Biochemical and Biophysical Research Communications 458 (2015) 656–662

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

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

Ziyi Zhou [a, 1], Xiaobai Wei [b, 1], Jun Xiang [c], Junpeng Gao [d], Lixin Wang [a], Jinsong You [a], Yefeng Cai [a, **], Dingfang Cai [c, *]

[a] Department of Neurology, Second Affiliated Hospital, Guangzhou University of Chinese Medicine and Guangdong Provincial Academy of Chinese Medical Sciences, Guangdong Provincial Hospital of Chinese Medicine, No. 111 Dade Road, Guangzhou 510120, China
[b] Department of Chinese Integrative Medicine, Huashan Hospital, Fudan University, No.12 Mid. Wulumuqi Road, Shanghai 200040, China
[c] Laboratory for Neurological Research of the Institute of Chinese Integrative Medicine, Zhongshan Hospital, Fudan University, No.180 Fenglin Road, Shanghai 200032, China
[d] Department of Chinese Integrative Medicine, Guangzhou First People's Hospital, Guangzhou Medical University, No. 1 Panfu Road, Guangzhou 510180, China

ARTICLE INFO

Article history:
Received 29 January 2015
Available online 13 February 2015

Keywords:
Erythropoietin
Neurovascular unit
Connexin43
Cerebral ischemia
Gap junctional intercellular communication

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.

© 2015 Elsevier Inc. All rights reserved.

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].

* Corresponding author. Fax: +86 21 64041912.
** Corresponding author. Fax: +86 20 39358023.
E-mail addresses: caiyefeng@126.com (Y. Cai), caidingfang@163.com (D. Cai).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2015.02.020
0006-291X/© 2015 Elsevier Inc. All rights reserved.

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.

Membranes were then incubated for 1 h at room temperature with horseradish peroxidase labeled goat anti-rabbit secondary antibody (1:4000, Invitrogen, Carlsbad, CA, USA). The membranes were placed into ECL solution for 5 min, and then exposed. The intensity of blots was quantified using the Leica Image Processing and Analysis System. β-Actin was used as an internal control.

2.9. Immunofluorescence

The sections were sequentially washed, blocked by 10% goat serum, incubated overnight at 4 °C with anti-Cx43, anti-p-Cx43 (both 1:500, Sigma–Aldrich), anti-neuron-specific nuclear protein (NeuN), anti-glial fibrillary acidic protein (GFAP) and anti-CD31(all 1:500, Millipore, Billerica, MA, USA) and then incubated 1 h a 37 °C with fluorescein isothiocyanate (FITC) coupled secondary goat anti-rabbit IgG (1:500, Invitrogen). The nuclei in Cx43 and p-Cx43 were highlighted by stained with 4', 6-diamidino-2-phenylindole (DAPI).

2.10. Cell counting

For determining cell counting, coronal sections of 10 μm were cut from the optic nerve to mamillary body. Every tenth section was chosen after the infarction area was first spotted and six sections were selected altogether. Then six nonoverlapping fields per section in the peri-infarct zone under high power lens (× 200 in NeuN and GFAP staining and × 400 in CD31 staining) were randomly chosen. The average was regarded as the NeuN, GFAP or CD31 staining cell numbers.

2.11. Statistical analysis

All data were presented as mean ± SEM and analyzed with SPSS12.0. Differences between groups were compared by using one-way analysis of variance (ANOVA). When the ANOVA identified significant between-group differences, Tukey's honestly significant difference (HSD) tests were used for intergroup comparisons. Differences were considered significant at $P < 0.05$. All analyses were conducted by an observer blinded to the genotype of the mice.

3. Results

3.1. Expression of Cx43 in each group of rats

Both Western blot and immunofluorescence revealed an increase of Cx43 in the peri-infarct area at 1 d, 3 d, and 7 d after MCAO ($p < 0.01$). RhEPO had no effect on Cx43 protein expression after

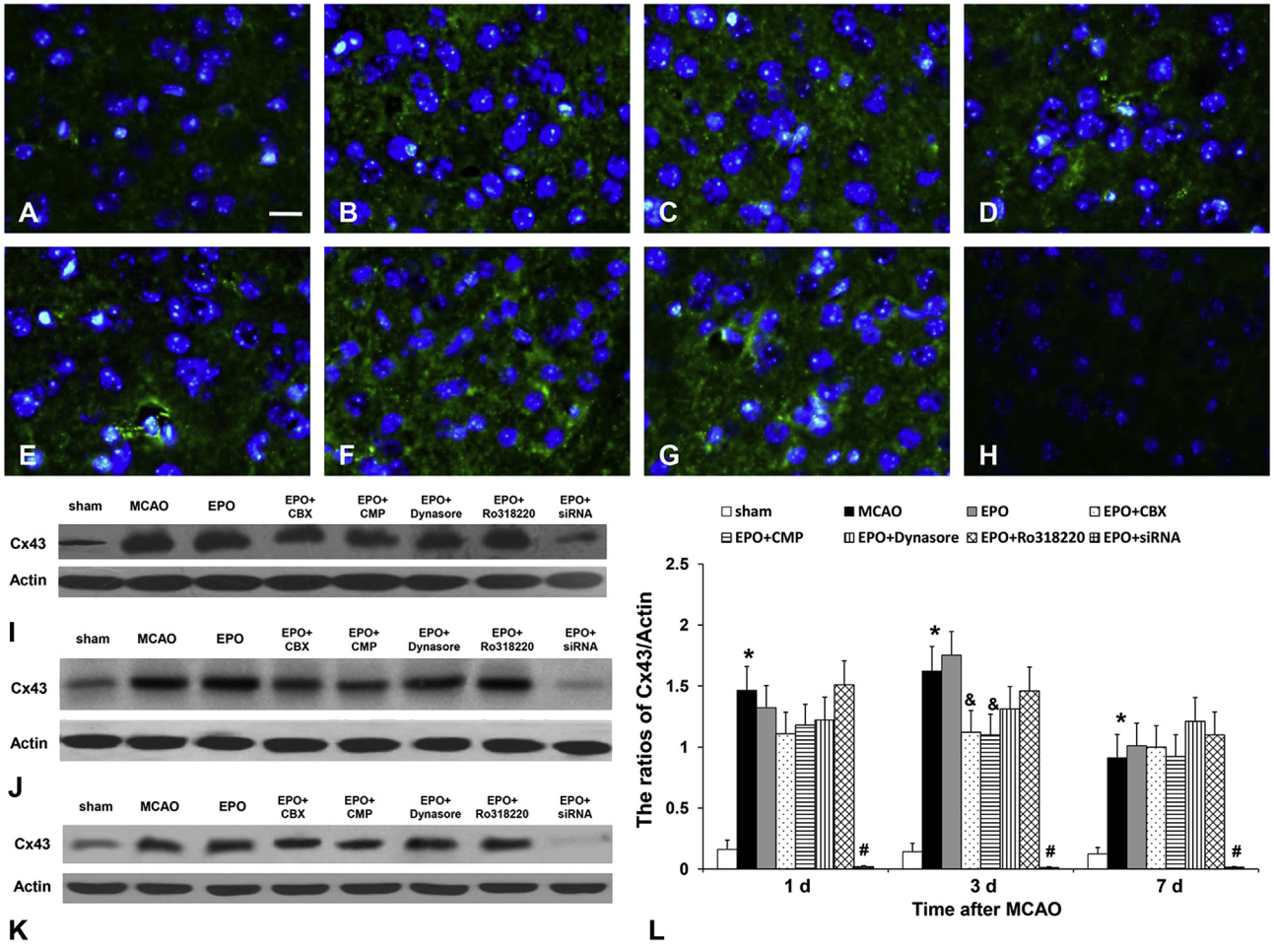

Fig. 1. Expression of Cx43 in each group of rats. (A–H) Immunofluorescence of Cx43 protein at 3 d after MCAO in groups of sham operation, MCAO, EPO, EPO + CBX, EPO + CMP, EPO + Dynasore, EPO + Ro318220, EPO + Cx43 siRNA respectively. Nuclei are highlighted by DAPI staining. Scale bar: 50 μm. (I–L) Western blot showed Cx43 was up-regulated at 1 d, 3 d, and 7 d after MCAO (n = 6, $^{*}p < 0.01$ vs. sham operation), which was not changed by rhEPO. There is no difference between the inhibitors including CBX, CMP, Dynasore and Ro318220 groups and EPO group, except a slight down-regulation by CBX and CMP at 3 d after MCAO (n = 6, & $p < 0.05$ vs. EPO). Cx43 protein was hardly detected after RNA interference (n = 6, #$p < 0.01$ vs. EPO). (I–K) Western blot images of 1 d, 3 d, and 7 d after MCAO. (L) Semi-quantitive analysis.

MCAO at any time point. Administration of inhibitors including CBX, CMP, Dynasore and Ro318220 did not change rhEPO's such effect, except a slight down-regulation by CBX and CMP at 3 d after MCAO ($p < 0.05$). Cx43 protein was hardly detected after RNA interference. The statistical differences were found in semi-quantitive analysis of Western blot (Fig. 1).

3.2. Expression of p-Cx43 in each group of rats

Similar to the section before, p-Cx43 protein was strongly up-regulated after MCAO ($p < 0.01$). However, both Western blot and immunofluorescence showed intraperitoneal injection of rhEPO significantly increased p-Cx43 in the peri-infarct area at 1 d, 3 d, and 7 d after MCAO ($p < 0.01$), which was far different from total Cx43. Only intracerebroventricularly injected of Ro318220 and Cx43 siRNA counteracted the effect of p-Cx43 up-regulation by rhEPO ($p < 0.01$). Also, semi-quantitive analysis of Western blot provided statistical differences (Fig. 2).

3.3. Neurological scores and EB extravasation amount in each group of rats

Neurological scores and EB extravasation amount were increased at 1 d, 3 d, and 7 d after MCAO, while rhEPO attenuated these effects ($p < 0.01$). Intracerebroventricularly injected of inhibitors containing CBX, CMP, Ro318220 and Cx43 siRNA blocked protective effects on neurological function and BBB integrity of rhEPO at all the three time points ($p < 0.01$) (Fig. 3A, B).

3.4. i[Ca^{2+}] and glutamate levels in each group of rats

To investigate the effect of rhEPO on GJIC, we selected two important neurotoxic substances Ca^{2+} and glutamate, which pass into or out of cells though Cx43. As the neurotoxicity, an intensive increase of them was detected at 1 d, 3 d, and 7 d after MCAO ($p < 0.01$). Levels of i[Ca^{2+}] and glutamate were reduced rhEPO at the three time points ($p < 0.01$). Furthermore, such neuropretective effects were greatly weakened by CBX, CMP, Ro318220 and Cx43 siRNA ($p < 0.01$), only except Dynasore (Fig. 3C, D).

3.5. NeuN, GFAP, and CD31 staining in each group of rats

We evaluated neuronal injury using NeuN staining and positive cells were survival neurons. GFAP is an astrocyte specific protein and was used to label astrocyte activation in our study. CD31 is predominantly expressed in neovascular endothelial cells, so we used it to assess changes of these cells. The changing trend of NeuN staining cells can be found in microscopic images and quantitive analysis was made by means of positive cell counting. There was a decrease of NeuN with an increase of GFAP and CD31 staining at 1 d,

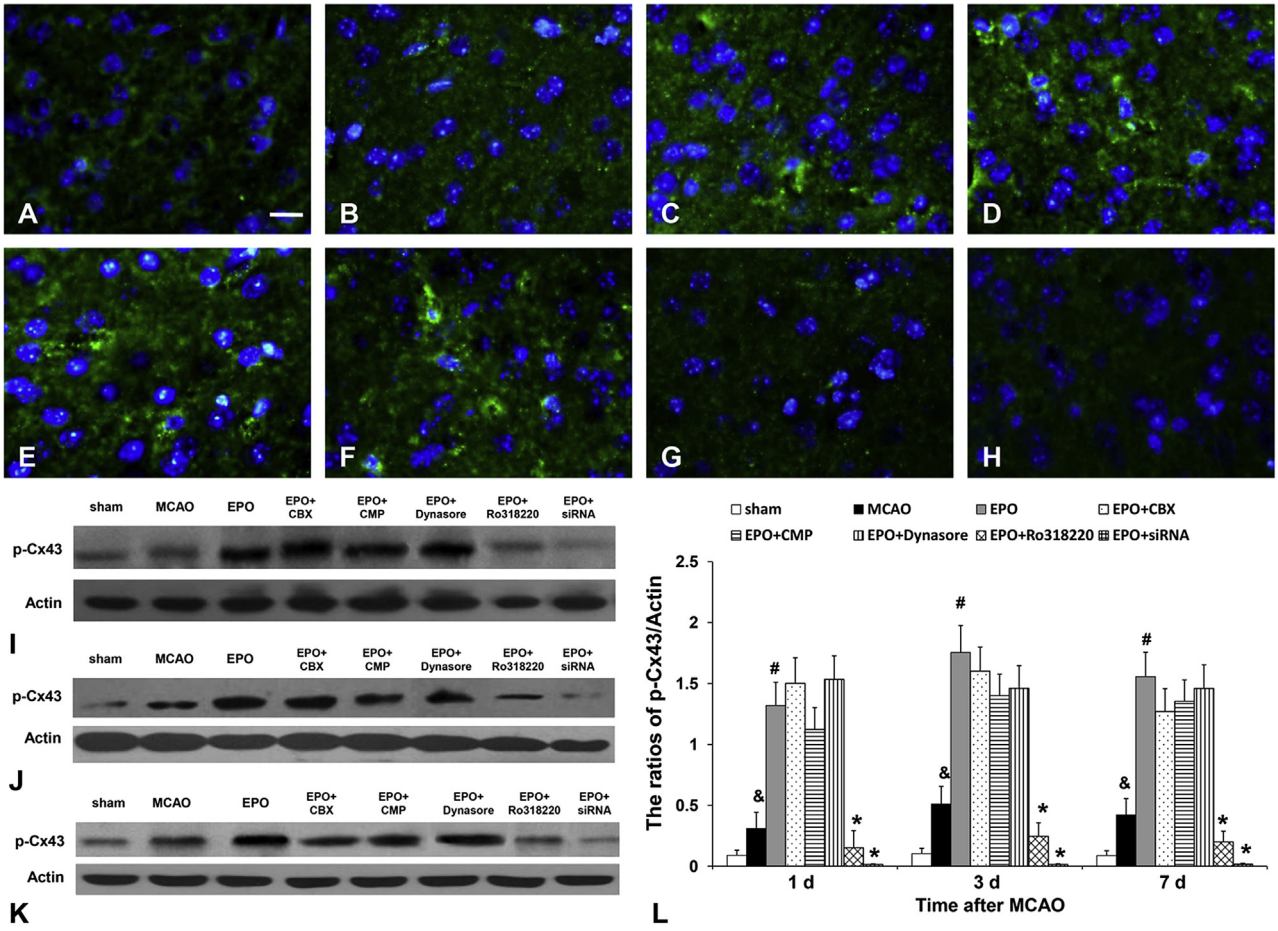

Fig. 2. Expression of p-Cx43 in each group of rats. (A–H) Immunofluorescence of p-Cx43 protein at 3 d after MCAO in groups of sham operation, MCAO, EPO, EPO + CBX, EPO + CMP, EPO + Dynasore, EPO + Ro318220, EPO + Cx43 siRNA respectively. Nuclei are highlighted by DAPI staining. Scale bar: 50 μm. (I–L) It was showed an up-regulation of p-Cx43 at 1 d, 3 d, and 7 d after MCAO by Western blot (n = 6, & $p < 0.01$ vs. sham operation), which was further increased by rhEPO (n = 6, #$p < 0.01$ vs. MCAO). Only Ro318220 and Cx43 siRNA counteracted rhEPO's effect (n = 6, *$p < 0.01$ vs. EPO). (I–K) Western blot images of 1 d, 3 d, and 7 d after MCAO. (L) Semi-quantitive analysis.

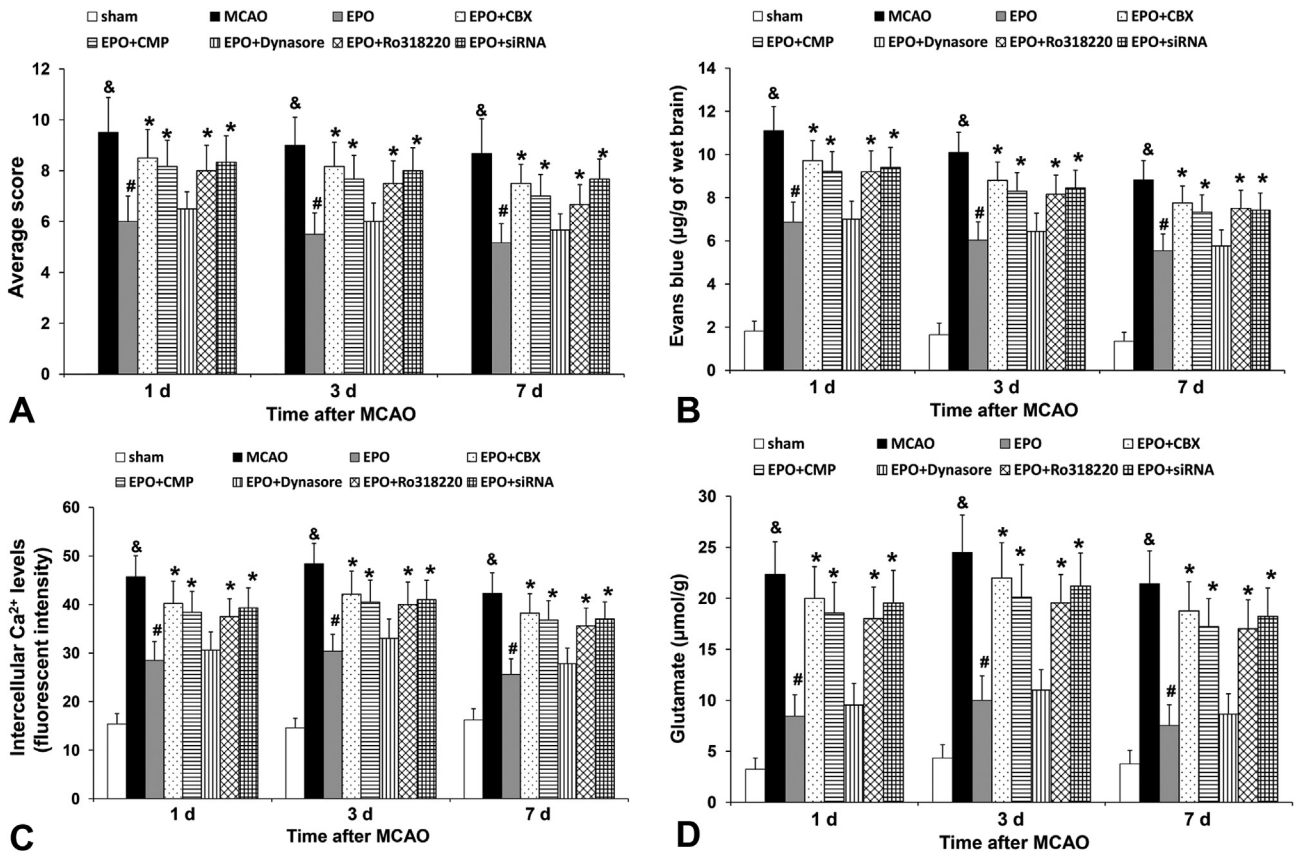

Fig. 3. Neurological scores, EB extravasation amount, i[Ca^{2+}] and glutamate levels in each group of rats. All the four parameters were increased at 1 d, 3 d, and 7 d after MCAO (n = 6, & $p < 0.01$ vs. sham operation). RhEPO significantly reduced neurological scores, EB extravasation amount, i[Ca^{2+}] and glutamate levels at each time point (n = 6, #$p < 0.01$ vs. MCAO), which was blocked by the inhibitors containing CBX, CMP, Ro318220 and Cx43 siRNA (n = 6, *$p < 0.01$ vs. EPO). (A) Neurological scores. (B) EB extravasation amount. (C) i[Ca^{2+}] levels. (D) Glutamate levels.

3 d, and 7 d after MCAO ($p < 0.01$). RhEPO increased NeuN and CD31, while reduced GFAP staining ($p < 0.01$), which was attenuated by the inhibitors containing CBX, CMP, Ro318220 and Cx43 siRNA ($p < 0.01$) (Fig. 4).

4. Discussion

In this study, we demonstrated EPO reduced neuronal death, blocked astrocyte activation and protected structure and function of endothelial cells in focal cerebral ischemic model. In the previous research, similar results were revealed by separate experiments in different CNS injury models [2–4,13]. Since neuron, astrocyte and endothelial cell are most important components in NVU and our results are in accord with previous work, we first disclosed EPO had protective effects on NVU in one single model. Moreover, the three time points selected in our study: 1 d, 3 d and 7 d after MCAO, are just in phases of NVU injuries starting, aggravation and restoration respectively, thus making possible to continuous observation of NVU damage after MCAO in different typical pathological phases. As the above effects were observed, we continuously investigated the involved mechanisms.

Our results further indicated EPO reduced levels of i[Ca^{2+}] and glutamate, which are essential neurotoxic substances passing into or out of cells through GJIC to damage NUV after cerebral ischemia [22]. It suggests regulation of GJIC may mediate EPO's protective effects on ischemic NVU injuries. Of the gap junction proteins, Cx43 is the most ubiquitously expressed and predominantly found in astrocyte end-foot processes along blood vessels thus impacting on BBB disruption and neuronal death [11]. So we focused the target on Cx43. We demonstrated EPO had no effect on total Cx43 expression in peri-infarct area via immunofluorescence and Western blot. The result is inconsistent with previous research, which is probably owing to differences of drug injection ways (intraperitoneal versus invenous) and pathological states (cerebral ischemia versus myocardial Infarction) [8]. Because phosphorylation is an important activated form, we further observed p-Cx43 expression and found EPO significantly up-regulation p-Cx43 after MCAO. Moreover, effects of Cx43 itself on cerebral ischemia are still controversial. It was found Cx43 deletion worsened neuronal apoptosis after MCAO [9]. However, other studies revealed that in ischemic models, Cx43 blockade improved outcomes and Cx43 knockout reduced neuronal apoptosis [10,14]. So Cx43 seems to have dual influence on cerebral ischemia and the influence, to some degree, is complex and comprehensive. For this reason, we chose multiple inhibitors to explore effects of Cx43 through different ways.

We selected five different inhibitors in total. CBX is well known as a common GJIC blocker, used to block cell-to-cell communication [23]. CMP is a mimetic peptide corresponding to a short linear sequence in the first extracellular loop of Cx43, used to block intracellular communication by Cx43 [24]. Dynasore is an inhibitor of dynamin GTPase activity, which reduced the association between dynamin2 and Cx43. Considering dynamin2 may control Cx43 invagination, endocytosis, recycling and degradation, we used Dynasore to block Cx43 redistribution [25]. Increasing PKCε content was associated with an increasing Cx43 phosphorylation and PKC

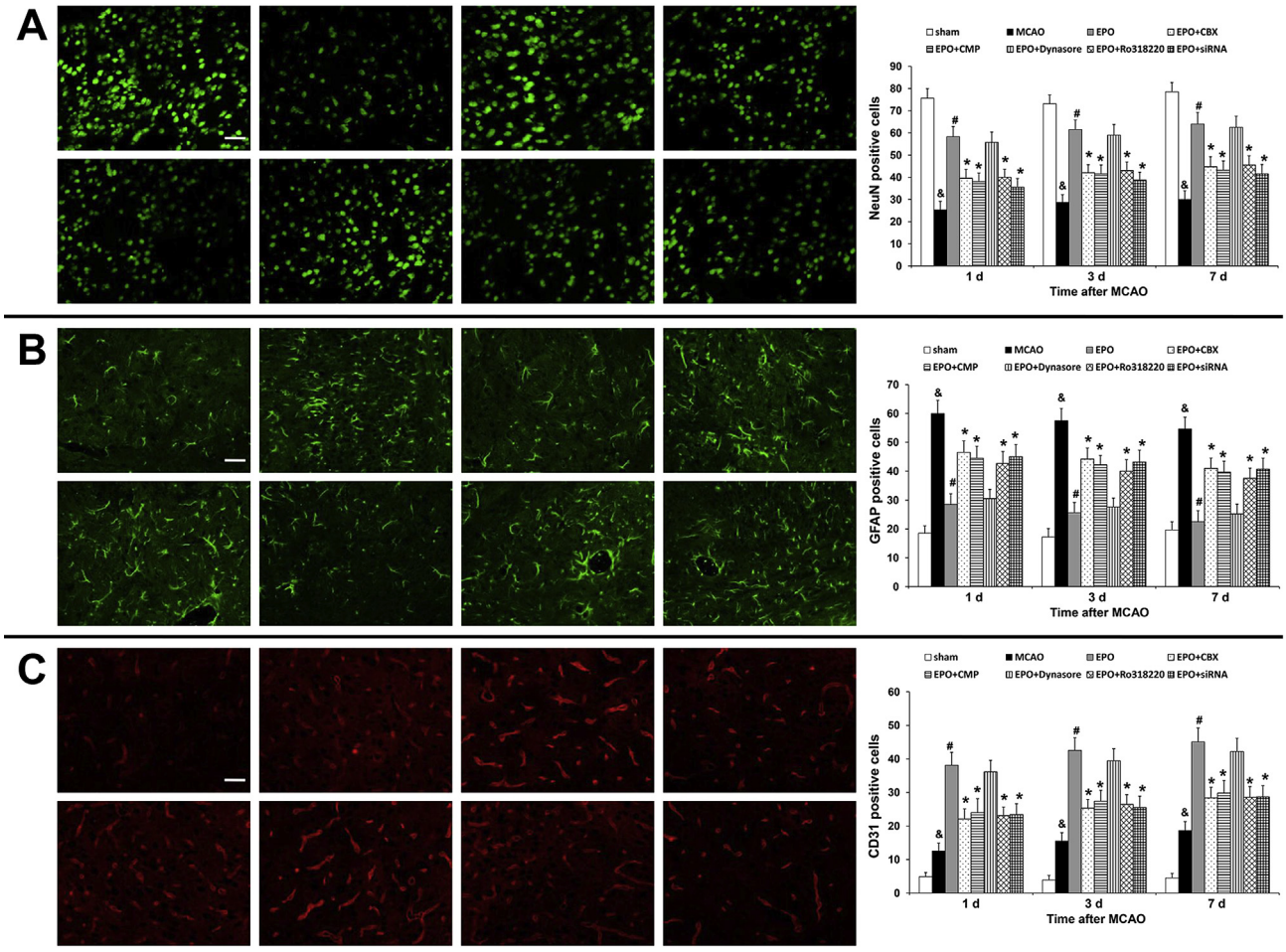

Fig. 4. NeuN, GFAP and CD31 staining in each group of rats. A marked reduction of NeuN staining as well as increase of GFAP and CD31 staining at 1 d, 3 d, and 7 d after MCAO was demonstrated (n = 6, & $p < 0.01$ vs. sham operation). Intraperitoneal injection of rhEPO increased NeuN and CD31 staining, while decreased GFAP staining (n = 6, #$p < 0.01$ vs. MCAO), which was blocked by the inhibitors containing CBX, CMP, Ro318220 and Cx43 siRNA, but Dynasore excluded (n = 6, *$p < 0.01$ vs. EPO). (A) NeuN staining. (B) GFAP staining. (C) CD31 staining. The immunofluorescence images represent groups of sham operation, MCAO, EPO, EPO + CBX, EPO + CMP, EPO + Dynasore, EPO + Ro318220, EPO + Cx43 siRNA successively. Statistical graphs of cell counting are displayed in the right. Scale bar: 100 μm (A, B); 50 μm (C).

inhibitor blocked Cx43 phosphorylation [26]. Thus PKCε inhibitor Ro318220 was used to block Cx43 phosphorylation in the current study. As is known, Cx43 siRNA can suppress Cx43 expression and our results also showed Cx43 protein and phosphorylation were hardly detected after administration of Cx43 siRNA. We demonstrated CBX, CMP and Cx43 siRNA markedly attenuated EPO's positive effects on NVU after MCAO, suggesting the intercellular communicating function of Cx43-GJIC is involved in the above effects. In addition, it was found in our study Ro318220 also weakened EPO's effect, while Dynasore had no influence. Combined with the up-regulation of p-Cx43 by EPO, it indicates EPO elicits its protective effects on NVU after cerebral ischemia depending on increase of p-Cx43 rather than Cx43 redistribution.

Cx43, as the most abundant and ubiquitously expressed connexin, is known to be a phosphoprotein. Phosphorylation of Cx43 can regulate connexin assembly into gap junctions, gap junction turnover and channel gating, thus is important to elicit its biological effects [27]. Previous studies showed decrease of Cx43 phosphorylation after ischemia/hypoxia, leading to astrocytic death [28]. It suggests Cx43 phosphorylation can develop neuroprotective effects. Additionally, other neuroprotective factors, e.g. nerve growth factor, also counteract the negative effects of ischemic events by increasing Cx43 phosphorylation [29]. Meanwhile, different changing trend of phosphorylated and unphosphorylated Cx43 by the same drug was also observed [30]. All the above findings were accordant with our results.

In conclusion, we demonstrate that EPO has protective effects on NUV injuries following cerebral ischemia, which is closely related with increase of p-Cx43, thus improve GJIC to reduce neurotoxic substances. The current study provides double targets to treatment of cerebral ischemia, which can present new insights to drugs development. Future work may further verify the above effects resorting to Cx43 knockout mice. Moreover, similar effects can be investigated using cell culture models to obtain more evidences and the related signal transduction pathways.

Conflict of interest

None.

Acknowledgments

This research was supported by grants from the National Natural Science Foundation of China (81403214). There are no conflict of interest to report.

References

[1] Y. Li, Z. Lu, C.L. Keogh, S.P. Yu, W. Li, Erythropoietin-induced neurovascular protection, angiogenesis, and cerebral blood flow restoration after focal ischemia in mice, J. Cerebr. Blood F. Met. 27 (2007) 1043–1054.

[2] F. Zhang, A.P. Signore, Z. Zhou, S. Wang, G. Cao, J. Chen, Erythropoietin protects CA1 neurons against global cerebral ischemia in rat: potential signaling mechanisms, J. Neurosci. Res. 83 (2006) 1241–1251.

[3] Y.J. Kim, Y.W. Jung, Systemic injection of recombinant human erythropoietin after focal cerebral ischemia enhances oligodendroglial and endothelial progenitor cells in rat brain, Anat. Cell. Biol. 43 (2010) 140–149.

[4] L. Vitellaro-Zyccarello, S. Mazzetti, L. Madaschi, P. Bosisio, E. Fontana, A. Gorio, S. De Biasi, Chronic erythropoietin-mediated effects on the expression of astrocyte markers in a rat model of contusive spinal cord injury, Neuroscience 151 (2008) 452–466.

[5] M.V. Frantseva, L. Kokarovtseva, J.L. Perez Velazquez, Ischemia-induced brain damage depends on specific gap-junctional coupling, J. Cerebr. Blood F. Met. 22 (2002) 453–462.

[6] G.J. Zoppo, Inflammation andthe neurovascular unit in the setting of focal cerebral ischemia, Neuroscience 158 (2009) 972–982.

[7] H. Tanigami, T. Okamoto, Y. Yasue, M. Shimaoka, Astroglial integrins in the development and regulation of neurovascular units, Pain Res. Treat. 2012 (2012) 1–10.

[8] S. Chua, S. Leu, Y.C. Lin, J.J. Sheu, C.K. Sun, S.Y. Chung, H.T. Chai, F.Y. Lee, Y.H. Kao, C.J. Wu, L.T. Chang, S.F. Ko, H.K. Yip, Early erythropoietin therapy attenuates remodeling and preserves function of left ventricle in porcine myocardial infarction, J. Investig. Med. 59 (2011) 574–586.

[9] T. Nakase, S. Fushiki, C.C.G. Naus, Astrocytic gap junctions composed of connexin 43 reduce apoptotic neuronal damage in cerebral ischemia, Stroke 34 (2003) 1987–1993.

[10] J.O. Davidson, C.R. Green, L.F.B. Nicholson, S.J. O'Carroll, M. Fraser, L. Bennet, A.J. Gunn, Connexin hemichannel blockade improves outcomes in a model of fetal ischemia, Ann. Neurol. 71 (2012) 121–132.

[11] S.S.L. Chew, C.S. Johnson, C.R. Green, H.V. Danesh-Meyer, Role of connexin43 in central nervous system injury, Exp. Neurol. 225 (2010) 250–261.

[12] J.L. Solan, P.D. Lampe, Connexin43 phosphorylation: structural changes and biological effects, Biochem. J. 419 (2009) 261–272.

[13] H. Chu, H. Ding, Y. Tang, Q. Dong, Erythropoietin protects against hemorrhagic blood-brain barrier disruption through the effects of aquaporin-4, Lab. Invest 94 (2014) 1042–1053.

[14] M. Xie, C. Yi, X. Luo, S. Xu, Z. Yu, Y. Tang, W. Zhu, Y. Du, L. Jia, Q. Zhang, Q. Dong, W. Zhu, X. Zhang, B. Bu, W. Wang, Glial gap junctional communication involvement in hippocampal damage after middle cerebral artery occlusion, Ann. Neurol. 70 (2011) 121–132.

[15] H.L. Xu, L. Mao, S. Ye, C. Paisansathan, F. Vetri, D.A. Pelligrino, Astrocytes are a key conduit for upstream signaling of vasodilation during cerebral cortical neuronal activation in vivo, Am. J. Physiol. Heart Circ. Physiol. 294 (2008) H622–H632.

[16] Y.Y. Li, X.N. Chen, X.X. Fan, Y.J. Zhang, J. Gu, X.W. Fu, Z.H. Wang, X.F. Wang, Z. Xiao, Upregulated dynamin 1 in an acute seizure model and in epileptic patients, Synapse 69 (2015) 67–77.

[17] J.A. Loweth, R. Svoboda, J.D. Austin, A.M. Guillory, P. Vezina, The PKC inhibitor Ro31-8220 blocks acute amphetamine-induced dopamine overflow in the nucleus accumbens, Neurosci. Lett. 455 (2009) 88–92.

[18] Q. Xu, Y.K. Cheong, S.Q. He, V. Tiwari, J. Liu, Y. Wange, S.N. Raja, J. Li, Y. Guan, W. Li, Suppression of spinal connexin 43 expression attenuates mechanical hypersensitivity in rats after an L5 spinal nerve injury, Neurosci. Lett. 566 (2014) 194–199.

[19] J. Chen, Y. Li, L. Wang, Z. Zhang, D. Lu, M. Lu, M. Chopp, Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats, Stroke 32 (2001) 1005–1011.

[20] H. Chu, Y. Tang, Q. Dong, Protection of vascular endothelial growth factor to brain edema following intracerebral hemorrhage and its involved mechanisms: effect of aquaporin-4, Plos. One 8 (2013) e66051.

[21] R.M. Paredes, J.C. Etzler, L.T. Watts, W. Zheng, J.D. Lechleiter, Chemical calcium indicators, Methods 46 (2008) 143–151.

[22] L. Bambrick, T. Kristian, G. Fiskum, Astrocyte mitochondrial mechanisms of ischemic brain injury and neuroprotection, Neurochem. Res. 29 (2004) 601–608.

[23] G.D. Sagar, D.M. Larson, Carbenoxolone inhibits junctional transfer and upregulates connexin43 expression by a protein kinase A-dependent pathway, J. Cell. Biochem. 98 (2006) 1543–1551.

[24] T. Desplantez, V. Verma, L. Leybaert, W.H. Evans, R. Weingart, Gap26, a connexin imetic peptide, inhibits currents carried by connexin43 hemichannels and gap junction channels, Pharmacol. Res. 65 (2012) 546–552.

[25] J. Gilleron, D. Carette, C. Fiorini, J. Dompierrec, E. Maciad, J.P. Denizote, D. Segretain, G. Pointis, The large GTPase dynamin2: a new player in connexin 43 gap junction endocytosis, recycling and degradation, Int. J. Biochem. Cell. B 43 (2011) 1208–1217.

[26] N. Duquesnes, M. Derangeon, M. Métrich, A. Lucas, P. Mateo, L. Li, E. Morel, F. Lezoualc'h, B. Crozatier, Epac stimulation induces rapid increases in connexin43 phosphorylation and function without preconditioning effect, Eur. J. Physiol. 460 (2010) 731–741.

[27] L. Márquez-Rosado, J.L. Solan, C.A. Dunn, R.P. Norris, P.D. Lampe, Connexin43 phosphorylation in brain, cardiac, endothelial and epithelial tissues, Biochim. Biophys. Acta 1818 (2012) 1985–1992.

[28] W. Li, E.L. Hertzberg, D.C. Spray, Regulation of connexin43-protein binding in astrocytes in response to chemical ischemia/hypoxia, J. Biol. Chem. 280 (2005) 7941–7948.

[29] P. Cushing, R. Bhalla, A.M. Johnson, W.J. Rushlow, S.O. Meakin, D.J. Belliveau, Nerve growth factor increases connexin43 phosphorylation and gap junctional intercellular communication, J. Neurosci. Res. 82 (2005) 788–801.

[30] J. Aravindakshan, D.G. Cyr, Nonylphenol alters connexin 43 levels and connexin 43 phosphorylation via an inhibition of the p38-mitogen-activated protein kinase pathway, Bio. Reprod. 72 (2005) 1232–1240.