Bi-directionally protective communication between neurons and astrocytes under ischemia

Xiao-Mei Wu, Christopher Qian, Yu-Fu Zhou, Yick-Chun Yan, Qian-Qian Luo, Wing-Ho Yung, Fa-Li Zhang, Li-Rong Jiang, Zhong Ming Qian, Ya Ke

1. Introduction

The brain is very sensitive to ischemia, which can be caused by cerebrovascular diseases such as stroke. In addition to neurons, astrocytes, the main supporting cells in the brain, can also be irreversibly injured [1]. The damage of these cells lead to lethal consequences or permanent neurological defects. For this reason, extensive research has been aimed at finding effective strategies and drugs to ameliorate or prevent brain ischemic injury, although few have achieved a satisfactory effect. One strategy that has been shown to provide effective and powerful protection against such harmful stress is ischemic/hypoxic preconditioning [2,3], which was first described in a dog model of myocardial injury in which sublethal ischemia enabled a remarkable reduction in LDH and an increase in cell viability in ischemic astrocytes in vitro. Selective neuronal loss by kainic acid injection induced a significant increase in apoptotic astrocyte numbers in the brain of ischemic rats in vivo. Furthermore, TUNEL analysis, DNA ladder assay, and the measurements of ROS, GSH, pro- and anti-apoptotic factors, anti-oxidant enzymes and signal molecules in vitro and/or in vivo demonstrated that IP neurons protect astrocytes by an EPO-mediated inhibition of pro-apoptotic signals, activation of anti-apoptotic proteins via up-regulation of anti-oxidant enzymes. We demonstrated the existence of astro-protection by IP neurons under ischemia and proposed that the bi-directionally protective communications between cells might be a common activity in the brain or peripheral organs under most if not all pathological conditions.

Abbreviations: CNS, central nervous system; EPO, erythropoietin; ERK, extracellular signal-regulated kinase; GFAP, glial fibrillary acidic protein; HIF-1alpha, hypoxia-inducible factor-1 alpha; IP, ischemia-preconditioning; IPcNCM, ischemia-preconditioned neuron culture medium; JAK-2, Janus kinase-2; KA, kainic acid; MAP2, microtubule-associated protein 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OGD, oxygen glucose deprivation; PI3K, phosphatidylinositol 3-kinase; rhEPO, recombinant human EPO; STAT5, signal transducer and activator of transcription 5

* Corresponding authors.

E-mail address: qianzhongming@fudan.edu.cn (Z.M. Qian).

These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.redox.2017.05.010

Received 12 April 2017; Received in revised form 16 May 2017; Accepted 19 May 2017

Available online 20 May 2017

2213-2317/ © 2017 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).
reperfusion-induced injury.

However, it is unknown whether preconditioned neurons have a role in protecting astrocytes from lethal ischemia/hypoxia injury. In the last decade, knowledge on the bi-directional communication between astrocytes and neurons in the brain has been dramatically expanded. It has been demonstrated that there is not only chemical transmitter-mediated astrocyte-to-neuron modulation but also neurotransmitter-mediated neuron-to-astrocyte signaling in the brain under physiological conditions [11–14]. It has also been shown that IP neurons, like astrocytes, can release protective factor(s) and hence protect un-preconditioned neurons against lethal ischemia/hypoxia injury [7,9]. These published data made us speculate that there may also be bi-directional protective communication between neurons and astrocytes under pathological (ischemic) conditions. We hypothesized that not only preconditioned astrocytes can protect neurons, but also preconditioned neurons can protect astrocytes from ischemia/hypoxia injury. In the present study, we tested this hypothesis and demonstrated for the first time that ischemia-preconditioned medium from neurons (IPcNCM) has a significant role in protecting astrocytes from ischemia-induced injury.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals were obtained from the Sigma Chemical Co., St. Louis, MO, USA. Primary polyclonal rabbit anti-Akt, phosphorylated Akt (p-Akt), extracellular signal-regulated kinase (ERK) 42/44, phosphorylated ERK 42/44 (p-ERK42/44), Bad, phosphorylated Bad Ser112 (112p-Bad), phosphorylated Bad Ser136 (136p-Bad), Bcl-2, cleaved caspase-3, caspase-3, phosphorylated signal transducer and activator of transcription 3 (p-STAT3) and 5 (p-STAT5) antibodies were purchased from Cell Signaling Technology, Beverly, MA, USA; primary monoclonal mouse anti-hypoxia-inducible factor-1 alpha (HIF-1 alpha) antibody from Novus Biologicals, Inc., Littleton, CO, USA; primary polyclonal rabbit anti-EPO antibody from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; antibodies against neuron microtubule-associated protein 2 (MAP2) and astrocyte glial fibrillary acidic protein (GFAP) from Chemicon International Ltd, Hampshire, UK; and both mouse anti-Histone 3 monoclonal antibody and Ab175819-8 isoprostane from Abcam, Cambridge, UK. The TUNEL detection kit was purchased from Roche Applied Science, Indianapolis, IN, USA; Bradford assay kit from Bio-Rad, Hercules, CA, USA, goat anti-rabbit or anti-mouse IRDye 800 CW secondary antibody from Li-Cor, Lincoln, NE, USA; Superoxide dismutas (SOD), catalase (CAT) and glutathione peroxidase (GSH-PX) assay kit from Jiancheng Bioengineering Institute, Nanjing, JS, China; and EPO ELISA kit from BioScience, Minneapolis, MN, USA.

2.2. Animals

Rats were supplied by the Centralized Animal Facilities of The Chinese University of Hong Kong (CUHK), housed in stainless steel cages at 21 ± 2 °C and had free access to food and water. The animal rooms were in a cycle of 12-h of light (7:00 to 19:00) and darkness (19:00 to 7:00). The Departments of Health of Hong Kong and the Shanghai Government and the Animal Research Ethics Committees of The Chinese University of Hong Kong and Fudan University approved the experimental procedures of this study.

2.3. Primary cortical neurons

Primary cortical neurons were prepared from 15 to 16 day-old rats embryos (E15-16) as described previously [15]. The purity of the neurons was assessed by staining with neuron-specific antibody against MAP2. In our case, over 98% of cells obtained were positively stained.

2.4. Primary cortical astrocytes culture

Primary cortical astrocytes were prepared from newborn SD rats at 1–3 days postnatal as described previously [16]. The purity of the astrocytes was assessed via anti-GFAP antibody (1:5000), reaching approximately 99%.

2.5. Oxygen glucose deprivation (OGD)

To mimic ischemic preconditioning, cells were exposed to OGD which was achieved by culturing cells in serum-free DMEM without glucose in a dedicated chamber (NAPCO 7101FC-1) with 1% O2, 94% N2 and 5% CO2 at 37 °C for a given period, as previously described [17].

2.6. Ischemia-preconditioned neuron culture medium (IPcNCM)

To prepare IPcNCM, neurons were exposed to OGD for 0, 0.5, 1 or 2-h and then incubated in a normoxic incubator for 24-h. Afterwards, the media were collected and referred to as IPcNCMs; IPc-0h, IPc-0.5h, IPc-1h or IPc-2h NCM respectively.

2.7. A neuronal loss model in vivo

To find out whether neurons have a protective effect on astrocytes in IP rats (270–280 g) in vivo, a neuronal loss model was established by injecting 0.5 nmol of kainic acid (KA) (1.5 μl of a 0.333 mM solution with PBS) stereotaxically into the cortical regions of rats at the following coordinates: 1.8 mm anterior to bregma, 2.0 mm lateral to the midline and 1.8 mm ventral to the dura. This dosage of KA had been reported to selectively destroy neurons but not astrocytes [18,19]. This model was verified by immunocytochemistry staining against MAP2 and GFAP in brain slices. 24-h after injection of KA, the rats were treated with IP (forebrain ischemia for 4-min) and then subjected to forebrain ischemia for 20-min, followed by reperfusion for 24-h (1/R). The sham-operation rats underwent an identical surgery but did not have KA injection, IP and ischemic injury. Forebrain ischemia was induced by bilateral common carotid artery occlusion plus hypotension, by removal of blood until 50 mm Hg from the jugular vein into heparinized sterile tubing before carotid clamping [20].

2.8. MAP2 and GFAP double staining

Rats were deeply anesthetized and transcardially perfused with normal saline solution, followed by 4% paraformaldehyde in 0.1 M PBS 24-h after ischemia-reperfusion. The brains were removed and post-fixed in 4% paraformaldehyde for 4-h, then transferred into 30% sucrose solution, until they sank to the bottom of the container. Coronal sections (20 μm) were made using a Leica CM3050S cryostat (Leica Microsystems, Wetzlar, Germany). Sections were blocked with 3% normal goat serum (diluted in PBS containing 0.3% Triton X-100) for 1-h and incubated with primary antibodies (anti-MAP2 and anti-GFAP, 1:1000, Chemicon) overnight at 4 °C. After rinsing with PBS, sections were incubated with rhodamine-conjugated goat anti-rabbit IgG (for MAP2, Millipore) and FITC-conjugated goat anti-mouse IgG (for GFAP, Invitrogen) as secondary antibodies (1:200) for 1-h. Fluorescent images were captured by a Nikon C-1 confocal imaging system (Nikon, Japan).

2.9. MTT assay

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was conducted as described previously [21]. Optical density was measured at the 570 nm wavelength by the use of an ELX-800 microplate assay reader (Bio-Tek, USA).
2.10. LDH release

The quantity of LDH (unit ml⁻¹ min⁻¹) released into the medium was determined by the decrease in absorbance at an optical density of 340 nm (Mod-756) for NADH disappearance within 3 min [22].

2.11. Determination of dichlorofluorescein and 8-isoprostane

After diffusion into the cell, 2',7'-dichlorofluorescein diacetate (DCFHDA) is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2', 7'-dichlorofluorescin (DCF). It was measured in the present study using a CytoFlor 4000 fluorescence spectrophotometer with excitation at 485 nm and emission at 530 nm, as described previously [23]. Background fluorescence was corrected by the inclusion of parallel blanks. ROS production was quantified from a DCF standard curve and expressed as pmol DCF formed/mg protein/min. 8-isoprostane, a group of prostaglandin-like compounds resulting from the peroxidation process of arachidonic acid induced by ROS [24], was determined by using an ELISA Kit (ab175819). Cells were processed according to the manufacturer’s instructions. Each ELISA sample was tested in duplicates according to the manual of the 8-isoprostane ELISA Kit. Absorbance readings at 450 nm were normalized to readings of Maximum Binding Control, and quantified into pg/ml using an 8-isoprostane standard curve [24].

2.12. Measurements of GSH, superoxide dismutase (SOD), CAT and GSH-PX

Astrocytes receiving different treatments were washed three times with ice-cold PBS and collected into eppendorf tubes, followed by centrifugation at 2000 rpm for 4 °C for 5-min. The pellets were then resuspended in 100 µl PBS and sonicated. After centrifugation at 4000 rpm for 10 min, the supernatants were collected and stored at −80 °C until assayed. The level of GSH and the activities of total SOD, manganese superoxide dismutase (Mn-SOD), copper & zinc superoxide dismutase (CuZn-SOD), CAT, and GSH-PX were measured spectrophotometrically by assay kits according to the manufacturers' instructions.

2.13. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The astrocytes grown on poly-D-lysine pre-coated glass slides were fixed, permeabilized and then treated with 30 µl of labeling solution, containing 3 µl of terminal deoxynucleotidyl transferase (TdT) for 1-h at 37 °C according to the manufacturer’s instructions. Subsequently, the slides were washed and nuclei staining was performed by incubation in 1 µg/ml DAPI for 10-min. The green fluorescein-labeled DNA was visualized by using a Nikon D-Eclipse confocal microscope (Nikon, UK). The percentages of nuclei displaying green fluorescence were calculated as a percentage of total nuclei visible in the field [23].

In the in vivo experiments, the brain slices were fixed, permeabilized and then treated with 30 µl of labeling solution, containing 3 µl of terminal deoxynucleotidyl transferase (TdT) for 1-h at 37 °C according to the manufacturer's instructions. Subsequently, the slides were washed and nuclei staining was performed by incubation in 1 µg/ml DAPI for 10-min. The green fluorescein-labeled DNA was visualized by using a Nikon D-Eclipse confocal microscope (Nikon, UK).

2.14. DNA laddering assay

Astrocytes were washed and lysed in 500 µl lysis buffer followed by centrifugation at 7000 rpm for 15-min (4 °C). The supernatants were removed and the pellet was mixed with equal volumes of phenol (pH 8.0) followed by centrifugation at 13,000 rpm for 10-min (4 °C). The upper aqueous layer was transferred into a fresh vial and mixed with 100 µl TE buffer followed by centrifugation at 13,000 rpm for 10-min (4 °C). DNA in the upper layer was extracted by a solution of phenol: chloroform:isoamyl alcohol (25:24:1) and then precipitated by 40 µl of 3 M NaOAc, pH 5.2 and 1 ml of ice-cold 100% ethanol. After centrifugation at 14,000 rpm for 15-min (4 °C), the DNA pellet was washed with 70% ethanol, resuspended in 15 µl TE buffer and incubated with 1 µl RNase (10 mg/ml) at room temperature for 1-h. Finally, DNA samples were run in 2% TAE (Tris-acetate-EDTA) agarose gel, containing 0.5 µg/ml ethidium bromide.

2.15. Enzyme-linked immunosorbsent assay (ELISA)

The concentrations of EPO in IPcNCM (in vitro) and the contents of EPO in KA or PBS injected-areas (in vivo) were measured by ELISA kits according to the manufacturer’s instructions. Optical density was read at a wavelength of 450 nm by an ELX-800 microplate assay reader (BioTek, USA).

2.16. Western blot analysis

Astrocytes were washed, homogenized with lysis buffer and subjected to sonication using a Soniprep 150 (MSE Scientific Instruments, London, UK) followed by centrifugation at 12,000g for 10-min at 4 °C [25]. The supernatants were then collected and stored at −80 °C for future use. The protein concentrations were determined using the Bradford protein assay kit. The primary antibodies used were mouse anti-HIF-1 alpha (1:500), rabbit anti-Bad (1:500), anti-112p-Bad, anti-136p-Bad, rabbit anti-Akt, anti-p-Akt, anti-Bcl-2, anti-Bax, anti-Bcl-XL, anti-caspase-3, anti-cleaved caspase-3, anti-p-STAT3, anti-p-STAT5 (1:1000), and 8-isoprostane antibodies (ab175819). Cells were processed according to the manufacturer’s instructions. Optical density was read at a resolution of 169 µm (Li-Cor). Anti-β-actin (1:20000) and anti-Histone 3 monoclonal antibodies (1:10000) were used as internal protein controls.

2.17. Statistical analysis

Statistical analyses were performed using Graphpad Prism. Data were presented as mean ± SEM. The differences between the means were all determined by two-way analysis of variance (ANOVA). A probability value of p < 0.05 was taken to be statistically significant.

3. Results

3.1. Effects of different durations of ischemia on cell viability and hypoxia-inducible factor-1 alpha expression in neurons

In order to find out an optimal and effective IP treatment for neurons, we first investigated the effects of the different durations (0, 0.5, 1, 2 or 4-h) of ischemia on cell viability and also HIF-1 alpha expression in neurons. The MTT assay showed that 0.5 or 1-h ischemia did not lead to any significant reduction in neuronal viability as compared with the control (0-h ischemia) (Fig. 1A). A significant reduction in neuronal viability began to appear after 2-h ischemia, which amounted to an 18% decrease in comparison with the control. More serious injury was observed when the neurons were exposed to 4-h ischemia, reflected by a decrease of cell viability to about 68% of the control. The levels of HIF-1 alpha were dramatically increased to 5.8, 6.1 and 2.5-fold of the control in neurons after 0.5, 1 and 2-h ischemia respectively, but only 1.6-fold after ischemia for 4-h (Fig. 1B and C).
Thus, 0.5–2-h ischemia were used to precondition the neurons, as this represents the period in which hypoxia-induced HIF production takes place while the neurons remain intact.

3.2. Ischemia-preconditioned neurons protect astrocytes against ischemic injury

To explore whether IP neurons could protect astrocytes against OGD injury, astrocytes were pre-incubated with IPC-0h, IPC-0.5h, IPC-1h or IPC-2h NCM for 48-h before being subjected to 12-h OGD. OGD (IPC-0h + Isch) induced a marked increase in LDH (2.3-fold of the control) and a significant reduction in astrocyte viability (53% of the control) (Fig. 1D & E). However, in astrocytes treated with 12-h OGD pre-treatment with IPCNCMs for 48-h induced a significant increase in cell viability (Fig. 1D), as well as a decrease in LDH (Fig. 1E) as compared with the cells treated without IPCNCM (IPC-0h + Isch). Astrocytes were
significantly preserved by pre-treatment with IPcNCMs as reflected by the increased cell viability, to 79% (IPc-0.5h + Isch), 85% (IPc-1h + Isch) and 75% (IPc-2h + Isch) of the control (IPc-0h + Isch) respectively (Fig. 1D). LDH leakage from astrocytes pretreated with IPc-0.5h, IPc-1h or IPc-2h NCM were 1.3, 1.2 and 1.5-fold respectively of the control (Fig. 1E), significantly less than those of the OGD astrocytes pretreated with IPc-0h NCM.

3.3. Inhibition of apoptosis confers the protective effect of ischemia-preconditioned neurons on astrocytes

In order to understand the mechanisms by which IP neurons protect astrocytes against OGD injury in vitro, we examined the effects of pre-treatment with IPcNCMs on OGD-induced apoptosis in astrocytes. As shown in Fig. 1F and G, OGD dramatically increased the number of TUNEL positive astrocytes to 62% of the total cell population (3.7% in the control). However, pre-treatment with IPc-0.5h, IPc-1h or IPc-2h NCM for 48-h induced a significant decrease in the number of TUNEL positive astrocytes. This finding was also verified by DNA ladder assay in which the IPcNCM displayed a similar anti-apoptotic profile (Fig. 1H). We then examined the effects of the IPc-1h NCM on pro-apoptotic factor caspase-3 and anti-apoptotic factors Bcl-2, Bad and Bcl-xL in the astrocytes treated with 12-h OGD. The IPc-1h NCM was used because 1-h of IP achieved the maximal protective effect (Fig. 1D and E) and anti-apoptotic effect (Fig. 1F-H). It was found that OGD induced a significant activation of caspase-3 (2.2-fold of the control), while IPc-1h NCM markedly suppressed this increase by almost 75% (Fig. 2A). On the other hand, treatment with IPc-1h NCM caused a 2-fold increase in expression of anti-apoptotic factors Bcl-2 (Fig. 2B) and Bcl-xL (Fig. 2C), which was paralleled by greatly enhanced phosphorylation of pro-apoptotic protein Bad on serine 136 (136p-Bad) (Fig. 2D) and 112 sites (112p-Bad) (Fig. 2E) in OGD astrocytes.

3.4. Anti-apoptosis induced by IP neurons is an EPO-mediated process

To elucidate why IP neurons can affect expression of apoptotic factors, we then investigated the role of EPO because EPO has been reported to protect neurons by preventing apoptosis [7,9,26,27] and we speculated that similar mechanisms might also operate in astrocytes. First, the EPO levels in IPc-0.5h, IPc-1h and IPc-2h NCMs were found to be significantly higher than those in IPc-0h NCM (Fig. 2F). The maximum effect was achieved by 1-h IP, with a nearly 3-fold increase in EPO content. This showed that IP could induce a significant increase in EPO release from neurons. Second, astrocyte viability was significantly lower (Fig. 2G) and LDH significantly higher (Fig. 2H) in 12-h OGD astrocytes treated with IPc-1h NCM plus anti-EPO (aEPO + IPc-1h + Isch) than in those treated with IPc-1h NCM only (IPc-1h + Isch), implying that the astro-protective effect of IPc-1h NCM could be largely blocked by anti-EPO. Third, rEPO, at a concentration comparable to that of EPO in the IPc-1h NCM has a similar astro-protective effect as revealed by MTT (Fig. 2G) and LDH assays (Fig. 2H). Finally, the number of TUNEL positive astrocytes (Fig. 2I and J) and caspase-3 (Fig. 2A) was significantly lower and the expression of Bcl-2 (Fig. 2B), Bcl-xL (Fig. 2C), 136p-Bad (Fig. 2D) and 112p-Bad (Fig. 2E) remarkably lower in 12-h OGD astrocytes treated with IPc-1h NCM and anti-EPO (aEPO + IPc-1h + Isch) than in those treated with IPc-1h NCM only (IPc-1h + Isch).

3.5. Activation of ERK, P13K and STAT5 pathways are involved in EPO-mediated protection of ischemic astrocytes by IP neurons

Next, we explored the signaling pathways involved in EPO-mediated anti-apoptosis. We first determined whether Jak2 dependent signal transduction pathways are involved in the protective effect of IP neurons, as Jak2 and its downstream effectors have been demonstrated to play a central role in EPO-mediated neuroprotection against ischemic injury in the brain [28]. As shown in Fig. 3A, the improved viability of 12-h OGD astrocytes induced by IPc-1h NCM was significantly attenuated by pre-treatment of the cells with Jak2 inhibitor AG490 (25 µM), Jak2 downstream PI3K inhibitor LY294002 (10 µM), ERK inhibitor PD98059 (25 µM) or STAT5 inhibitor 573108 (50 µM) for 20-min. However, no change was detected when NF-κB inhibitor BAY11-7082 (2 µM) and p38 MAPK inhibitor SB203580 (50 µM) were applied. These results were corroborated by the increase in the measurements of LDH release (Fig. 3B).

We then examined the effects of anti-EPO on the phosphorylation of Akt, ERK, STAT3 and STAT5 in astrocytes pre-treated with IPcNCM. Western blot analysis showed that IPc-1h NCM induced a significant increase in phosphorylation of Akt, ERK, STAT3 and STAT5 in astrocytes, all of which were however markedly inhibited by anti-EPO (Fig. 3C-F). Also, treatment with IPc-1h NCM significantly increased Bcl-2 expression and Bad and Bcl-xL phosphorylation in astrocytes, while these effects were also blocked by anti-EPO (Fig. 3G-J).

Also, we investigated the effects of the pathway-specific inhibitors on expression of pro- and anti-apoptotic factors. It was found that pre-treatment with LY294002 inhibited the IPc-1h NCM-induced increase in Bcl-2 and 136p-Bad, but not 112p-Bad and Bcl-xL levels in astrocytes (Fig. 3K-N). Pre-incubation with PD98059 blocked the effects of IPc-1h NCM on Bcl-2 and 112p-Bad but not 136p-Bad and Bcl-xL contents (Fig. 3K-N). The inhibitor 573108 could significantly inhibit the IPc-1h NCM-induced increase in Bcl-xL expression but had no effects on the other anti-apoptotic factors (Fig. 3K-N).

3.6. Ischemia-preconditioned neurons protect astrocytes against ischemia-induced apoptosis via up-regulation of EPO in the rat cortex in vivo

To find out whether IP neurons can protect astrocytes against I/R-induced apoptosis in vivo, a neuronal loss model was established by injecting kainic acid (KA) stereotaxically into the cortex. After 24-h of injection, the rats were treated with IP and then subjected to I/R. TUNEL-positive cells were counted by using rectangular grids placed randomly on the investigated areas. Data from 2 sections (40 µm of interval) of each investigated area from both sides were averaged for each animal (n = 6 rats in each group). It was found that the number of apoptotic astrocytes was increased to 89.28 ± 19.55 cells/0.1 mm² in the rats treated with 20-min forebrain ischemia (I) followed by reperfusion (R) for 24 h, in comparison with 3.76 ± 0.97 cells/0.1 mm² in the sham rats (Fig. 4A-C). However, the number of apoptotic astrocytes was significantly reduced, to 32.56 ± 9.59 cells in the I/R rats pre-treated with 4-min forebrain ischemia (IP + I/R). These indicated that 4-min of forebrain ischemia as IP successfully protected astrocytes against subsequent severe injury, induced by I/R via an anti-apoptotic effect. It was also found that the number of apoptotic astrocytes in the KA + IP + I/R group (112.55 ± 11.66 cells/0.1 mm², the absence of neurons) was significantly higher than that of the IP + I/R rats (32.56 ± 9.59 cells/0.1 mm², the presence of neurons). There were no significant differences in the numbers of apoptotic astrocytes between KA + IP + I/R and KA + I/R (123.48 ± 12.57 cells/0.1 mm²) groups (Fig. 4C). These data indicate that IP neurons were able to protect astrocytes against ischemia-induced apoptosis in vivo as well.

In some experiments, KA and PBS were injected into the left and right cerebral cortices of rats at corresponding coordinates before treatment with IP, and the tissues of the injection area were then harvested for measurement of EPO by ELISA. It was found that EPO content in PBS-injected areas was highly elevated in the IP rats (572.5 mIU/mg protein) compared to that of the non-IP rats (385.8 mIU/mg protein) (Fig. 4D). On the other hand, EPO content in the KA-injected areas was significantly lower (234.5 mIU/mg protein) than in the PBS-injected areas. There was no significant difference in EPO content in the KA-injected areas between KA (234.5 mIU/mg protein) and IP + KA (253.8 mIU/mg protein) rats (Fig. 4D). These
Fig. 2. Effects of EPO secreted by ischemia-preconditioned neurons on apoptosis of astrocytes in ischemia. Astrocytes were incubated with IPC-0 h or IPC-1 h NCM in the presence or absence of EPO antibody (aEPO) or recombinant human EPO (rhEPO, 75 pg/ml) followed by treatment with ischemia. The contents of cleaved/active caspase-3 (A), Bcl-2 (B), Bcl-xL (C), 136p-Bad (D), 112p-Bad (E), cell viability (G), LDH release (H), TUNEL-positive cells (I, Representative photographs of TUNEL (upper) and DAPI (lower) staining; J, The percentage of apoptotic cells in total cells) were measured as described in ‘Methods’. F, EPO contents in IPCNCMs measured by ELISA. Data were Mean ± SD (A-E: n = 4; F: n = 6). *p < 0.05, **p < 0.01, ***p < 0.001 versus ‘IPc-0h’, ‘IPc-0h + Isch’ or ‘IPc-1h + Isch’ (Isch = Ischemia).
Figure 1: Effects of IPC and aEPO on cell viability and LDH release.

(A) Cell viability after 24 hours of IPC and aEPO treatment. IPC and aEPO significantly increased cell viability compared to control.

(B) LDH release after 24 hours of IPC and aEPO treatment. IPC and aEPO significantly reduced LDH release compared to control.

Figure 2: Western blot analysis of p-Akt/Akt, p-Erk/Erk, p-STAT3/STAT3, p-STAT5/STAT5, Bcl-2/Bcl-xL, and Bcl-2/β-actin.

(C) p-Akt/Akt levels following IPC and aEPO treatment. IPC and aEPO significantly increased p-Akt/Akt levels.

(D) p-Erk/Erk levels following IPC and aEPO treatment. IPC and aEPO significantly increased p-Erk/Erk levels.

(E) p-STAT3/STAT3 levels following IPC and aEPO treatment. IPC and aEPO significantly increased p-STAT3/STAT3 levels.

(F) p-STAT5/STAT5 levels following IPC and aEPO treatment. IPC and aEPO significantly increased p-STAT5/STAT5 levels.

(G) Bcl-2/β-actin levels following IPC and aEPO treatment. IPC and aEPO significantly increased Bcl-2/β-actin levels.

(H) Bcl-xL/β-actin levels following IPC and aEPO treatment. IPC and aEPO significantly increased Bcl-xL/β-actin levels.

Figure 3: Western blot analysis of p-Bad/Bad.

(I) p-Bad/Bad levels following IPC and aEPO treatment. IPC and aEPO significantly increased p-Bad/Bad levels.

(J) Bcl-2/Bcl-xL levels following IPC and aEPO treatment. IPC and aEPO significantly increased Bcl-2/Bcl-xL levels.
results indicate that the reduced EPO content in the cerebral cortex of KA and KA + IP rats was due to KA-induced neuronal loss, and implied that neurons are an important source of EPO in IP, and that astroprotection by IP neurons in vivo is also EPO-associated.

3.7. Ischemia-preconditioned neurons inhibited oxidative stress induced by ischemia/reperfusion in astrocytes

We also examined the effects of IP neurons on ROS and GSH levels
in astrocytes treated with I/R by pre-treatment of astrocytes with IPCNCMs for 48-h before being exposed to ischemia (I) for 12-h followed by reperfusion (R) for 24-h. The ROS and GSH contents were determined, and fluorescence intensities were observed under confocal microscopy and quantified by using a fluorescent spectrophotometer as described in Methods. A, Representative confocal images indicating ROS levels in astrocyte which were measured by a fluorometric assay with 2′,7′-dichlorofluorescin diacetate (DCFH-DA); B, DCF intensity (% IPc-0h, n = 10); C, 8-isoprostane contents (% IPc-0h, n = 10); D, GSH contents (n = 6). E and F: Astrocytes were incubated with IPc-0h or IPc-1h NCM in the presence or absence of EPO antibody (aEPO) followed by treatment with ischemia, and DCF intensity (% IPc-0h, n = 10) (E) and 8-isoprostane contents (% IPc-0h, n = 10) (F) were then determined as described in Methods. G: Effects of ischemia-preconditioned neurons on expression of anti-oxidant enzymes in astrocytes. Astrocytes were incubated with IPc-0h or IPc-1h NCM for 48-h, and the contents of T-SOD, Mn SOD, CuZn SOD, CAT (catalase) and GSH-PX were then measured (n = 6). Data were Mean ± SD. *P < 0.05, **P < 0.01 vs. IPc-0h or IPc-0h + I/R.
and 112 sites (112p-Bad) in OGD astrocytes. Activation of caspase-3 is a key event in the execution of apoptotic cascade in CNS diseases, partly associated with the anti-apoptotic ability of IP neurons. We demonstrated that pre-treatment of astrocytes with IPcNCM can significantly inhibit the increase in the number of TUNEL positive cells, and at the same time cause a significant increase in the expression of anti-apoptotic factors Bcl-2 and Bcl-xL, as well as the phosphorylation of pro-apoptotic protein Bad on serine 136 (136p-Bad) and 112p-Bad [31,32] also provide evidence for the involvement of an anti-apoptotic role in the astro-protective effects of IP neurons.

Existing evidence has shown that EPO protects neurons by preventing apoptosis [7,9,26,27]. We therefore speculated that similar mechanisms might also operate in astrocytes. This hypothesis was strongly supported by the following findings; first, IP (1-h) led to a dramatic increase in HIF-1 alpha content in neurons as well as in EPO in the culture medium, evidencing that IP could lead to a remarkable increase in EPO release from neurons via increased HIF-1 alpha. Second, rEPO, at a concentration comparable to that of EPO in the IPc-1h NCM, has a similar astro-protective effect as IPc-1h NCM. Third, the astro-protective effect of IPcNCM could be largely blocked by anti-EPO. Fourth, the anti-apoptotic properties of IPc-1h NCM, including reduction of the number of TUNEL positive astrocytes and caspase-3 level, and the increase in expression of Bcl-2 and BclxL and phosphorylation of 136p-Bad and 112p-Bad, could also be largely inhibited by anti-EPO. Finally, pre-injection with KA to destroy neurons induced a significant reduction in EPO content, as well as an increase in the number of apoptotic astrocytes in the brain of IP rats treated with I/R in vivo. Taken together, these results strongly imply that the anti-apoptotic effect on ischemic astrocytes is mediated by the up-regulation of EPO in IP neurons.

JAK-2 is a key kinase in the signal transduction pathways activated by EPO [7,33]. The significant inhibition by Jak2 inhibitor AG490 of the IPcNCM-induced astro-protection strongly suggests that JAK-2 also plays a central role in EPO-mediated protection by IP neurons of astrocytes against ischemic injury, under our experimental conditions. Downstream from JAK-2, at least three different signaling pathways have been implicated in EPO-mediated anti-apoptosis: PI3K, MAPK and STAT. Bad is one major target of Akt, linking the PI3K pathway directly to the apoptotic machinery [34], and 136p-Bad has been shown to be a substrate of Akt kinase and thereby is sufficient to promote cell survival [26]. We showed that the inhibition of PI3K by LY294002 and the blockade of EPO by anti-EPO both could significantly diminish the IPcNCM-induced increase in phosphorylation of the serine-threonine protein kinase Akt, Bcl-2, 136p-Bad and 112p-Bad contents. The data indicate that the PI3K/Akt/136p-Bad and Bcl-2 pathway is involved in the EPO-mediated anti-apoptotic effect of IP neurons on astrocytes.

We also found that ERK inhibitor PD98059 significantly attenuated the improvement in astrocyte viability, Bcl-2 expression and 112p-Bad content induced by IPcNCM. Also, anti-EPO markedly blocked the significantly increased ERK phosphorylation, Bcl-2 expression and Bad and Bcl-xL phosphorylation in OGD astrocytes pre-treated with IPcNCM. These findings imply that the ERK/112p-Bad/Bcl-2 pathway is also associated with the EPO-mediated astro-protection induced by IP neurons. So far, only STAT5 has been demonstrated in the anti-apoptotic signaling of EPO [35,36], and only STAT3 is expressed constitutively throughout the brain in glial cells as well as in neurons [37]. Here, we demonstrated that anti-EPO could markedly block the activation of STAT3 and STAT5 induced by IPcNCM. Also, STAT5 inhibitor 573108 could significantly inhibit the IPcNCM-induced increase in Bcl-xL expression. These suggest that the STAT5/Bcl-xL signal pathway might also be involved in the EPO-mediated anti-apoptotic effect of IP neurons on astrocytes.

It is well known that the irreversible damage to both neurons and astrocytes induced by reperfusion can be caused by oxidative stress [38,39]. A large amount of ROS was generated during the reperfusion period through various pathways [40]. ROS can directly oxidize lipids, proteins, and nucleic acids or indirectly cause cellular damage through signaling pathways [41]. Because of the detrimental role of ROS, successful suppression of ROS levels can rescue neurons and astrocytes from oxidative damage induced by I/R. We therefore examined the effects of IP neurons on ROS levels in astrocytes treated with I/R and demonstrated that IPcNCM markedly suppressed increased ROS levels.
and oxidative stress in astrocytes exposed to I/R. We also investigated the effects of IPcNCM on GSH content in astrocytes treated with I/R, because GSH directly reacts with ROS to form oxidized glutathione disulfide (GSSG) and is an indicator of ROS, such that lower GSH content indicates higher ROS levels. It was found that treatment with IPcNCM induced a further increase in GSH levels in I/R astrocytes. These results imply that IP neuron-induced astro-protection against I/R-induced injury is achieved by an ability to inhibit I/R-induced oxidative stress. This inhibition by IP neurons is partially mediated by EPO released from the IP neurons, as the addition of anti-EPO antibody was found to partially block the increased anti-oxidant effects induced by the IP neurons in astrocytes.

It is well established that IP can up-regulate the activities of anti-oxidant enzymes in the brain. To understand the mechanisms involved in the inhibition by IP neurons of I/R induced-oxidative stress, we investigated the effects of IPcNCM on the activities of total superoxide dismutase (T-SOD), including mitochondrial manganese superoxide dismutase (Mn-SOD) and cytosolic copper, zinc superoxide dismutase (CuZn-SOD), catalase (CAT) and glutathione peroxidase (GSH-PX) in astrocytes, and demonstrated that treatment with IPc-1 h NCM could significantly increase all of these anti-oxidant enzymes as measured. These findings evidence that IP neurons have the ability to enhance the activities of anti-oxidant enzymes in astrocytes. A number of studies have demonstrated that superoxide, hydrogen peroxide and other peroxides are the major reactive oxygen species over-produced during reperfusion, and are responsible for oxidative damage. Evidence has also shown that CAT and GSH-PX can specially scavenge hydrogen peroxides and other peroxides, while SOD mainly detoxifies superoxide anions. Therefore, it is reasonable to believe that the up-regulation of these anti-oxidant enzymes is associated with the ability of IP neurons to inhibit I/R induced-oxidative stress or reduce ROS levels in astrocytes when exposed to I/R.

It has been demonstrated that EPO expression can be induced by hypoxia in astrocytes as well as in neurons, and that EPO receptor (EpoR) can also be detected in astrocytes as well as neurons. These results are in agreement with our finding of the significant increase in EPO in the cultured medium of IP neurons. The studies in our laboratories were funded by the Competitive Earmarked Grants of The Hong Kong Research Grants Council (GRF466713, GRF4106914 and GRF4111815 - KY), National Natural Science Foundation of China (31371092 - KY, 31300035, 31271132 and 31571195 - ZMQ) and National 973 Programs (2014CB541604 - ZMQ).

Conflict of interests

The authors declare no conflict of interest.

Funding sources

Acknowledgements

The authors conceived, organized and supervised the study; X.M.W., C.Q., Y.F.Z., Y.Y.C., Q.Q.L., F.L.Z. and L.R.J. performed the experiments; W.H.Y. and X.M.W. contributed to the analysis and interpretation of data. Y.K., Q.C. and Z.M.Q. revised the manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the
References

[1] L.J. Martin, A.M. Brambrink, C. Lehmann, C. Portera-Cailliau, R. Koehler, J. Rothstein, R.J. Traystman, Hypoxia-ischemia causes abnormalities in glutamate transporters and death of astroglias and neurons in newborn striatum, Ann. Neurol. 42 (1997) 325–348.

[2] J.M. Giddad, Cerebral preconditioning and ischaemic tolerance, Nat. Rev. Neurosci. 7 (2006) 437–448.

[3] U. Dinnagh, K. Becker, A. Meisel, Preconditioning and tolerance against cerebral ischaemia: from experimental strategies to clinical use, Lancer Neurol. 8 (2009) 398–412.

[4] C.E. Murray, R.B. Jennings, K.A. Reimer, Preconditioning with ischaemia: a delay of lethal cell injury in ischaemic myocardium, Circulation 74 (1986) 1124–1136.

[5] D.J. Hausenloy, D.M. Yellon, Ischaemic conditioning and reperfusion injury, Nat. Rev. Cardiol. 13 (2016) 193–209.

[6] S.V. Narayanan, K.R. Dave, M.A. Perez-Pinzon, Ischemic preconditioning and clinical scenarios, Curr. Opin. Neurol. 26 (2013) 1–7.

[7] K. Buscher, D. Freyer, M. Karches, N. Issev, D. Megow, B. Sawitzki, J. Piller, U. Dinnagh, A. Meisel, Erythropoietin is a parasite mediator of ischemic tolerance in the brain: evidence from an in vitro model, J. Neurosci. 22 (2002) 10291–10301.

[8] G. Trendelenburg, U. Dinnagh, Neuroprotective role of astrocytes in cerebral ischemia: focus on ischemic preconditioning, Glia 50 (2005) 307–320.

[9] J.C. Chavez, O. Baranova, J. Lin, P. Pichuile, The transcriptional activator hypoxia inducible factor 2 (HIF-2/EPAS-1) regulates the oxygen-dependent expression of erythropoietin in cortical astrocytes, J. Neurosci. 26 (2006) 9471–9481.

[10] B.A. Swanson, W. Ying, T.M. Kauppinen, Astrocyte influences on ischemic neuronal death, Curr. Mol. Med. 4 (2004) 193–205.

[11] A. Araque, G. Cangimotto, P.G. Haydon, Dynamic signaling between astrocytes and neurons, Annu. Rev. Physiol. 63 (2001) 795–815.

[12] M. Nederhaard, B. Ransom, S.A. Goldman, New roles for astrocytes: neurovascular coupling, Physiol. Rev. 86 (2006) 1009–1031.

[13] A. Verkhratsky, J.J. Rodriguez, V. Parpura, Neurotransmitters and integration in functional architecture of the brain, Trends Neurosci. 26 (2003) 523–530.

[14] P.G. Haydon, G. Cangimotto, Astrocyte control of synaptic transmission and neurovascular coupling, Physiol. Rev. 86 (2006) 1099–1103.

[15] A. Verkhratsky, J.J. Rodriguez, V. Parpura, Neurotransmitters and integration in neuronal-astroglial networks, Neurochem. Res. 37 (2012) 2236–2238.

[16] K.P. Ho, L. Li, L. Zhao, Z.M. Qian, Genistein protects primary cortical neurons from iron-induced lipid peroxidation, Mol. Cell. Biochem. 247 (2003) 219–222.

[17] Z.M. Qian, Y. Fan, P.L. Yang, Y.M. Fenn, Transferrin receptors on the plasma membrane of cultured rat astrocytes, Exp. Brain Res. 129 (1999) 473–476.

[18] X.M. Wei, Z.M. Qian, Y. Ke, F. Du, L. Zhu, Ginkgolide B preconditioning protects neurons against ischaemia-induced apoptosis, J. Cell. Mol. Med. 13 (2009) 4474–4483.

[19] G. Lenz, L. Manoero, S. Gottardo, M. Achaval, C. Salbego, R. Rodnith, Temporal profiles of the in vitro phosphorylation rate and immunoreactivity of glial fibrillary acidic protein (GFAP) after kainic acid-induced lesions in area CA1 of the rat hippocampus: demonstration of a novel phosphoprotein associated with gliosis, Brain Res. 764 (1997) 188–196.

[20] A. Vezzani, D. Moneta, C. Richichi, M. Aliprandi, S.J. Burrows, T. Ravizza, T. Miyawaki, T. Mashiko, D. Ofengei, R.J. Flannery, K.M. Noh, S. Fujisawa, Up-regulation of HIF-1alpha expression by hypoxia in normal cells and in hypoxic brains by two-photon imaging, J. Cereb. Blood Flow. Metab. 30 (2010) 734–738.

[21] E. Candelario-Jill, R.B. Fiebel, Cytochrome c reduction in ischemia and global brain injury, Curr. Pharm. Des. 14 (2008) 1401–1418.

[22] P.H. Chan, Reactive Oxygen Radicals in Signaling and Damage in the Ischemic Brain, J. Cereb. Blood Flow. Metab. 21 (2001) 2–14.

[23] J.A. Leopold, Y.Y. Zhang, A.W. Scribner, R.C. Stanton, J. Loscalzo, Glucose-6-phosphate dehydrogenase overexpression decreases endothelial cell oxidant stress and increases bioavailable nitric oxide, Arter. Thromb. Vas. Biol. 23 (2003) 476–482.

[24] M. Bergeron, J.M. Gidday, A.Y. Yu, G.L. Semenza, D.M. Ferriero, F.R. Sharp, Role of hypoxia-impaired cerebral ischaemic tolerance in neonatal rat brain, Ann. Neurol. 48 (2000) 285–298.

[25] L. Zhu, K. Yu, W. Xiao-Mei, Y. Lei, L. Yang, Q.Z. Ming, Ginkgolides protect PC12 cells against hypoxia-induced injury by p42/p44 MAPK pathway-dependent upregulation of HIF-1alpha expression and HIF-2alpha binding activity, J. Cell Biochem. 103 (2008) 564–575.

[26] M. Digicyanglou, S.A. Lipton, Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades, Nature 412 (2001) 641–647.

[27] T.P. Obrenovitch, Molecular physiology of preconditioning-induced brain tolerance to ischemia, Physiol. Rev. 88 (2008) 211–247.

[28] E. Kilic, U. Kilic, J. Soliz, C.L. Bassetti, M. Gazzano, D.M. Hermann, Brain-derived erythropoietin protects from focal cerebral ischemia by dual activation of ERK-1/2 and Akt pathways, FASEB J. 19 (2005) 2026–2028.

[29] A. Ashkenazi, V.M. Dixit, Death receptors: signaling and modulation, Science 281 (1998) 1305–1308.

[30] S.M. Man, T.D. Kanneganti, Converging roles of caspases in inflammation, apoptosis and cell death in innate immunity, Nat. Rev. Immunol. 16 (2016) 7–21.

[31] M.F. van Delft, D.C. Huang, How the Bcl-2 family of proteins interact to regulate apoptosis, Cell Res. 16 (2006) 203–213.

[32] X.M. Wu, Z.M. Qian, L. Zhu, F. Du, W.H. Yung, Q. Gong, Y. Ke, Neuroprotective effect of luteolin against ischaemia-reperfusion injury via up-regulation of erythropoietin and down-regulation of RTK2801, Br. J. Pharmacol. 164 (2011) 332–343.

[33] H.E. Brivexomy, Erythropoietin: multiple targets, actions, and modifying influences for biological and clinical consideration, J. Exp. Med. 210 (2013) 205–208.

[34] S.R. Datta, A. Brunet, M.E. Greenberg, Cellular survival: a play in three Akts, Genes Dev. 13 (1999) 2905–2927.

[35] M. Socolovsky, A.E. Fallon, S. Wang, C. Brugnara, H.F. Lodish, Fetal anaemia and apoptosis of red cell progenitors in STATA/STA; -; mice: a direct role for STAT5 in Bcl-2-mediated induction, Cell 98 (1999) 181–191.

[36] S.N. Constantinescu, L.J. Huang, H. Nam, H.F. Lodish, The erythropoietin receptor cytosolic juxtamembrane domain contains an essential, precisely oriented, hydrophobic motif, Mol. Cell 7 (2001) 377–385.

[37] S. Murata, N. Usuda, A. Okano, S. Kobayashi, T. Suzuki, Occurrence of a transcription factor, signal transducer and activators of transcription 3 (STAT3) in the postsynaptic density of the rat brain, Mol. Brain Res. 78 (2000) 80–90.

[38] C.A. Danilov, K. Chandrasekaran, J. Racz, L. Soane, C. Zieleck, G. Fiskum, Sulforaphane protects astrocytes against oxidative stress and delayed death caused by oxygen and glucose deprivation, Glia 57 (2009) 645–656.

[39] D.E. Bragin, B. Zhou, P. Ramamoorthy, W.S. Muller, J.A. Connor, H. Shi, Differential changes of glutathione levels in astrocytes and neurons in ischemic brains in two-photon imaging, J. Cereb. Blood Flow. Metab. 30 (2010) 734–738.

[40] M. Bergeron, J.M. Gidday, A.Y. Yu, G.L. Semenza, D.M. Ferriero, F.R. Sharp, Role of hypoxia-impaired cerebral ischaemic tolerance in neonatal rat brain, Ann. Neurol. 48 (2000) 285–298.