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INTRASTRIATAL ADMINISTRATION OF ERYTHROPOIETIN PROTECTS DOPAMINERGIC NEURONS AND IMPROVES NEUROBEHAVIORAL OUTCOME IN A RAT MODEL OF PARKINSON'S DISEASE

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Abstract—Erythropoietin (EPO), a hematopoietic cytokine, has recently been demonstrated to protect nigral dopaminergic neurons in a mouse model of Parkinson's disease (PD). In the present study, we tested the hypothesis that recombinant human erythropoietin (rhEPO) could protect dopaminergic neurons and improve neurobehavioral outcome in a rat model of PD. rhEPO (20 units in 2 μl of vehicle) was stereotaxically injected into one side of the striatum. 6-Hydroxydopamine (6-OHDA) was injected into the same side 1 day later. Another group of rats received rhEPO (5000 u/kg, i.p.) daily for 8 days, and unilateral injection of 6-OHDA in the striatum 3 days after systemic administration of rhEPO. We observed that intrastriatal administration, but not systemic administration of rhEPO significantly reduced the degree of rotational asymmetry. The rhEPO-treated rats also showed an improvement in skilled forelimb use when compared with control rats. The number of tyrosine hydroxylase (TH)-immunoreactive (IR) neurons in the ipsilateral substantia nigra (SN) was significantly larger in intrastriatal rhEPO-treated rats than that in control rats. TH-IR fibers in the 6-OHDA-lesioned striatum were also increased in the intrastriatal rhEPO-treated rats when compared with control rats. In addition, there were lower levels of expression of major histocompatibility complex (MHC) class II antigens and a smaller number of activated microglia in the ipsilateral SN in intrastriatal rhEPO-treated rats than that in control rats at 2 weeks, suggesting that intrastriatal injection of rhEPO attenuated 6-OHDA-induced inflammation in the ipsilateral SN. Our results suggest that intrastriatal administration of rhEPO can protect nigral dopaminergic neurons from cell death induced by 6-OHDA and improve neurobehavioral outcome in a rat model of PD. Anti-inflammation may be one of mechanisms responsible for rhEPO neuroprotection.

Key words: neuroprotection, inflammation, immunocytochemistry, striatum, substantia nigra, tyrosine hydroxylase.

Erythropoietin (EPO) has recently been demonstrated to provide neuroprotection of a variety of neurons including nigral dopaminergic neurons against experimental insults (Csete et al., 2004; Demers et al., 2005; Genc et al., 2001, 2002, 2004; Kanaan et al., 2006; McLeod et al., 2006; Signore et al., 2006). However, the mechanisms underlying EPO neuroprotection are largely unknown. EPO is naturally produced by fetal liver and adult kidney, and it can stimulate erythropoiesis in the bone marrow in response to hypoxia. For more than a decade, EPO has been used clinically in treating anemia resulting from chronic renal failure or from cancer chemotherapy. It has been shown that EPO protein and EPO receptors exist in brain neurons (Csete et al., 2004; Morishita et al., 1997). In response to hypoxia or ischemia, both the levels of EPO protein and expression of EPO receptors were reported to be upregulated (Chin et al., 2000; Liu et al., 2005), suggesting a close relationship between EPO and brain repair. Indeed, recombinant human erythropoietin (rhEPO) has been shown to protect cultured neurons from hypoxia (Lewczuk et al., 2000; Liu et al., 2006; Meloni et al., 2006; Siren et al., 2001), oxygen glucose deprivation-induced ischemia (Ruscher et al., 2002), glutamate (Morishita et al., 1997) and nitric oxide (NO) (Yamasaki et al., 2005) excitotoxicity. As rhEPO can cross the blood–brain barrier (Brines et al., 2000), systemic administration of rhEPO has been reported to reduce neuronal injury in animal models of focal or global ischemic stroke (Wang et al., 2004; Wei et al., 2006; Zhang et al., 2006), traumatic brain injury (Lu et al., 2005), and spinal cord injury (Celik et al., 2002).

rhEPO has also been found to protect dopaminergic neurons from hypoxia–ischemia (Demers et al., 2005), 6-hydroxydopamine (6-OHDA) lesioning *in vitro* (Csete et al., 2004; Signore et al., 2006) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Genc et al., 2001) in a mouse model of Parkinson's disease (PD). Using a 6-OHDA-induced mouse model of PD, Signore et al. (2006) showed that rhEPO prevented the loss of nigral dopaminergic neurons and maintained striatal catecholamine levels, resulting in significantly reduced rotational asymmetry. Recent studies have also shown that rhEPO can increase the survival of nigral grafts and improve graft function in a rat model of PD (Kanaan et al., 2006; McLeod et al., 2006). It has been suggested that EPO may exert its neuroprotective effects through multiple mechanisms in-

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Abbreviations: Alb, albumin; ANOVA, factor analysis of variance; CR3, complement receptor type 3; DAT, dopamine transporter; EPO, erythropoietin; GFAP, glial fibrillary acidic protein; IL-1β, interleukin-1beta; IR, immunoreactive; MHC, major histocompatibility complex; MPTP, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine; MTN, medial terminal nucleus of the accessory optic tract; NO, nitric oxide; PBS, phosphate-buffered solution; PD, Parkinson's disease; rhEPO, recombinant human erythropoietin; ROS, reactive oxygen species; SN, substantia nigra; TH, tyrosine hydroxylase; TNF-α, tumor necrosis factor-alpha; 6-OHDA, 6-hydroxydopamine.

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cluding anti-apoptosis (Siren et al., 2001; Signore et al., 2006), anti-inflammation (Villa et al., 2003), inhibition of glutamate release, reactive oxygen species (ROS) formation (Liu et al., 2006), activation of Akt/protein kinase B (PKB) via the phosphoinositide 3-kinase pathway (Signore et al., 2006), and activation of Janus kinase-2 (Jak2) and nuclear factor-kappaB (NF-κB) signaling pathways (Digicaylioglu and Lipton 2001).

Inflammation has recently been implicated as a critical mechanism responsible for the progressive neurodegeneration in PD (Block and Hong, 2005). Microglia, the resident innate immune cells play a major role in the inflammatory process in the brain. Activated microglia release various pro-inflammatory cytokines (interleukin-1β, IL-1β; tumor necrosis factor-α, TNF-α; interleukin-6, IL-6), NO, and superoxide, which can have deleterious effects on neurons (Wu et al., 2002). The phagocytosis by activated microglia removes cell debris, but it can also damage neighboring intact neurons. Numerous studies have demonstrated that the microglial response in experimental models of PD contributes to the degeneration of dopaminergic neurons (Cicchetti et al., 2002; Depino et al., 2003; He et al., 2001). By using minocycline, an anti-inflammation agent, He et al. (2001) demonstrated that the inhibition of microglial activation led to the protection of nigral dopaminergic neurons from 6-OHDA neurotoxicity in a mouse model of PD. This study highlighted that inflammation is a significant component of dopaminergic neuron degeneration. rhEPO was found to dramatically attenuate microglial and astrocytic activation, and selectively reduce pro-inflammatory cytokines following cerebral ischemia (Villa et al., 2003). Anti-inflammatory properties of EPO may be involved in attenuating toxic effects of 6-OHDA on dopaminergic neurons.

In the present study, we examined rhEPO neuroprotective effects in a 6-OHDA-induced rat model of PD. The study was designed to address the following questions: 1) whether intrastriatal administration of rhEPO could protect dopaminergic neurons and lead to the improvement of more complex behavioral responses such as paw reaching; 2) whether systemic administration of rhEPO could also protect dopaminergic neurons from 6-OHDA-induced neurotoxicity; 3) whether rhEPO elicited its neuroprotection via an anti-inflammatory mechanism. The neuroprotective effects of rhEPO were determined by counting dopaminergic neurons in the substantia nigra (SN), by measuring dopamine levels in the striatum, and by testing motor behavior. Inflammation in the striatum and the SN was also examined by assessing the levels of major histocompatibility complex (MHC) class I and class II antigen expression and infiltration of activated microglia and astrocytes.

EXPERIMENTAL PROCEDURES

Experimental design

Young adult female Sprague–Dawley rats (Charles River Laboratories, Inc., Wilmington, MA, USA), weighing 225–250 g at the beginning of this experiment, were housed under a 12-h light/dark cycle with *ad libitum* access to food and water in the Animal Core Facility of LSU Health Sciences Center, Shreveport. All animal procedures were done following the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Use and Care Committee of LSU Health Sciences Center. The number of animals used was the minimum required for statistical analysis, and all precautions were taken to minimize animal suffering.

A total of 72 rats were used in three experiments. Experiment 1: To examine rhEPO neuroprotective effects on dopaminergic neurons in a rat model of PD, five groups of rats were assigned. Group 1 (n=7, denoted as EPO+6-OHDA): rats received an injection of rhEPO (EPOGEN, Amgen, Inc., Thousand Oaks, CA, USA) into the right striatum and then an injection of 6-OHDA (Sigma-Aldrich, St. Louis, MO, USA) in the same side 1 day later. Group 2 (n=7, 6-OHDA): rats received only striatal injection of 6-OHDA. Group 3 (n=7, albumin (Alb)+6-OHDA): rats received an injection of the same volume of solution containing 0.25% human Alb (ZLB Behring AG, Berne, Switzerland) into the striatum and an injection of 6-OHDA in the same side 1 day later. These rats served as a vehicle control since rhEPO solution contains 0.25% human Alb. Group 4 (n=7, saline+6-OHDA): rats received an injection of the same volume of saline into the striatum prior to 6-OHDA injection. Group 5 (n=8, EPO i.p.+6-OHDA): rats received an i.p. injection of rhEPO (5000 units/kg) daily for 8 days. Three days after the systemic administration of rhEPO, rats also received an injection of 6-OHDA in the striatum. At 3 and 10 weeks after the 6-OHDA lesion, rotational asymmetry induced by *d*-amphetamine (Sigma-Aldrich) was tested. At 10 weeks, paw-reaching behavior was also tested. At the end of the experiment, all rats were killed and brain sections were prepared for tyrosine hydroxylase (TH) immunocytochemistry. The number of TH-immunoreactive (IR) cells was counted in the SN and the optical density of TH-IR fibers was evaluated in the striatum. Experiment 2: To examine local inflammation induced by rhEPO and human Alb in the striatum, three groups of rats only received rhEPO (n=4), human Alb (n=4) or saline (n=4). Injected rats were killed 1 day later and brain sections were prepared for complement receptor type 3 (CR3, a cell surface marker for microglia and macrophages) and glial fibrillary acidic protein (GFAP, a marker for astrocytes) immunocytochemistry. The accumulation of activated astrocytes, microglia and macrophages in the injected striatum was rated in a semi-quantitative fashion. In addition, brain sections from the 12 rats were also processed for dopamine transporter (DAT) immunocytochemistry to determine if rhEPO affects DAT activity in the dopaminergic terminals in the striatum, thereby blocking the uptake of 6-OHDA by dopaminergic neurons. Experiment 3: To examine if rhEPO exerted its neuroprotective effects on dopaminergic neurons via an anti-inflammation mechanism, 24 rats were assigned into the three groups: EPO+6-OHDA (n=8), Alb+6-OHDA (n=8) and Saline+6OHDA (n=8). The rats in each group were further divided into 4-day and 2-week subgroups. Brain sections were prepared for MHC class I and class II, CR3, and GFAP immunocytochemistry. The expression of MHC class I and class II antigens, and the infiltration of activated astrocytes, microglia and macrophages in the injected striatum and the SN were rated in a semi-quantitative fashion.

rhEPO injections

For the intrastriatal injections, 20 units of rhEPO, dissolved in 2 μl of vehicle, were injected unilaterally into the striatum of equithesin (3 ml/kg, i.p.) -anesthetized rats fixed in a Kopf stereotaxic frame using a 10 μl Hamilton microsyringe (Hamilton Co., Reno, NV, USA) fitted with a steel cannula. The dose of rhEPO was chosen based on a previous study (Genc et al., 2001). Injections were made at the following stereotaxic coordinates: 1.0 mm rostral to bregma; 3.0 mm lateral to the midline; 4.5 mm ventral to the dura; with tooth bar set up at zero. The microinjections were carried out at a rate of 0.25 μl/min. After injection, the cannula remained *in situ* for additional 4 min before withdrawn. For systemic administration, rhEPO (5000 units/kg, body weight) was intraperitoneally