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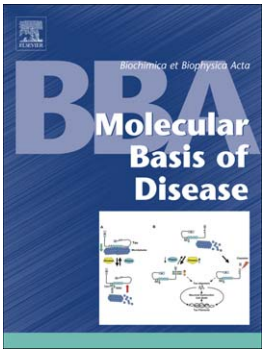


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**Original Article** **For Submission to Biochimica et Biophysica Acta (MBD)**



# Hyperthermic preconditioning protects astrocytes from ischemia/reperfusion injury by up-regulation of HIF-1 alpha expression and binding activity

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## ABSTRACT

It has been demonstrated that hypoxia-inducible factor-1 alpha (HIF-1 alpha) mediates ischemic tolerance induced by hypoxia/ischemia or pharmacological preconditioning. In addition, preconditioning stimuli can be cross-tolerant, safeguarding against other types of injury. We therefore hypothesized HIF-1 alpha might also be associated with ischemic tolerance induced by hyperthermic preconditioning. In the present study, we demonstrated for the first time that 6 hours of hyperthermia (38°C or 40°C) could induce a characteristic 'reactive' morphology and a significant increase in the expression of bystin in astrocytes. We also showed that pre-treatment with 6 hours of hyperthermia resulted in a significant increase in cell viability and a remarkable decrease in lactate dehydrogenase (LDH) release and apoptosis development in the astrocytes that were exposed to 24 hours of ischemia and a subsequent 24 hours of reperfusion. Analysis of mechanisms showed that hyperthermia could lead to a significant increase in HIF-1 alpha expression and also the HIF-1 binding activity in the ischemia/reperfusion astrocytes. The data provide evidence to our hypothesis that the up-regulation of HIF-1 alpha is associated with the protective effects of hyperthermic preconditioning on astrocytes against ischemia/reperfusion injury.


Keywords: Hyperthermiac preconditioning; ischemia/reperfusion; reactive astrocytes; hypoxia-inducible factor-1 alpha (HIF-1 alpha); apoptosis; cell viability; lactate dehydrogenase (LDH)

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## 1. Introduction

Preconditioning was first described in a dog model of myocardial injury in which sublethal ischemia enabled cells to better tolerate subsequent usually lethal ischemia [1]. It is now recognized that this phenomenon can be induced not only by ischemia but also a number of other stimuli including hypothermia and hyperthermia in the central nervous system [2]. During the past years, the phenomenon has received much attention because of its potential therapeutic importance [2,3].

A number of studies have demonstrated that preconditioning induced by hyperthermia (hyperthermic preconditioning) can produce a significant protecting effect in experimental animals. It has been reported that hyperthermic preconditioning protects against subsequent forebrain ischemic cell damage [4], spinal cord ischemic injury [5,6] and the lethal effects of endotoxin [7] in rats or mice. The studies also showed that hyperthermia prevents disruption of blood-brain barrier and ameliorates hypoxic-ischemic neuronal damage in newborn rat [8], and reduces the volume of middle cerebral artery occlusion (MCAO)-induced cerebral infarction [9] and the kainic acid-induced neuronal cell loss [10] in rats. In addition, it has been demonstrated that hyperthermia provides a continuous long-term and constant inhibitory effect on postischemic injury of the liver [11] and protects pig islet grafts from early inflammation in mice [12].

The accumulated data suggested that these beneficial effects might be associated with an increased expression of heat shock proteins (HSPs) [6,7,13,14] and heme oxygenase (HO-1) [11,15], the central adenosine receptors [9] and the upregulation of the polysialylation of neural cell adhesion molecule [10] in the rat brain. However, study on the

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mechanisms of the protective effects induced by hyperthermic preconditioning in the brain is still in its infancy. In addition to the above pathways, it is clear that cells have other mechanisms that have not yet been elucidated [16].

Preconditioning stimuli can be cross-tolerant, safeguarding against other types of injury [2]. Thus, there may be some overlapping mechanisms in preconditioning and unraveling these pathways may uncover an arsenal of neuroprotective therapeutic targets [2]. Accumulated evidence has demonstrated that hypoxia-inducible factor-1 alpha (HIF-1 alpha) mediates ischemic tolerance induced by hypoxia/ischemia preconditioning [17,18]. Our recent studies showed that pharmacological preconditioning (ginkgolides) can mimic the effects of hypoxic preconditioning to protect primary cultured neurons, astrocytes, PC12 and C6 cells against ischemic injury by up-regulation of HIF-1 alpha [17-20]. In addition, HSPs and HO-1 both are the target genes of HIF-1 alpha [21-23] and their expression is significantly increased in hyperthermia [7,13,14]. Based on these facts, we therefore hypothesized that the up-regulation of HIF-1 alpha might also be associated with the protective effects of hyperthermic preconditioning on brain cells. To test the hypothesis, we investigated effects of hyperthermia on the expression of HIF-1 alpha in the ischemia/reperfusion-astrocytes in the present study.

## 2. Materials and Methods

### *2.1. Materials*

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Company, St. Louis, MO, USA. Anti-HIF-1 alpha monoclonal antibody was purchased from Novus Biologicals, Littleton, CO, USA and the secondary antibodies (HRP-conjugated goat anti-

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rabbit IgG and goat anti-mouse IgG) were from Pierce, Rockford, IL, USA. Heat-inactivated fetal bovine serum and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco BRL, Gaithersburg, MD, USA and acrylamide and bisacrylamide were purchased from Bio-Rad Laboratories, Richmond, CA, USA. The solutions, buffers and media used in cell culture were desterilized by passing through a 0.22-μm Millipore and autoclaved at 121°C at 15 PSI for 20 min. ICR mice were supplied by the Animal House of Nantong University Medical Center. The Health Department of Nantong Government and Animal Ethics Committee of Nantong University approved the use of animals for this study. All the animals were housed in pairs in stainless steel cages at 21±2°C with relative humidity of 60–65% and alternating 12-h periods of light (7:00–19:00) and darkness (19:00–7:00).

### *2.2. Mouse astrocytes*

The mouse cerebrocortical astrocytes were prepared from newborn ICR mice by a procedure previously described [24,25]. Briefly, cerebral cortex was cut into small cubes (<1mm$^3$) and digested with 0.25% trypsin (Sigma, USA) for 30 min at 37°C. Trypsinization was terminated by an addition of DMEM containing 10% fetal bovine serum, followed by the mechanical trituration with a flame-polished Pasteur glass pipette. Cell suspensions were sieved through a 40-mm cell strainer and the filtrate was allowed preadherence for 30 min to remove any contamination from fibroblast before being seeded at a density of approximately one cerebrum per 50 cm$^2$ flask (Corning, USA) or six-well plates (NUNC, Denmark). The plated cells were incubated in a 5% $CO_2$ incubator (NAPCO 5400) at 37°C. After the cultures reached confluence (12–14 days), they were sub-cultured 3 times every 4 days and allowed pre-adherence for 30 min before being seeded in each subculture. The purity of the astrocyte cultures was assessed by staining for the astrocyte marker glial fibrillary acidic protein (GFAP) (Chemicon International Ltd., UK), which was approximately

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99%.

### *2.3. Experimental design*

Ischemic cortical astrocytes were prepared as previously described [20,26]. Briefly, astrocytes in Hank's solution without glucose and serum (oxygen–glucose deprivation, OGD) were exposed to 1% $O_2$ in a dedicated incubator (NAPCO 7101FC-1) at 37°C for 24 h. Reperfusion was conducted by the treatment of the cells in normal cultural medium (DMEM containing 10% fetal bovine serum) with nomoxia (21% $O_2$) in a humidified incubator at 37°C for 24 h.

To observe the effects of hyperthermia preconditioning on the viability of ischemic astrocytes, the cells were incubated at 37°C (normothermia), 38 °C or 40 °C (hyperthermia) for 6 h based on our preliminary experiments, and then exposed to ischemia and reperfusion respectively for 24 h. After the above treatments, cell viability was determined.

To understand the mechanism involved in the protective effect of hypothermia preconditioning on astrocytes from ischemia / reperfusion injury, we investigated HIF-1alpha expression in astrocytes pre-treated with hyperthermia. The cells were exposed to normothermia (37°C) or hyperthermia (38 or 40°C) for 6 h and then lysed and the expression of HIF-1alpha was measured using Western blot analysis. The binding activity of HIF-1alpha was also determined by a Electrophoretic Mobility Shift Assays (EMSA) method.

### *2.4. Morphological observation*

Effects of hypothermia preconditioning on morphological changes of astrocytes were observed under phase contrast microscopy as described previously [26].

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### *2.5. Cell viability*

The cell viabilities of astrocytes that received different treatments were measured using an MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolinum bromide) assay as described by He et al [13]. Briefly, a total of 25 μL MTT (1 g/L in PBS) was added to each well before the conduction of incubation at 37°C for 4 h. The assay was stopped by the addition of a 100 μL lysis buffer (20% SDS in 50% N'Ndimethylformamide, pH 4.7). Optical density (OD) was measured at the 570 nm wavelength by the use of an ELX-800 microplate assay reader (Bio-tek, USA) and the results were expressed as a percentage of absorbance measured in the control cells.

### *2.6. Lactate dehydrogenase*

The quantity of lactate dehydrogenase (LDH) release in the medium was determined by the decrease in absorbance at 340 nm for NADH disappearance within 3 min [27]. Briefly, the cells were treated as described in 'Experimental design', 500 μl of supernatant was then collected from each well and mixed with 1.3 ml of NADH (0.217 mmol/l) and 1.3 ml of sodium pyruvate (1.77 mmol/l) in the modiWed Krebs–Henseleit buffer (118 mmol/l NaCl, 4.8 mmol/l KCl, 1 mmol/l $KH_2PO_4$, 24 mmol/l $NaHCO_3$, 3 mmol/l $CaCl_2$, 0.8 mmol/l $MgPO_4$, pH 7.4) at 37°C for 30 s. The activity was spectrophotometrically measured by the use of an ELX-800 microplate assay reader (Elx800, Bio-tek, USA) at OD 340 nm.

### *2.7. Hoechst 33342 staining*

Hoechst 33342 staining was used to detect the morphological features of apoptotic cell death [20]. Astrocytes that received different treatments were stained with Hoechst 33342 (1 mg/ml) for 1 h. The cells were then examined under a fluorescent microscope (Leica, Wetzlar, Germany) equipped with a SPOT cool CCD camera (Canon, Japan) with a 350-

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nm excitation laser. Undamaged cell nuclei were large and diffusely stained whereas apoptotic nuclei showed chromatin that was condensed and fragmented.

### *2.8. Western blot analysis*

Western blot analysis was performed as described previously [17,18]. The cells that received different treatments were washed twice with ice-cold phosphate-buffered saline (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 at 4°C for 15 min. The supernatant was collected and the protein contentwas determined using the Bradford assay kit (Bio-Rad, Hercules, CA, USA). The proteins were separated by 10% SDS-polyacrylamide gels, transferred to the polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) previously blocked with 5% non-fat milk in TBS-T (20 mM 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-1 alpha (1:500 dilution, Novus Biologicals, Littleton, CO, US), bystin (1:10000 dilution, a gift from Jiawei Zhou, Shanghai Institutes for Biological Sciences, China) or primary monoclonal mouse anti-GFAP (1:20000 dilution, Chemicon International Ltd., UK) and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1000 dilution, Pierce Chemical Company, USA) for 2 h at room temperature. Immunolabeling was detected by using the Super Signal West Femto Maxium Sensitivity Substrate (Pierce Biotechnology, Inc., Rockford, IL, USA). The intensity of each band was quantified by using Scion Image software (Scion Corp., Frederick, MD, USA). To ensure even loading of the samples, the samemembrane was probed with the anti-mouse beta-actin monoclonal antibody (Sigma–Aldrich, MO) at 1:10,000 dilution.

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### *2.9. Electrophoretic Mobility Shift Assays*

Nuclear extracts were prepared using the NE-PER kit (#78833, Pierce Biotechnology, Rockford, IL,USA) according to the instructions of the manufacturer. Using the lightshift chemiluminescent EMSA kit (#20148, Pierce Biotechnology, Rockford, IL ,USA), the binding reaction mixtures were mixed in a volume of 15 μl with a final concentration of 5 μg nuclear proteins, 0.2 μg poly (dI·dc), 2 μl 10×binding buffer, the bio-labeled oligonucleotide probe (20 ng in 2μl) and incubated for 30 min at room temperature [18]. For specific competition EMSA, a 200-fold excess of the cold (unlabled) oligonucleotides (4 μg in 2μl) was added simultaneously during the incubative period. A 200-fold excess of the unlabeled probes (4 μg in 2 μl) harboring mutated HIF-binding site (HRE) was used as the nonspecific competition EMSA. The bio-labeled oligonucleotide probe harboring mutated HRE (20 ng in 2μl) was also used to test the specificity of the binding reaction. To separate from unbound DNA, the DNA-protein complexes were resolved on 6% native polyacrylamide gels [18].

### *2.10. Statistical analysis*

Statistical analyses were performed using SPSS 10.0. Data are presented as Mean ±S.E.M. 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 hyperthermia on the morphology and the expression of GFAP and*

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***bystin in astrocytes*.**

We first observed the hypothermia preconditioning-induced morphological changes of astrocytes using a phase contrast microscopy. Treatment of the cells with hyperthermia (Fig 1B: 38°C or Fig 1C: 40°C) for 6 h induced a characteristic 'reactive' morphology, ie. hypertrophic processes and stellate-shaped cell bodies. There were no significant changes in the morphology of astrocytes pre-exposed to 37°C (normothermia) for 6 h (Fig 1A).

Our recent study demonstrated that bystin is a much more sensitive marker in activated astrocytes than GFAP [20]. Therefore, we then examined effects of hyperthermia on the expression of bystin and also GFAP in astrocytes. Western blot analysis showed that treatment of the cells with 40°C (hyperthermia) for 6 h induced a significant increase in the expression of GFAP (Figs 1D and E; $p<0.05$, vs. the control), but no significant difference in the expression of GFAP was found between the cells incubated at 38°C (hypothermia) and 37°C (the control). However, in the case of the expression of bystin, the significant differences were found between the cells incubated not only at 40°C and 37°C (the control) but also 38°C and 37°C (the control) (Figs 1F and G, $p<0.05$, vs. the control).

***3.2. Hyperthermia preconditioning protected astrocytes from the injury induced by ischemia and reperfusion***

To find out whether there are any protective effects of hyperthermia preconditioning on astrocytes against ischemia/reperfusion injury, the cells were incubated at 37°C (normothermia), 38°C or 40°C (hyperthermia) for 6 h before exposed to ischemia and reperfusion respectively for 24 h, after which cell viability, LDH release and morphological changes were observed. It was found that ischemia and reperfusion (I/R) induced a significant decrease in the viability of astrocytes (Fig 2A, $p < 0.01$ vs. the control) and increase in LDH release (Fig 2B, $p < 0.01$ vs. the control). The pre-treatement of the cells

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with 38ºC or 40ºC (hyperthermia) for 6 h led to a significant increase in the viability (Fig 2A,) and decrease in LDH release (Fig 2B) in the astrocytes treated with I/R. The viability was significantly higher and the LDH release significantly lower in the cells of “38D+IR” and “40D+IR” groups (38ºC or 40ºC for 6h first and then I/R for 24h) than in the cells of I/R group (All $p < 0.01$ vs. I/R). In agreement with the above results, treatments of the astrocytes with ischemia and reperfusion for 24 h induced a significant change in cell morphology. Cell bodies began to shrink and processes decurtated or even disappeared. Relics of the dead cells could be found under a light microscope (Fig. 2C-II). The astrocytes treated with hyperthermia preconditioning (38ºC, Fig. 2C-III, or 40ºC, Fig. 2C-IV) for 6 h before exposed to ischemia (24 h) and reperfusion (24 h) acquired reactive morphology. The data imply that hyperthermia preconditioning has protective effect on astrocytes against ischemia/reperfusion-induced injury.

### *3.3. Hyperthermia preconditioning enhances anti-apoptotic abilities in I/R astrocytes.*

To understand the mechanisms involved in the protective effect of hyperthermia preconditioning on astrocytes from I/R injury, we first investigated whether hyperthermia preconditioning has any anti-apoptotic abilities in I/R astrocytes. Fig. 3 presents the results of Hoechst 33342 staining analysis. In Fig. 3A-I (the control), apoptotic nuclei were absent. However, in the I/R astrocytes (Fig. 3A II), nearly all nuclei were condensed and densely stained with a much brighter blue fluorescence as compared with the controls in Fig. 3A-I. The apoptosis induced by ischemia and reperfusion was effectively reversed by pre-treatment with hyperthermia (38ºC, Fig. 3A-III or 40ºC, Fig. 3A-IV) for 6 h. The nuclei of astrocytes in these two groups were relatively normal although apparent bigger nucleus could be found in the cells pre-treated with 40ºC for 6 h (Fig. 3A-IV). The pre-treatment of astrocytes with 38ºC or 40ºC (hyperthermia) for 6 h before I/R led to a significant decrease

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in apoptotic nuclei/total nuclei (%) (Fig 3B). The apoptotic nuclei/total nuclei (%) was significantly lower in the cells of “38D+IR” and “40D+IR” groups than in the cells of I/R group (All $p < 0.01$, vs. I/R). These findings imply that hyperthermia enhances the anti-apoptotic abilities in I/R astrocytes.

### *3.4. Effects of hyperthermic preconditioning on the expression of HIF-1 alpha and the binding activity of HIF-1 in nuclear proteins to the HRE of erythropoietin gene*

To better understand the mechanisms involved in the protective role of hyperthermia preconditioning, we also investigated effects of hyperthermic preconditioning on the HIF-1 alpha expression using Western blot analysis and binding activity in nuclear proteins to the HRE of erythropoietin (EPO) gene by an EMSA method. Western blot data showed that treatment with 38°C or 40°C (hyperthermia) for 6 h could lead to a significant increase in the expression of HIF-1 alpha in the astrocytes. The contents of HIF-1 alpha in the cells treated with 38°C or 40°C for 6 h were significantly higher than those in the cells treated with 37°C for 6 h (Figs 4A and B, All $p < 0.01$ vs. 37°C). There are no statistical differences in HIF-1 alpha expression between the astrocytes treated with 38°C and 40°C hyperthermia ($p = 0.05457$).

The test of EMSA aims to identify transcription factor complexes binding to the functional HRE within the EPO enhancer element. Nuclear extracts from astrocytes treated with hyperthermia (38 °C or 40 °C) for 6 h were incubated with bio-labeled probes harboring the HRE. There are significant bands shifted upward in the groups of hyperthermia (38°C or 40°C), the most significant being in the hyperthermia 40°C group (Fig 4C, line 4) the control group (37°C, line 2) and free group (no protein) (Fig 4C, the 1st line). These EMSA results demonstrated that the binding activity of HIF-1 in nuclear proteins to the HRE of EPO gene increases in astrocytes treated with hyperthermia.

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## 4. Discussion

Astrocytes are increasingly recognized for their impact on neuronal function and viability in health and disease [28]. By influencing the glutamate excitotoxicity, oxidative stress and acidosis which are primary mediators of neuronal death during ischemia and reperfusion [29-31], astrocytes could effectively protect neuron from ischemia and reperfusion-induced injury. Astrocytes have the ability to take up glutamate and reduce excitotoxic glutamate in the extracellular space of the brain [32], release ascorbate and take up its oxidized form (dehydroascorbate) to influence neuronal antioxidant status [29], and secret nitric oxide, TNF alpha, matrix metalloproteinase and other factors to delay neuronal death [29].

A number of studies have demonstrated that preconditioning induced by hyperthermia could produce a significant protecting effect in experimental rats and mice [4-6,8,10,33]. The protecting effects on astrocytes induced by hyperthermic preconditioning have also been reported by some recent studies in experiments in vitro [13,34]. In the present study, we demonstrated for the first time that 6 hours of hyperthermia (38°C or 40°C) could induce a characteristic 'reactive' morphology (hypertrophic processes and stellate-shaped cell bodies) and a significant increase in the expression of bystin which is a sensitive marker of the activation of astrocytes. Our findings also demonstrated that pre-treatment with 6 hours of hyperthermia resulted in a significant increase in the viability and a remarkable decrease in LDH release and apoptosis development in the astrocytes that had been exposed to 24 hours of ischemia and a subsequent 24 hours of reperfusion. These findings provided further evidence for the protecting effects of hyperthermic preconditioning on astrocytes against ischemia/reperfusion injury.

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Although it has been reported that HSP70 [13] and the activation of MAPK pathways including extracellular signal-regulated protein kinase (ERK), c-Jun NH(2)-terminal kinase (JNK), and p38 MAPK [34] might be involved in, the mechanism of the protective effects of hyperthermic preconditioning on astrocytes is still unclear. Oxygen availability is crucial for cellular viability. HIF-1 is the major oxygen homeostasis regulator that plays an essential role in cellular oxygen homeostasis, functioning as a widely operative transcriptional control system responding to physiological levels of cellular hypoxia [35-37]. The regulation of the HIF-1 activity depends mostly upon the alpha subunit and the up-regulation of HIF-1 alpha has been associated with the cell-protective roles of hypoxic preconditioning and pharmacological preconditioning [17-29]. Our findings showed that 6 hours of hyperthermia could lead to a significant increase in the expression of HIF-1 alpha and also the binding activity of HIF-1alpha in the ischemia/reperfusion astrocytes, providing direct evidence to our hypothesis.

Thomas et al [13] demonstrated that upon exposure to elevated temperature (39$^{o}$C), cultured astrocytes from rat diencephalon express high levels of HSP70, which plays important roles in maintaining cellular integrity and viability, and are less vulnerable to a subsequent oxidative stress. It has also been reported that hyperthermic preconditioning induces strong and long-lasting HO-1/HSP32, HSP72 and HSP90 expression in rat kidneys [15] and HO-1/HSP32 and HSP70 expression in the liver [11]. Both HSPs and HO-1 are the target genes of HIF-1 alpha [21-23]. Therefore, it is highly likely that the up-regulation of HSPs and HO-1 reported by these laboratories might be due to the increased expression and binding activity of HIF-1 alpha. It is also possible that the increased expression of HSPs and HO-1, induced by up-regulation of HIF-1 alpha, might also be involved in the protective effects on astrocytes from ischemia/reperfusion injury found in

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the present study although we did not directly measure the expression of HSPs and HO-1.

It has been reported that bystin, a protein potentially involved in embryo implantation, is markedly up-regulated in the reactive astrocytes of both 6-hydrodopamine-lesioned nigrostriatum and stablesioned cerebral cortex of adult rats in vivo, and in postnatal cortical astrocytes treated with pro-inflammatorymediators lipopolysaccharide and interleukin-1b in vitro [38]. In a recent study, we found that the response of this protein to ischemia and hypoxia was much more sensitive than GFAP. This suggested that bystin is a much more sensitive marker than GFAP in the activation of astrocytes induced by ischemia and hypoxia [26]. Bystin might be a key factor in the formation of reactive astrocytes induced by not only hypoxia and ischemia [26] but also hyperthermic preconditioning. Reactive astrocytes secrete erythropoietin, a paracrine messenger that could stimulate the Janus kinase-2 (JAK-2) and nuclear factor-kappaB (NF-κB) signaling pathways to prevent programmed cell death or apoptosis after ischemic injury or excitotoxic stress [28,28]. This mechanism might also be involved in the increased anti-apoptotic abilities of astrocytes induced by hyperthermia preconditioning in the present study against I/R injury.

In summary, the findings in the present study provided further evidence that hyperthermic preconditioning (6 hours of hyperthermia) plays a significant role in protecting astrocytes against ischemia/reperfusion injury. Analysis of the mechanisms showed that the protecting effects of hyperthermic preconditioning might be associated with the up-regulation of HIF-1 alpha and the increased anti-apoptotic abilities of astrocytes.

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## LEGENDS FOR FIGURES

**Fig 1. Effects of hyperthermia on the morphology and the expression of GFAP and bystin of astrocytes.** Primary astrocytes were exposed to either normothermia (37°C) or hyperthermia (38°C or 40°C) for 6 h. The morphological changes were then observed under a light microscope and the expression of GFAP and bystin was determined by Western blot analysis as described in 'Materials and Methods'. A. the control (normothermia, 37°C); B. hyperthermia (38°C) for 6 h; C. hyperthermia (40°C) for 6 h (scale bar = 10 μm); D and F. a representative experiment of Western blot analysis of GFAP (D) and bystin (F) respectively. E and G. Quantifications of expression of GFAP (E) and bystin (G) in astrocytes. The values were the Mean ± SEM (n=4). Temp (D): temperature (°C). *$p<0.05$ vs. normothermia group.

**Fig 2. Effects of hyperthermic preconditioning on cell-viability, LDH release and the morphology of astrocytes.** The astrocytes were pre-incubated at 37°C (normothermia), 38°C or 40°C (hyperthermia) for 6 h before exposed to 24 h of ischemia and a subsequent 24 h of reperfusion. The relevant observation and measurements were then conducted as described in 'Materials and Methods'. A. cell-viability; B. LDH release; and C.

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morphological changes: I. the control [pre-treated with 37°C for 6 h,]; II. I/R [24 h of ischemia and 24 h of reperfusion]; III. 38D+I/R [pre-treated with 38°C for 6 h]; and IV. 40D +I/R [pre-treated with 40°C for 6 h] (scale bar=10 μm). The values were the Mean ± SEM. **P<0.01, vs. the control; ## P<0.01, vs. I/R group.

**Fig 3. Effects of hyperthermic preconditioning on apoptosis induced by ischemia and reperfusion in astrocytes.** The astrocytes were pre-incubated at 37°C (normothermia), 38°C or 40°C (hyperthermia) for 6 h before exposed to 24 h of ischemia and a subsequent 24 h of reperfusion. Hoechst 33342 staining was then conducted as described in 'Materials and Methods'. A. Fluorescent photographs of Hoechst 33342 staining analysis: I. the control [pre-treated with 37°C for 6 h,]; II. I/R [24 h of ischemia and 24 h of reperfusion]; III. 38D+I/R [pre-treated with 38°C for 6 h]; and IV. 40D+I/R [pre-treated with 40°C for 6 h] (scale bar=50 μm), B. apoptotic nucleus/ total nucleus (%). The values were the Mean ± SEM (Six view sights of each group were counted and each group was repeated for three times). **P<0.01, vs. the control; ## P<0.01, vs. I/R group; @ P< 0.05, vs. 38D+I/R group.

**Fig 4. Effects of hyperthermic preconditioning on expression of HIF-1alpha and binding activity of HIF-1 in nuclear proteins to the HRE of EPO gene in astrocytes.** The astrocytes were incubated at 37°C (normothermia), 38°C or 40°C (hyperthermia) for 6 h and then Western blot analysis and EMSA test were conducted as described in 'Materials and Methods'. A. a representative experiment of Western blot analysis of HIF-1 alpha (a single band with a molecular weight of ~120 kDa); B. Quantifications of HIF-1 alpha expression; C. binding activity. The values were the Mean ± SEM (n=3, three independent experiments). Temp (D): temperature (°C). **P<0.01 vs. 37 (37°C, normothermia).

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Fig 1.

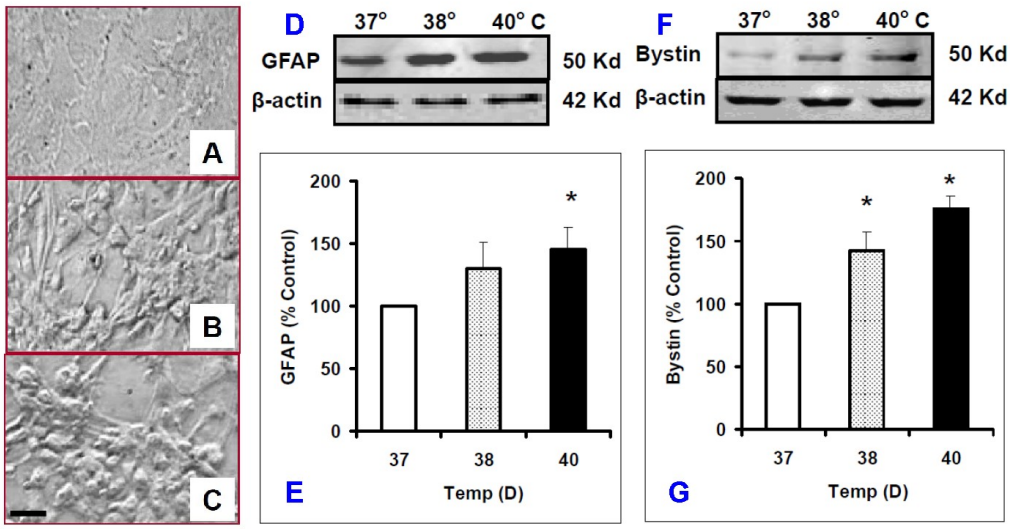


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Fig 2.

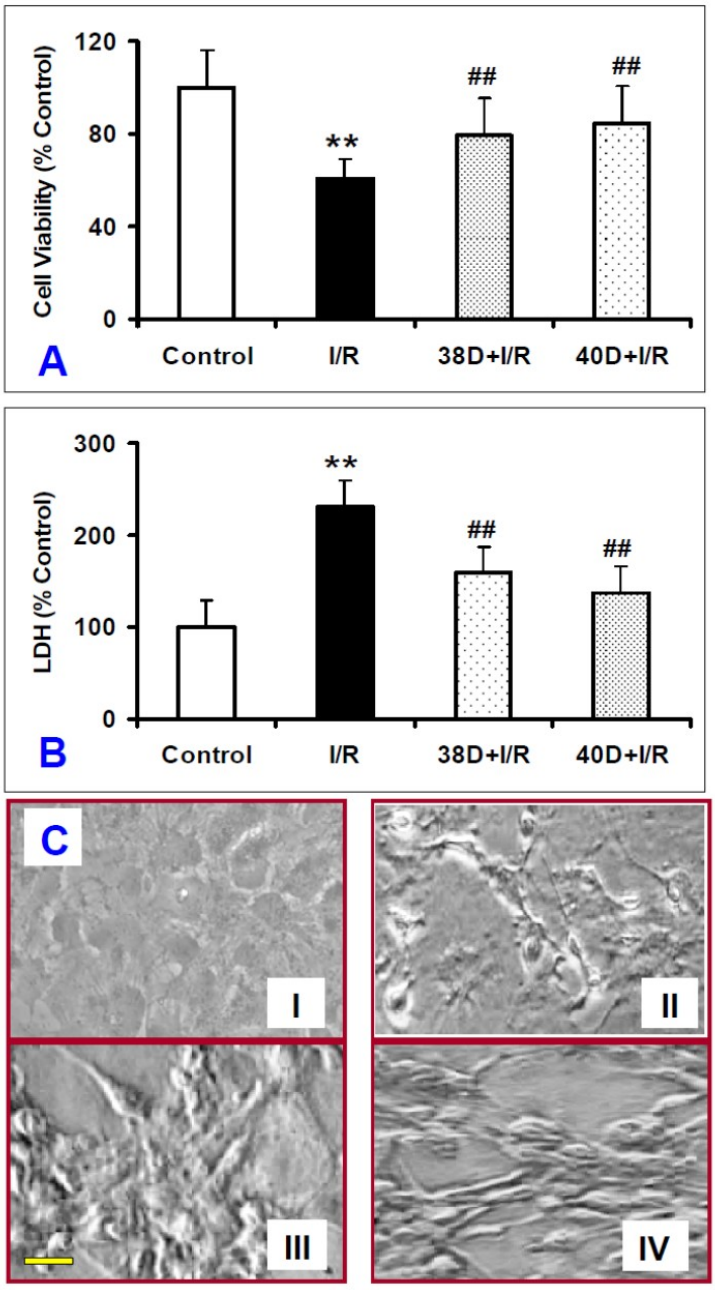

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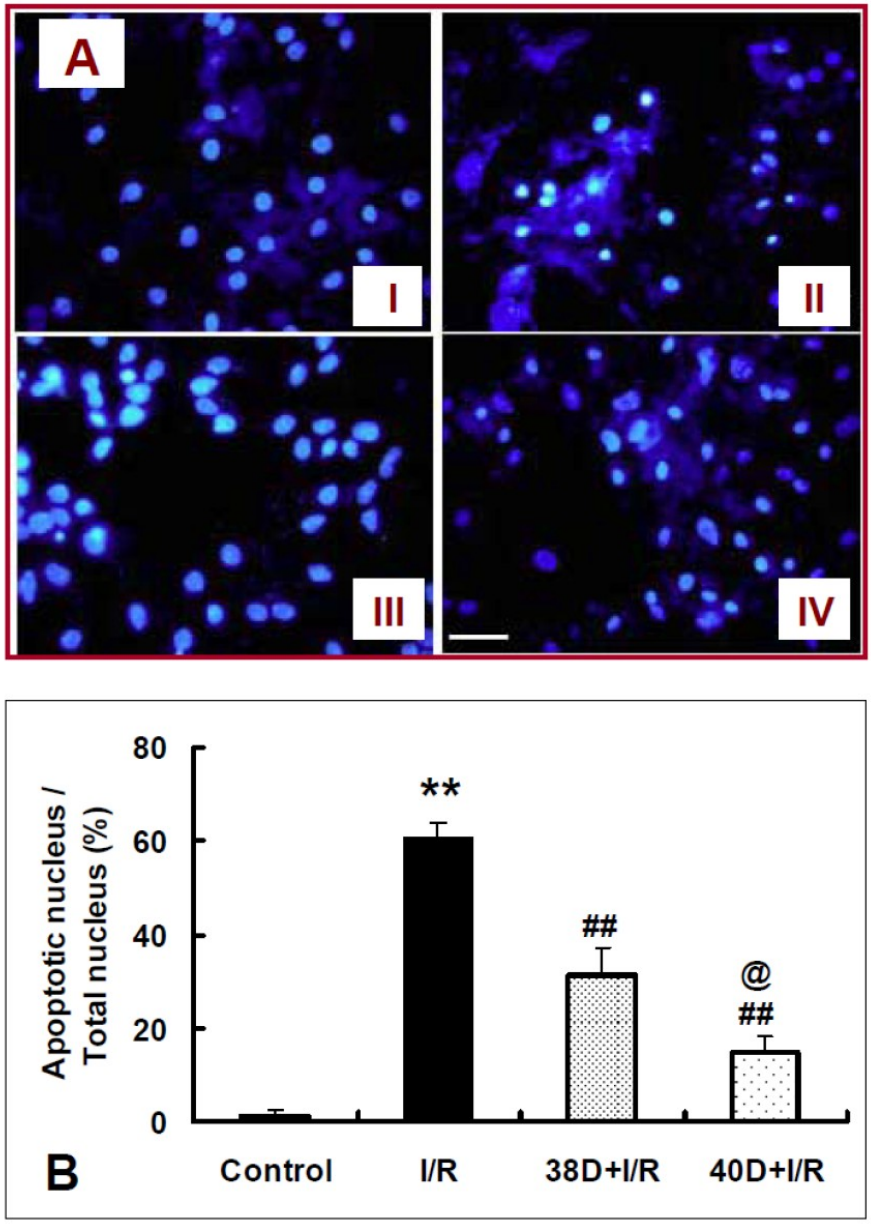
Fig 3.



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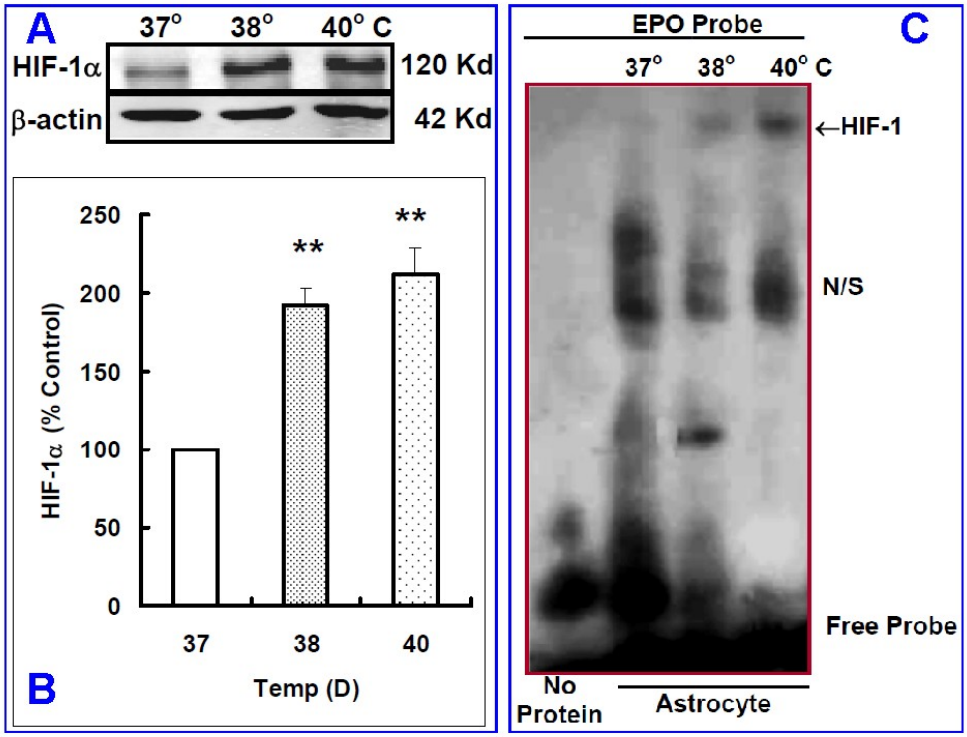
Fig 4.