 1.0 mm; DV: 7.8 mm) with a 28-gauge Hamilton syringe (Paxinos and Watson, 1998). Sham-operated rats received vehicle (1 μl DMSO) instead of rotenone (Xiong et al., 2009). The needle was left in place for an additional 2 minutes for complete diffusion of the drug. All animals were treated by i.p. penicillin to prevent postsurgical infection. After surgery, rats were monitored daily for behavior and health conditions. All rotenone-treated rats developed PD-like symptoms such as hypokinesia, freezing and flexed posture within the 10 days following stereotaxical procedure. The extent of the dopaminergic neuron loss was assessed 10 days after rotenone injection by challenge with apomorphine.

2.3. Apomorphine-induced rotations

Ten days after stereotaxic infusions of rotenone and vehicle, animals were injected with apomorphine hydrochloride (2 mg/kg, i.p.) to induce rotational behavior. Apomorphine solution was freshly prepared before the trial to minimize the oxidation effect. Full 360° unilaterally turns in a 10 min period were recorded. The rats, which turn unilaterally more than 7 cycles/min, were accepted as successful for PD model (Jin et al., 2008). Apomorphine-induced rotation test was repeated at the end of EPO and saline administration.

2.4. EPO treatment

The valid PD rats were randomly divided into two groups: group 1 ($n = 7$) was administered saline (1 ml/kg/day, i.p.) and group 2 ($n = 7$) was administered EPO (Eprex, Santa Farma) 2500 U/day (i.p.) for 28 days. Following EPO and saline treatment, behavioral testing was performed and then animals were perfused intracardially with 4% formaldehyde for histology and quantitative immunohistochemistry.

2.5. Histology and quantitative immunohistochemistry

For histological and immunohistochemical studies, all animals were anesthetized by an i.p. of ketamin (40 mg/kg, Alfamine®, Ege Vet, Alfasan International B.V., Holland)/xylazine (4 mg/kg, Alfazyne®, Ege Vet, Alfasan International B.V., Holland) and perfused with 200 ml of 4% formaldehyde in 0.1 M phosphate-buffer saline (PBS). Formalin-fixed brain sections (5 μm) were stained with hematoxylin and eosin Nissl staining. After that, sections were incubated in primary antibodies (tyrosine hydroxylase, Chemicon; 1/1000) for 24 h at 4 °C. Antibody detection was carried out with the Histostain-Plus Bulk kit (Invitrogen) against rabbit IgG and 3,3′ diaminobenzidine (DAB) was used to visualize the final product (Xiong et al., 2009). All sections were washed in PBS and photographed with Olympus C-5050 digital camera mounted on Olympus BX51 microscope. Immunoreactivity in the striatal sections was quantified by measuring intensity per area using Image J software (National Institutes of Health, Bethesda, MD). The intensity measurements were performed in three sections per studied group and 8–10 microscopic fields in each section. The extent of the rotenone lesion was examined quantitatively in the striatum by Nissl staining. Total neuron counts were performed in six sections *per* studied group by an image analysis system (Image-Pro Express 1.4.5, Media Cybernetics, Inc. USA).

2.6. Measurement of brain lipid peroxidation (MDA)

Lipid peroxidation was determined in tissue samples by measuring MDA levels as thiobarbituric acid reactive substances (TBARS) (Demougeot et al., 2000). Briefly, trichloroacetic acid and TBARS reagent were added to the tissue samples, then mixed and incubated at 100 °C for 60 min. After cooling on ice, the samples were centrifuged at 3000 rpm for 20 min and the absorbance of the supernatant was read at 535 nm. MDA levels were calculated from the standard calibration curve using tetraethoxypropane and expressed as nmol/g protein.

2.7. Measurement of brain protein levels

Total protein concentration in brain samples was determined according to Bradford's method using bovine serum albumin as standard (Bradford, 1976).

2.8. Detection of TNF-α in brain tissue

The frozen brain tissue was homogenized with a glass homogenizer in 1 ml of buffer containing 1 mmol/L of PMSF, 1 mg/L of pepstatin A, 1 mg/L of aprotinin, and 1 mg/L of leupeptin in PBS solution (pH 7.2), and then was centrifuged at 12,000 rpm for 20 minutes at 4 °C. The supernatant was then collected and the total protein was determined utilizing the Bradford method. The levels of TNF in the tissue supernatants were measured using an ELISA kit (eBioscience, Inc, San Diego, CA) specific for known rat TNF-α. The measurement of TNF-α was performed in a step-by-step fashion consistent with the protocol booklet of the ELISA kit. According to the specifications given by the manufacturer, the inter-assay and intra-assay coefficients of variation for TNF-α were 7.9–8.2% and