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Effects of hypoxic preconditioning on the expression of iron influx and efflux proteins in primary neuron culture

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ABSTRACT

The mechanisms of neuroprotection induced by hypoxic preconditioning (HP) and the effects of HP on iron metabolism proteins in the brain have not been fully elucidated. Based on the accumulated information, we hypothesized that HP would be able to affect the expression of iron metabolism proteins in the brain and that the changes in the expression of these proteins induced by HP might be partly associated with the HP-induced neuroprotection. Here, we demonstrated for the first time that HP could induce a significant increase in the expression of HIF-1alpha as well as iron uptake (TfR1 and DMT1) and release (Fpn1) proteins and thus increase transferrin-bound iron (Tf-Fe) and non-transferrin-bound iron (NTBI) uptake and iron release, and also a progressive increase in cellular iron content in the cultured neurons. We concluded that HP has the ability to speed iron transport rate and proposed that the increase in iron transport rate and cellular iron in neurons might be one of the mechanisms involved in neuroprotection in the HP neurons. We also demonstrated that Fpn1 expression was significantly affected by HIF-1alpha, implying that the gene encoding this iron efflux protein is hypoxia-inducible.

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1. Introduction

Preconditioning was first described in a dog model of myocardial injury in which sub-lethal ischemia enabled cells to better tolerate the usually lethal subsequent ischemia (Murray et al., 1986). It is now recognized that this phenomenon can be induced not only by ischemia but also by hypoxia in the central nervous system (Gustavsson et al., 2005; Ran et al., 2005; He et al., 2007). In the past few years, the phenomenon has received much attention because of its potential therapeutic importance (Stone, 2003). A number of studies have demonstrated that preconditioning induced by hypoxia can produce a significant effect to protect neurons in cells, animals and humans (Matsushima and Hakim, 1995; Vannucci et al., 1998; Gidday et al., 1999; Wu et al., 2005; Wagner et al., 2002). However, the mechanisms of neuroprotection induced by hypoxic preconditioning (HP) have not yet been fully elucidated although the involvement of hypoxia-inducible factor-1alpha (HIF-1alpha) in this process is confirmed.

Adaptation to hypoxia in cells and tissues leads to the transcriptional induction of a series of genes, including several with important functions in iron metabolism (Ke and Costa, 2006). It has been known for a long time that transferrin (Tf) and transferrin receptor (TfR1) are two key proteins involved in iron metabolism under physiological conditions (Qian et al., 1997). Studies have demonstrated that hypoxia can increase iron uptake by cells as well as the expression of Tf and TfR1, both of which have been identified as hypoxia-inducible genes (Rolfs et al., 1997; Bianchi et al., 1999; Lok and Ponka, 1999). It has also been reported that several other proteins that are involved in iron transport or regulation, such as ceruloplasmin (Cp) (Mukhopadhyay et al., 2000), iron regulatory protein 1 (IRP1) (Hanson et al., 1999) and 2 (IRP2) (Hanson and Leibold, 1998), are regulated by HIF-1 in response to hypoxic conditions. Furthermore, it has been demonstrated that the gene encoding hepcidin, a newly discovered iron regulatory hormone,

Abbreviations: Cp, ceruloplasmin; DMEM, Dulbecco's modified Eagle's medium; DMT1, divalent metal transporter 1; DMT1 + IRE, divalent metal transporter 1 with iron response element; DMT1−IRE, divalent metal transporter 1 without iron response element; FBS, fetal bovine serum; Fpn1, ferroportin1; GFAP, glial fibrillary acidic protein; HIF-1alpha, hypoxia-inducible factor-1alpha; HP, hypoxic preconditioning; HP/R, hypoxic preconditioning/re-oxygenation; IRP1, iron regulatory protein 1; IRP2, iron regulatory protein 2; MAP2, microtubule-associated protein 2; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-monotetrazolium bromide; NTBI, non-transferrin-bound iron; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; Tf-Fe, transferrin-bound iron; TfR1, transferrin receptor.

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is regulated by hypoxia (Nicolas et al., 2002). In our recent studies (Zhu et al., 2008; Qian et al., 2011), we demonstrated that divalent metal transporter 1 (DMT1), another key iron uptake protein under physiological conditions, is a hypoxia-inducible gene.

Currently, we know very little about the effects of HP on the expression of iron metabolism proteins in the brain. Based on the above findings, however, we speculated that the responses of the expression of iron metabolism proteins in neurons or other brain cells to HP might also be associated with the HP-induced neuroprotective role or the increased hypoxic tolerance. We also proposed that the expression of ferroportin1 (Fpn1), a key iron efflux protein, might also be regulated by HIF-1alpha. In this study, we therefore investigated systematically the effects of HP on the expression of iron transport proteins in primary cultured neurons. Our findings showed for the first time that HP could induce a significant increase in the expression of iron uptake (TfR1 and DMT1) and release (Fpn1) proteins and thus speed iron transport rate in neurons. Iron is a transition metal that is essential for oxygen transport, cell growth and survival (Zhu et al., 2008). Therefore, we believed that the increase in iron transport rate in neurons might be one of the mechanisms involved in the neuroprotection role induced by HP.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA. The scintillation cocktail and tubes were purchased from Beckman Coulter Company, Fullerton, CA, USA and $^{55}FeCl_3$ from Perkin–Elmer Company, Wellesley, MA, USA. The mouse anti-rat TfR1 monoclonal antibody was obtained from Zymed Laboratories, South San Francisco, CA, USA and antibodies against DMT1 with (DMT1 + IRE) or without (DMT1−IRE) iron response element and Fpn1 were purchased from Alpha Diagnostic International Company, San Antonio, TX, USA. The primary monoclonal mouse anti-HIF-1alpha was obtained from Novus Biologicals, Littleton, CO, USA. Goat anti-rabbit or anti-mouse IRDye 800 CW secondary antibodies were purchased from Li-COR Biosciences, Lincoln, NE, USA. The specific antibodies against neuron microtubule-associated protein 2 (MAP2) and astrocyte glial fibrillary acidic protein (GFAP) were purchased from Chemicon International Ltd., UK. Bradford assay kit was bought from Bio-Rad, Hercules, CA, USA and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-monotetrazolium bromide (MTT) was from BDH Chemicals Ltd., Poole, England. The Health Department of Hong Kong Government and the Animal Ethics Committee of the Chinese University of Hong Kong approved the use of animals for this study.

2.2. Primary cortical neuron cultures

The primary cortical neuronal cultures were prepared using a method described previously (Du et al., 2009). In brief, the pregnant SD rats were killed by using isoflurane deep anesthesia and the cortices were aseptically removed from the embryonic day 16–17 (E16–17) fetuses, minced with sterile surgical blades and incubated in 0.25% trypsin for 30 min. Dissociated cortical cells were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% inactivated fetal bovine serum (FBS) with penicillin (100 U), and then seeded on 6-, 12-, 24- or 96-well plates pre-coated with poly-L-lysine. The cultures were maintained at 37 °C in a humidified environment with 5% CO_2 in a CO_2 incubator (NAPCO 5400). Non-neuronal cell division was inhibited by an exposure to cytarabine for 48 h. The purity of these cultures was assessed by staining for the neuron specific antibodies against MAP2 and the astrocyte marker GFAP. After 7 days in culture, it was confirmed that the cells were predominantly neuronal cells (>98%). All experiments were performed after the cells were in culture for 7 days.

2.3. Experimental design

To investigate the effects of hypoxic preconditioning (HP) on the expression of iron influx proteins TfR1 and DMT1 and iron efflux protein Fpn1 as well as iron uptake and release in primary neuron culture, the cells in DMEM without serum were exposed to hypoxia (1% O_2) in a dedicated incubator (NAPCO 7101FC-1) with 1% O_2, 94% N_2 and 5% CO_2 at 37 °C for 0 (normoxia), 1, 2, 4 or 12 h. To investigate the effect of hypoxic preconditioning/re-oxygenation (HP/R), the cells were exposed to hypoxia (1% O_2) for different periods as the above, and then exposed to normoxia (21% O_2) for 24 h. After treatment with HP or HP/R, cell viabilities were assayed by a MTT assay, protein contents of TfR1, DMT1, Fpn1 and HIF-1alpha were determined by Western blot analysis, respectively, and transferrin-bound iron (Tf-Fe) and non-transferrin-bound iron (NTBI) entry into and iron release from neurons were determined by the radioisotope measurements.

2.4. Cell viability

The cell viability was assessed using a MTT assay as described previously (Du et al., 2010) in which the yellow MTT is reduced to a purple formazan by mitochondrial dehydrogenase in live cells. Briefly, a total of 25 ml MTT (1 g/L in phosphate-buffered saline, PBS) was added to each well and another 4 h of incubation at 37 °C was conducted. The assay was stopped by the addition of 100 ml lysis buffer (20% SDS in 50% non-dimethylformamide, pH 4.7). Optical density (OD) was measured at the 570 nm wavelength by the use of an ELX-800 microplate assay reader (Elx800, Bio-tek, USA). Results were expressed as a percentage of absorbance measured in control cells.

2.5. Measurement of total iron content in neurons

The cells were dissolved in 50 mM NaOH (100 μl/well, 6-well plate) and a 50 μl aliquot was subjected to detect the cellular protein concentration. Standard curves ranging from 0 to 40 ppb were constructed by diluting iron standard (1 mg iron/ml, Alpha Products, Danvers, MA). Both standards and samples were read in triplicate by injecting 50 μl aliquots into the graphite furnace of the GFAAS machine (Perkin Elmer SIMAA 6000, Rautaruukki Ltd., Raahe, Finland). Iron analysis (absorbance readings) were recorded at 248.3 nm, slit at 0.2 nm, pretreatment temperature at 1400 °C, and atomization temperature at 2400 °C. Iron content could be calculated from the standard curve and intracellular iron contents were further normalized with the cellular protein concentrations.

2.6. Western blot analysis

Western blot analysis was performed as described previously (Ke et al., 2005; Qian et al., 2007). The cells that received different treatments were washed twice with ice-cold PBS. The proteins were extracted with 150 ml cold lysis buffer (50 mmol/L Tris–HCl with pH 6.8, 1 mmol/L EDTA, 1% SDS, 1% Nonide P-40, 1 mmol/L Na_3VO_4, 1 mmol/L NaF, 5% bmercaptoethanol, 0.4 mmol/L phenylmethylsulfonyl fluoride (PMSF), 2 mg/L Aprotinin, 2 mg/L Leupeptin, and 2 mg/L Pepstain). Lysates were kept in ice for 30 min and centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was collected and the protein content was determined using the Bradford assay kit. A total of 30 μg protein (for detecting TfR1, Fpn1, DMT1 + IRE and DMT1−IRE) or 120 μg protein (for detecting

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