RESEARCH ARTICLE

Neuroprotective mechanisms of DNA methyltransferase in a mouse hippocampal neuronal cell line after hypoxic preconditioning

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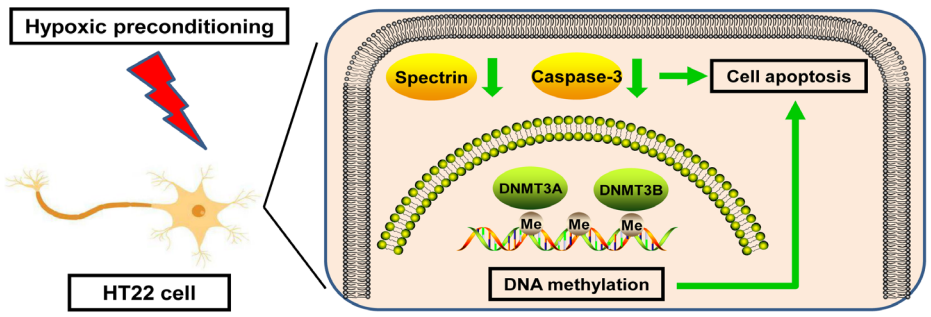
Graphical Abstract

Hypoxic preconditioning regulates the expression of DNA methyltransferases (DNMTs) which may be related to cell cycle and cell apoptosis in HT22 cells



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Abstract

Hypoxic preconditioning has been shown to improve hypoxic tolerance in mice, accompanied by the downregulation of DNA methyltransferases (DNMTs) in the brain. However, the roles played by DNMTs in the multiple neuroprotective mechanisms associated with hypoxic preconditioning remain poorly understood. This study aimed to establish an *in vitro* model of hypoxic preconditioning, using a cultured mouse hippocampal neuronal cell line (HT22 cells), to examine the effects of DNMTs on the endogenous neuroprotective mechanisms that occur during hypoxic preconditioning. HT22 cells were divided into a control group, which received no exposure to hypoxia, a hypoxia group, which was exposed to hypoxia once, and a hypoxic preconditioning group, which was exposed to four cycles of hypoxia. To test the ability of hypoxic preadaptation to induce hypoxic tolerance, cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium assay. Cell viability improved in the hypoxic preconditioning group compared with that in the hypoxia group. The effects of hypoxic preconditioning on the cell cycle and apoptosis in HT22 cells were examined by western blot assay and flow cytometry. Compared with the hypoxia group, the expression levels of caspase-3 and spectrin, which are markers of early apoptosis and S-phase arrest, respectively, noticeably reduced in the hypoxic preconditioning group. Finally, enzyme-linked immunosorbent assay, real-time polymerase chain reaction, and western blot assay were used to investigate the changes in DNMT expression and activity during hypoxic preconditioning. The results showed that compared with the control group, hypoxic preconditioning downregulated the expression levels of DNMT3A and DNMT3B mRNA and protein in HT22 cells and decreased the activities of total DNMTs and DNMT3B. In conclusion, hypoxic preconditioning may exert anti-hypoxic neuroprotective effects, maintaining HT22 cell viability and inhibiting cell apoptosis. These neuroprotective mechanisms may be associated with the inhibition of DNMT3A and DNMT3B.

Key Words: *caspase-3; cells; growth; injury; plasticity; recovery; regeneration; repair*

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Introduction

Hypoxia can occur in many extreme environments, including plateau, diving, and aerospace, and may occur during many diseases (Li et al., 2017; Cree et al., 2018; Chen et al., 2019). The brain is extremely sensitive to different forms of hypoxia, including transitory global cerebral ischemia (which can cause ischemic hypoxia), hypoxemia, and serious hypotension, which can damage fragile neurons, accompanied by neurological deficits and behavioral dysfunction (Cheng et al., 1999; Li et al., 2019a). Therefore, cells in the body must be able to respond or adapt to hypoxia during physiological and pathological conditions. More than three decades ago, Schurr et al. (1986) reported that hypoxic/ischemic preconditioning, an endogenous protective mechanism, can relieve neuronal damage caused by hypoxia in hippocampal slices. Hypoxic preconditioning (HPC) in the brain describes a phenomenon through which transient hypoxic exposure confers neuroprotection against a subsequent, prolonged, and injurious hypoxic event (Shao et al., 2006). HPC exerts a protective effect against brain injuries induced by ischemia/hypoxia (Xu et al., 2019). Therefore, the neuroprotective effects induced by HPC may potentially be utilized as a therapeutic strategy for hypoxic/ischemic diseases that affect the brain (Li et al., 2017). However, the molecular mechanisms underlying the neuroprotective effects of HPC are not fully understood.

To clarify the neuroprotective mechanisms associated with HPC, various HPC animal models have been developed (Cantagrel et al., 2003; Giusti and Fiszer de Plazas, 2012; Shao and Lu, 2012). Our group developed an HPC animal model of repeated autohypoxia to systematically study the effects and mechanisms of HPC (Shao et al., 2005; Zhang et al., 2014). We found that DNA methyltransferases (DNMTs) may be associated with hypoxic tolerance using this animal model (Zhang et al., 2014). DNA methylation, which is catalyzed by DNMTs, regulates chromatin structure and gene expression in the central nervous system (Mehler, 2008). Apoptosis induced by hypoxia may be associated with changes in the activity and expression of DNMTs (Li et al., 2019b). However, the relationships between the neuroprotective effects associated with HPC and DNMT expression and activity have not been investigated outside of our animal model. Therefore, the establishment of a cell model is important to provide additional insights into the roles played by DNMTs in HPC.

To elucidate the role played by DNMTs in HPC-induced neuroprotection, we developed an *in vitro* cell model of HPC using the mouse hippocampal neuronal HT22 cell line (He et al., 2013), which has been used as a hippocampal neuronal cell model in many studies (Koh, 2011; Shen et al., 2018; Xie et al., 2018). The *in vitro* model is simple, making detection of molecular mechanisms easy to accomplish. For the first time, HT22 cells were exposed to hypoxia repeatedly, which was designed to mimic our animal model of repeated autohypoxia.

HPC results in endogenous neuroprotective effects, but the mechanisms that underly this process are not completely clear. This study established a repeated-hypoxia HT22 cell model to explore the potential involvement of DNMTs in neuronal responses during HPC and demonstrate the effects of DNMTs during HPC-mediated neuroprotection *in vitro*.

Materials and Methods

Cell culture

HT22 cells were purchased from the National Laboratory of Molecular Oncology, Cancer Institute and Hospital (Chinese Academy of Medical Sciences and the Peking Union Medical College, Beijing, China) and were cultured in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco), in a humidified atmosphere of 5% CO_2/95% air at 37°C.

A 24-hour incubation with modified DMEM containing 100 mM dibutyryl cyclic adenosine monophosphate (Sigma, St. Louis, MO, USA), 100 mM phorbol 12,13-dibutyrate (Sigma), 50 ng/mL nerve growth factor-beta (Sigma), and 1× N_2 supplement (Gibco) was used to induce the differentiation of HT22 cells into cells with neuronal characteristics and functions (Zhao et al., 2016).

Repeated hypoxia protocol for cell culture

The cultured cells were divided into three groups: the control group, the hypoxia group, and the HPC group. Hypoxia and oxygenation were performed as previously described (Wasa et al., 2005). Cells in the control group were exposed to normoxia (5% CO_2 + 95% air) for 24 hours. Cells in the hypoxia group were exposed to 1% O_2 + 5% CO_2 + 94% N_2 for 13 hours and then cultured in normoxia for 6 hours. Cells in the HPC group were exposed to four cycles of 1% O_2 + 5% CO_2 + 94% N_2 for 30 minutes and 21% O_2 + 5% CO_2 + 74% N_2 for 30 minutes, followed by exposure to 1% O_2 + 5% CO_2 + 94% N_2 for 13 hours, and then cultured in normoxia for 6 hours (Shao et al., 2005). All groups were treated in a Forma Steri-Cult CO_2 Incubator (Thermo Fisher Scientific, Marietta, OH, USA). When the cell viability of the HPC group reached 90%, as detected by MTS assay, the model was considered to be successfully generated. In addition, the numbers of cytoplasmic vacuolizations (round and shiny giant vacuoles presented in the cytoplasm) per 100 HT22 cells, in 10 fields of view (original magnification, 40×), were counted in each group, to analyze the morphological changes in HT22 cells.

3-(4,5-Dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA). According to the manufacturer's protocol, HT22 cells were seeded into 96-well plates, at a density of 2000 cells per well, and cultured under normoxic conditions (5% CO_2 + 95% air) at 37°C for 24 hours. Cells were either exposed to hypoxia or maintained under normoxic conditions. After hypoxia, MTS was added and evaluated every half hour. Experiments were performed as previously

described (Tian et al., 2019).

Analysis of cell cycle and apoptosis

Cultured cells were collected and washed twice with phosphate-buffered saline. The cells were centrifuged at 1000 rpm/min, for 5 minutes, and cell pellets were obtained by discarding the supernatant. For cell cycle analysis, cell pellets fixed were with 70% ethanol and stained with 5 μL propidium iodide (Becton Dickinson, Franklin Lakes, NJ, USA) containing 0.1% Triton X-100 and 0.1 mg/mL RNase A. For cell apoptosis analysis, cell pellets were re-suspended with 10X Annexin V Binding Buffer (0.1 M Hepes/NaOH, 1.4 M NaCl, 25 mM $CaCl_2$) and stained with 5 μL fluorescein isothiocyanate (FITC) Annexin V (Becton Dickinson) and/or 2 μL propidium iodide. The percentages of cells in different phases and the percentage of apoptotic cells were analyzed by FACScan flow cytometry (Becton Dickinson) using a ModFit 3.0 computer program (Becton Dickinson).

Real-time quantitative polymerase chain reaction

The experiment was performed after hypoxia exposure, in each group. Cultured cells were collected and total RNA was isolated and reverse transcribed as previously described (Tian et al., 2019). Real-time quantitative polymerase chain reaction (PCR) was performed to measure the relative mRNA expression levels of DNMT1, DNMT3A, and DNMT3B using an ABI7900 system (Applied Biosystems, Foster City, CA, USA). The relative mRNA levels of *DNMT1*, *DNMT3A*, and *DNMT3B* were analyzed compared with *β-actin* gene expression, which served as the internal standard. Changes in target gene expression among the groups were analyzed using the $2^{-\Delta\Delta Ct}$ method (Zhang et al., 2015). All procedures were performed according to the instructions included with SYBR Premix Ex Taq II (Takara, Kyoto, Japan). The primers used are listed in **Table 1**.

Western blot assay

Protein expression levels were determined by western blot assay as previously described (Tian et al., 2019). Cultured cells were collected and washed in phosphate-buffered saline before incubation with radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China) on ice for 10 minutes. The protein concentrations were estimated using the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gels electrophoresis and transferred to nitrocellulose membranes (Roche Diagnostics, Indianapolis, IN, USA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline. The membranes were incubated with primary antibodies at 4°C for 12 hours as follows: rabbit anti-mouse DNMT1, DNMT3A, and DNMT3B polyclonal antibodies (1:1000; all from Novus Biologicals, Littleton, CO, USA), rabbit anti-mouse β-actin monoclonal antibody (1:1000; Sigma), rabbit anti-mouse caspase-3 polyclonal antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA), and rabbit anti-mouse spectrin polyclonal antibody (1:1000; Cell Signaling Technology). Afterward, the membranes were incubated with horseradish peroxidase-goat anti-rabbit secondary antibody (1:5000; Beyotime Biotechnology, Shanghai, China) for 1 hour at 37°C. The grey densitometric values of the protein bands were quantified using an image analysis system, with ImageJ (Scion Corporation, Torrance, CA, USA).

Table 1 Primer sequences

Gene	Sequence (5′–3′)	Product size (bp)
β-Actin	Forward: GGC TGT ATT CCC CTC CAT CG	154
	Reverse: CCA GTT GGT AAC AAT GCC ATG T	
DNMT1	Forward: CCT GGC TAA AGT CAA GTC CCT	60
	Reverse: GTG TGT GTT CCG TTC TCC AAG	
DNMT3A	Forward: GGC CGA ATT GTG TCT TGG TG	80
	Reverse: GTG TGT GTT CCG TTC TCC AAG	
DNMT3B	Forward: AAG CTC CCG GCT GTC TAA GA	52
	Reverse: CTG CGT GTA ATT CAG AAG GCT	

DNMT: DNA methyltransferase.

DNMT activity assay

Nuclear proteins from HT22 cells were isolated, using a commercial kit (Epigentek, Brooklyn, NY, USA). According to the manufacturer's instructions, the DNA methyltransferase activities of total DNMT, DNMT1 and DNMT3B were detected using the corresponding EpiQuik DNMT activity/inhibition assay Ultra kit (Epigentek). Briefly, the reaction was initiated by adding 10 μg of nuclear extract, containing active DNMTs, to the unique, cytosine-rich DNA substrate-coated enzyme-linked immunosorbent assay plate, followed by incubation for 60 minutes at 37°C. The methylated DNA was recognized by the anti-5-methylcytosine antibody. The amount of methylated DNA, which was proportional to the enzyme activity, was calorimetrically quantified at 450 nm.

Statistical analysis

All analyses were conducted using SPSS Version 17.0 software (SPSS Inc., Chicago, IL, USA). The experimental data are expressed as the mean ± standard deviation (SD). One-way analysis of variance, followed by Tukey's honest significant difference *post hoc* test, was applied for statistical analysis. $P < 0.05$ was considered significant.

Results

HPC improves the hypoxic tolerance of HT22 cells

Morphological changes in the HT22 cells in the control, hypoxia, and HPC groups were observed by inverted light microscopy (**Figure 1A**). **Figure 1B** shows that the refraction of the cells in the control group illustrated minimal cytoplasmic vacuolization. Compared with the control group,

cytoplasmic vacuolization was more severe in the hypoxia groups ($P < 0.05$). Compared with the hypoxia group, the number of cytoplasmic vacuoles was significantly reduced in the HPC group ($P < 0.05$). Although the number of cytoplasmic vacuoles in the HPC group was higher than that in the control group, this difference was not significant ($P > 0.05$).

Cell viability was measured by MTS assay. Compared with the control group, cell viability in the hypoxia group was significantly decreased ($P < 0.05$). However, after repetitive hypoxic exposure, cell viability recovered significantly in the HPC group compared with that in the hypoxia group ($P < 0.05$; **Figure 1C**). In addition, cell viability in the HPC group recovered and approached the level observed in the control group, with no significant difference observed between the HPC and control groups ($P > 0.05$). These results indicate that HPC may improve the hypoxic tolerance of HT22 cells.

HPC decreases the degradation of spectrin

Normal HT22 cells possessed substantial levels of spectrin (**Figure 2A**). Spectrin cleavage leads to the elevated generation of spectrin breakdown products of 145/150 kDa (indicative of necrotic and excitotoxic neuronal death) and 120 kDa (indicative of apoptotic cell death; Lu et al., 2018). The expression of spectrin breakdown products at 145/150 kDa increased in the hypoxia group compared with the control group ($P < 0.05$). The expression of spectrin breakdown products at 145/150 kDa decreased in the HPC group compared with the hypoxia group ($P < 0.05$; **Figure 2B**). However, the expression of spectrin breakdown products at 145/150 kDa was not significantly different between the control and HPC groups ($P > 0.05$). The expression of spectrin breakdown products at 120 kDa was not found to be different among the three groups ($P > 0.05$; **Figure 2C**).

HPC decreases cleaved caspase-3 levels

The effects of HPC on HT22 cells was measured by evaluating the levels of pro-caspase-3 and the p17/19 subunit of cleaved caspase-3 using western blot assay (**Figure 3A** and **B**). Compared with the levels in the control group, pro-caspase-3 levels were downregulated ($P < 0.05$; **Figure 3A**) and cleaved caspase-3 were upregulated ($P < 0.05$; **Figure 3B**) in the hypoxia group. Compared with the levels in the hypoxia group, the expression level of pro-caspase-3 was increased ($P < 0.05$; **Figure 3A**) and the cleaved caspase-3 level was decreased ($P < 0.05$; **Figure 3A**) in the HPC group. In the HPC group, the expression level of pro-caspase-3 was lower and the cleaved caspase-3 level was higher than that in the control group. However, the expression levels between the two groups were very close, and no significant difference was observed between the two groups ($P > 0.05$). These results suggested that HPC can decrease cleaved caspase-3 levels caused by hypoxia.

Effects of HPC on cell cycle and cell apoptosis

Flow cytometry was used to examine the cell cycle distribution and cell apoptosis rates of each group. The data showed that compared with the control group, the proportion of S phase cells and the cell apoptosis rate both increased significantly in the hypoxia group ($P < 0.05$; **Figure 4**). The proportions of S phase cells and the cell apoptosis rate in the HPC group decreased significantly compared with those in the hypoxia group ($P < 0.05$; **Figure 4**). However, the proportions of S phase cells and the cell apoptosis rate in the HPC group did not recovered to the levels observed in the control group. HPC may effectively decrease the number of cells arrested in the S phase and reduce the level of cellular apoptosis caused by hypoxia.

DNMT expression and activity in HPC HT22 cells

The expression levels of DNMT1, DNMT3A, and DNMT3B mRNA and protein in HT22 cells were detected by real-time PCR and western blot assay, respectively. No significant differences were observed among the three groups for DNMT1 mRNA or protein levels ($P > 0.05$; **Figure 5A** and **D**). Compared with the control and hypoxia groups, the DNMT3A and DNMT3B mRNA and protein levels significantly decreased in the HPC group ($P < 0.05$; **Figure 5B**, **C**, **E**, and **F**). The total DNMT activity in HT22 cells was lower in the HPC group than in the control and hypoxia groups ($P < 0.05$; **Figure 5G**). No significant differences in DNMT1 activity were observed among the three groups ($P > 0.05$; **Figure 5H**). Compared with the activity in the control group, the DNMT3B activity was significantly lower in the hypoxia and HPC groups ($P < 0.05$; **Figure 5I**).

Discussion

Lu et al. (1963) described the hypoxic preconditioning phenomenon as "a kind of induced tolerance of tissue-cells to hypoxia", using a repeated autohypoxia animal model, more than 50 years ago. We found that DNMT, an enzyme that regulates DNA methylation levels, was downregulated in the hippocampus and that it may be involved in the development of hypoxic tolerance using an autohypoxia animal model (Zhang et al., 2014). Several factors that may affect DNMT expression in the brain of a whole-body model (Lundberg et al., 2009; Kolodkin and Auger, 2011). To further clarify the neuroprotective mechanism of DNMTs during the development of hypoxic tolerance, we developed a simple *in vitro* model.

An *in vitro* model removes many of the fluctuating factors that can affect a whole-body hypoxic/ischemic model, including the removal of beneficial factors that may protect cells from experiencing hypoxic/ischemic injury following oxygen deprivation in culture (Akaneya et al., 1993). Zitta et al. (2016) used a human neuronal IMR-32 cell line as an *in vitro* model of hypoxic/ischemic cell injury to investigate the neuroprotective mechanisms of 2-iminobiotin. Nervous cells were isolated from a brain exposed to controlled levels of hypoxia (Feber et al., 2016). Coronal slices from neonatal have also been used as a model for the study of pericytes within the intact neurovascular unit under hypoxic conditions (Zehendner et al., 2013). Therefore, neuronal cell lines, primary nerve cells, and brain slices can be used as *in vitro* models for the detection of neuronal injuries and protective mech-

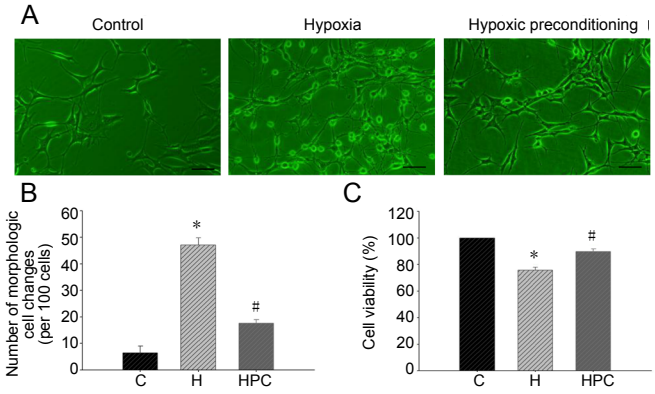

Figure 1 Effects of HPC on HT22 cell morphology and cell viability.
Cell morphology and cell viability were measured by light microscopy and MTS assay, respectively. HPC relieved the morphologic changes observed following hypoxia and increased cell viability. (A) Light microscopic examinations of HT22 cell morphology (original magnification, 40×), scale bars: 10 μm. (B) The number of morphologic cell changes. Data are presented as the number of morphologic changes per 100 cells. Compared with the hypoxia (H) group, the number of cytoplasmic vacuolization was markedly reduced in the HPC group. (C) MTS assay to determine HT22 cell viability. Data are expressed as a percentage of the control (C) group, calculated as $OD_{treated}/OD_{control} \times 100\%$. *$P < 0.05$, *vs.* C group; #$P < 0.05$, *vs.* H group. Data are expressed as the mean ± SD ($n = 3$; one-way analysis of variance followed by Tukey's honest significant difference *post hoc* test). The experiment was conducted in triplicate. C: Control; H: hypoxia; HPC: hypoxic preconditioning; MTS: 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium; OD: optical density.

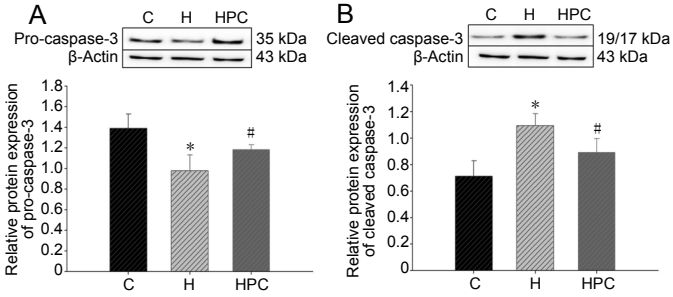

Figure 3 Effects of HPC on cleaved caspase-3 levels in HT22 cells.
(A) Western blot assay and semi-quantitative analysis of changes in the protein levels of pro-caspase-3: HPC decreased caspase-3 activity. (B) Western blot assay and semi-quantitative analysis of changes in cleaved caspase-3 levels. *$P < 0.05$, *vs.* C group; #$P < 0.05$, *vs.* H group. The grey densitometric values of western blot assays were normalized against the loading control (β-actin). Data are expressed as the mean ± SD ($n = 3$; one-way analysis of variance followed by Tukey's honest significant difference *post hoc* test). The experiment was conducted in triplicate. C: Control; H: hypoxia; HPC: hypoxic preconditioning.

anisms under hypoxic/ischemic conditions. In this study, an *in vitro* HPC cell model was generated using the immortalized mouse hippocampal neuronal cell line HT22, which was subcloned from the parental HT4 primary mouse hippocampal neuronal culture and are characterized by stable growth and easy availability (Liu et al., 2009; He et al., 2013). Changes in gene and protein expression in HT22 cells under hypoxic conditions should be similar to those observed in primary mouse hippocampal neuron culture owing to the origination of this cell line. Thus, HT22 cells may represent a good *in vitro* model for hypoxic tolerance in hippocampal neurons induced by HPC without requiring the sacrifice of parous mice. HT22 cells have been used as an *in vitro* oxy-

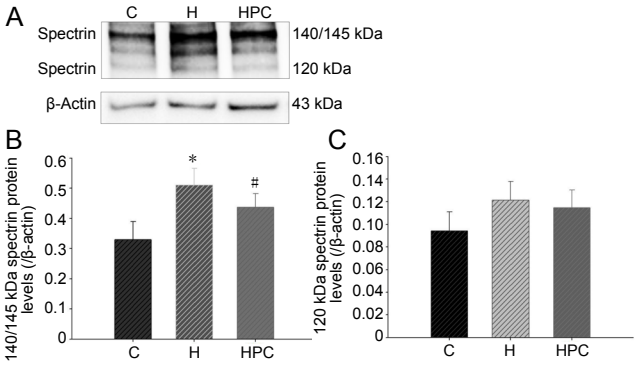

Figure 2 Effects of HPC on the degradation of spectrin in HT22 cells.
(A) The protein levels of 140/145 kDa spectrin and 120 kDa spectrin were measured by western blot assay. HPC decreased the levels of necrotic and excitotoxic neuronal death (associated with 140/145 kDa spectrin). (B) Semi-quantitative analysis of the changes in 140/145 kDa spectrin protein levels. (C) Semi-quantitative analysis of changes in the protein levels of 120 kDa spectrin. *$P < 0.05$, *vs.* C group; #$P < 0.05$, *vs.* H group. The grey densitometric values of western blot assays were normalized against the loading control (β-actin). Data are expressed as the mean ± SD ($n = 3$; one-way analysis of variance followed by Tukey's honest significant difference *post hoc* test). The experiment was conducted in triplicate. C: Control; H: hypoxia; HPC: hypoxic preconditioning.

gen-glucose deprivation (OGD)-reoxygenation cell culture model to examine the cellular mechanisms that mediate ischemia/reperfusion injury and cytoprotection (Ryou and Mallet, 2018). Thus, our HPC cell model may function as a simple and efficient tool for understanding the complex molecular mechanisms underlying hypoxic tolerance induced by acute repeated hypoxia in mice.

Reoxygenation following hypoxia/ischemia is known to exacerbate cytotoxic effects (Hess and Manson, 1984). We attempted different pilot experiments examining hypoxia/reoxygenation conditions and found that 13 hours of hypoxia followed by 6 hours of reoxygenation can injure HT22 cells, as assessed by analyses of morphology, apoptosis, and cell death-related molecules, such as cleaved caspase-3. Different hypoxia/reoxygenation cycles and cycle durations have been used to induce neuroprotection in whole-body animals or cell cultures (Zhang et al., 2004; Yao et al., 2011). In this study, the HPC HT22 cell group was treated with four cycles of 30 minutes in 1% O_2 and 30 minutes in 21% O_2, which mimicked our repeated hypoxia animal model and HPC was able to reduce the cell damage caused by hypoxia/reoxygenation. Magill et al. (2012) have developed a novel *in vitro* model of ischemic preconditioning using a human skeletal muscle cell line, and they found that ischemic preconditioned cells treated with 2 cycles of 10 minutes at 1% O_2 and 10 minutes at 21% O_2 were resistant to ischemia/reperfusion injury. Although different cell lines and precondition stress protocols were utilized, these results and the results from the present study demonstrated that cycles of short-term hypoxia/reoxygenation can stimulate protection in cells.

In the present study, the DNMT3A and DNMT3B levels in HT22 cells decreased after HPC. Dynamic changes in DNA methylation, catalyzed by DNMTs, are necessary to mediate brain plasticity and function (Einstein et al., 2010; Sweatt, 2016; Kader et al., 2018). Three types of DNMTs (DNMT1,

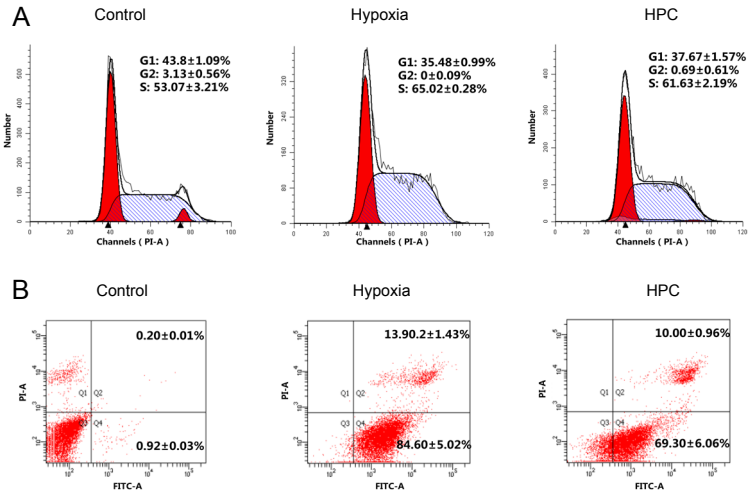

Figure 4 Effects of HPC on cell cycle progression.
HPC can relieve S-phase arrest and apoptosis caused by hypoxia. (A) Cell cycle, detected by flow cytometry. (B) Cell apoptosis, detected by flow cytometry. The cell cycle was analyzed with propidium iodide staining and flow cytometry. FITC-A: Fluorescein isothiocyanate-area; HPC: hypoxia preconditioning; PI-A: propidium iodide-area.

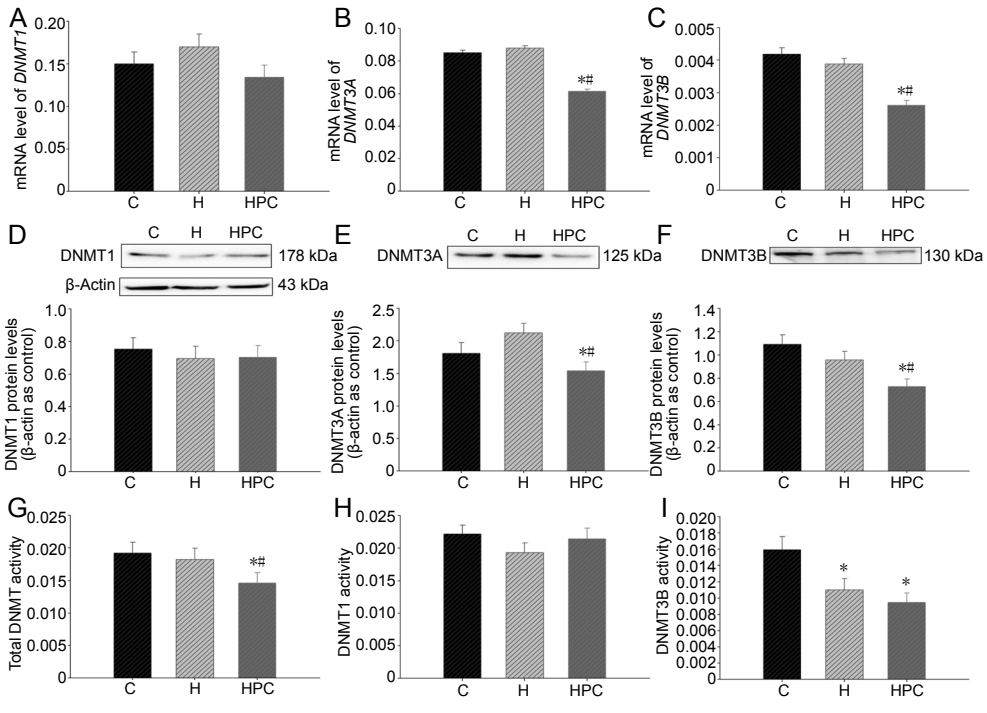

Figure 5 Influence of HPC on the expression levels and activities of DNMTs.
DNMT1, *DNMT3A*, and *DNMT3B* mRNA expression levels were measured by real-time polymerase chain reaction. β-Actin was used as the reference gene. DNMT1, DNMT3A, and DNMT3B protein expression levels were measured by western blot assay. The grey densitometric values of the western blot assay were normalized against the loading control (β-actin). The total DNMTs, DNMT1, and DNMT3B activities were measured by the DNMT activity assay. HPC decreased DNMT3A and DNMT3B mRNA and protein levels. The total DNMT and DNMT3B activities were decreased after HPC. (A) mRNA level of *DNMT1*. (B) mRNA level of *DNMT3A*. (C) mRNA level of *DNMT3B*. (D–F) Western blot assay and semi-quantitative analysis of changes in DNMT1 (D), DNMT3A (E), and DNMT3B (F) levels. (G) Total DNMT activity. (H) DNMT1 activity. (I) DNMT3B activity. $*P < 0.05$, *vs.* C group; $\#P < 0.05$, *vs.* H group. Data are expressed as the mean ± SD ($n = 3$; one-way analysis of variance followed by Tukey's honest significant difference *post hoc* test). The experiment was conducted in triplicate. C: Control; DNMT: DNA methyltransferase; H: hypoxia; HPC: hypoxic preconditioning.

DNMT3A, and DNMT3B) execute different functions during the regulation of DNA methylation, which regulates gene expression in mammalian cells. Decreased DNMT3A expression significantly reduced post-OGD cell death *in vitro* and decreased the post-ischemic infarct volume *in vivo* (Pandi et al., 2013). Reducing DNMT1 levels in post-mitotic neurons can protect against ischemic brain injury (Endres et al., 2001). Therefore, the decreased DNMTs in the brain may be associated with neuroprotection. Our previous report showed that HPC can reduce DNMT3A and DNMT3B levels in the hippocampus of an acute, repeated hypoxia mouse model (Zhang et al., 2014). The DNMT levels observed in the *in vitro* model in this study were similar to those observed in the whole-body animal model. Simultaneously, HPC markedly increased the number of S-phase cells in the current study. HT22 cells treated with 5-aza-cdR, a DNMT inhibitor, demonstrated fewer S-phase cells (Yang et al., 2017). We proposed that acute repeated hypoxia can protect neuronal cells from hypoxic injury through decreased DNMT3A and DNMT3B levels, in both *in vivo* and *in vitro* models.

However, we should also examine the effects of upregulated DNMT expression, to confirm the roles played by DNMTs in HPC-induced neuroprotection. Moreover, in addition to improving hypoxic tolerance and inhibiting cell apoptosis, DNMTs may be involved in other neuroprotective effects associated with HPC, such as learning and memory and neural regeneration effects, which require further study.

In summary, repeated hypoxia can protect nerve cells from damage, as demonstrated using the mouse hippocampal neuronal cell line HT22. This protection was associated with decreased DNMT3A and DNMT3B levels. The neuroprotection and changes in DNMT expression observed in HT22 cells were similar to those observed in the hippocampus of

an acute, repeated hypoxia mouse model. The use of this *in vitro* cell model can clarify the role played by DNA methylation regulated by DNMTs during neuroprotection.

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