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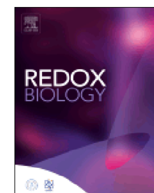

Research Paper

Bi-directionally protective communication between neurons and astrocytes under ischemia

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ABSTRACT

The extensive existing knowledge on bi-directional communication between astrocytes and neurons led us to hypothesize that not only ischemia-preconditioned (IP) astrocytes can protect neurons but also IP neurons protect astrocytes from lethal ischemic injury. Here, we demonstrated for the first time that neurons have a significant role in protecting astrocytes from ischemic injury. The cultured medium from IP neurons (IPcNCM) induced 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 the P13K/ERK/STAT5 pathways and activation of anti-oxidant 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.

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 deficits. 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 cells to better tolerate subsequent, usually lethal ischemia [4]. A number of studies have demonstrated that preconditioning induced by ischemia or hypoxia can produce a significant effect for protection of neurons or brain tissues in experimental animals and humans [5]. The discovery of preconditioning has opened a window for utilizing the endogenous protection mechanisms of the body for treating patients of stroke and other central nervous system (CNS) disorders [6].

Although the molecular mechanisms underlying preconditioning have not been completely elucidated, it has been well confirmed that ischemic preconditioned (IP) astrocytes play a significant role in the protection of neurons or brain tissues against ischemia/hypoxia or oxygen glucose deprivation (OGD)-induced injury [7–9]. During past years, a number of studies have also been conducted to investigate the mechanisms involved in neuro-protection by IP astrocytes. By influencing glutamate excitotoxicity, oxidative stress and acidosis, which are primary mediators of neuronal death during ischemia and reperfusion [10], IP astrocytes could effectively protect neurons from ischemia and

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

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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 Ser^{112} (112p-Bad), phosphorylated Bad Ser^{136} (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 acadic 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% N_2 and 5% CO^2 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 (I/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-dimethylthazol-2-yl)-2,5-diphenyltetrazolinum 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).

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