Turkish Journal of Geriatrics
2006; 9 (1): 13-18

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RESEARCH

ERYTHROPOIETIN DECREASES APOPTOSIS INDUCED BY 6-HYDROXYDOPAMINE IN PC12 CELLS

ABSTRACT

Aim: In the present study, we investigated whether Erythropoietin (Epo) has a protective effect against cytotoxicity and apoptotic cell death induced by 6-hydroxydopamine (6-OHDA) in PC12 cell line cultures.

Material and Methods: Epo exerted significant protective effect against 6-OHDA-induced cell injury and apoptosis as confirmed by lactate dehydrogenase cytotoxicity assay, anti-single strand DNA immunostaining and annexin-V staining.

Results: Epo significantly decreased 6-OHDA-induced DP5 pro-apoptotic protein mRNA expression and increased bcl-XL anti-apoptotic protein mRNA expression. Any effect of Epo on CREB transcription factor phosphorylation was not observed.

Conclusion: These results suggest that Epo exerts neuroprotective effect against 6-OHDA-induced cell death at least partially via the differential regulation of the expression of genes involved in the apoptotic process.

Key words: Erythropoietin; Apoptosis; PC12 cell line; 6-hydroxydopamine; DP5; CREB; Parkinson's disease

ARAŞTIRMA

ERİTROPOETİN PC12 HÜCRELERİNDE 6-HİDROKSİDOPAMİN İLE İNDÜKLENEN APOPTOZU AZALTMAKTADIR

ÖZ

Amaç: Sunduğumuz çalışmamızda, PC12 hücre hattında 6-hidroksidopamin(6-OHDA)'nin oluşturduğu hücre toksisitesine ve apoptotik hücre ölümüne karşı Eritropoetin(Epo)'in etkisi olup olmadığı araştırıldı.

Gereç ve Yöntem: Epo'nun 6-OHDA ile indüklenen hücre hasarına ve apoptozise karşı belirgin protektif etki gösterdiği laktat dehidrogenaz hücre toksisite analizi, tek iplikli DNA'ya karşı antikorla immunhistokimya ve Aneksin V boyaması ile değerlendirildi.

Bulgular: Epo 6-OHDA ile indüklenen DP5 pro-apoptotik proteininin mRNA artışını azaltmakta ve bcl-XL anti-apoptotik proteininin mRNA ekspresyonunu arttırmaktadır. Epo'nun CREB transkiripsiyon faktörünün fosforilasyonuna etkisi gözlenmemiştir.

Sonuç: Bu sonuçlar 6-OHDA ile indüklenen hücre ölümünde Epo'nun nöroprotektif etkisini kısmen apoptotik yoldaki gen ekspresyonlarını regüle ederek yaptığını düşündürmektedir.

Anahtar sözcükler: Apoptoz, PC12 hücre hattı, 6-hidroksidopamin, DP5, CREB, Parkinson hastalığı

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Geliş Tarihi: 30/10/2005
(Received)

Kabul Tarihi: 20/12/2005
(Accepted)

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INTRODUCTION

One of the neurotoxins classically used to create animal models of Parkinson's disease (PD) is 6-hydroxydopamine (6-OHDA) (1). In vitro, this neurotoxin induces apoptosis of various catecholaminergic cell lines (2,3,4). Determining the agents that may prevent neuronal death induced by 6-OHDA in vitro might contribute to establish effective treatment strategies for PD. A candidate agent that can offer neuroprotection against 6-OHDA-induced injury may be erythropoietin (Epo). Many in vitro and in vivo studies have demonstrated that Epo has neuroprotective effects against various insults such as hypoxia, ischemia, trauma and inflammation (reviewed in 5). In the mouse model of PD induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, Epo is neuroprotective when given directly by stereotaxis into the brain (6). Epo promotes dopaminergic differentation from CNS stem cells in vitro (7). However, the in vitro effect of Epo on 6-OHDA toxicity has never been investigated. Therefore, we investigated whether Epo has protective effect against cytotoxicity and apoptotic cell death induced by 6-OHDA in PC12 cell line cultures. The possible modulatory effect of Epo on 6-OHDA-induced pro-apoptotic and anti-apoptotic gene expression alterations and CREB phosphorylation was also evaluated.

MATERIALS AND METHODS

PC12 cells were grown in RPMI 1640 supplemented with 10% horse serum (HS), 5% fetal bovine serum (FBS), 50 units penicillin/ml and 100 mg streptomycin/ml. Cells were incubated at 37∞C in 5% CO_2 and the medium was changed every 2-3 days. After confluency, cells were removed from culture flasks by pipeting and resuspended in a complete medium containing RPMI1640 medium, 2% HS, 1% FBS, 50 units penicillin/ml and 100 mg streptomycin/ml. Cells were plated in 25 cm^2 poly-$_D$-lyzine (PDL)-coated flasks for reverse transcriptase-polymerase chain reaction (RT-PCR), 6 well culture plates for annexin-V-propidium iodide (PI) and anti-single strand DNA (ssDNA) staining assays, 96 well culture plates for lactate dehydrogenase (LDH) cytotoxicity assay and 100 mm steril petri dishes for western blotting (WB). 0.1-1.0 U/ml recombinat murine Epo (rmEpo) (Roche) was added to some cultures and incubated for 24 h. Next day 10-200 mM 6-OHDA were added to cultures and incubated 4, 10 and 24 h for RT-PCR, 24 h for cytotoxicity and apoptosis assays and 15 min for WB. No agents were added to control cultures.

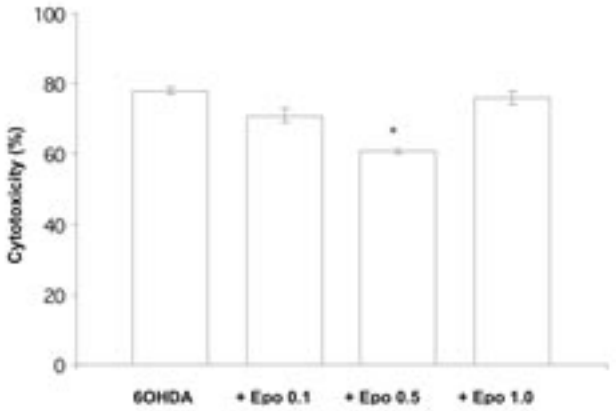
Cytotoxicity was quantified by spectrophotometrically measuring release of LDH with a commercially available kit (Cytotoxicity Detection Kit; Roche) according to the manifacturer's instructions. From each well 50 µl of the culture supernatant from each well was collected for measuring released LDH activity. The cells were then lysed by adding Triton X-100, and a second 50 µl aliquot was collected for determining intracellular LDH activity. The absorbance of the samples was measured at 490 nm using an ELISA plate reader. The reference wawelength was 620 nm. LDH release was expressed as the percentage (%) of the total LDH activity (released LDH activity in the medium plus intracellular LDH activity) per well.

To evaluate whether Epo effects apoptosis induced by 6-OHDA, anti-ssDNA immunostaining was performed according to the manifacturer's instructions (Apostain anti-ssDNA antibody; Alexis Immunochemicals) as previously described (8). After anti-ssDNA staining, nuclei were labelled with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI)-antifade (Oncor). Slides were examined under a Nikon fluorescence microscope. Negative control coverslips were stained by omitting primary antibody. The ratio of apostain positive cells to total cells was calculated by counting 100 cells in each of five randomly-selected low power fields. Annexin-V-PI staining was performed according to the manufacturer's instructions (Annexin-V-Fluos; Roche). 10 000 cells were analyzed on a flow cytometer (Becton-Dickinson). An aliquot of each sample was also evaluated by fluorescence microscopy.

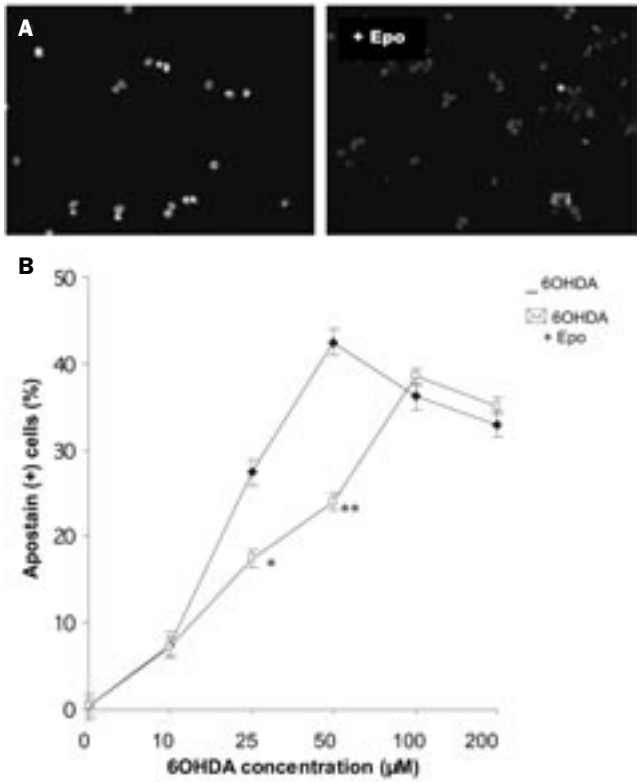
For RT-PCR analysis of bcl-2, bcl-XL, bax and DP5 mRNA expression, RNA was isolated from PC12 cells using Nucleospin RNA isolation kit (Macherey Nagel) as described by the manufacturer. cDNA was synthesized from 5 mg RNA by using M-MLV reverse transcriptase (Promega). cDNA was amplified using the rat primers for DP5, bcl-2, bcl-XL and bax [5]. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) gene was used as an internal standart. The steps of PCR amplification were 95°C for 3 min, 95°C for 30 s, 55°C 30 s, 72°C 1 min, 72°C 5 min for 32 cycles. 28 cycles were used for GAPDH. PCR products were resolved on 2% agarose gels. Optic density of each band was calculated using the Multianaylist software (1.1. version) and Densitometer GS700 (BioRad). The results are expressed by density ratios to GAPDH. WB analysis was performed as previously described (9). CREB1 and phosphorylated CREB (pCREB) protein expressions were evaluated using polyclonal rabbit anti-CREB1 (Santa Cruz) and phosphorylated CREB (pCREB) antibodies (Upstate Biotechnology).

RESULTS

Results are presented as mean ± SEM of three different separate experiments performed with separate cell cultures.



Figur 1— The protective effect of Epo pre-treatment in PC12 cells against cytotoxicity induced by 6-OHDA. The results of the LDH assay reveal that Epo added to the cultures at 0.5 U/ml concentration significantly increases cell viability after 50 µM 6-OHDA exposure for 24 h. The mean viability value in the 0.5 U/ml Epo-treated condition is significantly higher than the control condition without Epo (*p < 0.05). The results represent mean ± SEM of triplicate conditions obtained from three independent experiments. Error bars represent SEM.



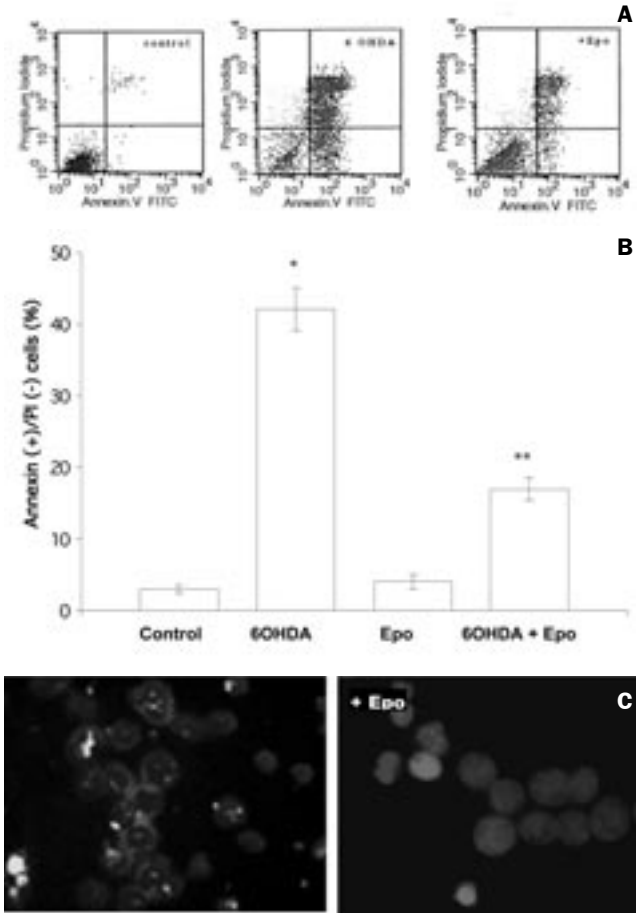
Figur 2— *A,* 6-OHDA (50 µM) exposure for 24 h induces apoptotic cell death in PC12 cells as shown by anti-ssDNA immunostaining. Apostain positive apoptotic cells stained green. DAPI counterstaining stained all nuclei blue. When compared with the without-Epo condition, Epo pre-treatment significantly decreases apoptotic cell death (1000 X). *B,* 0.5 U/ml Epo pre-treatment significantly decreases apoptotic cell death induced by 25 and 50 µM 6-OHDA exposure for 24 h (*p < 0.05; **p<0.05).

Each condition was triplicated in each experiment. Multiple group comparisons of the differences in quantitative measurements were made by ANOVA followed by *t* test was used for statistical analysis and $p < 0.05$ was considered to be significant. LDH assay revealed that pretreatment by Epo at a moderate dose (0.5 U/ml) significantly decreased cytotoxicity induced by a moderate dose of 6-OHDA (50 µM) (Figur 1). Epo treatment simultaneously with 6-OHDA exposure did not show any protection in PC12 cells, also heat-inactivated Epo did not show any effect on cell survival (data not shown). Epo could not prevent the damage caused by high dose of 6-OHDA (100 and 200 mM) (data not shown). We next examined whether the neuroprotective effect of Epo against 6-OHDA toxicity implicates an anti-apoptotic mechanism. Pretreatment with Epo (0.5 U/ml) attenuated apoptotic cell death induced by a 24 exposure to low doses of 6-OHDA (25 and 50 µM) as revealed by anti-ssDNA staining (Figur 2A and B). Epo also significantly decreased 6-OHDA-induced externalization of membrane phosphatidylserine residues as confirmed by annexin-V-PI staining (Figur 3A and B) and annexin-V-DAPI staining (Figur 3C).

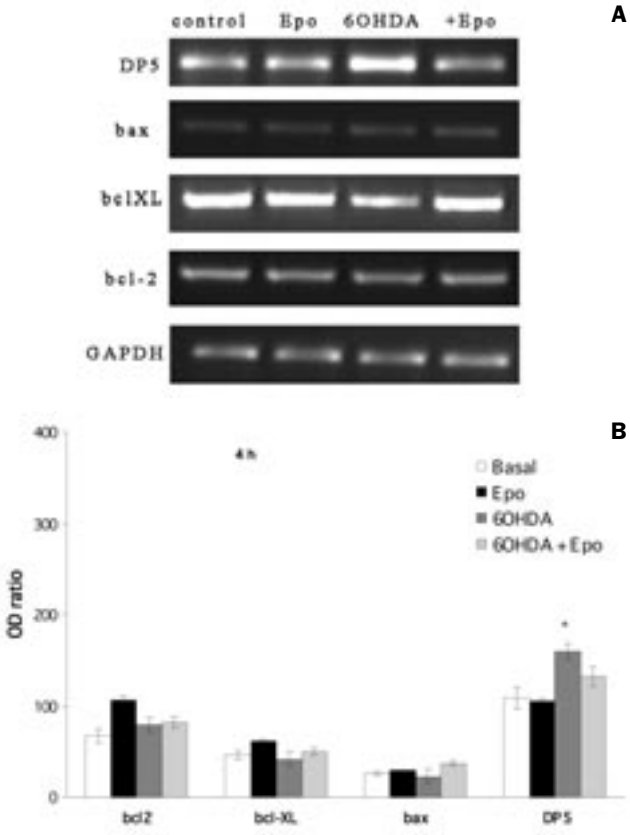
As Epo exerted anti-apoptotic action in the 6-OHDA-induced toxicity model, it was of interest to examine the effect of Epo and 6-OHDA on the expression of bcl2 family protein mRNA levels. Gene expression analysis showed that Epo decreased 6-OHDA-induced DP5 mRNA expression and increased bcl-XL mRNA expression decreased by 6-OHDA (Figur 4A-D). In contrast, there were no significant changes in bcl-2 and bax mRNA expression upon 6-OHDA and Epo exposure. We also evaluated the possible effect of 6-OHDA and Epo on the phosphorylation of transcription factor CREB and the expression of CREB1 protein. Neither Epo, nor 6-OHDA changed CREB1 protein expression. However, 6-OHDA significantly increased CREB phosphorylation in PC12 cells. No effect of Epo on CREB phosphorylation was observed (Figur 5A and B).

DISCUSSION

In the present study, the protective effect of Epo against 6-OHDA cytotoxicity was investigated in vitro using the PC12 cell line. 6-OHDA is a neurotoxin which leads to apoptosis of

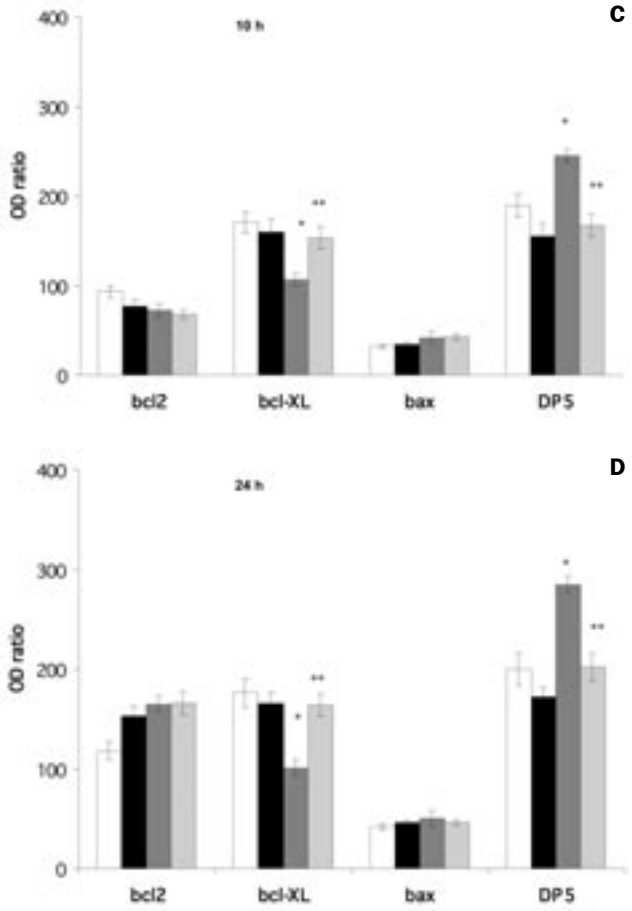


Figur 3— *A,* Representative flow cytometric analysis of 6-OHDA-induced cell death. Dot plot diagrams represent typical apoptotic and necrotic cell populations detected by annexin-V-FITC and PI staining. (a) Untreated PC12 cells. (b) PC12 cells were treated with 50 μM 6-OHDA for 24 h. (c) PC12 cells were pre-treated with 0.5 U/ml rmEpo for 24 h and then 50 μM 6-OHDA for 24 h. The lower left quadrant of each panels shows the viable (intact) cells, which exclude PI and are negative for annexin-V staining. The upper right quadrants contain the nonviable, necrotic cells, positive for annexin-V-FITC staining and for PI uptake. The lower right quadrants represent the apoptotic cells, positive for annexin-V-FITC and negative for PI. *B,* 6-OHDA exposure significantly increases annexin-V positive cells as compared with control cultures (*p < 0.05). Epo treatment (0.5 U/ml) significantly decreases the percentage of the annexin-V positive cells as compared with the 6-OHDA alone condition (**p < 0.05). *C,* 6-OHDA (50 μM) exposure for 24 h induces annexin-V staining in PC12 cells as shown by annexin-V fluos-DAPI staining. Annexin-V positive apoptotic cells stained green. DAPI counterstaining stained all nuclei blue. As compared with without-Epo condition, Epo pre-treatment significantly decreases annexin-V positive cells (1000 X).

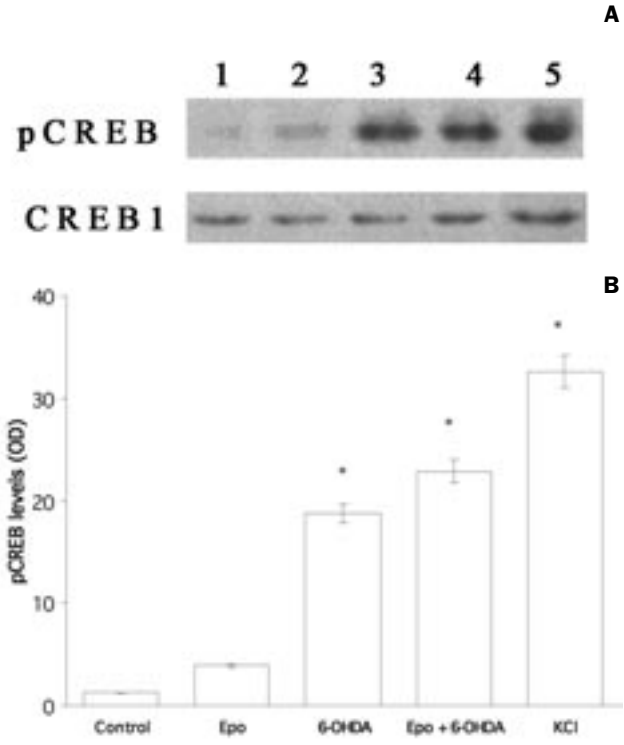


Figur 4— Semiquantitative time course analysis of mRNA levels of the members of bcl-2 family proteins in three study groups. Representative gel pictures are given in A. Effects of rmEpo (0.5 U/ml) applied for 24 h before 6-OHDA (50 μM) on the expression of bcl-2, bcl-XL, bax and DP5 mRNA expression were evaluated at 4 h (B), 10 h (A and C), and 24 h (D) after 6-OHDA exposure. The expression levels of the individual mRNAs were normalized to GAPDH mRNA expression by densitometric evaluation and presented as optic density (OD) ratio. (*p < 0.05, significantly different from baseline (control) levels; **p < 0.05, significantly different from 6-OHDA-treated group at the same point). No significant transcriptional regulation was observed for bcl-2 and bax mRNA when compared with the expression of the housekeeping gene GAPDH. Error bars represent SEM.

catecholaminergic cells and is classically used to create animal models of PD (2, 3, 4, 10). We observed that 6-OHDA-treated cells exhibit both apoptosis and necrosis, although apoptosis predominated at moderate concentrations. Ultrastructural analysis of 6-OHDA treated PC12 cells showed that neuronal lesions produced by this neurotoxin are complex. Low doses of 6-OHDA produces apoptosis and prenecrosis, whereas higher doses produce necrosis. However, apoptosis is also present when high doses of 6-OHDA are applied (10). The mechanisms underlying the effectiveness of Epo against



Figur 4, cont.



Figur 5— Effects of Epo and 6-OHDA on CREB1, CREB2 and pCREB protein expression levels in PC12 cells. *A,* PC12 cells were exposed to 0.5 U/ml rmEpo (lane 2), 50 µM 6-OHDA (lane 3) or combination of these agents (lane 4), or 60 mM KCl (lane 5) for 30 min. No agent was added to control culture (lane 1). *B,* Densitometric analysis of 42 kDa pCREB. Values indicate the mean ± SEM of three experiments. *p < 0.05 as compared with control value.

6-OHDA neurotoxicity is not known but may involve several events such as direct scavenging of oxidized 6-OHDA or of oxygen species derived from 6-OHDA, because Epo is known to be an anti-oxidant (1). However, the fact that Epo concentrations higher than 0.5 U/ml could not prevent the toxic effects of 6-OHDA argues against a simple stoichiometric reaction. This bell-shaped neuroprotection pattern is typical of antioxidative drugs such as vitamin C, apomorphine and dopamine (11). We examined whether the protective effect of Epo against 6-OHDA-induced cell death implicates an anti-apoptotic mechanism. Our results showing a marked decrease in DNA fragmentation in Epo-treated cultures suggest that the nature of the protection includes an anti-apoptotic mechanism. Interestingly, we did not find any anti-apoptotic effect of Epo against toxicity induced by high doses of 6-OHDA. This might suggest the presence of different apoptosis inducing mechanisms triggered by different concentrations of 6-OHDA and modified by Epo. Further studies are needed to clarify this issue. Epo also significantly decreased 6-OHDA-induced externalization of membrane phosphatidylserine residues as confirmed by annexin-V-PI staining. A similar effect has been reported in a different toxicity paradigm in primary neuron cultures (5).

Epo is known to promote cell survival via the bcl-2 family genes in non-neural tissues and regulates the expression of apoptosis-related genes from bcl-2 family in different experimental paradigms (5,12,13). In our system, pretreatment of cultures with Epo is required to confer protection, suggesting induction of a gene expression program. We found that Epo reversed gene expression changes induced by 6-OHDA. Similar stimulatory effect of Epo on bcl-XL mRNA and protein expression was reported in various experimental paradigms (3,12,13). The present study is also the first in vitro demonstration of 6-OHDA-inducible DP5 mRNA expression in PC12 cells. DP5 is the ortholog of the human gene, Harakiri (Hrk),

identified as a molecule that by blocking the anti-apoptotic action of Bcl-2 and Bcl-XL induces apoptosis (14, 15). DP5 expression is highly specific to the nervous system in rat and mouse (16). Modulation of DP5 pro-apoptotic molecule expression at the trancriptional level by Epo as shown here may mediate its neuroprotective action.

CREB is a transcription factor which is implicated in neuroprotection, and the transcriptional activation of CREB depends on its phosphorylation at Ser-133. We observed a significant increase in CREB phosphorylation upon 6-OHDA exposure. A similar effect was reported in vivo and may reflect a compensatory response against injury (17). However, we could not find any effect of Epo on CREB phosphorylation. In contrast, a downregulatory effect of Epo on CREB phosphorylation was reported in mature rat oligodendrocytes (18). Thus, the modulatory effect of Epo on CREB phosphorylation may be cell specific.

PC12 cells are frequently used to model PD-like cell loss as they secrete dopamine and possess a dopamine transporter. Yet, it should be noted that the generalizability to neurons of data obtained from PC12 cells is limited. Although the in vitro model used in the present study could be relevant to study the neuroprotective effect of Epo, it is however, necessary to confirm these results in vivo. In conclusion, the results of the present study suggest that Epo exerts a neuroprotective effect against 6-OHDA-induced injury and differential regulation of the expression of genes involved in apoptotic processes by Epo may mediate this effect.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Dokuz Eylul University Research Foundation and The Scientific and Technical Research Council of Turkey. Authors thank Dr. Alexis Brice (INSERM U289 in Paris) for PC12 cell line and Dr. Tolga F. Koroglu for reviewing the language of the article.

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