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Erythropoietin ameliorates early brain injury after subarachnoid haemorrhage by modulating microglia polarization via the EPOR/JAK2-STAT3 pathway

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ABSTRACT

Inflammatory modulation mediated by microglial M1/M2 polarization is one of the main pathophysiological processes involved in early brain injury (EBI) after subarachnoid haemorrhage (SAH). Previous studies have shown that recombinant human erythropoietin (rhEPO) alleviates EBI following experimental SAH. However, the mechanisms of this beneficial effect are still poorly understood. Recent research has suggested that EPO shows anti-inflammatory properties. Therefore, we tried to analyse whether rhEPO administration influenced microglial M1/M2 polarization in early brain injury after SAH and to identify the underlying molecular mechanism of any such effect. We found that treatment with rhEPO markedly ameliorated SAH-induced EBI, as shown by reductions in brain cell apoptosis, neuronal necrosis, albumin exudation and brain edema. Moreover, the expression levels of *p*-JAK2 and *p*-STAT3 were significantly increased in the cortex after SAH induction and were further increased by EPO treatment; in addition, the *p*-JAK2 inhibitor AZD1480 impaired the protective effect of EPO against SAH-induced EBI in vivo. Furthermore, EPO promoted the polarization of microglia towards the protective M2 phenotype and alleviated inflammation. In cultured microglia under oxyhemoglobin (OxyHb) treatment, EPO up-regulated the expression of the EPO receptor (EPOR), which did not occur in response to OxyHb treatment alone, and EPO magnified OxyHb-induced increases in *p*-JAK2 and *p*-STAT3 and modulated OxyHb-challenged microglial polarization towards M2. Interestingly, the effect of EPO on microglia polarization was cancelled by EPOR knockdown or by *p*-JAK2 or *p*-STAT3 inhibition, suggesting a core role of the EPOR/JAK2/STAT3 pathway in modulating microglial function and phenotype. In conclusion, the therapeutic effect of rhEPO on the early brain injury after SAH may relate to its modulation of inflammatory response and microglia M1/M2 polarization, which may be mediated in part by the EPOR/JAK2/STAT3 signalling pathway. These results improved the understanding of the anti-inflammatory effect of EPO on microglia polarization, which might optimize the therapeutic modalities of EPO treatment with SAH.

1. Introduction

Subarachnoid haemorrhage (SAH) is a severe condition characterized by high mortality and disability. Early brain injury (EBI) is now considered one of the most important causes of disability and death in SAH patients [1,2]. To date, there are no truly effective treatments for this condition, and numerous experimental and clinical studies have been performed for the potential development of effective therapeutic strategies. Several studies on experimental models of SAH have demonstrated that recombinant human erythropoietin (rhEPO) can prevent vasospasm [3–7]. Our previous studies found that EPO can also alleviate hypoxic-ischaemic injury or intracerebral haemorrhage by restoring cerebral blood flow and attenuating inflammatory factors [8,9]. Chen et al. also found that EPO could reduce EBI through

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modulating cerebral oxidative stress by inducing antioxidant and detoxifying enzymes after SAH in rats [10]. Experimental evidence suggests the efficacy of EPO-based therapy in SAH [11–15]. However, the role and mechanism of EPO treatment with SAH still need to be explored and clarified.

An increasing number of studies, including our previous studies, have convincingly demonstrated that EPO plays an important role not only in neurovascular protection but also in immune and inflammatory regulation [16–18]. The immunomodulatory capabilities of EPO may present a new approach to the use of EPO treatment for SAH. Increasing evidence from preclinical and clinical studies indicates that not only innate immune cells but also adaptive immune cells are recruited and activated in SAH [19], suggesting activation of the whole immune system in SAH.

Microglia are the frontline soldiers of immune defence in the central nervous system, serving as tissue-resident macrophages that influence immune response to injury and repair [20]. Blood components, including heme, thrombin, platelets and leukocytes, activate the resident microglia, leading to robust production of cytokines. Chemokines stimulate peripheral immune cells such as neutrophils and monocytes; these cells rapidly infiltrate to the location of injury, where resident microglia and infiltrated immune cells release cytokines and trigger the inflammatory cascade [3]. Compared with peripheral macrophages, microglia present similar characteristics in regard to phenotypic transformation and changes in immunological function. In response to different stimuli, microglia assume diverse phenotypes, including the two phenotypes of M1 and M2 [21,22]. The M1 phenotype tends to release pro-inflammatory cytokines that aggravate tissue injury; by contrast, the M2 phenotype preferentially releases anti-inflammatory cytokines and neurotrophic factors that promote inflammation resolution and tissue repair [23,24]. Notably, simply suppressing all types of microglial activation not only fails to produce a better outcome but also may exacerbate neuronal injury [25]. Given this, modulating microglia polarization towards the M2 phenotype may be the best way to mitigate inflammatory injury induced by the unbalanced activation of microglia [26].

It has been demonstrated that EPO can modulate macrophage M1/M2 polarization through enhancement of EPOR expression in acute pulmonary inflammation and can reduce pulmonary and intestinal inflammation [17,18]. Furthermore, Chen's research has indicated that the JAK2/STAT3 pathway participates in the protective effect of EPO in SAH [27]. Thus, the aim of the current study was to determine whether rhEPO treatment could change EPOR/JAK2/STAT3 activity, microglial polarization, and expression of anti-inflammatory genes in early brain injury following SAH. We hypothesized that the effect of rhEPO on the modulation of inflammatory response and microglia polarization through the EPOR/JAK2/STAT3 pathway may be one mechanism by which rhEPO decreases the degree of early brain injury.

2. Materials and methods

2.1. Animals

C57BL/6 J mice (25–28 g, 10–12 weeks old) were purchased from the Animal Center of the Third Military Medical University, Chongqing, China. The mice were housed in temperature- and humidity-controlled animal quarters, with a 12-h/12-h light/dark cycle. All animal experimental treatments were approved by the Animal Care and Use Committee of the Third Military Medical University and were performed in accordance with the guidelines of the National Institutes of Health on the care and use of animals.

2.2. Mouse SAH model

A well-characterized model of experimental SAH [28] was implemented. Each mouse was anaesthetized with isoflurane, and its head was fixed in a stereotactic frame. We made an 8-mm incision and drilled a 0.9-mm hole into the skull 4.5 mm anterior to the bregma. Subsequently, we advanced a spinal needle to the chiasmatic cistern, and 100 μL of arterial blood was injected into the intracranial space for 60 s. Mice in the sham group were injected with an equal volume of saline. The scalp incision was closed. Animals that died after the operation were excluded. At the end of the experiments, some of the animals were perfused with 4 °C paraformaldehyde (4%) under deep anaesthesia, and their brains were subsequently postfixed and dehydrated overnight at 4 °C. Eight-micrometre-thick coronal sections were cut on a freezing microtome for later Nissl, TUNEL, and immunofluorescence staining analysis. The rest of the animals were decapitated under deep anaesthesia, and their brains were harvested for brain edema measurement, qRT-PCR and Western blot analysis.

2.3. Cell cultures and stimulation

BV-2 cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, streptomycin (100 U/ml), and penicillin (100 U/ml) (KeyGen). For in vitro simulation of SAH-like activation, cells were seeded in Petri dishes or 24-well plates for 48 h, and the medium was changed to serum-free medium. The cells were then treated with oxyhemoglobin (OxyHb, Sangon Biotech, Shanghai, China) with or without rhEPO (Sunshine Pharmaceutical, Shenyang, China) for 24 h. Subsequently, cells were analysed by Western blot, qRT-PCR and immunofluorescence staining.

2.4. Groups and drug administration

In experiment 1, 72 mice (a total of 78 mice were used, of which 6 died after induction of SAH) were assigned randomly into three groups: the sham group, the SAH group, and the SAH + EPO group (n = 24 for each group). rhEPO was dissolved in sterile saline and injected intraperitoneally 5 min after SAH induction. The dose of rhEPO used was 5 U/g body weight [29]. It has been reported that the activation of microglia reaches a peak at 24 h after SAH [30]. Therefore, the animals that underwent experimental SAH were killed 24 h after blood injection.

In experiment 2, 96 mice (a total of 104 mice were used, of which 8 died after induction of SAH) were assigned randomly into four groups: the sham group, the SAH + Veh group, the SAH + EPO + Veh group, and the SAH + EPO + AZD1480 (Selleck Chemicals, Houston, TX, USA) group (n = 24 for each group). The mice in the SAH + EPO + AZD1480 group received a single injection of AZD1480 intraperitoneally (30 μg/g body weight) [31] immediately after induction of SAH. Mice in the vehicle groups were treated with an equal volume of solvent. All the mice in experiment 2 were sacrificed 24 h after SAH induction.

In experiment 3, BV-2 cells were divided into 7 groups: Vehicle, OxyHb + Vehicle, OxyHb + EPO + Vehicle, OxyHb + EPO + siRNA-scr (Santa Cruz, CA, USA), OxyHb + EPO + siRNA-EPOR (Santa Cruz), OxyHb + EPO + AZD1480, OxyHb + EPO + Stattic (Selleck Chemicals). Cells in the siRNA-transfection groups were transfected with siRNA-EPOR (100 nM) or scrambled-sequence siRNA (concentration equal to siRNA-EPOR). Forty-eight hours after siRNA transfection, cells were treated with OxyHb (20 μM), rhEPO (40 U/ml) [18], AZD1480 (5 μM), and/or Stattic (10 μM) in fresh medium and incubated for 24 h (at 37 °C, 5% CO_2), after which the samples were subjected to analysis.

2.5. Brain water content

Brain edema was assessed with the wet/dry method. Briefly, brain samples were rapidly removed from the skull, placed separately into preweighed glass vials, and weighed to acquire wet weight. The vials