

Erythropoietin may modify tyrosine kinase activity for its neuroprotective action in mice

M. G. Tyagi

Department of Pharmacology, Christian Medical College, Vellore 632002, TN, India
tyagi239@yahoo.co.in

Abstract

The neuroprotective effect of erythropoietin (EpO) was evaluated against $MgCl_2$ induced cerebral ischemia. EpO (500 IU/kg i.p) prevented the $MgCl_2$ (0.5M) induced cerebral ischemia in the mice. EpO also reduced the bleeding time in mice by 7.64 %. Pretreatment with tyrosine kinase inhibitor, Genistein (6mg/kg i.p) attenuated the neuroprotective effect of EpO and reversed the effects of EpO on bleeding time in mice. These studies suggest that EpO is protective against $MgCl_2$ induced cerebral ischemia and a role for tyrosine kinase enzyme in this action is suggested based on the results of this study.

Keywords: Tyrosine kinase, erythropoietin, cerebral ischemia, mice

Introduction

Cerebral ischemia and hemorrhagic strokes resulting in ischemic cell death represent major cause of cerebrovascular disorders. Various potential treatment approaches have been developed to reduce the extent of tissue injury, approaches that have been derived from experimental models of cerebral ischemia (Tyagi & Parthiban, 2003). A pathological result of cerebral ischemia is the hyperactivity of specific neuronal synapses and mechanisms targeting attenuation of excitotoxicity at the molecular receptor, and / or ion channel, protein and enzyme changes continue to be explored for therapeutic implications.

In the brain a nonlethal ischemic event can induce tolerance against subsequent, more severe ischemia, ischemic preconditioning or ischemic tolerance (Bernaudin *et al.*, 1999). EpO was originally identified as the principal regulator of erythroid progenitor cells, which are responsible for the formation of red blood cells. EpO might act as a protective agent against hypoxia, indeed, infusion of soluble EpO and EpOR expression change significantly protected during brain development, thus indicating the importance of EpO/EpOR system in neurodevelopment. Moreover hypoxia responsive production of EpO found in the adult brain suggests it may act as a neuroprotective factor after brain injury (Ruscher *et al.*, 2002; Yenari *et al.*, 2008). Finally, the EpO induced release of neurotransmitter suggests that the hormones can directly or indirectly influence neurotransmission.

Receptor and cytosolic tyrosine kinases play an important role in the control of most fundamental cellular processes including the cell cycle, cell migration and cell metabolism and survival, as well as cell proliferation and differentiation. All receptor tyrosine kinases contain an extracellular ligand binding domain that is usually glycosylated cytoplasmic domains by a single transmembrane helix. EpO may modulate protein tyrosine phosphatase 1B and have effects on DNA synthesis and cell proliferation (Chong *et al.*, 2002). Genistein inhibits the activities of tyrosine specific protein kinases. One of the earliest responses detected with in cells upon ligand induced homodimerization of the receptor is a transient increase in

tyrosine phosphorylation of cellular proteins including the receptor EpO-R like other hemopoietic cytokine receptors associates with and activates protein tyrosine kinases in order to transmit a signal. The EpO-R binds the cytoplasmic protein tyrosine kinase Janus kinase 2 (JAK2) which has been implicated in signal transduction by a number of cytokine receptors. Like JAK 1, JAK 3 and Tyk 2 the other members of the Janus kinase family JAK 2 is composed of a C-terminal kinase domain, a kinase like protein kinase domain of unknown function (Callero *et al.*, 2007; Ingley, 2009). On the other hand the importance of Mg^{2+} in homeostatic balance is well known. Mg^{2+} is an important ion for regulation of myogenic tone of cerebral blood vessels and it is believed to play an important role in the autoregulation of cerebral blood flow. We utilized $MgCl_2$ for inducing cerebral ischemia in this study. $MgCl_2$ induced reversible global cerebral ischemia in mice is an excellent *in vivo* model.

The main purpose of the study was to determine role of tyrosine kinase in the neuro protective actions of EpO and characterize the actions of EpO-R responsible for neuro protection. This study was conducted to elucidate neuroprotective role of erythropoietin against $MgCl_2$ induced cerebral ischemia.

Materials and methods

Drugs used for this study

Normal saline (Fresenius Kabi, India Ltd), $MgCl_2$ (Glaxo India Ltd.), Erythropoietin (Ethnor Pharma, India), Genistein (Sigma Chemical Co., USA).

Animals care

Albino Swiss mice of either sex weighing between 25 and 35 g were kept under standard laboratory conditions and given food and water *ad libitum*. A 12 hour dark: light cycle was also maintained.

Induction of cerebral ischemia

Swiss albino mice were utilized for induction of cerebral ischemia. Global cerebral ischemia was induced by the intramuscular injection (0.1ml) of 0.5 M $MgCl_2$. The indicative symptoms of cerebral ischemia were gasping, sedation and loss of righting reflex. The total time duration of ischemic episode beginning with the onset and recovery of ischemic episode was noted (Tyagi & Parthiban, 2003).

Bleeding time in mice

Bleeding time in the mice was estimated by a modified technique. Tail vein was punctured with the help of a lancet and submerged in a beaker filled with 0.9 % saline. The time taken for the bleeding to stop was recorded (Tyagi & Namboodri, 2005).

Statistical evaluation

The data are represented as mean \pm SE. Statistically significant difference was ascertained by 'P' value which is considered significant of $P < 0.05$ and highly significant of $P < 0.01$ as comparison of different groups were done using ANOVA and individual groups of students's 't' test.

Table 1. Effect of intraperitoneal injection of EpO on MgCl₂ induced cerebral ischemia in mice

Pretreatment (Dose, ip)	Treatment (Dose, ip)	MgCl ₂ induced cerebral ischemia (Minutes)	'P' Value
Saline (0.1ml)	Saline	16.43 \pm 3.46	1: 2 - NS
Saline (0.1ml)	EpO(500 IU)	11.29 \pm 2.95	
Saline (0.1ml)	EpO (1000 IU)	10.63 \pm 1.81	1: 3 - NS
Genistein	EpO (500 IU)	22.21 \pm 3.12	1:4 - P<0.05
Saline	Genistein	16.91 \pm 3.27	1: 5 - NS

The animals were treated with EpO (500, 1000 IU/kg i.p) and or pretreated with Genistein (6mg/kg, ip) 40 minutes before intramuscular injection of MgCl₂ (0.5M). Values $P > .05$ were considered to be non significant and depicted as NS while on the other hand values $P < .05$ were considered to be statistically significant.

Results

The results of this study are depicted in Table 1 and Fig. 1. The effect of the tyrosine kinase inhibitor, Genistein and EpO was ascertained on the MgCl₂ induced cerebral ischemia and bleeding time. The control group values for cerebral ischemia were 16.43 minutes which increased marginally to 16.91 minutes after pretreatment with Genistein. The pretreatment with Genistein (6mg/kg i.p) caused an EpO mediated increase in cerebral ischemia by 26.1 % to 22.21 minutes. The effect of the tyrosine kinase inhibitor, Genistein (6 mg/kg i.p) and EpO was also evaluated on the bleeding time.

The mean bleeding time was 68.35 secs. Genistein caused an increase of 11.21% while EpO caused a decrease in the bleeding time by 7.64%. However, pretreatment with Genistein 40 minutes before EpO injection produced a reversal and increase in bleeding time by 9.71 %.

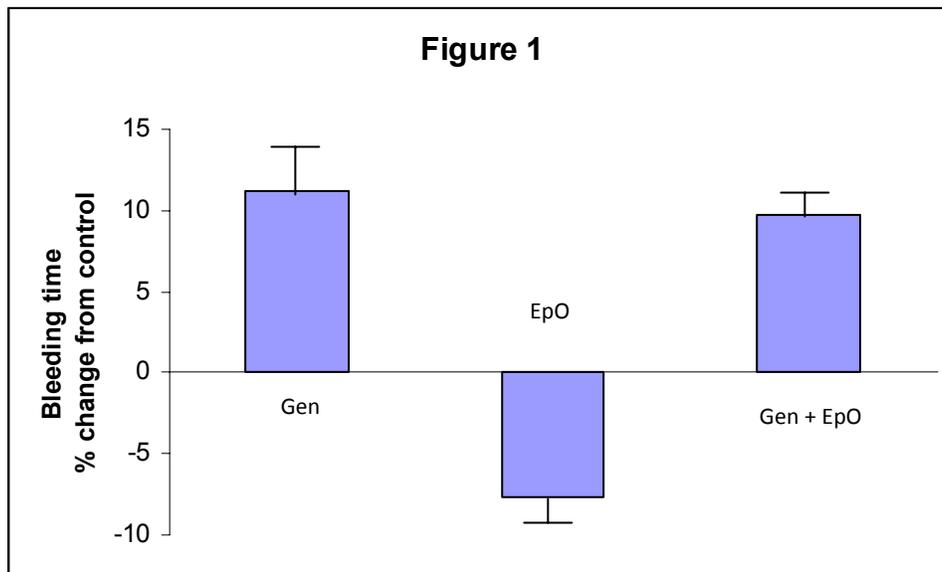
Discussion

The results of our study demonstrate that EpO produces neuroprotective action against MgCl₂ induced cerebral ischemia in mice. These results are shown in Table 1. EpO has been shown to prevent programmed cell death in neuronal systems. Initiation of the cascades that modulate protection by EpO and its receptor may begin with the activation of the Janus tyrosine kinase 2 protein. Subsequent down stream mechanisms appear to lead to the activation of

multiple signal transduction pathways that include transcription factor STAT5, protein tyrosine phosphatases and nuclear factor kB. One of the mechanisms by which EpO may protect against MgCl₂ induced ischemia is that EpO preserves cardiac function during ischemia/perfusion (Lipsic *et al.*, 2006). Previous studies identified several key pathways by EpO that were critical for protection against neuronal apoptosis (Digicaylioglu *et al.*, 1995). Receptor tyrosine kinases play an important role in the control of most fundamental cellular processes including the cell cycle, cell migration, cell metabolism and survival. Pretreatment with tyrosine kinase inhibitor, Genistein prevented the neuroprotective action of erythropoietin and prolonged the cerebral ischemia time and the bleeding time. Genistein is a highly specific inhibitor for tyrosine kinases and inhibits the activity of serine and threonine kinase and other ATP analogue-related enzymes *in vitro*. Genistein injected intraperitoneally has been used in previous studies to inhibit tyrosine kinase activity and is an effective tool for *in vivo* studies (Morris *et al.*, 1999). Further more, Genistein was revealed to inhibit EGF- stimulated phosphorylation in cultured A431 cells (Akiyama *et al.*, 1987). Thus EpO induced neuroprotection could also be due to antiapoptotic and better cerebral vascular perfusion. This may result in improving the uptake and availability of O₂ in the neural cells. It is also known that EpO increases intracellular calcium in neuronal cells, which in turn leads to increase in tyrosine phosphorylation (Miller *et al.*, 1994).

EpO and EpOR expression change significantly during brain development, thus indicating the importance of the EpO/EpOR system in neurodevelopment. Research has shown that EpO receptors are expressed in many areas of the brain, including the cortex, hippocampus, midbrain and endothelial cells (Linnekin *et al.*, 1992; Masuda *et al.*, 1993). Moreover the hypoxia responsive production of EpO found in the adult brain suggests it may act as a neurotrophic and neuroprotective factor after brain injury. Although we could not define further the cellular mechanisms underlying neuroprotection by EpO the results are consistent with the hypothesis that EpO acts in the central nervous system primarily as a direct protective factor with additional properties of reducing the bleeding time. It can be stated that EpO has neuroprotective action against MgCl₂ induced cerebral ischemia and tyrosine kinase enzyme mediated actions contribute significantly to this action. Intramuscular injection of MgCl₂ causes a state of reversible global ischemia characterized by shallow breathing, sedation and loss of righting reflex. The effects are also secondary to the effects of MgCl₂ on the heart. The animals recover after a brief duration. Thus it is a safe and effective method of inducing cerebral ischemia. Magnesium has been implicated in central nervous injury thus these results are novel in this regard (Vink & Cernak, 2000). These results can be extrapolated for possible therapeutic application for the treatment of stroke patients. Initiation of brain intrinsic protective mechanisms may be another novel strategy to future successful approaches to provide neuroprotection against hypoxia/ischemia (van der Kooij *et al.*, 2008; Guo *et al.*, 2006).

Fig. 1. Influence of Genistein (6mg/kg ip) and EpO (500 IU/kg ip) alone and in combination on bleeding time in the mice. The results are expressed as mean \pm SE of six observations



In this study, a single dose EpO before the MgCl₂ insult, preserved and protected against cerebral ischemia. Thus signifying that EpO could be affecting astroglial lineage in the neurogenic zones and tyrosine mediated actions on glial derived factors may also be involved in these mechanisms. In conclusion, we present experimental evidence for the functional role of intrinsic EpO in hypoxia injury *in vivo*, demonstrated the beneficial effects of an optimal therapeutic time-window of extrinsic EpO in neuroprotection against ischemia/reperfusion injury in the mice model.

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