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Hypoxic preconditioning increases iron transport rate in astrocytes

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ABSTRACT

The mechanisms involved in the neuroprotection induced by hypoxic preconditioning (HP) have not been fully elucidated. The involvement of hypoxia-inducible factor-1 alpha (HIF-1alpha) in such neuroprotection has been confirmed. There is also evidence showing that a series of genes with important functions in iron metabolism, including transferrin receptor (TfR1) and divalent metal transporter 1 (DMT1), are regulated by HIF-1alpha in response to hypoxia in extra-neural organs or cells. We therefore hypothesized that HP is able to affect the expression of iron metabolism proteins in the brain and that changes in these proteins induced by HP might be associated with the HP-induced neuroprotection. We herein 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 (ferroportin1) proteins, and thus increase tansferrin-bound iron (Tf-Fe) and non-transferrin-bound iron (NTBI) uptake and iron release in astrocytes. Moreover, HP could lead to a progressive increase in cellular iron content. We concluded that HP has the ability to increase iron transport speed in astrocytes. Based on our findings and the importance of astrocytes in neuronal survival in hypoxic/ischemic preconditioning, we proposed that the increase in iron transport rate and cellular iron in astocytes might be one of the mechanisms associated with the HP-induced neuroprotection. We also demonstrated that ferroportin1 expression was significantly affected by HIF-1alpha in astrocytes, implying that the gene encoding this iron efflux protein might be a hypoxia-inducible one.

1. Introduction

Preconditioning induced by hypoxia can produce significant protective effects on neurons in experimental cells, animals and humans [1–6]. The involvement of hypoxia-inducible factor-1 alpha (HIF-1alpha) and the increased expression of its target genes in the neuroprotection induced by hypoxic preconditioning (HP) have been well confirmed. In addition, adaptation to hypoxia of cells and tissues leads to the transcriptional induction of a series of genes, several of which have important functions in iron metabolism [7]. Transferrin (Tf) and transferrin receptor (TfR1) are two key proteins involved in iron uptake by mammalian cells [8]. 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 [9–11]. It has also been reported that several other iron transport or regulation proteins, including ceruloplasmin (Cp) [12], iron regulatory protein 1 (IRP1) [13] and 2 (IRP2) [14], and hepcidin [15], are regulated by HIF-1 in response to hypoxic conditions. Our recent studies [16,17] have demonstrated that divalent metal transporter 1 (DMT1) is a hypoxia-inducible gene.

Currently, we know very little about the effects of HP on the expression of iron transport proteins in the brain. Moreover, the mechanisms involved in the neuroprotection induced by hypoxic preconditioning (HP) have not yet been fully elucidated. Based on the foregoing findings about the effects of HP on the expression of iron metabolism proteins obtained from the extra-neural organs or cells, we speculated that HP is also able to affect the expression of iron metabolism proteins in the brain. We hypothesized that the responses of iron metabolism proteins in astrocytes or other brain cells to HP might be associated with the HP-induced neuroprotection. We also proposed that the expression of ferroportin1 (Fpn1), a key iron efflux protein, might 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 astrocytes. Astrocytes were chosen in this study because of their importance in neuronal survival in the post-ischemic period [18], and because of their key function as a central integrator in the brain [19]. We demonstrated 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 up the rate of iron transport in astrocytes, and proposed that the increase in the rate of iron transport in astrocytes might be one of the mechanisms involved in the neuroprotection induced by HP.

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2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co., St. Louis, MO, 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, US. Goat anti-rabbit or anti-mouse IRDye 800 CW secondary antibodies were purchased from LI-COR Biosciences, Lincoln, NE, USA. The specific antibody against astrocyte glial fibrillary acidic protein (GFAP) was purchased from Chemicon International Ltd, UK. Bradford assay kit was bought from Bio-Rad, Hercules, CA, USA and $^{55}FeCl_3$ from Perkin-Elmer Company, Wellesley, MA, USA. 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 astrocyte culture

All experiments were carried out with cortical astrocytes prepared from newborn SD rats at postnatal 1–3 days by a procedure previously described [20,21] with minor modifications. Cortices were cut into small cubes (<1 mm^3) and digested with 0.125% trypsin for 15 min at 37 °C. Trypsinization was terminated by the addition of Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml). Cell suspensions were passed through a 40 μm cell strainer. The filtrate was allowed pre-adherence for 1 h to remove any contamination from fibroblasts before being seeded at a density of 1×10^6/ml in 25 or 50 cm^2 flasks. The cells were then incubated in a 5% CO_2 incubator at 37 °C. After the cultures reached confluence (for about 7 days), the cells were subcultured and allowed pre-adherence for 30 min in the subculture process. The purified astrocytes were seeded on 6, 12, 24 or 96-well plates precoated with poly-L-lysine at a density of 1×10^6/ml and cultured for another 7 days before use. The purity of the astrocytes was assessed as the percentage of cells with GFAP (1:5000), which was approximately 99%.

2.3. Experimental design

To investigate the effects of hypoxic preconditioning (HP) on the expression of iron transport proteins (TfR1, DMT1 and Fpn1) as well as iron uptake and release in primary astrocyte culture, the cells in DMEM without serum were exposed to hypoxia (1% O2) in a dedicated incubator (NAPCO 7101FC-1) with 1% O_2, 94% N_2 and 5% CO_2 at 37 °C for 0, 1, 2, 4 or 12 h. To investigate the effect of hypoxic preconditioning/re-oxygenation (HP/R), the astrocytes were exposed to hypoxia (1% O_2) for different durations as mentioned earlier, and then exposed to nomoxia (21% O_2) for 24 h. After treatment with HP or HP/R, cell viabilities were assayed by a MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-monotetrazolium bromide, BDH Chemicals Ltd., England.) assay, protein contents of TfR1, DMT1, Fpn1 and HIF-1alpha were determined by western blot analysis, and transferrin-bound iron (Tf-Fe) and non-transferrin-bound iron (NTBI) entry into and iron release from astrocytes were determined by radioisotope measurements.

2.4. Cell viability

The cell viability was assessed using a MTT assay in which the yellow MTT is reduced to a purple formazan by mitochondrial dehydrogenase in live cells as described previously [22]. Briefly, a total of 25 ml MTT (1 g/l in phosphate-buffered saline, PBS) was added 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% N0N-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 the control cells.

2.5. Measurement of total iron content in astrocytes

The astrocytes were dissolved in 50 mM NaOH (100 μl/well, 6-well plate) and a 50 μl aliquot was subjected to detection of 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 analyses (absorbance readings) were recorded at 248.3 nm, slit at 0.2 nm, with pretreatment temperature at 1400 °C and atomization temperature at 2400 °C. Iron content could be calculated from the standard curves 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 [23,24]. 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 at 4 °C for 15 min. 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 HIF-1alpha) was separated by 10% SDS-polyacrylamide gels (SDS-PAGE), transferred to the polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) previously blocked with 5% non-fat milk in 20 mM TBS-T (Tris-buffered saline with pH 7.5 and 0.1% Tween-20), and incubated overnight at 4 °C with the indicated primary monoclonal mouse anti-HIF-1alpha (1:500), rabbit anti-rat DMT1 + IRE (1:5000), DMT1 − IRE1 (1:5000), mouse anti-rat TfR monoclonal antibody (1:2500), and rabbit anti-mouse Fpn1 (1:5000) overnight at 4 °C, and then incubated with the goat anti-rabbit (for detecting DMT1 + IRE, DMT1 − IRE and Fpn1) or anti-mouse (for detecting TfR1 and HIF-1alpha) IRDye 800 CW secondary antibody (1:5000) for 1 h at room temperature. To ensure even loading of the samples, the same membrane was probed with rabbit anti-rat β-actin monoclonal antibody at 1:20,000 dilutions for 6 h and then incubated with the goat anti-mouse IRDye 800 CW secondary antibody for 30 min. Proteins were visualized using an Odyssey scanner and the intensities of the specific bands were analyzed by Odyssey infrared imagine system, with a resolution of 169 μm (Odyssey infrared imagine system).

2.7. Transferrin-bound iron uptake by astrocytes

Transferrin-bound iron (^{55}Fe-Tf) solution was prepared as previously described [25]. After different treatments, the astrocytes were incubated with 2 μM of ^{55}Fe-Tf in 0.5 ml serum-free DMEM medium at 37 °C for 30 min with gentle shaking. After that, they were washed with cold PBS twice and all un-bound Tf was stripped with an acid buffer (0.2 N acetic acid, 500 mM NaCl, 1 mM $FeCl_3$). The cells were then lysed with 500 μl lyses buffer and a 10 μl aliquot was subjected to detection of protein concentration. The cytosol was separated from the membrane by centrifugation at 10,000 *g* at 4 °C for 20 min, and the pellet (membrane fraction) was dissolved with 490 μl of 1%

SDS. Each fraction was added with 3 ml scintillation solution to count the cpm separately. The sum of the radioactivity in the cytosol and the membrane was referred to as the total cellular radioactivity. The cellular radioactivity was the total cellular radioactivity divided by the protein amount [i.e., cellular radioactivity = the sum of the radioactivity in the cytosol and the membrane (cpm)/protein amount (μg)], and the cpm value of each group was normalized with the individual control group which was taken as 100%.

2.8. Non-transferrin-bound iron uptake by astrocytes

The radio-labeled ^{55}Fe (NTBI) solution was prepared according to Qian and Morgen [26]. After different treatments, the astrocytes were incubated with 1.0 μM $^{55}FeCl_3$ in 0.5 ml serum-free DMEM medium at 37 °C for 30 min with gentle shaking. After that, they were washed with cold PBS twice and lysed with 500 μl of lyses buffer (1% SDS) and a 10 μl aliquot was subjected to detection of protein concentration. Three ml of scintillation solution were added to the remnant lysed fraction to count the cpm. The cellular radioactivity was defined as cellular radioactivity (cpm)/protein amount (μg), and the cpm value of each group was normalized with the individual control group.

2.9. Iron release assay

Iron release from astrocytes was measured as previously described [27]. The ^{55}Fe solution was freshly prepared according to Qian and Morgen [26]. After washing with PBS three times, the astrocytes were incubated with 0 or 2 μM of ^{55}Fe solution at 37 °C for 60 min with gentle shaking. The cells were then washed twice with PBS and treated with HP or HP/R as described in the "Experimental design". After that, the medium was collected and the cells were lysed. The radioactivity of both fractions was assayed by a scintillation counter (Perkin-Elmer) as cpm. The relative percentage of the radioactivity in the medium and in the cells was calculated. The percentage of ^{55}Fe (II) release was calculated with the following equation: ^{55}Fe (II) release (%) = [(cpm in medium)/(cpm in medium + cpm in cells)] × 100%. Each treatment group was normalized with the individual control group.

2.10. Statistical analysis

Statistical analyses were performed using SPSS 10.0. Data are presented as mean ± SEM. The difference between means was determined by one-way ANOVA followed by a Student–Newman–Keuls test for multiple comparisons. A probability value of $P<0.05$ was taken to be statistically significant.

3. Results

3.1. Effects of hypoxic preconditioning on cell viabilities

The effects of different durations of HP (0, 0.5, 1, 2, 4 or 12 h) or HP/R (0, 0.5, 1, 2, 4 or 12 h/24 h) on cell viability were first investigated in astrocytes. The MTT assay results (Fig. 1) indicated that HP induced a progressive increase in cell viability from HP-0.5 h to HP-4 h. The cell viability in the HP-2 h and HP-4 h was significantly higher than that in the control ($P<0.05$). The further increase in the time of HP (HP-12 h) induced a significant reduction in cell viability as compared with HP-4 h. The cell viability in the HP-12 h was significantly lower than that in HP-4 h ($P<0.05$), but not significantly different from the control. Unlike what we found in the HP-treated astrocytes, treatment with HP/R did not induce any significant effects on cell viability during the period from HP-0.5 h/R-24 h to HP-4 h/R-24 h. There were no significant differences among the groups of HP-0.5 h/R-24 h, HP-1 h/R-24 h, HP-2 h/R-24 h and HP-4 h/R-24 h with the control. However, with the increase in the time of treatments, HP-12 h/R-24 h also induced a significant reduction in cell viability. The viability of the cells treated with HP-12/R-24 h was significantly lower that in the control ($P<0.05$).

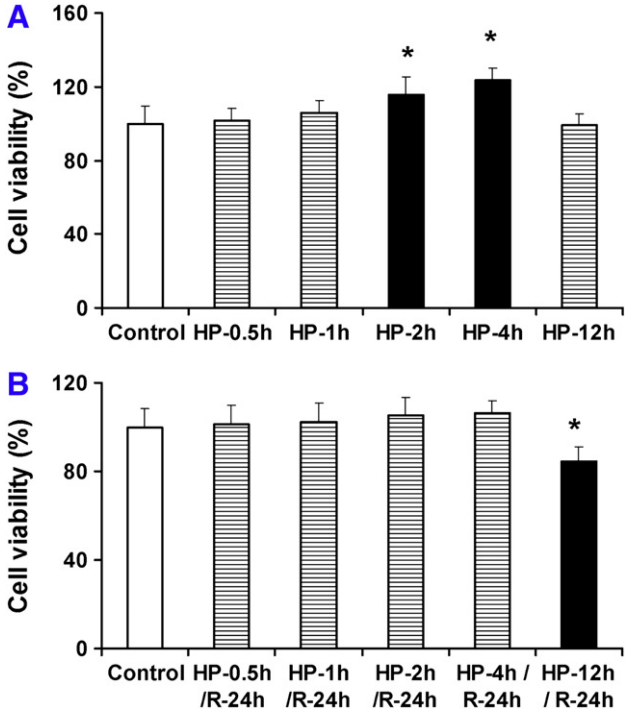


Fig. 1. Effects of hypoxic preconditioning on cell viabilities. The astrocytes were treated with hypoxic preconditioning (HP) for 0 (Control), 1, 2, 4 or 12 h with or without reoxygenation (R) (24 h) as described in the "Experimental design". Cell viabilities were then assayed by a MTT assay. A: Effects of HP, B: Effects of HP/R. Data are presented as mean ± SEM ($n=5$). *$P<0.05$ vs. the control.

3.2. Hypoxic preconditioning induced a significant increase in TfR1 expression and Tf-Fe uptake in astrocytes

We then investigated the effects of HP or HP/R on the expression of a major iron uptake protein TfR1 as well as Tf-Fe uptake in astrocytes. It was found that treatments of astrocytes with HP induced a progressive increase in TfR1 expression as well as Tf-Fe uptake during the period from HP-0.5 h to HP-4 h (Fig. 2A, B and D). The TfR1 expression and Tf-Fe uptake in the cells treated with HP-0.5 h, HP-1 h, HP-2 h or HP-4 h all were significantly higher than those in the control ($P<0.05$). The further increase in the time of HP (HP-12 h) induced a significant reduction not only in TfR1 expression but also in Tf-Fe uptake. Both the TfR1 expression and Tf-Fe uptake in the HP-12 h were significantly lower than those in the HP-4 h ($P<0.05$), but they were still significantly higher than those in the control. Moreover, treatments with HP/R induced a progressive increase in TfR1 expression as well as Tf-Fe uptake in astrocytes (Fig. 2A, C and E). The TfR1 expression and Tf-Fe uptake in the astrocytes treated with HP/R at all time points we had examined were significantly higher than those in the control ($P<0.05$). The highest expressions of TfR1 and Tf-Fe uptake were found in the cells treated with HP-12 h/R-24 h. The tendencies in the effect of HP or HP/R on Tf-Fe uptake were very similar to those on TfR1 expression. This implies that Tf-Fe uptake was mainly dependent on TfR1 expression in astrocytes treated with HP or HP/R.

3.3. Hypoxic preconditioning induced a significant increase in DMT1 expression and NTBI uptake in astrocytes

DMT1 is another major iron uptake protein. Therefore we also investigated the effects of HP or HP/R on the expression of this protein and on NTBI uptake in astrocytes as well. Treatments of astrocytes with HP-0.5 h, HP-1 h or HP-25 h induced a significant increase in the expression

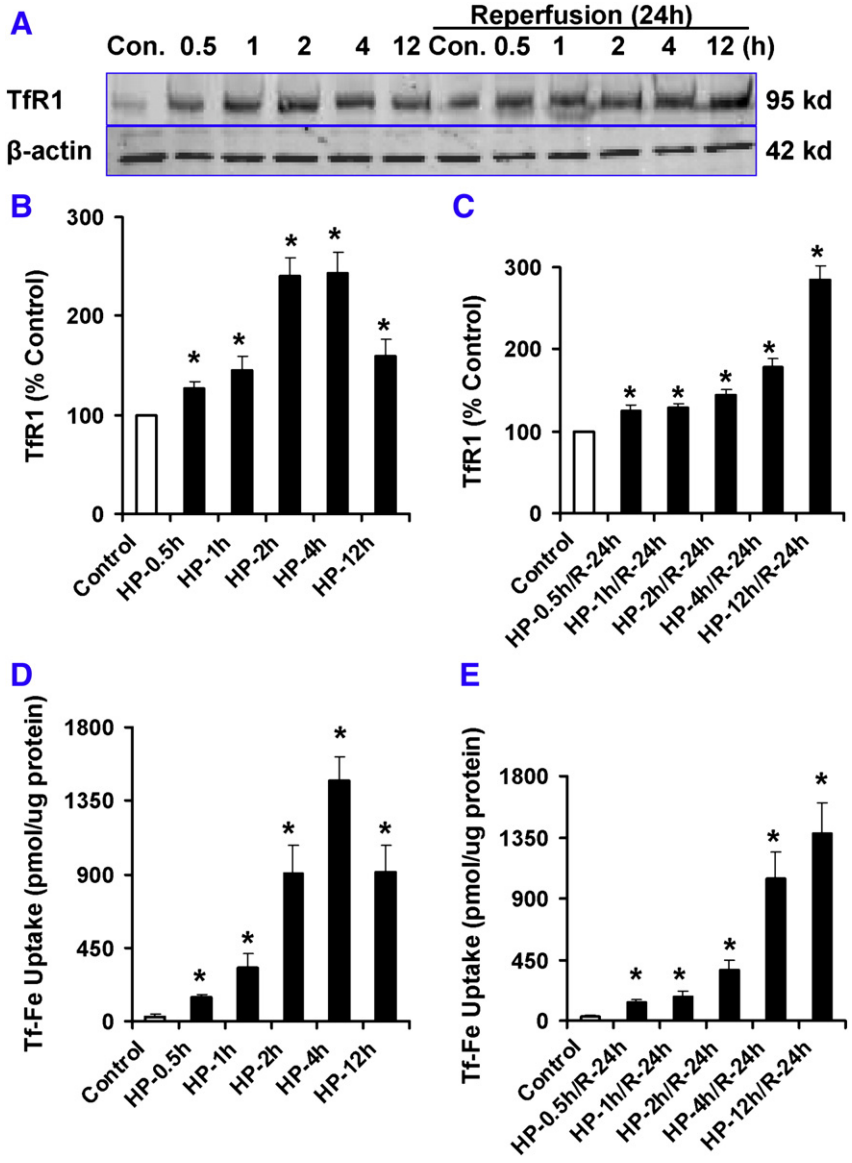


Fig. 2. Hypoxic preconditioning induced a significant increase in TfR1 expression and Tf-Fe uptake in astrocytes. The astrocytes were treated with hypoxic preconditioning (HP) for 0 (Control), 1, 2, 4 or 12 h with or without re-oxygenation (R) (24 h) as described in the "Experimental design", then TfR1 expression was determined by western blot analysis (A, B and C) and Tf-Fe uptake (D and E) by astrocytes by the radioisotope measurements. Data are presented as mean ± SEM (n = 6). *P<0.05 vs. the control.

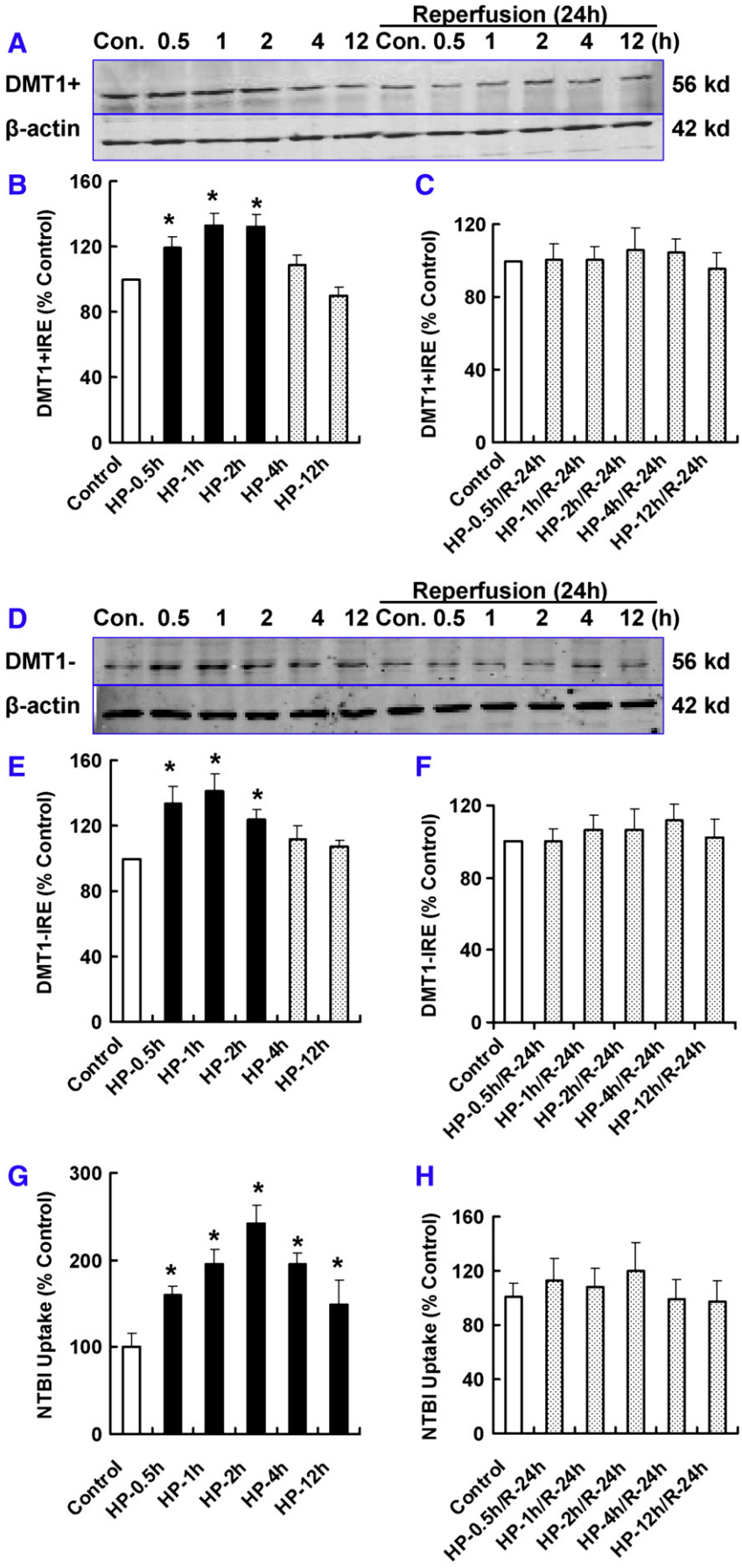
of DMT1 + IRE (Fig. 3A and B) as well as DMT1 − IRE (Fig. 3D and E). The contents of DMT1 + IRE and DMT1 − IRE in the cells treated with HP-0.5 h, HP-1 h or HP-2 h were significantly higher than those in the corresponding controls. The highest expression of both DMT1 + IRE and DMT1 − IRE was found in the cells treated with HP-1 h. However, the further increase in the time of HP induced a progressive decrease in the expression of DMT1 + IRE (Fig. 3A and B) as well as DMT1 − IRE (Fig. 3D and E). Although the contents of DMT1 + IRE in the cells treated with HP-4 h and DMT1 − IRE in the cells treated with HP-4 h or HP-12 h were still higher than those in the corresponding controls, no significant differences were found between these groups and the controls.

Furthermore, treatments of astrocytes with HP induced a progressive increase in NTBI uptake by astrocytes during the earlier stage: HP-0.5 h to HP-2 h (Fig. 3G). The NTBI uptake by the cells treated with HP-0.5 h, HP-1 h or HP-2 h was significantly higher than that in the control cells. The highest NTBI uptake was found in the cells treated with HP-2 h. However, the further increase in the time of HP (HP-2 h to HP-12 h) induced a progressive decrease in NTBI uptake by astrocytes. Although NTBI uptake in the astrocytes treated with HP-4 h or HP-12 h was significantly higher than that in the controls (Fig. 3G) while DMT1 + IRE and DMT1 − IRE in these HP cells were not significantly different from those of the control (Fig. 3A, B, D and E), the tendencies in the effects of HP on NTBI uptake (Fig. 3G) were basically similar to those on the expression of DMT1 + IRE (Fig. 3A and B) and DMT1 − IRE (Fig. 3D and E) in astrocytes (Fig. 3G). This might suggest that NTBI uptake was mainly determined by the the expression of DMT1 + IRE and DMT1 − IRE in the HP-treated astrocytes.

Unlike what we had found in the HP-cells, treatment of astrocytes with HP/R did not induce any significant changes in the expression of DMT1 + IRE (Fig. 3A and C), DMT-IRE (Fig. 3D and F) or NTBI uptake (Fig. 3H). There were no significant differences between the levels of the two forms of DMT1 between all the HP/R groups and their corresponding controls. Moreover, no significant differences were found between NTBI uptake by all HP/R cells and their control cells.

3.4. Hypoxic preconditioning induced a significant increase in Fpn1 expression and iron release in astrocytes

We also investigated the effect of HP or HP/R on the expression of Fpn1, a newly discovered trans-membrane iron export protein, and


A
Reperfusion (24h)
Con. 0.5 1 2 4 12 Con. 0.5 1 2 4 12 (h)
DMT1+
β-actin
56 kd
42 kd
B
DMT1+IRE (% Control)
Control
HP-0.5h
HP-1h
HP-2h
HP-4h
HP-12h
C
DMT1+IRE (% Control)
Control
HP-0.5h/R-24h
HP-1h/R-24h
HP-2h/R-24h
HP-4h/R-24h
HP-12h/R-24h
D
Reperfusion (24h)
Con. 0.5 1 2 4 12 Con. 0.5 1 2 4 12 (h)
DMT1-
β-actin
56 kd
42 kd
E
DMT1-IRE (% Control)
F
DMT1-IRE (% Control)
G
NTBI Uptake (% Control)
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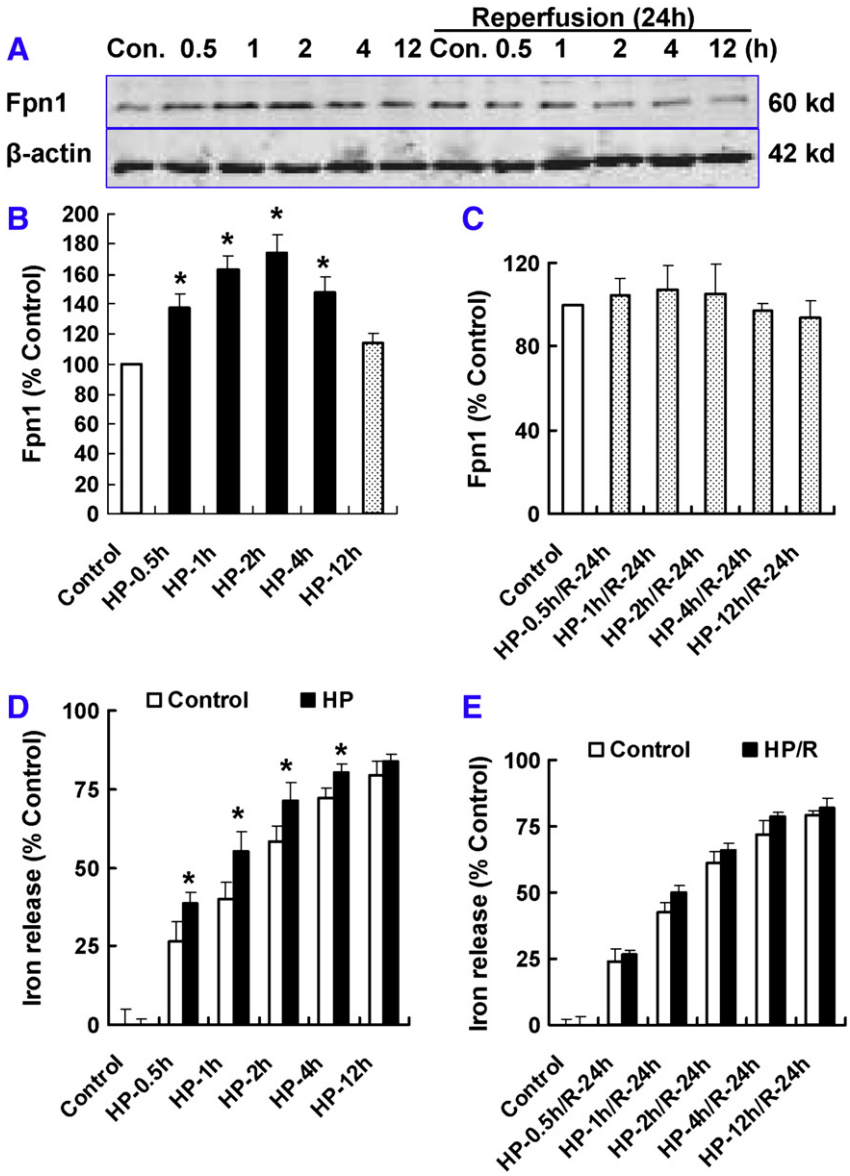

Fig. 4. Hypoxic preconditioning induced a significant increase in Fpn1 expression and iron release in astrocytes. The astrocytes were treated with hypoxic preconditioning (HP) for 0 (Control), 1, 2, 4 or 12 h with or without re-oxygenation (R) (24 h) as described in the "Experimental design", then the expression of Fpn1 was determined by western blot analysis (A, B and C) and iron release from astrocytes by the radioisotope measurements (D and E). Data are presented as mean ± SEM (n = 6). *P<0.05 vs. the control.

iron release in astrocytes. The existence of Fpn1 in the brain has been well documented [28,29]. Our findings demonstrated for the first time that HP could induce a progressive increase in the expression of Fpn1 during the period from HP-0.5 h to HP-2 h (Fig. 4A and B). The contents of Fpn1 in the cells treated with HP-0.5 h, HP-1 h, HP-2 h or HP-4 h were significantly higher than those in the control. The highest expression of Fpn1 was found in the cells treated with HP-2 h. However, the further increase in the time of HP (HP-2 h to HP-12 h) induced a progressive decrease in Fpn1 expression (Fig. 4A and B). There were no significant differences in Fpn1 expression between the HP-12 h cells and the control. The tendencies in the effect of HP on iron release (Fig. 4D) were very similar to those on Fpn1 expression. In consistent with the effect on Fpn1 expression, iron release in the cells treated with HP-0.5 h, HP-1 h, HP-2 h or HP-4 h was also significantly higher than that in the control and the highest release of iron was found in the cells treated with HP-2 h. This implies that Fpn1 is a major factor to determine iron release in astrocytes treated with HP. However, contrary to what we had found in the HP cells, treatments of astrocytes with HP/R did not induce any significant changes in Fpn1 expression (Fig. 4A and C) or also iron release (Fig. 4E). There were no significant differences in Fpn1 expression and iron release from astrocytes between all HP/R groups and their corresponding controls.

3.5. Hypoxic preconditioning induced a significant increase in the contents of hypoxia-inducible factor-1 alpha and iron in astrocytes

To find out the possible connection between HIF-1 and iron transport, we investigated the effects of HP or HP/R on the levels of HIF-1alpha and iron contents in astrocytes. It was found that HP induced a progressive increase in the contents of HIF-1alpha during the period

Fig. 3. Hypoxic preconditioning induced a significant increase in DMT1 expression and NTBI uptake in astrocytes. The astrocytes were treated with hypoxic preconditioning (HP) for 0 (Control), 1, 2, 4 or 12 h with or without re-oxygenation (R) (24 h) as described in the "Experimental design", then the expression of DMT1 + IRE (A, B and C) and DMT1 − IRE (D, E and F) was determined by western blot analysis and NTBI uptake (G and H) by astrocytes by the radioisotope measurements. Data are presented as mean ± SEM (n = 6). P<0.05 vs. the control.

from HP-0.5 h to HP-4 h (Fig. 5A). However, the further increase in the time of HP (HP-12 h) induced a significant decrease in HIF-1alpha. The content of HIF-1alpha in the group of HP-12 h was significantly lower than that in the group of HP-4 h and also not significantly different from the control. In the cells treated with HP/R, no significant differences were found in the levels of HIF-1alpha among the control and all HP/R groups (Fig. 5B). Iron contents in astrocytes were found to have increased progressively with the increase in the time of HP (Fig. 5C) or HP/R (Fig. 5D) although there were no significant differences among the groups of HP-0.5 h, HP-1 h, HP-2 h, HP-4 h and the control in the HP astrocytes (Fig. 5C) and the groups of HP-0.5 h/R-24 h and the control in the HP/R cells (Fig. 5D).

4. Discussion

Although it has been confirmed that a series of genes with important functions in iron transport, including TfR1 [9–11] and DMT1 [16,17], in extra-neural organs and cells are hypoxia-inducible genes, very little is known about the effects of HP on the expression of these proteins in the brain. Moreover, the mechanisms of the neuroprotection induced by HP have not yet been fully elucidated although the involvement of HIF-1alpha is confirmed. One of the major objectives of this study was to determine whether HP could affect the expression of iron transport proteins and iron transport in astrocytes as had been found outside of the brain. Our findings demonstrated for the first time that HP has the ability to induce a significant increase in the expression of 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 in astrocytes in addition to the increased cellular iron content, cell viability and expression of HIF-1alpha in cells. It has been well determined that astrocytes function as a central integrator in the brain [18] and play an important role in neuronal survival in the post-ischemic period [19]. Moreover, iron is a transition metal essential for oxygen transport, cell growth and survival [16]. The increase in the availability of cellular iron might be able to promote the synthesis of iron-associated molecules involved in oxygen transport and then increase the abilities of oxygen transport and the availability of oxygen in astrocytes. Therefore, the increase in the rate of iron transport and cellular iron in astrocytes might be one of the mechanisms involved in the neuroprotective role or the increased anoxic tolerance.

Both TfR1 and DMT1 are hypoxia-inducible genes [9–11]. In the HP astrocytes, we found a significant increase in the expression of not only iron uptake (TfR1 and DMT1) and release (Fpn1) proteins but also HIF-1alpha. This implies that the increased expression of iron transport proteins is probably due to the increased expression of HIF-1alpha. However, it was noticed that the tendencies in the effects of HP on the expression of HIF-1alpha are very similar to those on TfR1, but not to those on DMT1 and Fpn1. The HP induced a progressive increase in the expression of HIF-1alpha, and the highest expression was found in HP-

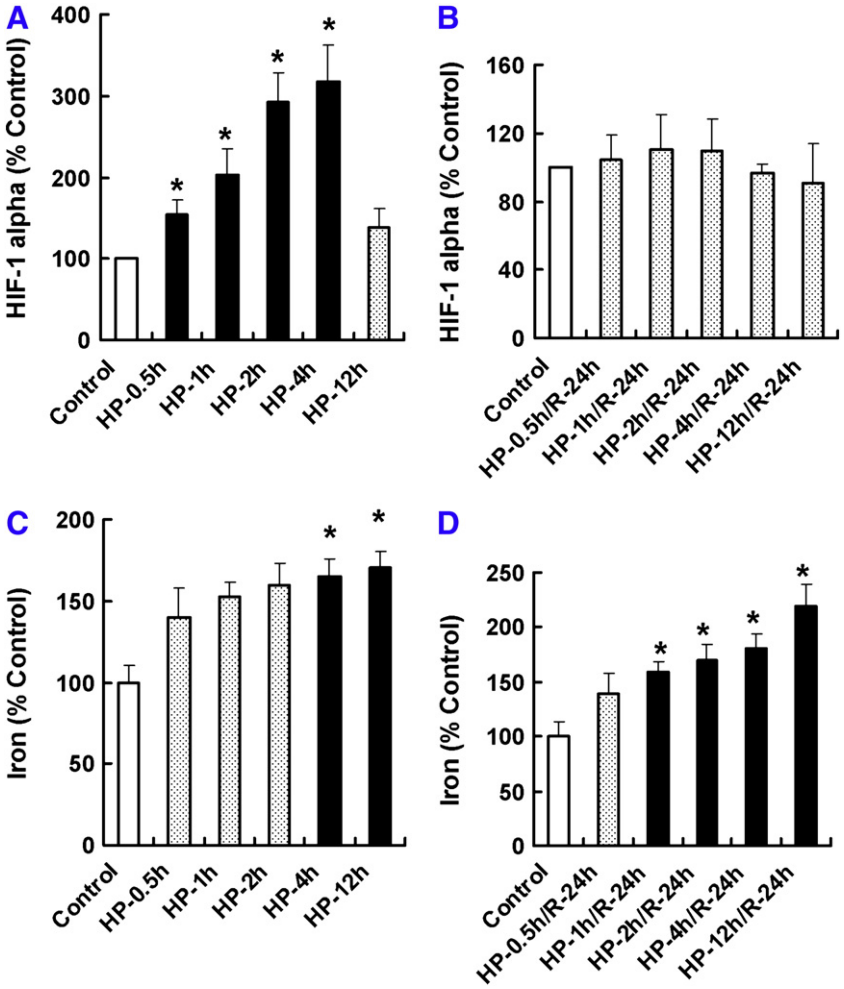

Fig. 5. Hypoxic preconditioning induced a significant increase in the contents of hypoxia-inducible factor-1 alpha and iron in astrocytes. The astrocytes were treated with hypoxic preconditioning (HP) for 0 (Control), 1, 2, 4 or 12 h with or without re-oxygenation (R) (24 h) as described in the "Experimental design", then the expression of HIF-1alpha was determined by western blot analysis (A and B) and iron contents in the astrocytes by a GFAAS method (C and D). Data are presented as mean ± SEM (n = 6). *P < 0.05 vs. the control.

4 h, while the highest expression of DMT1 (DMT1 + IRE and DMT1 − IRE) and Fpn1 was in HP-1 h and HP-2 h respectively. These results imply that HIF-1alpha plays a predominant role in TfR1 expression, but not in DMT1 or Fpn1 expression in the HP cells. There might be other factors that are also involved in the control of DMT1 and Fpn1 regulation in additon to HIF-1alpha in the astrocytes treated with HP.

However, the responses of the expression of HIF-1alpha, TfR1, DMT1 and Fpn1 to HP/R are different from those to HP. In the HP/R cells, no significant changes were found in the expression of HIF-1alpha, DMT1 (DMT1 + IRE and DMT1 − IRE) or Fpn1. Accordingly, treatment with HP/R did not induce any significant effect on NTBI uptake or iron release in astrocytes. The similar tendencies in the effects of HP/R on the expression of HIF-1alpha as well as DMT1 (NTBI uptake) and Fpn1 (iron release) might suggest that the expression of DMT1 and Fpn1 is mainly controlled by HIF-1alpha in the HP/R cells. However, TfR1 expression and Tf-Fe uptake were found to have increased with the time of HP/R treatments although HIF-1alpha did not respond to HP/R treatment. The inconsistency in the responses of the expression of HIF-1alpha and TfR1 to HP/R treatment showed that HIF-1alpha is not the only or major factor to regulate TfR1 expression in the HP/R cells. HR/P treatment might induce some molecules that can produce a significant effect on the expression of TfR1 in astrocytes. In both of the HP and HP/R cells, the cellular iron contents increased with the time of treatments, however, iron levels in the HP/R cells were relatively higher than those in the HP cells at almost all time points we measured. This was probably due to the increased expression of Fpn1 and then iron release in the HP cells while the expression of Fpn1 did not respond to the HR/P.

In addition, HIF-1alpha is very sensitive to oxygen availability. In the HP/R cells, HIF-1alpha might be up-regulated under HP and then returned to the control level after "R". Its up-regulation under HP may stimulate a numbers of target genes, some of which may be long-lasting such as TfR1 (and Tf-Fe uptake), while the others including DMT1 (and NTBI) may not in terms of the expression and/or function of these genes after HIF-1alpha stimulation. This possibility might also be associated with the inconsistency in the responses of the expression of HIF-1alpha, DMT1 (DMT1 + IRE and DMT1 − IRE), Fpn1 and TfR1 and also cellular iron levels to HP and HP/R treatments.

It is currently completely unknown whether the expression of Fpn1 is regulated by hypoxic preconditioning in the brain. In this study, we presented evidence for the first time that Fpn1 expression in astrocytes was significantly affected by HP, but not HP/R. Moreover, HP, but not HP/R, induced a significant effect on HIF-1alpha expression in the cells. These findings might imply that the gene encoding this iron efflux protein might be a hypoxia-inducible one. This possibility needs further investigations.

In summary, we investigated the effects of HP and HP/R on the expression of iron transport proteins as well as HIF-1alpha in astrocytes. 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, DMT1 − IRE and DMT1 + IRE) and release (Fpn1) proteins, and thus increase Tf-Fe and NTBI uptake and iron release, and also a progressive increase in cellular iron content. We concluded that HP has the ability to speed up the rate of iron transport in astrocytes and proposed that the increase in the rate of iron transport and cellular iron in astrocytes might be one of the mechanisms involved in the neuroprotective role or the increased anoxic tolerance induced by HP.

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
Fpn1	ferroportin1
GFAP	glial fibrillary acidic protein
HIF-1alpha	hypoxia-inducible factor-1 alpha
HP	hypoxic preconditioning
HP/R	hypoxic preconditioning/re-oxygenation
IRP1	iron regulatory protein 1
IRP2	iron regulatory protein 2
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-monotetrazolium bromide
NTBI	non-transferrin-bound iron
Tf-Fe	transferrin-bound iron
TfR1	transferrin receptor

Author contributions

Y.K. and M.F. conceived, organized and supervised the study and obtained funding; L.Y., F.D., Q.G., Z.G.B., Z.J.Z. and L.L.Z. performed experiments and performed statistical analysis; Y.K. wrote the paper.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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