Brain Research Bulletin 103 (2014) 66–71

Contents lists available at ScienceDirect

Brain Research Bulletin

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

Research report

Transferred inter-cell ischemic preconditioning-induced neuroprotection may be mediated by adenosine A1 receptors☆,☆☆

Jungyeon Yun[a,b], Jun Li[a,c], Zhiyi Zuo[a,*]

[a] Department of Anesthesiology, University of Virginia, Charlottesville, VA, United States
[b] Department of Anesthesiology, Research Institute and Hospital, National Cancer Center, Republic of Korea
[c] Department of Anesthesiology, Fourth Affiliated Hospital, Harbin Medical University, Harbin 150001, China

ARTICLE INFO

Article history:
Received 25 January 2014
Received in revised form 19 February 2014
Accepted 21 February 2014
Available online 6 March 2014

Keywords:
Adenosine A1 receptor
Akt
Inter-cell preconditioning
Neuroprotection

ABSTRACT

Ischemic preconditioning-induced neuroprotection is a well-known phenomenon. We hypothesize that this form of neuroprotection is transferable among the same type of cells. To test this hypothesis, human neuroblastoma SH-SY5Y cells were induced to become neuron-like cells. Primary rat cortical neuronal cultures were also used. These cells were subjected to various lengths of short oxygen-glucose deprivation (OGD, an *in vitro* simulation of ischemia) and then 1-h OGD. Some cells that were not exposed to a short episode of ischemia were incubated with culture medium from the cells that had 3- or 5-min OGD. Those cells were subjected to OGD for 1 h at 1 or 24 h after they were exposed to the medium. Cell injury was evaluated at 24 h after the 1-h OGD by lactate dehydrogenase release assay. In another experiment, cells subjected to a 3-min OGD or exposed to the medium from cells that had a 3-min OGD were harvested at 30 min after the OGD or the medium exposure for Western blotting of Akt, a prosurvival protein. Our study showed that a prior episode of ischemia lasting from 3 to 10 min significantly reduced the 1-h OGD-induced cell injury. Medium from cells subjected to a 3-min OGD also induced acute and delayed phases of neuroprotection in OGD-naïve human neuron-like cells and primary rat cortical neuronal cultures. Cells subjected to a 3-min OGD or incubated with the medium from cells exposed to a 3-min OGD had increased phosphorylated/activated Akt. The increased phosphorylated Akt and neuroprotection induced by medium transferring were inhibited by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), an adenosine A1 receptor inhibitor. The 3-min OGD-induced neuroprotection was inhibited by LY294002, an Akt activation inhibitor. These results suggest that ischemic preconditioning-induced neuroprotection is transferable among the cells. Small molecules, such as adenosine, may mediate this effect.

1. Introduction

Ischemic preconditioning is now a well-known phenomenon in which episodes of short ischemia induce robust protection against prolonged and detrimental ischemia in the same tissues or organs (Gidday, 2006; Murry et al., 1986). Multiple endogenous mechanisms including activating prosurvival proteins, such as Akt, have been considered to mediate ischemic preconditioning-induced protection (Gidday, 2006). This form of protection has been found in various organs including brain. Ischemic preconditioning-induced neuroprotection has been actively studied to identify potential targets/interventions to improve neurological outcome after stroke (Gidday, 2006; Iadecola and Anrather, 2011).

The initial studies on ischemic preconditioning have been focused on applying short episodes of ischemia to the same tissues that are subjected to a subsequent prolonged ischemia. However, preconditioning effect can be induced by applying ischemia to a different tissue. For example, subjecting a limb to short episodes

Abbreviations: DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LDH, lactate dehydrogenase; OGD, oxygen-glucose deprivation.

☆ The study was performed in and should be attributed to the Department of Anesthesiology, University of Virginia, USA.

☆☆ *Grant support*: This study was supported by grants (R01 GM065211 and R01 GM098308 to Z. Zuo) from the National Institutes of Health, Bethesda, Maryland, by a grant from the International Anesthesia Research Society (2007 Frontiers in Anesthesia Research Award to Z Zuo), Cleveland, Ohio, by a Grant-in-Aid from the American Heart Association Mid-Atlantic Affiliate (10GRNT3900019 to Z. Zuo), Baltimore, Maryland, and the Robert M. Epstein Professorship Endowment, University of Virginia.

* Corresponding author at: Department of Anesthesiology, University of Virginia Health System, 1 Hospital Drive, P.O. Box 800710, Charlottesville, VA 22908-0710, United States. Tel.: +1 434 924 2283; fax: +1 434 924 2105.
E-mail address: zz3c@virginia.edu (Z. Zuo).

http://dx.doi.org/10.1016/j.brainresbull.2014.02.008
0361-9230/

of ischemia can induce neuroprotection (remote ischemic preconditioning) (Zhao et al., 2012). This finding significantly increases the applicability of using ischemic preconditioning for neuroprotection because application of ischemia to a limb is more practical and safer than inducing ischemia in the brain. However, it is not really clear how a preconditioning stimulus applied to an organ/tissue can induce protection in another organ/tissue. Signals transmitted through nervous system or molecules in the circulating blood have been proposed as the mediators for this phenomenon (Lim and Hausenloy, 2012; Zhao et al., 2012). A third form of preconditioning has been found in the heart. The effluent from a preconditioned heart can induce protection in a naïve heart. This protection is called transferred inter-cardiac preconditioning (Dickson et al., 1999). The mechanisms for this preconditioning are largely unknown.

In this study, we hypothesize that ischemic preconditioning-induced neuroprotection is transferable among the same type of cells and that this transferable preconditioning goes through mechanisms similar to those for ischemic preconditioning in the same tissues. To test our hypotheses, we induced human neuroblastoma SH-SY5Y cells to become neuron-like cells. Oxygen-glucose deprivation (OGD) was used to simulate ischemia *in vitro*. Culture medium transferring was used to determine whether the preconditioning is transferable.

2. Materials and methods

2.1. SH-SY5Y cell culture

The human neuroblastoma SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). SH-SY5Y cells were cultured in a 1:1 mixture of Eagle's minimum essential medium and F-12 supplemented with 10% fetal bovine serum. They were kept at 37 °C in a humidified incubator gassed with 5% CO_2 and 95% air and sub-cultured when they were 70–80% confluent. The cells were fed twice each week.

For experiments, SH-SY5Y cells were plated at a density of 5×10^3 cells/cm^2 in 6-well plates. One day later, culture medium was changed into the Neurobasal medium (Invitrogen Life Technologies, GIBCO, Carlsbad, CA) supplemented with B27 supplement (Invitrogen Life Technologies) and L-glutamine (500 μM; Nacalai Tesque Inc., San Diego, CA). Retinoic acid (Sigma, St. Louis, MO) was added to the medium to make the final concentration at 10 μM for 3 days to induce SH-SY5Y cells to differentiate into a homogenous population of cells with neuronal morphology (Kume et al., 2008; Lin et al., 2011a,b). The cells were used in experiments the next day.

2.2. Primary rat cortical neuronal culture

These cells were purchased from Invitrogen Life Technologies and were isolated from 18-day old embryos of the Fischer 344 rats. They were plated at ~1.7×10^4 cells per well in 96-well plates and cultured in Neurobasal medium supplemented with 200 mM Glutamax-1 and B27. The cells were fed and maintained as we described before (Li and Zuo, 2011) and used in experiments on the fifth day after they were plated in our laboratory.

2.3. Experimental protocol

Two phases of neuroprotection, the acute and delayed phases, were determined. To investigate the acute phase, cells were exposed to OGD for 3, 5 or 10 min. One hour later, they were exposed to a 60-min OGD. To determine whether OGD preconditioning was transferable, cells were subjected to a 0-, 3- or 5-min OGD. Thirty minutes later, the culture medium was harvested and added to OGD-naïve cells whose culture medium was just removed. The OGD-naïve cells were subjected to the 60-min OGD 1 h later.

To determine the delayed phase of neuroprotection, the experimental protocol was the same as for studying the acute phase but the interval between the short episode of OGD or the medium transferring and the 60-min OGD was 24 h instead of 1 h.

2.4. Applications of drugs

The Akt activation inhibitor LY294002 (Sigma, St. Louis, MO) was administered to cells to make the final concentration of 10 μM immediately before a 3-min OGD or at 60 min before a 60-min OGD. The adenosine A1 receptor inhibitor 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was applied to the cells at the final concentration of 100 nM at 30 min after the 3-min OGD (just before the culture medium was harvested for transferring) or just before the 3-min OGD (for cells subjected to 3-min OGD and then 60-min OGD).

2.5. OGD

As we described before (Lin et al., 2011a,b), the culture medium was removed and the cells were washed with Dulbecco's phosphate-buffered saline (Invitrogen Life Technologies). Neurobasal-A medium (Invitrogen Life Technologies) that has no glucose, glutamine, or B27 was gassed in advance with 100% N_2 for 10 min to eliminate oxygen in the medium. After 2 ml of this pre-gassed Neurobasal-A medium was added to each well, the culture plates were placed into the Billups-Rothenberg air-tight chamber. The chamber was closed and flushed through a channel with 100% N_2 until O_2 concentration in the gases of the chamber reached 2% as detected by a Datex^{TM} infrared analyzer (Capnomac, Helsinki, Finland). The channel was tightly closed and cells were kept at 37 °C for 3, 5, 10 or 60 min. At the end of incubation, oxygen concentrations in the gases from the chambers were confirmed to be lower than 2% and then the cells were exposed to room air. Glucose, glutamine and B27 were added to the cells and the cells were incubated at 37 °C for the predetermined times.

2.6. Determination of lactate dehydrogenase (LDH) release

LDH activity in the culture medium and cells at 24 h after the 60-min OGD was determined by a colorimetric assay using LDH Cytotoxicity Detection Kit (Clontech Mountain View, CA). The culture medium was centrifuged at $100 \times g$ for 10 min and 100-μl cell-free supernatant was transferred to 96-well-plate. This supernatant was incubated with the same amount of the reaction mixture according to the manufacturer's protocol. The absorbance of samples to light was measured at 490 nm with the reference wavelength of 655 nm in a spectrophotometer (Bio-Rad Laboratories, Hercules, CA). Background absorbance from the cell-free buffer solution was subtracted from all absorbance measurements. To measure the LDH activity in the cells, culture medium was removed and 1.0% Triton X-100 lysing solution was applied to the cells. After 20 min of incubation, 100 μl of cell lysates was incubated with the same amount of the reaction mixture and the absorbance of the samples was measured. The LDH release in percentage was calculated using the following equation: LDH level in the incubation medium × 100/(intracellular LDH level + LDH level in the culture medium).

2.7. Sample preparation for Western blotting

In the first experiment, cells were subjected to or were not subjected to a 3-min OGD. Cells were harvested at 30 min after the OGD. In the second experiment, culture medium from cells exposed to a