Increased Expression of Erythropoietin Receptor in 1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine-induced Parkinsonian model

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Increased Expression of EPOR in MPTP-induced Parkinsonian model

# Equal Contribution

Summary

Erythropoietin (EPO), known for its role in erythroid differentiation, has been suggested have a direct protective role against a variety of neurotoxic insults. In the present study, we investigated the expression of EPO receptor (EPOR) and the number of EPOR positive cells in 3 encephalic regions (ventral mesencephalon, striatum, cortex) following 1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-lesion. C57BL/6 mice underwent intraperitoneal injection of MPTP at 24 h intervals for 5 days, and their brains were examined 1 day, 2days, 4days, 7days, 14days or 21 days after the last injection. Western blot and immunohistochemistry analysis revealed that EPOR was dramatically up-regulated in the ventral mesencephalon, 4d after MPTP insult until the 21d. In contrast, there was a baseline level of EPOR in the striatum and cortex. At subsequent time points after MPTP injury, the levels of EPOR in the two regions were not statistically different compared with those in normal animals. These results suggest that the regional specific up-regulation of EPOR at an early stage after MPTP stimulus may represent a pro-survival mechanism against neurotoxin injury in Parkinsonian model.

Keywords: EPOR; MPTP; C57BL/6 mice; Parkinson disease; ventral mesencephalon

1. Introduction

Erythropoietin (EPO) is a cytokine that acts in erythroid progenitor proliferation and differentiation. Research during the past years has clearly demonstrated that EPO is a potent promoter of neuronal survival. Studies in vitro provided most of the information related to the molecular pathways involved in EPO action. These data showed that EPO

might have a direct protective role against a variety of neurotoxic insults, such as hypoxic conditions (Marti et al., 1996), glutamate toxicity (Morishita et al., 1997), free-radical injury (Chong et al., 2003), and exposure to neurotoxicants (Villa et al., 2003; Shang et al., 2007). We have demonstrated that EPO substantially reduced 1-methyl-4-phenylpyridinium-induced cellular death in PC12 cells via its characteristics of an anti-oxidant and anti-apoptotic (Wu et al., 2007a; Wu et al., 2007b).

EPO exerts its neuroprotective function by binding with its receptor (EPOR), a cytokine belonging type I superfamily. In concert with the strong empirical basis for EPO's neuroprotective mechanisms, ample evidence suggests that the endogenous EPO and EPOR system is involved in neuronal protection. The abundant expression of EPOR in the central nervous system (Bernaudin et al., 1999; Juul et al., 1999; Siren et al., 2001) and the up-regulation of EPOR by several pathological conditions (Spandou et al., 2004; Grasso et al., 2005) suggest that this receptor is an important mediator of the brain's response to injury. But it is unknown whether its expression persists in the pathogenesis of neurodegenerative diseases such as Parkinson disease (PD) and whether it has a physiological role in the ventral mesencephalon.

Because the expression of the local EPOR is sparsely investigated, the aim of this study was to elucidate the diversity of the EPOR expression and its time course in a model of PD in C57BL/6 mice.

2. Methods

2.1. Animals and MPTP administration

Eight-week-old male C57BL/6 mice (Experimental Animal Center, Institute of organ

transplantation, Tongji Medical College), weighing 20-25 g, were used in all the experiments. The following animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (1985), NIH, Bethesda. The mice were group-housed (five mice per cage with five compartments) in a room maintained at 20–22 ℃ on a 12-h light–dark cycle with food and water available ad libitum. At the beginning of the experiment, the mice (5-7 per group) received an intraperitoneal (i.p.) injection of MPTP-HCl (30 mg/kg of free base; Sigma, St. Louis, MO, USA) in saline at 24 h intervals for 5 days (Dehmer et al., 2000; Hayley et al., 2004). The control group was injected with saline only at the same day. The mice were sacrificed at 1, 2, 4, 7, 14 and 21 days after the last MPTP or saline injection.

2.2. Western blot analysis

The procedure used to dissect the brain regions in mice for the MPTP studies can be performed step-by-step in accordance with supplementary information（Jackson-Lewis et al,. 2007）. Following treatment, the mice were decapitated and the ventral midbrain(consisting of the SN, VTA and some tissue immediately surrounding the area, excluding the basis pedunculi) , striatum and frontal cortex were rapidly dissected and homogenized in ice cold-lysis buffer (50 mmol/l Tris–HCl, pH 8.0, 100 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 1%Triton X-100, 0.1% SDS, 50 mmol/l sodium fluoride and 1 mmol/l sodium vanadate) containing a protease inhibitor cocktail to obtain whole cell protein. Lysates were cleared by centrifugation and protein concentration was determined by BCA kit. Equal amounts of proteins were fractionated by SDS-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane.

The membranes were blocked with 5% defatted milk in TBS-Tween (TBS-T) (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween-20) and incubated with anti-EPOR rabbit polyclonal antibodies (at 1:3000 dilution, Santa Cruz Biotechnology) overnight at 4 °C. The signals were detected using goat anti-rabbit horseradish peroxidase conjugated secondary antibody and enhanced chemiluminescence (ECL), then exposed to X-ray films (Fuji, Japan). The protein bands were quantified by densitometry. The densitometry values for the proteins of interest were corrected for protein loading using β-actin.

2.3. Immunohistochemistry

Animals were deeply anesthetized with sodium pentobarbital (120 mg/kg) and perfused transcardially with saline containing 0.5% sodium nitrite and 1000 U/100 ml heparin sulfate, followed by cold 4% formaldehyde generated from paraformaldehyde in 0.1 M sodium phosphate buffer (PBS; pH 7.2). The brains were post-fixed in the same solution for 1 h, embedded in paraffin, and processed for histological studies. Five micrometer thick sections were cut with a microtome, deparaffinized in xylene, and rehydrated in a graded ethanol series. Slides were steamed in 0.01 mol/L of sodium citrate buffer, pH 6, for 10 minutes in a microwave oven. Endogenous peroxidase activity was quenched by exposing the slides to 0.3% hydrogen peroxide in methanol for 15 minutes, after which the slides were rinsed in phosphate-buffered saline for a total of 10 minutes. Sections were washed in PBS containing 0.5% normal goat serum for 30 min and incubated overnight at 4°C with anti-EPOR rabbit polyclonal antibodies (1:200; Sigma, Santa Cruz Biotechnology). Slides were rinsed three times in phosphate-buffered saline for 10 minutes and incubated for 30 minutes with the specific biotinylated secondary antibody

(Vector Laboratories, Burlingame, CA). The slides were washed in phosphate-buffered saline and incubated for 30 minutes with the avidin-biotin complex (Vector Laboratories); they were then developed with 3, 3'-diaminobenzidine for 5 minutes. Sections were mounted on gelatin coated slides, dehydrated through graded ethanols, cleared in xylene and coverslipped with permount. Slides were examined microscopically to assess the EPOR immunoreactivity. All specimens were compared with negative controls performed using normal immunoglobulin G or phosphate-buffered saline as a primary antibody. An investigator blinded to the experimental groups from which each sample was obtained conducted this study. Data are calculated as the ratio of the number of EPOR–positive cells in the MPTP-treatment groups with that in the control group.

2.4. Statistical analysis

Data are expressed as the mean ± S.D. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's test for multiple comparisons. A probability of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Time-related increased EPOR expression in ventral mesencephalon

To analyze the effect of MPTP, EPOR protein level was examined by Western blot. In control mice, there was a baseline expression of EPOR in the ventral mesencephalon. MPTP itself, 1d later, did not produce any significant changes in expression of EPOR protein in this model (Fig. 1). EPOR protein level was increased compared to control value at 2d after MPTP treatment, although this difference was not statistical significant. In

contrast, 4d after MPTP insult, EPOR expression in the ventral mesencephalon increased 1.9-fold, indicating an up-regulation of this receptor ($P<0.05$, Fig. 1). 7 days after the last MPTP administration, EPOR in the ventral mesencephalon was even more increased compared to control value ($P<0.01$), and the tendency of accrescence last to the 21 day. This finding indicates that the main response of the C57BL/6 mice brain to MPTP is the up-regulation of the EPOR.

3.2 Time-related increased number of EPOR-immunostained cells in substantia nigra

1d and 2d after the last MPTP treatment, the number of EPOR–immunostained cells in substantia nigra was a little slightly increased compared with the observation made on normal animals. Immunohistochemistry results revealed that the number of EPOR positive cells was dramatically up-regulated in the substantia nigra, 4d after MPTP insult until the 21d (Fig. 2), the difference was statistical significant ($P<0.05$).

3.3. EPOR expression and the number of EPOR-positive cells in other encephalic regions

In control mice, there was a baseline expression of EPOR in the striatum. MPTP itself produced slight changes in expression of EPOR protein in this model (Fig. 3), but the difference was not statistical significant ($P>0.05$).

1d, 2d, 4d, 7d, 14d, and 21d after the last trauma, the numbers of EPOR positive cells in the striatum were observed. The histological sections revealed no statistical differences in EPOR positivity compared with the findings observed in normal animals ($P>0.05$) (Fig.4).

The tendency of EPOR expression in cortex was similar with that in striatum. One day after the last MTPT treatment, the EPOR expression in cortex were not statistically different compared with those in animals that did not undergo MPTP insult ($P>0.05$) (Fig. 5).These findings were unchanged during subsequent time points.

At subsequent time points after MPTP injury, the numbers of EPOR-immunostained cells in cortex were not statistical differences compared with those in animals that did not undergo MPTP insult ($P>0.05$) (Fig. 6).

4. Discussion

Despite a large body of evidence demonstrating that exogenous EPO administered in its recombinant form exerts neurotrophic and neuroprotective actions, the precise physiological role of EPOR in the CNS remains an open question. The presence of EPOR on adult dopaminergic neurons leads to the speculation that they might play a regulatory role in both neuroprotection and brain homeostasis of the pathogenesis in PD (Csete et al., 2004). The present study shows that EPOR is ubiquitously expressed in different encephalic region of C57BL/6 mice. Protein expression of EPOR in ventral mesencephalon and the number of EPOR positive cells in substantia nigra increased significantly, in mice after MPTP lesion, whereas, the expression of the same receptor have no change in the striatum and cortex. This is the first study to report the expression of EPOR in three brain regions in an animal model of PD.

A rapidly expanding body of literature attests to the fact that, distinct from their role in erythropoiesis, EPO-EPOR interactions confer significant cytoprotection to a host of neuronal and non-neuronal cell types within the mammalian CNS (Noguchi et al., 2007).

Antioxidant, antiapoptotic, anti-inflammatory, neurotrophic, angiogenic, and synaptogenic activities have been implicated as potentially important mechanisms mediating EPO-related neuroprotection (Noguchi et al., 2007). Particularly germane to the present study are recent reports that EPO potently protects rodent dopaminergic neurons from MPTP toxicity (Genc et al., 2001; Genc et al., 2002; Puskovic et al., 2006).

It has been hypothesized that EPOR expression in different type of cells in brain tissue parallels the occurrence of cellular responses associated with brain damage progression. Although weak EPOR expression in the brain under normal conditions, this receptor often serve as a protective mechanism under stress or many pathophysiological conditions. Previous studies have suggested that EPOR expression may be changed in different insults processes. Expression of EPOR can be induced by hypoxia in immature and mature brains (Juul et al., 1999; Lewczuk et al., 2000). Increased expression of EPOR has been also reported following ischemia in rodent and human brain (Brines et al., 2000; Sirén et al., 2001). In the model of compressive spinal cord injury of rats, a marked augment expression of EPOR was observed in neurons, vascular endothelium, and glial cells after injury (Grasso et al., 2005). EPOR on blood vessels in the human epileptogenic hippocampus (Eid et al., 2004) was strongly immunoreactive and EPOR was found to be increased in the hippocampus after status epilepticus (Chu et al., 2008). EPOR is also up-regulated in the hippocampus and temporal cortex of persons with sporadic Alzheimer's disease and mild cognitive impairment (Assaraf et al., 2007).

Parkinson’s disease is a debilitating neurological disorder, characterized by loss of dopaminergic neurons located in the substantia nigra pars compacta. 1-Methyl-4-phenyl-1,

2, 3, 6-tetrahydropyridine (MPTP), a neurotoxin that selectively damages dopaminergic cells in the substantia nigra, has been used to generate an animal model of PD （Langston et al.,1983; Heikkila et al., 1984）. The resultant functional change of the pathogenesis probably produces adaptive changes in some protein. Especially, the accrescence of EPOR expression in the ventral mesencephalon, an area particularly susceptible to MPTP-related damage, suggests that this up-regulation of expression of EPOR can be interpreted as the consequence of the self-protective, compensatory reaction of the body to exogenous or endogenous impairments.

The results from our current study suggest that the induction of EPOR may contribute to the ongoing pathogenesis, and raise the exciting possibility that EPOR in the ventral mesencephalon, could provide a target to the treatment of PD by the use of EPO. Although further studies on functional features of EPOR and the time courses of EPOR expression in neurons and glia respectively are needed, the data in the present study provided insights into neurontoxin-related changes in trophic support as well as basic knowledge about PD.

Acknowledgements

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Figure legends

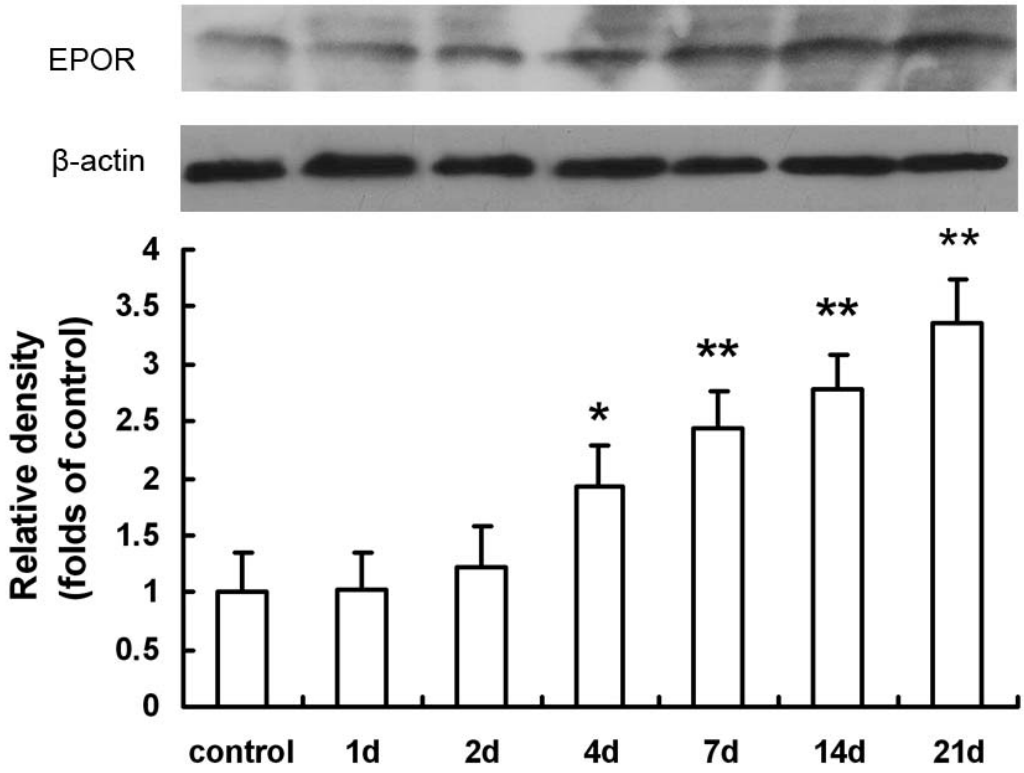

Fig1. Western blot analysis of EPOR in ventral mesencephalon from controls and MPTP-treated mouse. The top panel shows time course of EPO-R protein expression. EPO-R was detected in the normal ventral mesencephalon and increased after the last MPTP treatment. The bottom panel shows densitometric analysis of three separate experiments. Densitometric analysis demonstrated a significant increase by 4d (*****, $P<$ 0.05 vs. control; ******, $P<0.01$ vs. control).

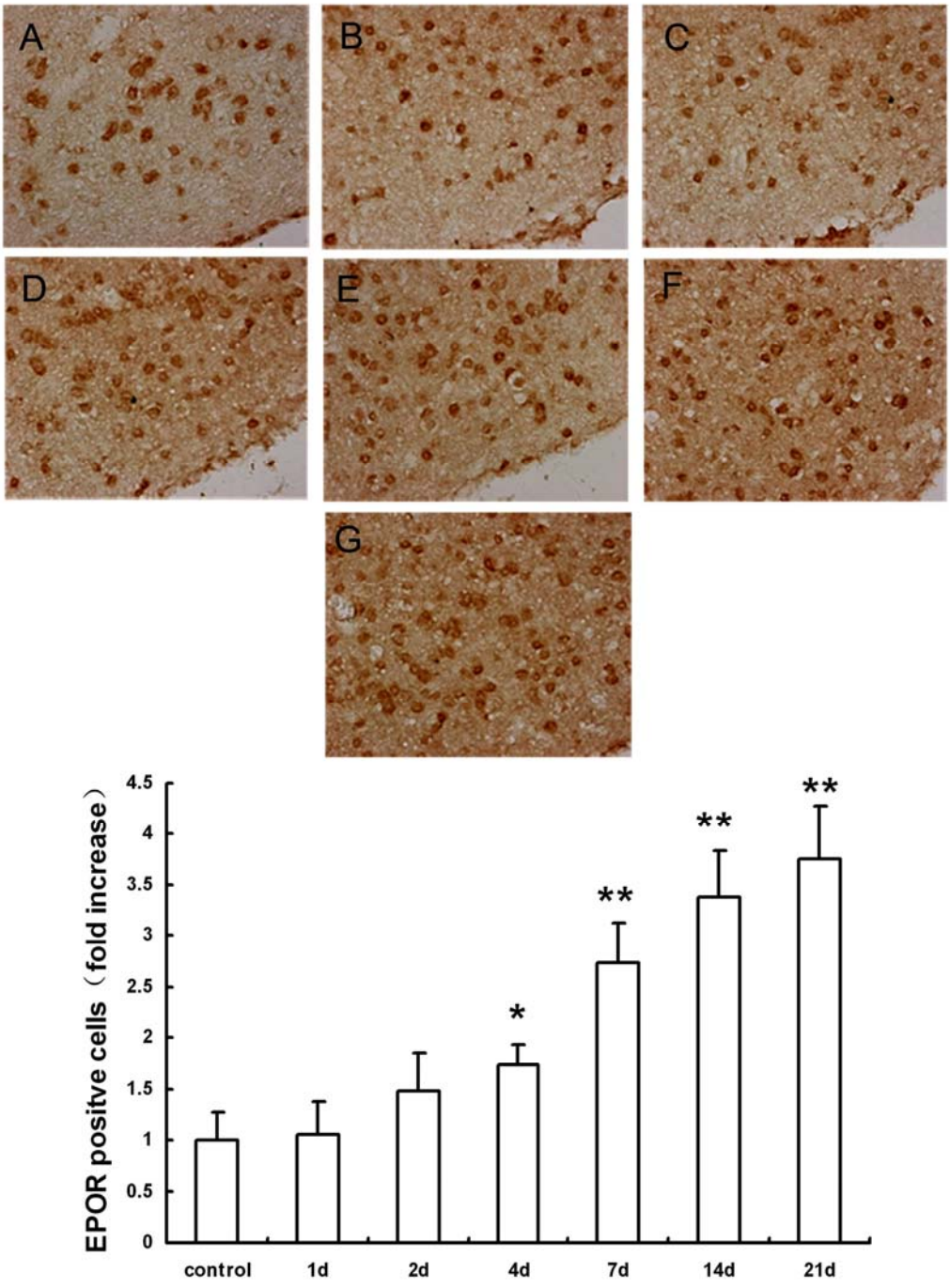

Fig 2. Photomicrographs representing EPOR immunoreactivity in ventral mesencephalon. Compared EPO-R expression in the ventral mesencephalon at control (A), 1 (B), 2 (C), 4 (D), 7(E), 14 (F) and 21 days (G) after the last MPTP treatment in C57BL/6 mice, immunohistochemistry section revealed that the number of EPOR positive cells was dramatically up-regulated in the ventral mesencephalon, 4d after MPTP insult until the

21d(*****, $P<0.05$ vs. control; ******, $P<0.01$ vs. control)..

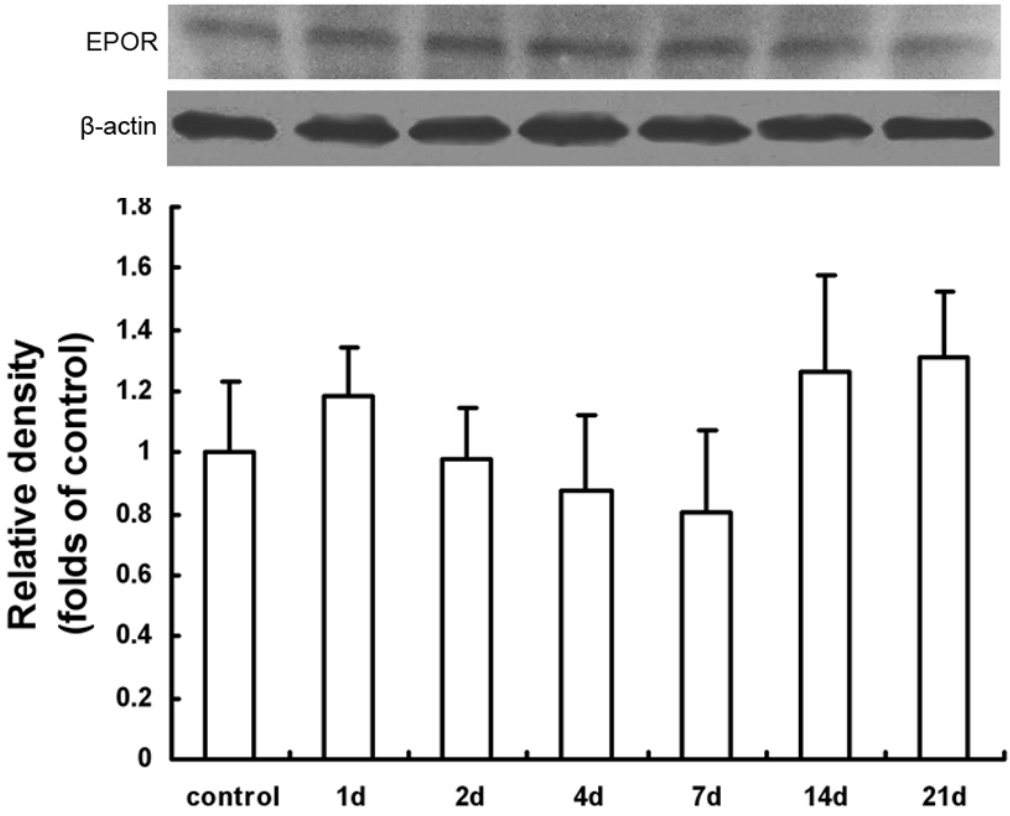

Fig 3. Western blot analysis of EPOR in striatum from controls and MPTP-treated mouse. The top panel shows Western blot analysis of EOPR of striatum from controls and MPTP-treated mouse. The bottom panel shows densitometric analysis of three separate experiments. Statistical analysis revealed no significant differences in EPO-R expression compared with the findings observed in controls.

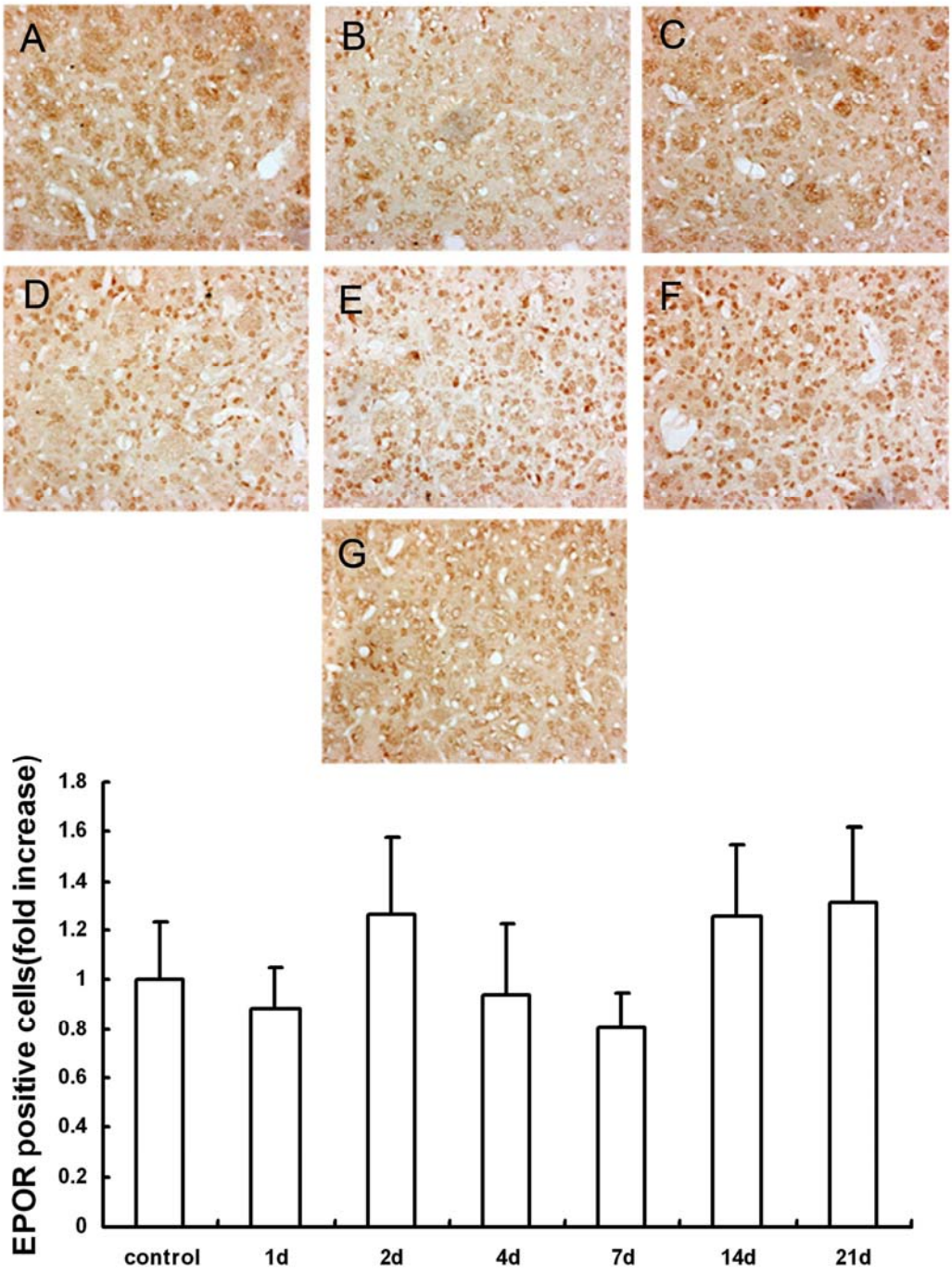

Fig 4. Photomicrographs representing EPOR immunoreactivity in striatum. 1d(B), 2d (C), 4d (D), 7d(E), 14d (F), and 21d (G) after the last MPTP treatment in C57BL/6 mice, the numbers of EPOR positive cells in the striatum were observed. The histological sections revealed no significant differences in EPOR positivity compared with the findings observed in normal animals (A).

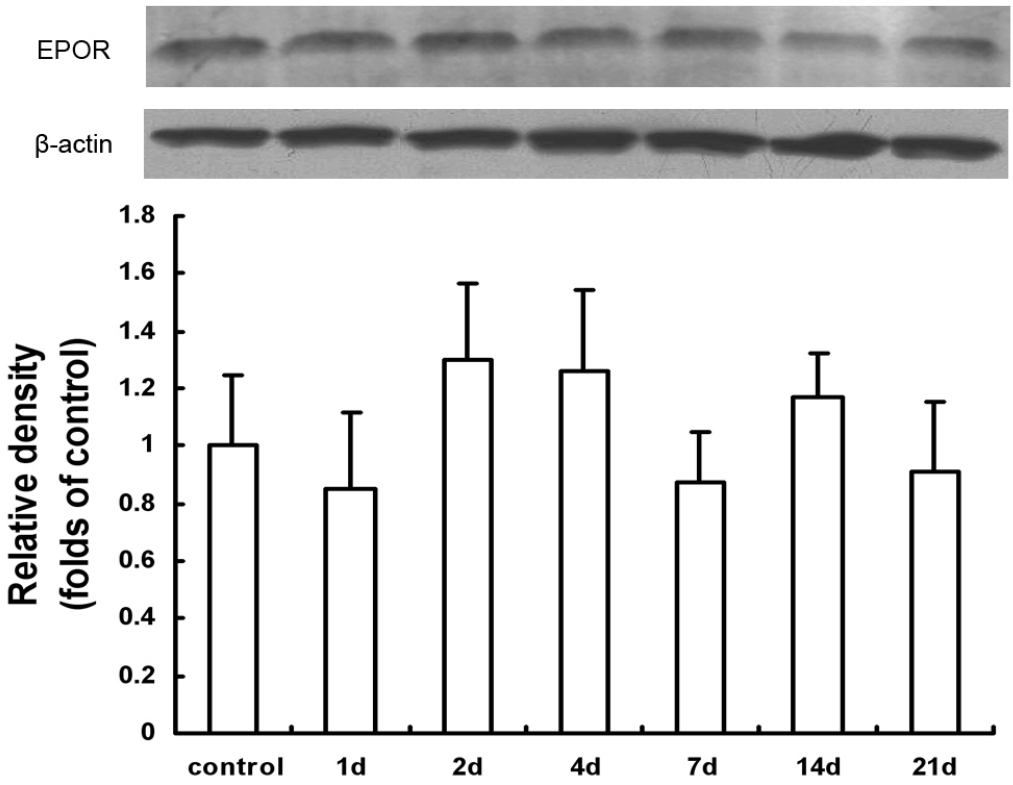

Fig 5. Western blot analysis of EPOR in cortex from controls and MPTP-treated mouse. The top panel shows Western blot analysis of EOPR of cortex from controls and MPTP-treated mouse. The bottom panel shows densitometric analysis of three separate experiments. Statistical analysis revealed no significant differences in EPO-R expression compared with the findings observed in controls.

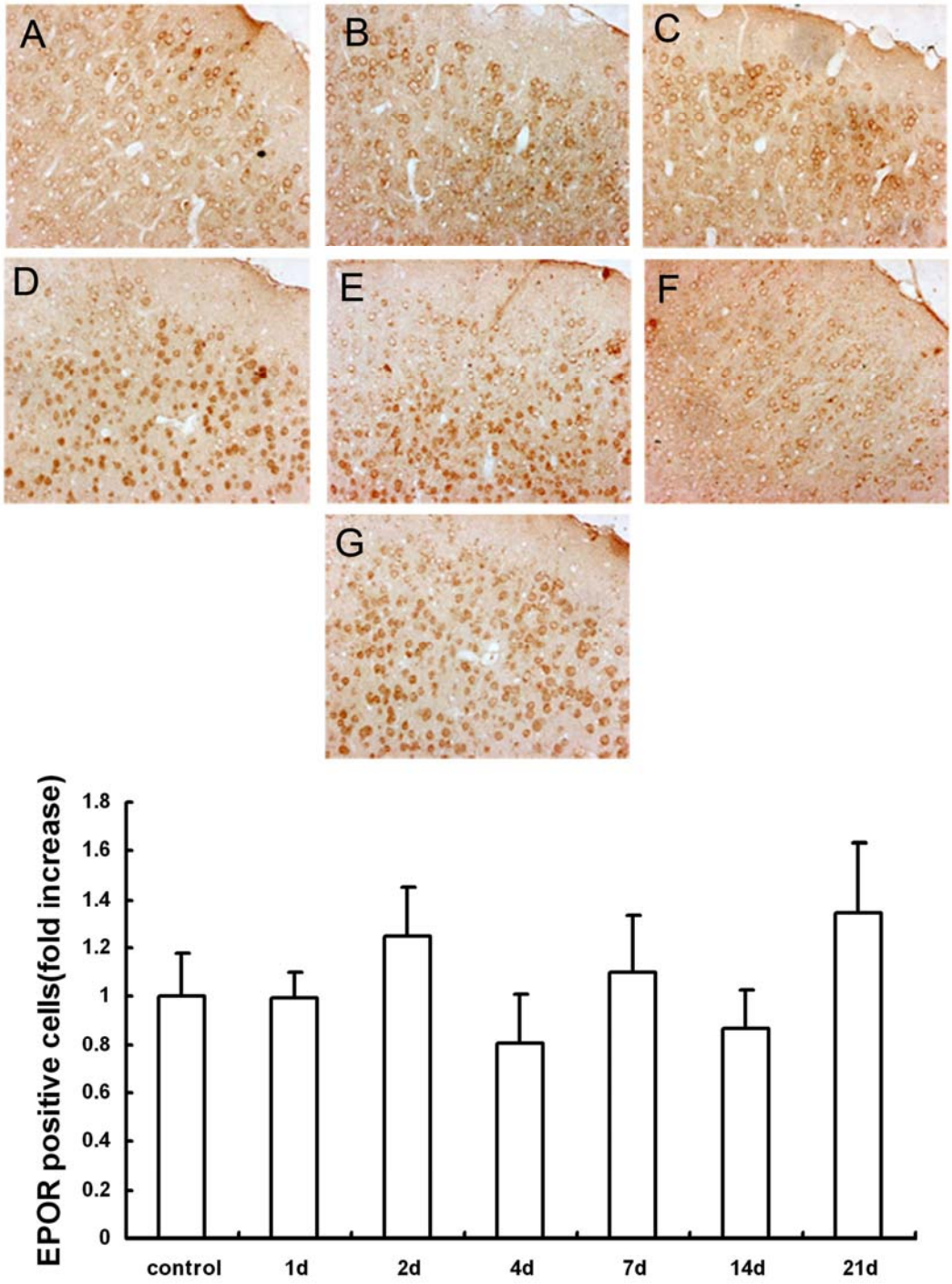

Fig 6. Photomicrographs representing EPOR immunoreactivity in cortex. 1d (B), 2d (C), 4d (D), 7d (E), 14d (F), and 21d (G) after the last MPTP treatment in C57BL/6 mice, the numbers of EPOR positive cells in the cortex were observed. The histological sections revealed no significant differences in EPOR positivity compared with the findings observed in normal animals (A).