Life Sciences xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Nephroprotection through the Akt/eNOS pathway by centrally administered erythropoietin in a rat model of fixed-volume hemorrhage

Behjat Seifi[a,*], Mehri Kadkhodaee[a], Mina Ranjbaran[a], Enayatollah Bakhshi[b]

[a] Department of Physiology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[b] Department of Biostatistics, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran

ARTICLE INFO

Keywords:
Hemorrhagic shock
Erythropoietin
Brain
Kidney
Akt/eNOS signaling

ABSTRACT

Aims: This study was designed to investigate the protective effects of centrally administered erythropoietin (EPO) on brain oxidative stress and inflammatory markers to protect the kidneys during hemorrhagic shock (HS).

Main methods: Animals were assigned into three groups (n = 6). Sham rats were subjected to cannulation of femoral artery and vein as well as stereotaxic surgery. In HS group, 50% of total blood volume was withdrawn and resuscitation was started 2 h later. In EPO group, stereotaxic surgery in lateral ventricle was performed one week before induction of HS for administration of EPO (2 IU) just before resuscitation. Plasma samples, kidney and brain tissues were allocated after a further 3 h in all animals.

Key findings: There was a significant increase in survival rate in the EPO group (69.3%) compared to the HS group (35.7%). Brain EPO administration significantly attenuated the rises in BUN, plasma Cr and NGAL, brain and renal MDA content and also increased SOD activity in the kidney and brain compared to the HS group. Brain, plasma and kidney TNF-α and IL-6 levels were significantly reduced by EPO compared to HS group. EPO increased the phosphorylation of Akt on Ser473 and eNOS mRNA expression in the kidney tissue compared to the HS group.

Significance: In conclusion, centrally administered EPO reduced pro-inflammatory and oxidative stress indices in the kidney and reduced apoptosis by activation of the Akt/eNOS signaling pathway. Hence, it can be hypothesized that EPO may play a major role in the central regulation of renal system as a neuromodulator.

1. Introduction

In many cases following hemorrhagic shock (HS), acute kidney injury develops which is still considered as a serious problem in clinic. Resuscitation may improve tissue perfusion and oxygen delivery to the tissues but it causes greater pro-inflammatory cytokines formation and intensifies tissue damage [1]. After trauma, brain function may be influenced by the systemic inflammatory response as a result of reduced blood flow. Maintenance of brain function is an important target of critical care in patients with ischemia and hypovolemia after trauma. During HS, several physiological responses attempt to protect neurons from hypoxia and hypo-perfusion by shifting blood flow from splanchnic organs [2]. An early onset of systemic inflammation causes some disturbances in the compensatory mechanisms, especially when large amounts of blood are lost or when the hemorrhage is prolonged. Low cerebral perfusion pressure and the systemic inflammatory response may cause neurological damage which leads to multiple organ dysfunctions. Whilst perfusion pressure could be managed by resuscitation fluids, therapeutic prevention of inflammation remains as a major challenge in clinical situation. Therefore, pharmacologic interventions to inhibit the initiation of brain injury during HS is expected to show some improvements in brain metabolism and further recovery in the other organs.

Erythropoietin (EPO) was recently introduced as a tissue-protective and anti-apoptotic cytokine in animal models of ischemia-reperfusion type injuries at various anatomical locations including the central nervous system, kidney and many other organs [3]. EPO appears to act in both autocrine and/or paracrine fashions, while the endocrine action of EPO is also discussed. There are evidences showing that EPO is synthesized in the brain [4,5]. EPO receptors are also found in the nervous system, including inside the neurons, astrocytes, and endothelial cells [6]. The presence of EPO/EPO receptors in brain suggests other roles than those usually assigned to this protein. There is strong evidence that EPO may also act as a neuroprotector or neuromodulator in the central nervous system. Hypoxia and ischemia are important driving forces of EPO expression in the brain. Hypoxia-induced gene

* Corresponding author at: Department of Physiology, School of Medicine, Tehran University of Medical Sciences, Tehran 1417613151, Iran.
E-mail address: b-seifi@tums.ac.ir (B. Seifi).

http://dx.doi.org/10.1016/j.lfs.2017.11.003
Received 10 July 2017; Received in revised form 25 October 2017; Accepted 5 November 2017

products of EPO might be part of a self-regulated physiological protection mechanism to prevent neuronal injury, especially under conditions of chronically reduced blood flow like prolonged HS [7].

These studies encouraged us to investigate the effect of centrally administered EPO to protect the peripheral organs during HS. Recently, we have shown that treatment of severely hypovolemic rats with systemic EPO protected kidney function and reduced systemic inflammatory responses [8]. Considering that the neurological dysfunction in hemorrhagic shock is also influenced by cerebral perfusion pressure and hypoxia, the goal of this study was to investigate whether the resuscitation with EPO in cerebroventricular fluid will have additive effect on brain metabolic activity and in the prevention of kidney damage in HS. To achieve this object, we evaluated cerebral and renal pro-inflammatory and oxidative stress indices in rats following HS induction and brain EPO administration. Moreover, we investigated the potential mechanisms behind this protective effect by evaluating the activity of Akt/eNOS/NO signaling pathway in the kidneys.

2. Methods

2.1. Animals

Male Wistar rats weighing 285–300 g were included in the experiments. Animals were housed in a light-controlled room with a 12 h light-dark cycle and were allowed ad libitum access to food and water. Experimental protocols and animal care methods in the experiment were approved by the Animal Experimental Committee at Tehran University of Medical Sciences.

2.2. In vivo experiments

2.2.1. Induction of hemorrhagic shock

All rats were anesthetized intraperitoneally by injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). PE-50 catheters were inserted into the left femoral artery for blood withdrawal and into the left femoral vein for blood resuscitation.

In order to induce hemorrhagic shock, 50% of total blood volume was withdrawn using the following formula [Weight (g) × 0.03 + 0.7 ml] [9]. Blood removal was carried out during 30 min via heparinized syringes through the femoral artery catheter. Two hours later, resuscitation was started with the shed blood and equal volumes of Ringer's lactate through the femoral vein catheter. Animals were followed up for a further 3 h and scarified at the end of this time.

2.2.2. Stereotaxic surgery

To inject erythropoietin into the left lateral ventricle, one week before the induction of hemorrhagic shock, the rats were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg). Animals were mounted onto a stereotaxic apparatus. A stainless steel guide cannula (Gauge 21) was placed within the left lateral ventricle. Coordinates for cannulas was obtained according to the rat brain in stereotaxic coordinates [10] that is, LV: AP 0.8 mm, LR 1.4 mm, and H 3.6 mm.

Just before resuscitation, erythropoietin (2 IU in a volume of 5 µl normal saline), was injected into the left lateral ventricle via a cannula connected to a Hamilton syringe with a polyethylene tube. The injection lasted for 30 s and the injection cannula was left in place for an additional 1 min to prevent backflow.

2.2.3. Experimental design

Animals were randomly assigned to three experimental groups (n = 6 in each). Sham group, rats were subjected to cannulation into femoral artery and vein and further stereotaxic surgery. HS group, animals were subjected to stereotaxic surgery in lateral ventricle and after 1 week recovery, hemorrhagic shock was induced. Isotonic saline was used as a vehicle. EPO group, 2 IU recombinant human erythropoietin (in 5 µl normal saline, into left lateral ventricle) was administered just before resuscitation. At the end of the procedure, plasma samples were stored for biochemical studies as well as kidney and brain tissues allocated for ex vivo measurements.

2.3. Ex vivo experimentations

2.3.1. Renal functional assessments

Blood samples were taken via femoral artery into heparinized syringes and were centrifuged (3500g for 10 min) to collect plasma. Plasma was used for blood urea nitrogen (BUN), creatinine (Cr) measurements by colorimetric methods (Hitachi 704 auto-analyzer, Japan) and neutrophil gelatinase-associated lipocalin (NGAL) assessment by ELISA (Abcam, Inc., UK).

2.3.2. Histological analysis of the kidneys

Renal tissue samples were fixed in 10% formaldehyde, embedded in paraffin, and cut into 4 µm sections. The sections were stained with hematoxylin and eosin. Histopathological changes documented photographically. To assess the presence of tubular injury, we evaluated the presence of cell necrosis, loss of brush borders from proximal tubules, cast formation, tissue congestion, infiltration and tubular dilation and/or obstruction.

2.3.3. Tissue oxidative stress assessments

Left brain hemisphere and renal tissue samples were collected and then stored at −70 °C after being snap-frozen in liquid nitrogen. Malondialdehyde (MDA) content and superoxide dismutase (SOD) activity were determined in the supernatant of the tissue homogenates.

MDA level was analyzed by the method of Esterbauer and Cheeseman [11]. MDA reacts with thiobarbituric acid to produce a pink pigment and the optical density was assayed at 532 nm. SOD activity was measured according to the method of Paoletti and Mocal [12]. In this assay, superoxide anion is generated from oxygen and oxidation of NADPH is related to the availability of superoxide anions in the tissue samples. The optical density of supernatants was assessed at 340 nm.

2.3.4. Pro-inflammatory indices assessments

Tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) concentration was assayed by enzyme-linked immunosorbent assay (ELISA). All reagents, standards dilutions, control and test samples were transported to room temperature and prepared according to the manufacturer's directions (R & D Systems, Inc., USA). The quantitative sandwich enzyme immunoassay technique was employed for this assessment. Reactions were quantified by optical density by a microplate reader (BioTek Instrument, ELX 800, Inc., USA) at a wavelength of 450/570 nm.

2.3.5. Protein extraction and western blotting

Briefly, rat kidney samples were homogenized in ice-cold lysis buffer and centrifuged at 13,000g for 15 min at 4 °C. Supernatants were removed and centrifuged again at 4 °C to obtain the cytosolic fraction. The resulting supernatants were carefully collected and the protein contents were determined by the use of Bradford buffer. Proteins (30 µg each) were separated by 12% SDS-PAGE and transferred to a polyvinyldenedifluoride (PVDF) membrane. Membranes were blocked with 2.5% skim milk, 2.5% glycerol in Tris buffered saline with 0.05% tween-20 (TTBS) and incubated individually with a primary antibody: rabbit anti-total Akt (Cell Signaling Technology, Danvers, MA, USA; 1:1000) and rabbit anti-pAkt Ser473 (Cell Signaling Technology, Danvers, MA, USA; 1:2000). After 3 times washing with TTBS, the membranes were incubated with horseradish-peroxidase (HRP) conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA; 1:2000) for 1 h at room temperature. Further, the membranes were washed again three times with TTBS and then the reactive bands were visualized using a chemiluminescence detection system (Image station 4000MM Pro, Kodak, USA). Blots were scanned and quantified