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Protection of erythropoietin against ischemic neurovascular unit injuries through the effects of connexin43

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ABSTRACT

Erythropoietin (EPO) has protective effects on many neurological diseases, including cerebral ischemia. Here, we aimed to test EPO's effects on ischemic neurovascular unit (NVU) injuries and examine whether the effects were dependent on connexin43 (Cx43) mediated gap junctional intercellular communication (GJIC). We detected the expression of Cx43 and phosphorylation of Cx43 (p-Cx43) at 1 d, 3 d, and 7 d after middle cerebral artery occlusion (MCAO). Meanwhile, we examined the effects of EPO on NVU injuries including neuronal survival, astrocyte activation and regeneration of endothelial cells as well as whether the effects were Cx43 dependent by using multiple inhibitors. We found EPO highly increased p-Cx43, but not total Cx43 at all chosen times. Importantly, EPO led to neurological and blood–brain barrier functions improvement by associating with promotion of angiogenesis as well as reduction of neuronal death, astrocyte activation and neurotoxic substances levels. Moreover, these effects were significantly weakened by the inhibitors blocking GJIC, Cx43 communicative function, phosphorylation and expression, only Cx43 redistribution inhibitor excluded. Our data suggest the protective effects of EPO on NUV injuries are highly associated with the increase of p-Cx43, which improves GJIC to reduce neurotoxic substances.

1. Introduction

Erythropoietin (EPO) was originally recognized as the major hormone that stimulated and regulated the production of red blood cells. Now, more and more research supports the protective effects of EPO in cerebral ischemic models with multiple mechanisms [1]. Over the past 10 years, studies of the blood vessel in cerebrovascular disease have expanded from consideration of only endothelial cells to include interactions with neurons, astrocytes, pericytes, and extracellular matrix, thus the term “neurovascular unit (NVU)” is established. Several studies have reported EPO protects neuron and endothelial cells as well as attenuates astrogliosis respectively [2–4]. However, little research has systematically investigated the effects of EPO on NVU as a whole and the involved mechanisms remain unknown.

Gap junctional intercellular communication (GJIC) mediates electronic coupling and permits rapid propagation among cell networks. Connexin43 (Cx43) is the primary component protein in astrocytic gap junctions, which allows the passage of ions and small molecules [5]. During focal cerebral ischemia, the predominant change of NVU is disruption of its structural integrity and enhancement of blood–brain barrier (BBB) permeability [6]. It is also reported Cx43 knockout mice exhibited mostly reduction of intercellular communication and weakened the BBB, suggesting Cx43 regulates the integrity of the BBB, as well as the homeostasis of NVU [7].

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It is investigated that EPO can up-regulate Cx43 expression [8]. Based on the evidences that EPO is closely related with Cx43, as well as both of them are essential to NVU, we speculate that the effect of EPO on NVU following cerebral ischemia may result from regulating Cx43 expression. However, it is still controversial how Cx43 acts on cerebral ischemia. It was shown that Cx43 knockout exacerbated ischemic injury [9]. On the other hand, Cx43 inhibitor was exhibited protective effects after cerebral ischemia [10]. More importantly, intracellular redistribution and phosphorylation of Cx43 may be two crucial forms to develop its function following cerebral ischemia [11,12]. Therefore, we suspect that EPO may influence ischemic NVU damage via changing the special forms of Cx43.

To test these hypotheses, the effect of EPO on NVU after ischemic injury was first investigated. We subsequently selected specific inhibitors to suppress GJIC, Cx43 communicative function, redistribution, phosphorylation and expression, then explored the involved mechanisms through observing the effects of these inhibitors.

2. Materials and methods

2.1. Animals and experimental groups

Male Sprague–Dawley (SD) rats weighing 280–300 g provided by the Experimental Animal Center of Guangzhou University of Chinese Medicine in China were housed in temperature (22 ± 2 °C) and humidity controlled (55 ± 5%) rooms. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Guangzhou University of Chinese Medicine.

Rats were randomly divided into eight groups: Group 1: Sham operation. Group 2: middle cerebral artery occlusion (MCAO). Group 3: MCAO plus EPO. Group 4: MCAO plus EPO plus carbenoxolone (CBX, Sigma–Aldrich, St. Louis, MO, USA, a GJIC inhibitor). Group 5: MCAO plus EPO plus Cx43 mimetic peptide (CMP, also called Gap26, APExBIO, Houston, TX, USA, a Cx43 inhibitor). Group 6: MCAO plus EPO plus Dynasore (Sigma–Aldrich, a Cx43 redistribution inhibitor). Group 7: MCAO plus EPO plus Ro318220 (Sigma–Aldrich, a protein kinase C (PKC) ε inhibitor). Group 8: MCAO plus EPO plus Cx43 small interfering RNA (siRNA). The parameters were detected at 1 d, 3 d and 7 d after MCAO and each parameter of each group contained six rats.

2.2. Rat MCAO model and drugs injection

Rats were anesthetized with chloral hydrate (350 mg/kg, intraperitoneally) and the left common carotid artery (CCA), the external carotid artery (ECA), and the internal carotid artery (ICA) were exposed. A length of 3.0 monofilament nylon suture (18.5–19.5 mm) with its tip rounded by heating near a flame, was advanced from the ECA into the lumen of the ICA until it blocked the origin of MCA. 1.5 h after MCAO, reperfusion was initiated by withdrawal of the suture until the tip cleared the lumen of the ECA. Sham-operated animals were subjected to the same surgical procedure but the suture was not introduced. Recombinant human EPO (rhEPO, Sunshine Pharmaceuticals, Shenyang, China) was injected intraperitoneally with a dose of 5000 IU/kg at 2 h after reperfusion and daily afterward for 7 d. The drugs including CBX (1.5 μg/g), CMP (3.5 μg/g), Dynasore (50 ng/g) and Ro318220 (4 ng/g) were injected intracerebroventricularly immediately after reperfusion with a stereotaxic frame (Alctt Biotech, Shanghai, China) at 1.5 mm lateral to the midline, 1.5 mm posterior to the coronal suture and at a depth of 3.5 mm from the surface of the brain. The doses of drugs were according to our preliminary experiments and previous reports [13–17].

2.3. SiRNA

This section was prepared as previously reported [18]. ON-TARGET plus SMARTpool Cx43 siRNA was purchased from Thermo Scientific (Grand Island, NY, USA). Single deprotected strands were resuspended with an isotonic buffer to a concentration of 1 μg/μl. The strands were incubated at 90 °C for 5 min and then at 37 °C for 1 h. SiRNA was prepared immediately before administration by mixing the RNA solution (1 μg/μl in annealing buffer) with the transfection reagent i-Fect (v/v: 1/3; Neuromics, Edina, MN, USA) to a final siRNA/lipid complex concentration of 0.25 μg/μl. Cx43 siRNA was infused intracerebroventricularly with 40 μl per rat at 1 d before MCAO.

2.4. Neurological testing

A standardized battery of behavioral tests was used to quantify neurological function at 1 d, 3 d and 7 d after MCAO as reported by Chen et al. [19]. The tests included motor tests, sensory tests, beam balance tests, reflexes absent and abnormal movements. Neurological function was graded on a scale of 0–18 (normal score: 0; maximal deficit score: 18) and the higher score, the more severe was the injury. Tests were conducted by an observer blinded to the treatment groups.

2.5. Determination of BBB permeability with the use of Evans blue

Evans blue (EB, Sigma–Aldrich) in normal saline was injected intravenously. The rats were anesthetized 1 h later and perfused with 200 ml of normal saline solution through the left cardiac ventricle. The rats were decapitated and the tissue samples in the peri-infarct area were obtained. The samples were homogenized in methylformamide (1 ml/100 mg brain tissue), incubated for 24 h at 60 °C, and centrifuged for 5 min at 1000 rpm. The absorbance (A) of supernatants was analyzed at 632 nm by spectrophotometry [20].

2.6. The intracellular free calcium ion concentration (i[Ca^{2+}]) examination

The determination of i[Ca^{2+}] was performed according to reported protocol [21]. The single brain cell suspension was non-invasively labeled with Fluo-3/AM at a concentration of 3 pM in phenol red-free medium at 37 °C for 1 h. This membrane non-fluorescent acetoxymethyl ester was converted to fluorescent form by intracellular esterases. It then exhibited a 40-fold increase in fluorescence intensity upon Ca^{2+} binding. Selected cells in each chamber were scanned by the TCS SP2 ($E_{\lambda X}$ = 488 nm; $E_{\lambda M}$ = 530/30 nm), and the fluorescent emission was monitored. Excitation and detection parameters were kept constant in all experiments.

2.7. Determination of glutamate level

Glutamate level was measured using glutamate colorimetric assay kit (BioVision, Milpitas, CA, USA). Sample homogenates preparation and assays were performed as recommended by the manufacturer. The level of glutamate in brain tissues was normalized and expressed as μmol per g of total protein.

2.8. Western blot

Proteins (50 μg) were loaded onto 4% stacking/12% separating SDS-polyacrylamide gels for electrophoresis, and then transferred onto nitrocellulose transfer membranes. After blocked, membranes were incubated overnight at 4 °C with anti-Cx43 and anti-p-Cx43 (both 1:1000, Sigma–Aldrich) rabbit polyclonal antibodies.

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