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Ethanol extract of *Portulaca oleracea L.* protects against hypoxia-induced neuro damage through modulating endogenous erythropoietin expression

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Abstract

In addition to its role in erythropoiesis, erythropoietin is also appreciated for its neuroprotective effects, and it has been suggested for treatment of some ischemic–hypoxic neurovascular diseases. The protective effects of endogenous erythropoietin in the brain give rise to the hypothesis that modulating erythropoietin expression might be a better way for treatment of ischemia–hypoxia neurovascular diseases. We have found that ethanol extract of *Portulaca oleracea L.* (EEPO) could increase erythropoietin expression in hypoxic mouse brain in our previous study. The present study is to investigate whether EEPO exerts its neuroprotective effects against hypoxia injury through regulating endogenous erythropoietin expression. The results demonstrated that EEPO decreased the serum neuron specific enolase level in hypoxia mice and the activity of caspase-3 in neuron, increased the neuron viability and attenuated the pathological damages caused by the hypoxia condition. Importantly, we also found that EEPO stimulated the endogenous erythropoietin expression at both mRNA and protein levels. Using the conditioned medium containing soluble erythropoietin receptor, we found that the neuroprotective effects of EEPO were dependent, at least partly, on erythropoietin expression. Although EEPO did not affect transcription of hypoxia inducible factor-1α (HIF-1α), it did stabilize expression of HIF-1α. It is concluded that EEPO has neuroprotective effects against hypoxia injury, which is at least partly through stimulating endogenous erythropoietin expression by stabilizing HIF-1α.

Keywords: Ethanol extract; *Portulaca oleracea L.* (EEPO); Hypoxia; Apoptosis; Erythropoietin; Hypoxia inducible factor-1 (HIF-1)

1. Introduction

Hypoxia is defined as a state in which oxygen is reduced in tissues of the body, and it has been associated with the pathology of acute mountain sickness, cardiovascular diseases and stroke, the leading causes of death in many countries [1]. The mammalian brain is exquisitely sensitive to neuronal damage caused by hypoxia. Among the cerebral neurons, pyramidal cells of the CA1 region are most vulnerable to hypoxia [2]. Death of neurons following hypoxia can result in a variety of neurological dysfunction. Therefore, increasingly more studies have been focusing on antihypoxic drugs. Identifying novel cellular pathways activated under hypoxia neuronal environments will cast new lights on the therapeutic strategies against hypoxia neurological diseases.

Erythropoietin (EPO) is mainly involved in stimulating erythroid cell production by supporting the survival, proliferation and differentiation of erythroid progenitor cells [3]. Studies found that, in addition to hematopoietic cells, other cell types including endothelial and neuronal cells also express erythropoietin receptor (EPO-R) and participate in EPO response [4,5]. It has been widely demonstrated that EPO was produced in brain by astrocytes and neurons and that the endogenous EPO in the brain had neuroprotective effects [6,7]. Recently, EPO has been suggested for clinical treatment of many ischemic–hypoxic cardiovascular and neurovascular diseases, although some studies found that it may have negative effect on patients with acute stroke [8–10]; all these indicate that modulating the expression of EPO might be a potential way for treatment of ischemia–hypoxia neurovascular diseases.

The *Portulaca oleracea L.* (PO) is a warm-climate annual with a cosmopolitan distribution. It is known as a "vegetable for long life" in Chinese folklore and is sold in shops as a vegetable in the United Arab Emirates and Oman [11–13]. *Portulaca oleracea L.* is also widely used as a traditional Chinese herbal medicine; the leaves and seeds of PO are eaten or applied topically to soothe skin [14]. A wide range of other pharmacological effects of PO, such as antibacterial, analgesic, anti-inflammatory and wound-healing activities, have also been reported [11–16]. Recently a neuropharmacological study by Zhang et al. [17] further confirmed the neuroprotective effect of PO. When searching for promising antihypoxic drugs, we noticed that ethanol extract of PO (EEPO) exhibited potent antihypoxic properties and that it increases EPO expression in the brain of hypoxia mice [18,19].

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Therefore, the aim of the present study is to understand whether the neuroprotective effects of EEPO under hypoxia condition involves the endogenous EPO expression.

In the present study, we investigated the hypoxia neuroprotective effects of EEPO and its effect on the EPO expression. Furthermore, we studied the possible mechanism by which EEPO modulates EPO expression. Our results demonstrated that EEPO had neuroprotective effects under hypoxia condition and that it increased neuronal EPO expression, possibly through stabilizing the hypoxia inducible factor-1α (HIF-1α) under hypoxia condition, which further indicates that PO may be used for treatment of ischemic–hypoxic neurovascular diseases.

2. Materials and methods

2.1. Chemicals

Neurobasal/B-27, horse serum and fetal calf serum were obtained from Invitrogen (Carlsbad, CA, USA). Cultures dishes were obtained from TPP or Falcon. Hoechst33342 was obtained from Molecular Probes (Eugene, OR, USA). Soluble EPO-R was obtained from R&D Systems Inc. (Minneapolis, MN, USA). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA) or Calbiochem (Merck KGaA, Darmstadt, Germany).

2.2. Plant materials

The aerial parts of PO were collected in Henan province, China, in October 2006 and authenticated by Prof. Zheng Han-chen. A voucher specimen has been deposited in the Department of Traditional Chinese Medicine, Second Military Medical University (20090829). The powdered aerial parts of PO (40 kg) were refluxed with 80% ethanol solution twice, each time for 1 h. The extract was concentrated under reduced pressure to 80 L and then centrifuged at 5000 rpm for 4 min, and the precipitation part (241.3 g) was used as test material.

2.3. Experimental animals and treatment

All animal procedures were done strictly in accordance with the international ethical guidelines and the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*, and all the experiments were carried out with the approval of the Experimental Animal Administration Committee of the Second Military Medical University. Experimental groups consisted of 10 ICR mice (18–22 g) per group. They were housed at 24°C±1°C under a 12-h light/12-h dark cycle with free access to standard pellet diet and tap water. After 2 days' adaptation, the mice were divided into control group, 6-h hypoxia exposure group, 12-h hypoxia exposure group and 24-h hypoxia exposure group. Each group was subdivided into distilled water group, low-dose EEPO group, medium-dose EEPO group and high-dose EEPO group; the mice were orally administrated with distilled water or with 0.5, 1, or 2 mg/kg (body weight) EEPO in 0.5 ml distilled water every day for 7 days. One hour after the last drug administration, mice in the hypoxia exposure groups were subjected to normobaric low oxygen environment (10% oxygen and 90% nitrogen) for 6, 12 or 24 h. Mice in the control group were sacrificed 1 h after the last drug administration, and those in the hypoxia exposure groups were sacrificed immediately after hypoxia administration; their cortices and serum were collected for analysis.

2.4. Cell preparation and treatment

The cerebral cortices were isolated from 1-day-old ICR mice with 0.25% trypsin in Hank's balanced salt solution. The primary cultured cortical neurons were plated into in a poly-L-lysine-coated six-well plates at a density of about 3×10^6 cells/ml. Cells were maintained in Neurobasal/B27 (Invitrogen) medium under a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Experiments were carried out with neurons cultured for 12–14 days. The cells were characterized by neuron specific enolase (NSE) staining and observation under microscope (supplement data 2 and 3). The results showed that more than 90% cells were NSE-positive cells, and they were used for later experiments. SK-N-SH cell line was obtained from American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GiBco BRL, Life Technologies) containing 10% fetal bovine serum.

2.5. Preparation of conditioned culture medium

The conditioned culture medium (CCM) was prepared as described by Ruscher et al. [20] with some modification. After the neurons were cultured for 13 days, equal volumes of EEPO (100 μg/ml) and DMSO were added at the beginning of hypoxia exposure. One hour later, the neurons were returned to the normal condition and kept for 6 h; then the culture medium was collected for later use (designated CCM-DMSO and CCM-EEPO, respectively). The blank culture media (BCM) were those with DMSO (BCM-DMSO) and with EEPO (BCM-EEPO) in the empty wells receiving the same treatment as for preparation of CCM. In order to understand whether the neuroprotective effect of CCM is associated with EPO, different concentrations of soluble EPO-R were added into CCM, and then the cell viability was examined.

2.6. Cell viability assay

Cell Counting Kit-8 (CCK-8, Dojindo, Japan) was used to determine the cell viability in different groups. Briefly, the culture medium was discarded, and the test solution of the kit (100 μl in 900 μl of DMEM) was added to each well. Plates were incubated at 37°C for another 2 h, and the absorbance at 450 nm was measured by a microplate reader (Synergy 2 BioTek instruments Inc., WI, USA).

2.7. Cell apoptosis examination

Apoptotic cells were quantified by Hoechst33258 staining as described above. Cells with condensed and/or fragmented chromatin were considered apoptotic and counted from at least three independent experiments. At least 1000 cells were individually examined in three or more culture wells for each experimental condition by a researcher who was blind to the experiment design. The typical morphological changes in apoptotic cell nuclei were analyzed by fluorescence microscopy with the nuclear dye Hoechst33342. Cells were fixed in ice-cold 4% paraformaldehyde/phosphate-buffered saline (PBS) for 15 min, washed twice in PBS and then incubated for 5 min at room temperature with 1 μM Hoechst33342. After washing, cells were viewed for nuclear chromatin morphology in a Leica DMRB microscope.

2.8. Cells transfection and luciferase assays

SK-N-SH cells were grown in DMEM with 10% fetal bovine serum supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were co-transfected with the mixture of indicated luciferase reporter plasmids; HRE-luciferase (Plasmid 26731, Addgene) and control reporter vector pRL-TK were used to co-transfect SK-N-SH cells. HRE-luciferase was composed of three hypoxia response elements (24-mers) from the Pgk-1 gene upstream of firefly luciferase. After treatment, the cells were lysed, and luciferase activity (which indicated the transcription activity of HIF-1) was assayed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's recommendations.

2.9. Activity of caspase-3

The activity of caspase-3 was measured using the colorimetric assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, cells were harvested and resuspended in cell lysis buffer (10^8 cells/ml). After being lysed, 10^6 cells were mixed with 32 μl of assay buffer and 2 μl of 10 mM Ac-DEVD-pNA substrate. After incubation at 37°C for 4 h, absorbance was measured using a microplate reader (Bio-Rad) at 405 nm. Absorbance of each sample was determined by subtraction of the mean absorbance of the blank from that of the sample and corrected by the protein concentration of the cell lysate. The results were described as relative absorbance to that of control group.

2.10. Enzyme-linked immunosorbent assay

The concentrations of serum NSE and EPO in the culture supernatant were assessed using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Inc., USA), with the absorbance read on a microplate reader (Bio-Rad) at a wavelength of 450 nm. The NSE and EPO concentrations of each group were normalized and described as the relative absorbance to that of control group.

2.11. Western blotting analysis of protein expression

Expressions of HIF-1α and EPO proteins were investigated by Western blotting analysis. The protein was lysed in phosphate extraction buffer, and the protein concentration was determined by a BCA protein assay kit (Beyotime Institute of Biotechnology, Nantong China). Equal amounts of proteins (40 μg) were resolved by using sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The blots were blocked with 2% bovine serum albumin and 0.1% Tween 20 in Tris–NaCl buffer for 1 h and incubated overnight at 4°C with the first antibody (Santa Cruz Biotechnology Inc.) at a dilution of 1:500. After extensive washing, the blots were incubated with the secondary horseradish-peroxidase-conjugated antibody (Santa Cruz Biotechnology Inc., 1:1000) for 2 h at 37°C. Immunoreactive bands were visualized by using an enhanced chemiluminescence detection system (Amersham Life Science, Arlington Heights, IL, USA). The GAPDH expression was used as loading control.

2.12. Real-time polymerase chain reaction for gene expression

Total RNA was extracted from neurons with the TRIzol reagent according to the recommendation of the manufacturer. Reverse transcription (RT) of RNA to cDNA was done using equal amount of total RNA by using a TaKaRa mRNA selective polymerase chain reaction (PCR) kit. The mixture was incubated at 40°C for 30 min, followed by

incubation at 85°C for 7 min. Subsequently, cDNA was quantified in duplicate on a Rotor-Gene RG3000 (Corbett Research, Sydney, Australia) using a SYBR green Premix Ex Taq kit (TaKaRa, Japan) according to the manufacturer's instructions. The primers used were as follows: VEGF forward 5′-CAAGATCCGCAGACGTGTAA-3′, reverse 5′-CGCCTTGGCTTGTCACAT-3′;GLUT-1 forward 5′-TATTGCTGTGGCTGGCTTCT-3′, reverse 5′-GCCTTTGGTCTCAGGGACTT-3′; EPO forward 5′-ACTCTCCTTGCTACTGATTCCT-3′, reverse 5′-ATCGTGACATTTTCTGCCTCC-3′; β-actin forward 5′-TGAGGAG-CACCCTGTGCT-3′+, reverse 5′-CCAGAGGCATACAGGGAC-3′ and HIF-1α forward 5′-ATTGAACGGAGCAAAAGACTATTAT-3′, reverse 5′-TTAACTTCACAATCGTAACTGGTCA-3′. Specificity of amplification products was verified by melting curve analysis and agarose gel electrophoresis. Relative mRNA expression of the target gene was calculated with the comparative CT method. The amount of target gene was normalized to that of the endogenous β-actin.

2.13. Statistical analysis

All results were expressed as mean±S.D. Statistical analysis was carried out by using SPSS 11.0. All values below the detection limits were set to zero in analyses. The *P* values less than .05 were considered statistically significant.

3. Results

3.1. EEPO attenuated hypoxia-induced brain damage in mice

As shown in Fig. 1A-a, and Fig. 1A-c, hypoxia condition (10% oxygen for 24 h) induced brain damages of mouse brain in control groups, while EEPO treatment greatly protected the mouse brain from hypoxia damage (Fig. 1A-b and Fig. 1A-d). The ELISA showed that the serum NSE level increased with the duration of hypoxia exposure in the control group (Fig. 1B), and EEPO decreased the serum NSE level in a concentration-dependent manner (Fig. 1B). Moreover, histopathology evaluation results showed less brain damage in EEPO group than that in the corresponding control group (supplement data 1).

3.2. EEPO protected primary cultured neurons from hypoxia exposure

Using CCK-8 assay, we measured cell viability and found that, in the same hypoxia environment (1% O_2 for 24 h), the cell viability was increased in EEPO-treated groups in a dose-dependent manner compared with that in the control group (Fig. 2A). As shown in Fig. 2B and C, under normoxia condition, there were few cells with densely stained nucleus by Hoechst33342 staining, a sign of apoptosis, in the cultured neurons treated with DMSO (Fig. 2B-a) and EEPO (Fig. 2B-c). After hypoxia exposure (1% oxygen for 24 h), more apoptotic cells were found in the control group (Fig. 2B-b, Fig. 2C) than in the EEPO group (Fig. 2B-d, Fig. 2C). Using special substrate, we found that the caspase-3 activity was inhibited in EEPO treatment neurons compared with control ones (Fig. 2D).

3.3. Protective effects of EEPO against hypoxia damage involved endogenous EPO expression

Western blotting and real-time RT-PCR were used to examine the effects of EEPO on the EPO mRNA and protein expression in cultured neurons. The results showed that, after hypoxia exposure for 12 and 24 h, the mRNA and protein levels of EPO were increased compared with those of normoxia condition (Fig. 3A and B). Compared with the control group, EEPO enhanced the EPO mRNA at 12 and 24 h after exposure to hypoxia (Fig. 3A). The EEPO also increased EPO protein expression after hypoxia exposure (Fig. 3B).

To further test whether the protective effects of EEPO are dependent on the endogenous EPO expression, we examined the EPO level in CCM by ELISA. We found that the EPO level of CCM-EEPO was about 1.62-fold of that in CCM-DMSO (Fig. 3C). Then, we changed the neurons culture medium with CCM 1 h before the hypoxia exposure. After hypoxia treatment, the culture medium was discarded, and the cell viability was tested by CCK-8. The results showed that the cell viability of the CCM-EEPO-treated group was higher than

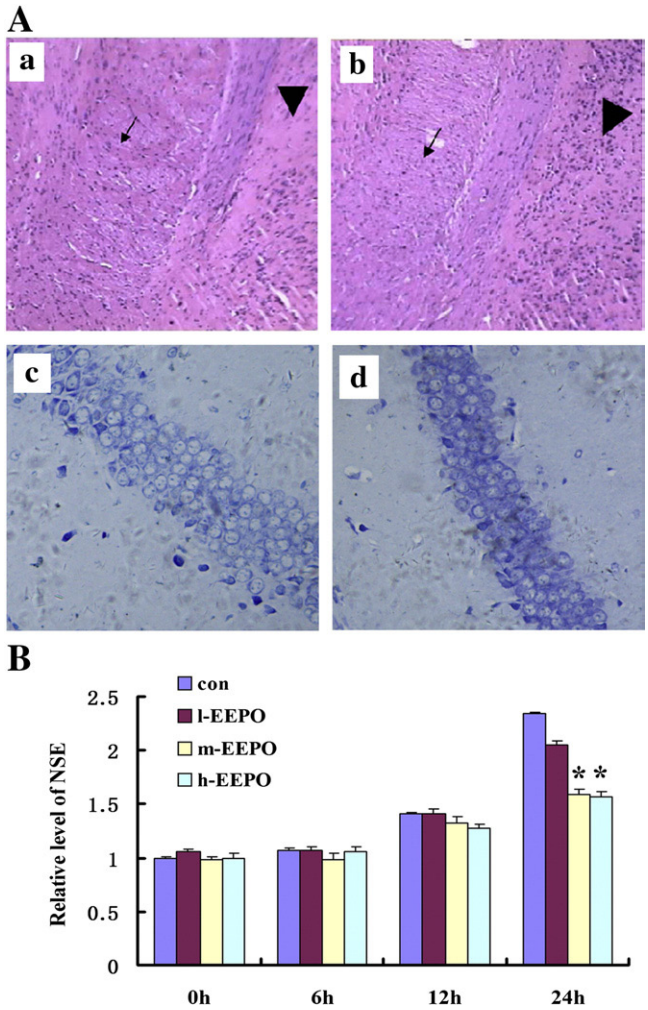

Fig. 1. Protective effects of EEPO on hypoxia mouse brain. The EEPO attenuated the histological damages of hypoxia mouse brain (A) and decreased the NSE in mouse serum (B). After hypoxia exposure for 24 h, more severe damage (un-uniform staining for cell swelling indicated by arrow and cell lost indicated by arrowhead) of the deep cerebral white matter near the CA2 region was found in the control group (A-a) compared with that in the h-EEPO group (A-b). In the control group, the cells in the CA2 region were aligned irregularly and stained slightly (A-c), and in the h-EEPO group, the cells were aligned regularly and stained moderately (A-d). The doses of EEPO in l-EEPO, m-EEPO and h-EEPO groups were 0.5, 1 and 2 mg/kg (body weight), respectively. Original magnification ×100 for hematoxylin and eosin staining and Nissl's staining panels. *P<.05 compared with the control group using the same doses of EEPO; n=8~10.

that of the CCM-DMSO-treated ones (Fig. 3D). We also found that sEPO-R, which can decrease the protective effect of exogenous EPO on neurons, dose dependently decreased the cell viability in both CCM-EEPO- and CCM-DMSO-treated neurons under the hypoxia condition (Fig. 3D).

3.4. EEPO stabilized the HIF-1α protein level and enhanced its transcription activity

Given that EPO is a target gene of HIF-1 and the HIF-1α is the main regulating subunit of HIF-1, we investigated the effects of EEPO on HIF-1α expression and the transcription activities of HIF-1. The real-time PCR results showed that EEPO had no effect on HIF-1α mRNA (data not show). Western blotting results demonstrated that, compared with the corresponding control group, the EEPO enhanced the HIF-1α protein expression in the cortices of hypoxia mice

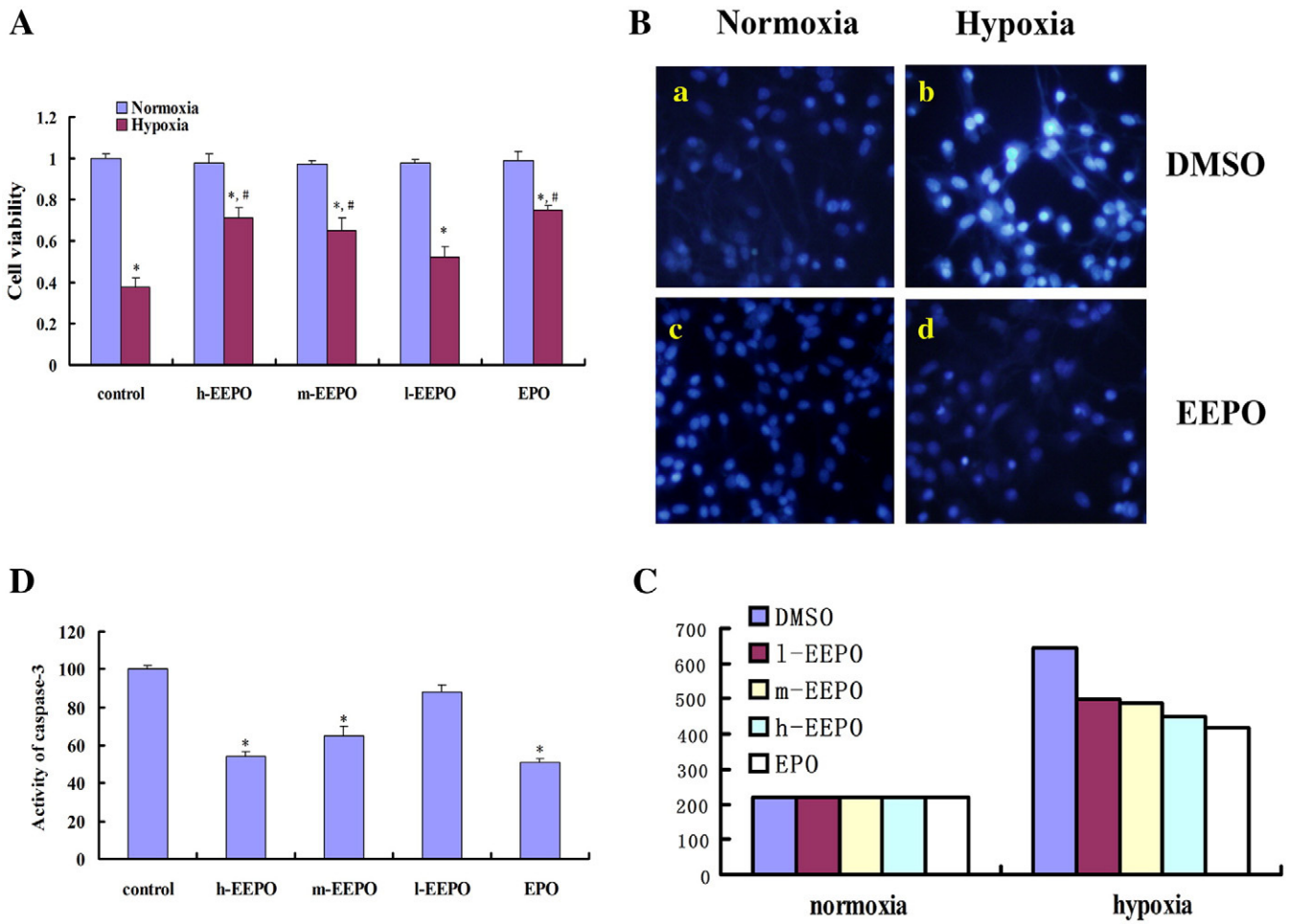

Fig. 2. Protective effects of EEPO on hypoxia neurons. The EEPO increased the cell viability (A), decreased the apoptosis (B) and (C) and inhibited the caspase-3 activity (D). The end concentrations of EEPO in the l-EEPO, m-EEPO and h-EEPO groups were 1, 10 and 100 μg/ml, respectively. Erythropoietin (end concentration was 50 U/ml) was used as positive control (A: * $P<.05$ compared with the normoxia control group, #$P<.05$ compared with the hypoxia control group, $n=6$, at least in three independence experiments. B: representative pictures of Hoechst33342 staining; original magnification ×100. C: the number of apoptotic cells/1000 cells in three wells, at least in three independence experiments. D: *$P<.05$ compared with the hypoxia control group, $n=3$, at least in three independence experiments).

(Fig. 4A) and in the hypoxia neurons (Fig. 4B). In the HRE assay, we found that EEPO enhanced the luciferase activity of HRE-luc in SK-N-SH cells with and without cobalt chloride stimulation (Fig. 4C), indicating that the EEPO promoted the transcription activity of HIF-1. Our Western blotting results also showed that the EEPO enhanced the hif-1α protein expression in cultured neurons (Fig. 4D). Furthermore, real-time PCR showed that EEPO enhanced the *vegf* and glucose transpoter-1 expression (Fig. 4E and F); since both of them were target genes of HIF-1, it further supported that EEPO could stimulate the HIF-1 transcription activity.

4. Discussion

The aim of the present study is to test whether the neuroprotective effects of EEPO against hypoxia insult involved the endogenous EPO expression and, if so, to explore the possible mechanisms. The results demonstrated that the EEPO protected the neuronal cells from hypoxia damage, at least partly, through increasing endogenous EPO expression *via* stabilizing HIF-1α protein.

In addition to its pharmacological and folkloric actions, PO may also help to reduce the risks of cancer and heart diseases [21]; this might be due to the flavonoids, the biologically active constituents of PO, which have been reported to have the following functions: antioxidation, antibacteria, antivirus, antiulcerogenic, anti-inflammatory, relieving cough and dispelling the phlegm [22]. Five flavonoids were found in the PO ethanol extracts by capillary electrophoresis and electrochemical detection methods [23]. Hypoxia can increase the mitochondrial reactive oxygen species, which is the main inducer of oxidative stress [24]. Oxidative stress plays an important role in the hypoxia-induced damage. The antioxidation functions of flavonoids have led to dense research in seeking antihypoxic drugs from natural sources with high potency and low toxicity, such as ginseng and gingko [25,26]. Our present study showed that EEPO decreased the hypoxia-induced damage in mouse cortices (Fig. 1) and cultured neurons (Fig. 2). Whether the neuroprotective effects were due to the flavonoids needs further research.

The hypoxic response is a stress response triggered by low ambient O_2 [27]. Adaptation to hypoxia leads to changes of a series of genes related to angiogenesis [28,29], iron metabolism [30], glucose metabolism [31] and cell proliferation/survival [32]. Erythropoietin expression is regulated mainly by hypoxia [33]. Erythropoietin, once believed to be produced only by the adult kidney and fetal liver [34], has also been found in astrocytes, in neurons [35] and in the brains of rodents and primate [36]. Therefore, in addition to its functions in erythropoiesis, EPO is also appreciated for its neuroprotective effects, and many studies have shown the neuroprotective effect of

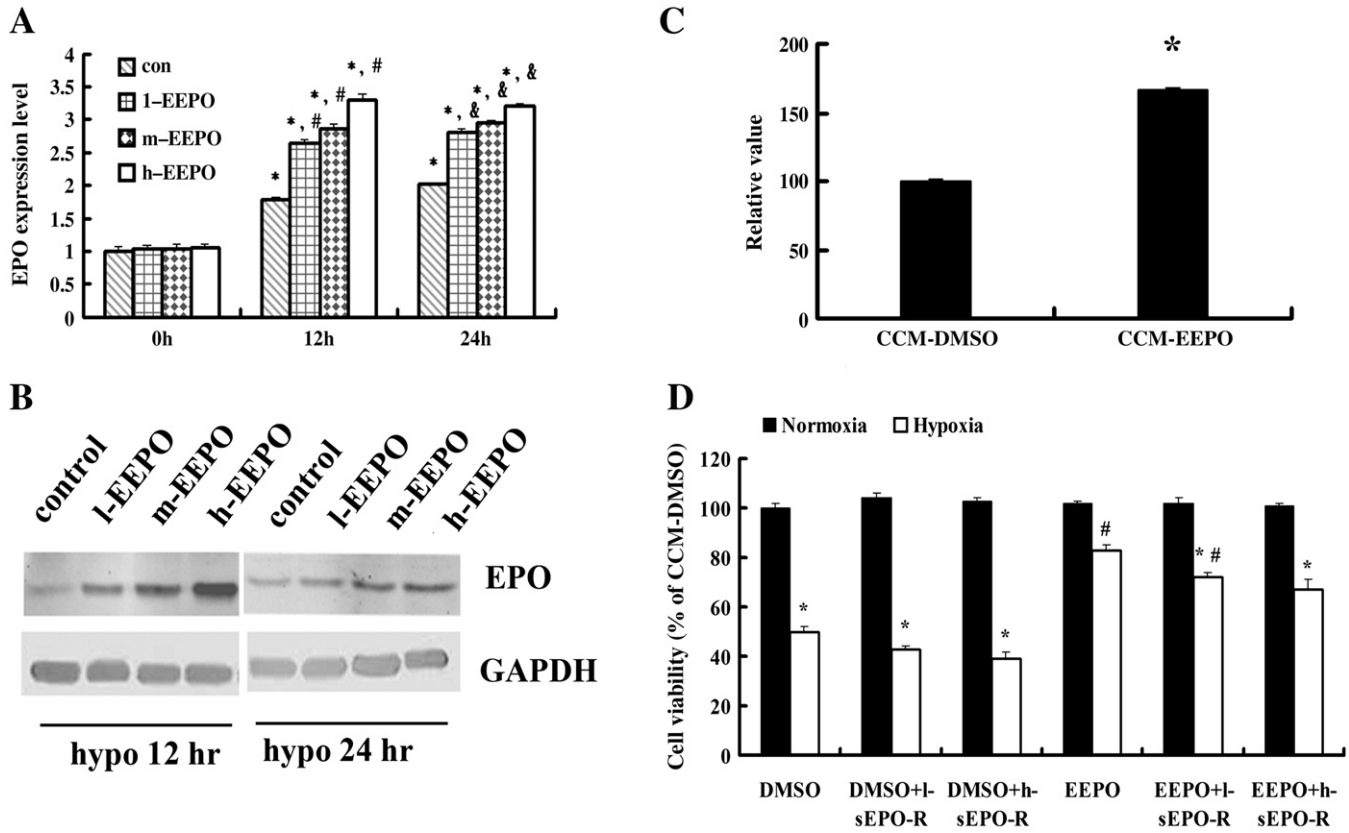

Fig. 3. Neuroprotective effects of EEPO involved modulation of endogenous EPO expression. The EEPO increased the EPO mRNA (A) and protein (B) expression in hypoxia neurons (1% oxygen). The EEPO increased the EPO level in the CCM (C). The CCM-EEPO protected hypoxia neurons by modulating endogenous EPO expression (D). The end concentrations of sEPO-R were 0.1 μg/ml (l-sEPO-R) or 0.5 μg/ml (h-sEPO-R). The end concentrations in the l-EEPO, m-EEPO and h-EEPO groups were 1, 10 and 100 μg/ml, respectively (A: *P<.05 compared with the normoxia control group receiving equal level of EEPO or DMSO. #P<.05 compared with the control group with 12-h hypoxia exposure. &P<.05 compared with the control group with 24-h hypoxia exposure, n=3~4. C: *P<.05 compared with CCM-DMSO, n=4. D: *P<.05 compared with normoxia control group, #P<.05 compared with the CCM-DMSO hypoxia group, n=6).

exogenous EPO [37]. Moreover, endogenous EPO is suggested to be a cytokine with a neurotrophic and a neuroprotective role in the nerve system, and it is becoming increasingly evident that EPO plays a critical role in the hypoxia preconditioning mediated responses in the nervous system [38]. Our present results showed that EEPO enhanced the EPO expression in hypoxia mouse cortices and cultured hypoxia neurons in a dose-dependent manner. It was reported that transfer of the culture supernatant from pretreated cells to OGD-challenged neurons protected the neurons *in vitro via* endogenously produced EPO, and administration of sEPO-R abolished the protective effect of EPO [20]. In the present study, we also found that there was a higher level of EPO in CCM-EEPO than in CCM-DMSO, and the cell viability of hypoxia neurons treated with CCM-EEPO was much higher than that of neurons treated with CCM-DMSO. Then, in order to test whether the protective effects are dependent on the EPO, we added sEPO-R into the culture medium to block EPO, and we found that the cell viability decreased in a dose-dependent manner with sEPO-R, indicating that the neuroprotective effects of EEPO are, at least partly, through modulating the endogenous EPO.

It has been widely accepted that the primary factor mediating hypoxia adaptation response is the HIF-1, an oxygen-sensitive transcriptional activator [39]. Hypoxia-inducible factor-1, belonging to Per-ARNT-SIM(PAS) superfamily, is characterized by the presence of PAS domain that controls dimerization [40]. Hypoxia-inducible factor-1 contains HIF1-α and HIF1-β [39], with HIF1-β constitutively expressed and HIF1-α tightly regulated by cellular oxygen concentration [41]. Hypoxia-inducible factor-1α is continuously degraded under normoxic condition by the ubiquitin–proteosome system but is stabilized by hypoxia when O_2-dependent prolyl hydroxylases that target its O_2-dependent degradation domain are inhibited [42]. Hypoxia-inducible factor-1 is the key regulator of EPO gene expression [43]. So we tested whether EEPO can modulate EPO expression by stimulating HIF-1. We found that EEPO enhanced the HIF1-α protein expression in hypoxia mouse cortices and hypoxia neurons, though EEPO did not affect HIF-1α mRNA expression. Additionally, EEPO enhanced HRE report gene activity, *vegf*, and glucose transporter-1 mRNA expression, which is modulated by HIF-1, suggesting that EEPO may enhance transcription of *vegf* and glucose transporter-1 *via* HIF-1a. These results indicate that EEPO can stimulate EPO expression in hypoxia neuronal cells and promote protein stabilization of HIF-1α. The posttranslational modulation of HIF-1α under hypoxia condition is a very complicated process [44]; further study is needed on the mechanism by which the EEPO affects degradation of HIF-1.

The following are the two limitations of the present study. First, the present research did not test individual components of the extracts. With gas chromatography–mass spectrometry method, we had investigated the chemical constituents of the end extracts and found that most of the extracts were the part with low polarity (about 60.12%); the other part includes alkaloids, tannins and flavonoids. In the future research, we will focus on the effective component of EEPO and their corresponding effective doses. Second, in this report, we aimed to study the antihypoxia effect of EEPO. It is obvious that pure hypoxia is a rare clinical condition compared to cerebral ischemic

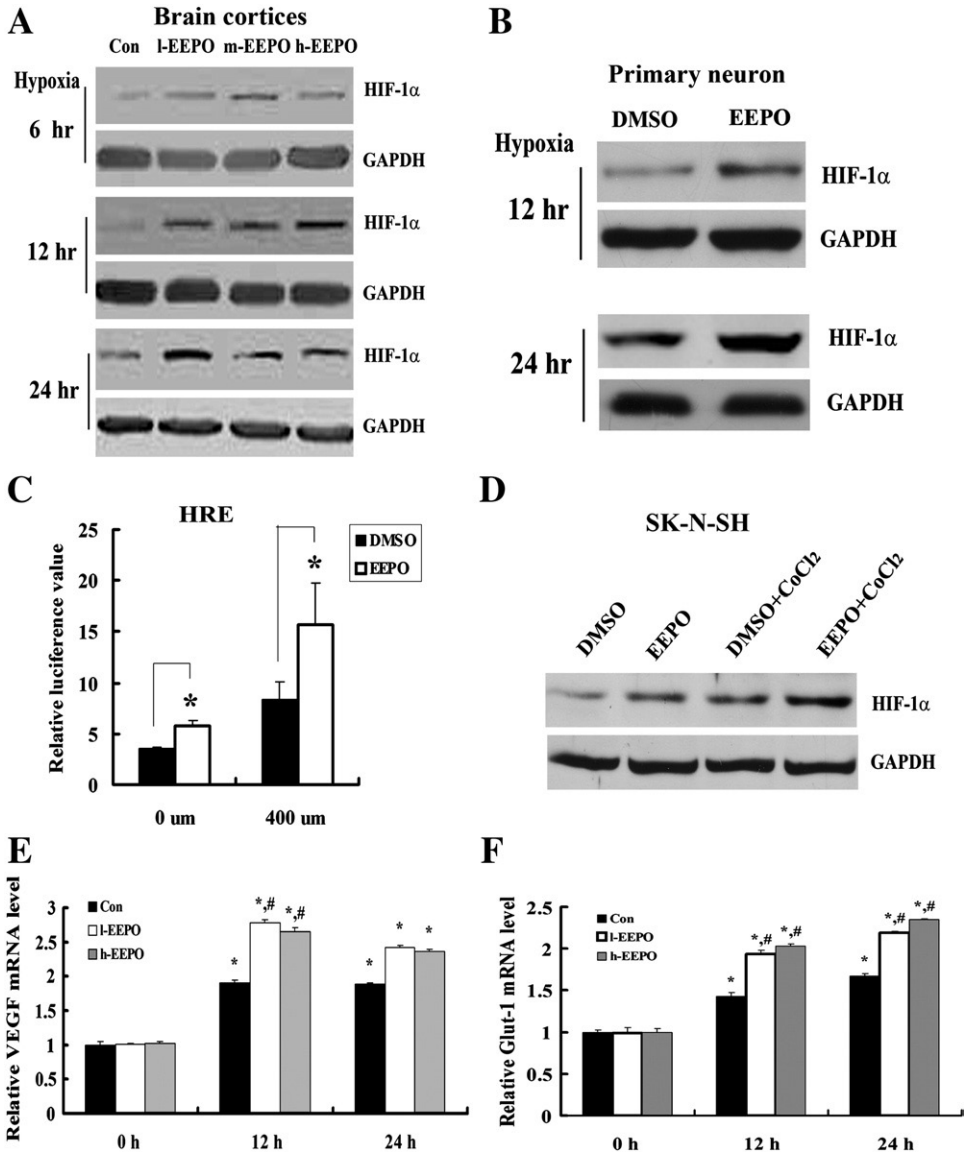

Fig. 4. The EEPO enhanced the HIF-1α protein expression and transcription activity. The EEPO enhanced HIF-1α protein expression in hypoxia mouse cortices (A) and in hypoxia neurons (B). The EEPO increased the HRE-luciferase reporter gene activities (C) and HIF-1α protein expression levels (D). The EEPO enhanced the *glu*-1(E) and *vegf* (F) expression in hypoxia neurons. The end concentrations of EEPO in the l-EEPO and h-EEPO groups were 1 and 100 μg/ml, respectively (D: *P<.05 compared with DMSO group, n=4. E and F: *P<.05 compared with the normoxia control group, #P<.05 compared with the control group under the same hypoxia condition, n=4).

events. For future clinical use of the EEPO, testing the neuroprotective effects against hypoxia/ischemia injury of the EEPO is of more significance.

In summary, our results confirm the protective effects of EEPO on hypoxia neural cells, which are, at least partly, through modulating of endogenous EPO expression, and the mechanism by which EEPO modulated the EPO expression might be related to the stabilization of HIF-1α. Our findings in the present study provide a useful and attractive candidate for developing neuroprotective agent for treatment of hypoxia-induced neurovascular diseases.

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References

[1] Kangae L, Robert AR, John JL. Hypoxia, drug therapy and toxicity. Pharmacol Ther 2007;113:229–46.
[2] Pulsinelli WA. Selective neuronal vulnerability: morphological and molecular characteristics. Prog Brain Res 1985;63:29–37.
[3] Naranda T, Wong K, Kaufman RI, Goldstein A, Olsson L. Activation of erythropoietin receptor in the absence of hormone by a peptide that binds to a domain different from the hormone binding site. Proc Natl Acad Sci U S A 1999;96:7569–74.
[4] Morishita E, Masuda S, Nagao M, Yasuda Y, Sasaki R. Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. Neuroscience 1997;76(1):105–16.
[5] Jelkmann W. Molecular biology of erythropoietin. Intern Med 2004;43:649–59.
[6] Marti HH. Erythropoietin and the hypoxic brain. J Exp Biol 2004;207(Pt 18):3233–42.

[7] Constance TN, Pundit A, Teng RF, Jia Y. Role of erythropoietin in the brain. Crit Rev Oncol/Hematol 2007;64:159–71.
[8] Cotena S, Piazza O, Tufano R. The use of erythropoietin in cerebral diseases. Panminerva Med 2008;50(2):185–92.
[9] Ehrenreich H, Weissenborn K, Prange H, Schneider D, Weimar C, Wartenberg K, et al. Recombinant human erythropoietin in the treatment of acute ischemic Stroke. Stroke 2009;40:e647–56.
[10] Minnerup J, Wersching H, Schäbitz WR. EPO for stroke therapy — is there a future for further clinical development? Exp Transl Stroke Med 2010;2:10.
[11] Hu LF, Xu XY, Wang BQ. Research and utilization situation of *Portulaca Oleracea L.* in China. Prac J Med Pharm 2003;20:315–6.
[12] Xiang L, Xing D, Wang W, Wang R, Ding Y, Du L. Alkaloids from P*ortulaca oleracea L.* Phytochemistry 2005;66(21):2595–601.
[13] Miller AG, Morris M. Plants of Dhofa, the Southern region of Oman. Traditional, economical and medicinal uses. The Office of the Advisor for Conservation of Environment, Diwan of Court, Sultanate of Oman; 1988.
[14] Ghazanfar SA. Handbook of Arabian medicinal plants. Boca Raton, FL: CRC Press; 1994. p. 176.
[15] Rashed AN, Afifi FU, Disi AM. Simple evaluation of the wound healing activity of a crude extract of *Portulaca oleracea L.* (growing in Jordan) in Mus musculus JVI-1. J Ethnopharmacol 2003;88:131–6.
[16] Zhang XJ, Ji YB, Qu ZhY, Xia JCh, Wang L. Experimental studies on antibiotic functions of *Portulaca oleracea L.* in vitro. Chinese J Microecol 2002;14: 277–80.
[17] Zhang HX, Yu NC, Huang GF, Shao JB, Wu YX, Huang HJ, et al. Neuroprotective effects of purslane herb aqueous extracts against d-galactose induced neurotoxicity. Chem-Biol Interact 2007;170:145–52.
[18] Wang WY, Gu LM, Wang X, Dong LW, Huang XQ, Li M. Protective effect of *Portulaca oleracea* extracts on hypoxic nerve tissue and its mechanism. Asia Pac J Clin Nutr 2007;16(Suppl 1):227–33.
[19] Chen ChJ, Wang WY, Wang XL, Dong LW, Yue YT, Xin HL, et al. Anti-hypoxic activity of the ethanol extract from *Portulaca oleracea* in mice. J Ethnopharmacol 2009;124:246–50.
[20] Ruscher K, Freyer D, Karsch M, Isaev N, Megow D, Sawitzki B, et al. Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an in vitro model. J Neurosci 2002;22:10291–301.
[21] Chen J, Shi YP, Liu JY. Determination of noradrenaline and dopamine in Chinese herbal extracts from *Portulaca oleracea L.* by high-performance liquid chromatography. J Chromatogr A 2003;1003:127–32.
[22] Jian JW, Xiao QX. Phytomedicine effective component book. Beijing: People's Hygiene Press; 1986.
[23] Xu XQ, Yu LS, Chen GN. Determination of flavonoids in *Portulaca oleracea L.* by capillary electrophoresis with electrochemical detection. J Pharm Biomed Anal 2006;41:493–9.
[24] Swamy M, Salleh MJ, Sirajudeen KN, Yusof WR, Chandran G. Nitric oxide (no), citrulline-no cycle enzymes, glutamine synthetase and oxidative stress in anoxia (hypobaric hypoxia) and reperfusion in rat brain. Int J Med Sci 2010;7(3): 147–54.
[25] Zhu L, Wu XM, Yang L, Du F, Qian ZM. Up-regulation of HIF-1alpha expression induced by ginkgolides in hypoxic neurons. Brain Res 2007;1166:1–8.
[26] Qian LB, Wang HP, Chen Y, Chen FX, Ma YY, Bruce IC, et al. Luteolin reduces high glucose-mediated impairment of endothelium-dependent relaxation in rat aorta by reducing oxidative stress. Pharmacol Res 2010;61(4):281–7.
[27] Wang GL, Semenza GL. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc Natl Acad Sci U S A 1993;90:4304–8.
[28] Giordano FJ, Johnson RS. Angiogenesis: the role of the microenvironment in flipping the switch. Curr Opin Genet Dev 2001;11(1):35–40.
[29] Semenza GL. Involvement of hypoxia-inducible factor 1 in human cancer. Intern Med 2002;41(2):79–83.
[30] Mukhopadhyay CK, Mazumder B, Fox PL. Role of hypoxia-inducible factor-1 in transcriptional activation of ceruloplasmin by iron deficiency. Biol Chem 2000;275(28):21048–54.
[31] Lu H, Forbes RA, Verma A. Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. J Biol Chem 2002;277(26):23111–5.
[32] Krishnamachary B, Berg-Dixon S, Kelly B, Agani F, Feldser D, Ferreira G, et al. Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. Cancer Res 2003;63(5):1138–43.
[33] Wenger RH. Mammalian oxygen sensing, signal and gene regulation. J Exp Biol 2000;203:1253–63.
[34] Semenza GL, Nejfelt MK, Chi SM, Antonarakis SE. Hypoxia-inducible nuclear factors bind to an enhancer element located 3′ to the human erythropoietin gene. Proc Natl Acad Sci U S A 1991;88(13):5680–4.
[35] Marti HH, Wenger RH, Rivas LA, Straumann U, Digicaylioglu M, Henn V, et al. Erythropoietin gene expression in human, monkey and murine brain. Eur J Neurosci 1996;8:666–76.
[36] Digicaylioglu M, Bichet S, Marti HH, Wenger RH, Rivas LA, Bauer C, et al. Localization of specific erythropoietin binding sites in defined areas of the mouse brain. Proc Natl Acad Sci U S A 1995;92:3717–20.
[37] Michael A, Kooij VD, Groenendaal F, Kavelaars A, Heijnen CJ, Bel FV. Neuroprotective properties and mechanisms of erythropoietin in in vitro and in vivo experimental models for hypoxia/ischemia. Brain Res Rev 2008;4:22–33.
[38] Liu J, Narasimhan P, Yu F, Chan PH. Neuroprotection by hypoxic preconditioning involves oxidative stress-mediated expression of hypoxia inducible factor and erythropoietin. Stroke 2005;36:1264–9.
[39] Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A 1995;92:5510–4.
[40] Huang ZJ, Ederry I, Rosbash M. PAS is a dimerization domain common to drosophila period and several transcription factors. Nature 1993;364:259–62.
[41] Semenza GL. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 1999;15:551–78.
[42] Salceda S, Caro J. Hypoxia-inducible factor 1α (HIF1-α) protein is rapidly degraded by the ubiquitin–proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem 1997;27: 222642–7.
[43] Jelkmann W, Wagner K. Beneficial and ominous aspects of the pleiotropic action of erythropoietin. Ann Hematol 2004;83(11):673–86.
[44] Giaccia A, Siim BG, Johnson RS. HIF-1 as a target for drug development. Nat Rev 2003;2:1–9.