Neurosci Bull June 1, 2013, 29(3): 287–294. http://www.neurosci.cn
DOI: 10.1007/s12264-013-1298-y

·Original Article·

Progesterone treatment before experimental hypoxia-ischemia enhances the expression of glucose transporter proteins GLUT1 and GLUT3 in neonatal rats

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ABSTRACT

Progesterone is an efficient candidate for treating stroke and traumatic brain damage. The current study was designed to investigate the effects of progesterone on glucose transporter proteins (GLUT1 and GLUT3) during hypoxic-ischemic injury in a neonatal rat model. We demonstrated strong staining for GLUT1 in the walls of blood vessels and GLUT3 immunoreactivity in hippocampal neurons after hypoxia-ischemia. Hypoxia-ischemia elevated GLUT1 and GLUT3 at both the mRNA and protein levels in the hippocampus, and pre-treatment with progesterone (8 mg/kg) further enhanced their accumulation until 24 h after hypoxic-ischemic injury. These results showed that progesterone treatment induced the accumulation of both GLUT1 and GLUT3 transporters, and an energy-compensation mechanism may be involved in the neuroprotective effect of progesterone during hypoxic-ischemic injury after cerebral ischemic attacks.

Keywords: hypoxic-ischemic injury; progesterone; GLUT1; GLUT3; stroke

INTRODUCTION

Focal cerebral ischemia, currently one of the leading causes of death and injury worldwide, has attracted considerable attention. Cerebral ischemia causes blood flow reduction and leads to impaired oxygen and glucose delivery[1]. Lethal brain damage mainly results from neuron death, and the shortage of metabolic energy supply is one cause for the death of neurons. Furthermore, oxygen and glucose depletion impairs ion transport, induces the change of membrane potential and finally leads to the depolarization of neurons[2-4]. Therefore, as a primary energy substrate for mammalian brain metabolism, glucose is essential for the maintenance of neuronal function, especially in focal cerebral ischemia. The delivery of energy substrates such as glucose from blood to brain always requires facilitation by the glucose transporter (GLUT) proteins. Six isoforms of GLUT proteins are expressed in the mammalian brain, among which GLUT1 and GLUT3 are predominant. GLUT1 transports glucose across the endothelial cells of the blood-brain barrier (BBB), and then GLUT3 helps glucose to pass through the neuronal cell membrane. Limitations in the functions of GLUTs lead to abnormal brain function and neuron death[5].

In order to model human stroke, middle cerebral artery thread-occlusion in the rat is used in stroke pathophysiology and therapeutic research. Many reports have shown that alterations in the expression of GLUTs occur in cerebral ischemia[5-7]. And the evolution of brain damage from hypoxic ischemia in the neonatal rat involves major changes in GLUT1 and GLUT3 mRNA expression[8]. Induced expression of GLUT1 is detectable at 12 h to 7 days of recovery in the rat cerebral cortex after transient global ischemia[8].

Moreover, reduction in GLUT1 mRNA and protein occur in glial cells and astroglia in animal models[6, 9]. Adult rats with middle cerebral arterial ischemia also show a progressive increase in brain GLUT3 concentration[10]. The reduction or increment in GLUT expression may serve as a molecular indicator of the stress of temporary reduction of blood flow.

A broad range of evidence shows that progesterone is neuroprotective under pathological conditions by modulating the BBB, interacting with the inflammatory cascade, decreasing the development of cerebral edema, limiting apoptosis, and protecting neurons distal to the injury that would normally die[11-13]. Clinically, progesterone treatment has neuroprotective benefits in several phase I and phase II clinical trials on traumatic brain injury and brain-injured children[12, 14]. A recent hypothesis suggests that the involvement of progesterone in protecting the fetus during development may recapitulate its effects in the treatment of traumatic and degenerative disorders of the brain[12].

The role of progesterone as a pleiotropic neurosteroid for brain injury has been well addressed. However, its regulatory effect on ischemic energy metabolism, especially on GLUT proteins, is still unrevealed. The current study aimed to investigate whether progesterone regulates the expression of GLUT proteins in the hippocampus after cerebral hypoxia-ischemia in infant rats.

MATERIALS AND METHODS

Animals

Timed pregnant Sprague-Dawley rats were housed individually in cages. Pups were housed with their dam after birth under a 12:12-h light-dark cycle in accordance with the guidelines and regulations of the Animal Care and Use Committee. Pups were randomized to the following groups: control, hypoxic-ischemic, sham-operated, and progesterone-pretreated hypoxic-ischemic (n = 12/group). Progesterone was dissolved in sesame oil and given as a single dose of 8 mg/kg by i.p. injection 30 min before the ischemia surgery. In the sham-operated group the same volume of sesame oil without progesterone was injected.

Induction of Hypoxia-Ischemia

Male and female 7-day-old rats were anesthetized by inhalation of 0.1% isoflurane in oxygen. The pups were kept at 37°C as the right common carotid artery was exposed and then ligated with surgical silk through a near-midline incision. After the wound was closed, all pups were allowed to recover in an incubator perfused with 8% oxygen-balanced nitrogen at 37°C for 2 h. In sham-operated rats, the right common carotid artery was exposed without ligation and they were not subjected to hypoxia. The operated pups were returned to the dams after hypoxic exposure. The animal study protocol was approved by the Institutional Animal Care and Use Committee.

Histological Examination

Pyramidal cell loss was assessed at 24 h after hypoxic-ischemic injury by hematoxylin and eosin staining. Pups were anesthetized with pentobarbital followed by transcardiac perfusion with 4% paraformaldehyde in PBS buffer. Brains were immersed in fixative and processed for paraffin embedding. Coronal sections (4 μm thick) were cut at the level of the hippocampus. Every sixth section was collected and stained with hematoxylin and eosin. Cells in the pyramidal layer of CA1 were counted under a light microscope at 400× magnification.

Immunohistochemistry Assay

Pups were killed under deep anesthesia and the brain was fixed in freshly-prepared paraformaldehyde (4%) for 40 min at 4°C. For immunohistochemical analysis, the tissue was cut into 16-μm sections on a freezing microtome. After heat-induced epitope retrieval and washing with TBS/Tween 20, sections were blocked with normal goat serum and then incubated with GLUT1 and GLUT3 rabbit polyclonal antibodies (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Negative controls were sections without the primary antibody. After washing, sections were incubated with biotinylated secondary antibody for 1 h in a humidified chamber. Antibody reactions were detected with the streptavidin-biotin-peroxidase reaction following standard procedures (Zhongshan Golden Bridge Biotechnology Co., Ltd, Beijing, China). Positive cells per cm^2 were counted in 30 views in each group, based on stereology measurements.

Western Blot Analysis

Pups were killed under anesthesia at 24 h after hypoxic-ischemic injury. The hippocampus was frozen in liquid nitrogen and then kept at −80°C. The tissue was homogenized in ice-cold lysis buffer containing 0.2 mol/L TBS, 1 mmol/L

PMSF, 5 mmol/L EDTA, and 1× protease inhibitor cocktail (Sigma, St. Louis, MO). The homogenate was then centrifuged at 1 000 g for 5 min to remove cellular debris. The supernatant was further centrifuged at 16 000 g for 15 min at 4°C. The supernatant was collected and aliquots were kept at −80°C until use. Protein concentration was measured by the Bradford assay using bovine serum albumin (BSA) as the control standard. Each 40 μg of protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. After washing with 0.2 mol/L TBS/0.2% Tween-20, membranes were incubated with blocking solution (TBS with 3% BSA and 2% Tween-20, 37°C, 1 h). Membranes were then washed twice with TBS and incubated with rabbit anti-GLUT1 (1:500 dilution, sc-7903, Santa Cruz Biotechnology), rabbit anti-GLUT3 (1:500 dilution, sc-30107, Santa Cruz Biotechnology) or rabbit anti-actin polyclonal antibody (1:1 000 dilution, sc-130657, Santa Cruz Biotechnology). Blots were washed, incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit IgG (1:1 000 dilution, Zhongshan Golden Bridge), followed by a second extensive wash. Reaction of alkaline phosphatase was developed by a solution containing 5-bromo-4-chloro-3-indolyl phosphate di-sodium salt/nitro blue tetrazolium chloride. The optical density was measured using a Gel Doc system and Multi-Analyst software (Bio-Rad, Philadelphia, PA). Actin was used as a loading control. Data are presented as relative protein content (measured as the optical density) with respect to that in the control group.

RNA Extraction and RT-PCR

We used ~100 mg of the right hippocampus for RNA extraction. Total RNA was extracted using an EZ-total RNA isolation kit [Sangon Biotech (Shanghai), Co., Ltd, Shanghai, China], according to the manufacturer's instructions. Commercial reagents (Single Step RT-PCR Kit from Sangon Biotech) and conditions were applied according to the manufacturer's protocol. RT-PCR reactions were performed in an ABI PCR thermocycler (Applied Biosystems). The primers for GLUT1, GLUT3 and actin were designed based on the NCBI database: actin forward: 5'- ATGGATGACGATATCGCTGCG-3', reverse: 5'-TCGTCCCAGTTGGTGACAATG-3'; GLUT1 forward: 5'-CAATCAAACATGGAACCACCG-3', reverse: 5'-CGATTGATGAGCAGGAAGCG-3'; GLUT3 forward: 5'-GAGTCATCAATGCGCCTGAG-3', reverse: 5'-AGCTCCTCAGAGCCCAGAAT-3'. All reactions were performed in duplicate.

Statistical Analysis

Data are expressed as mean ± SEM with significance determined by the *t*-test in SPSS software (SPSS, Chicago, IL).

RESULTS

Histological Staining of Hippocampal Sections

To evaluate the hypoxic-ischemic injury in neonatal rats, hippocampal sections were stained with hematoxylin and eosin. CA1 pyramidal neurons in the control rats showed a round cell body with a clearly stained nucleus (Fig. 1A). However, 2-h hypoxic-ischemic injury caused extensive death of hippocampal CA1 pyramidal neurons at 24 h (Fig. 1B). Stereology showed that ~42% of pyramidal neurons in the CA1 layer were lost after hypoxic-ischemic injury (Fig. 1C), suggesting that the procedure successfully induced

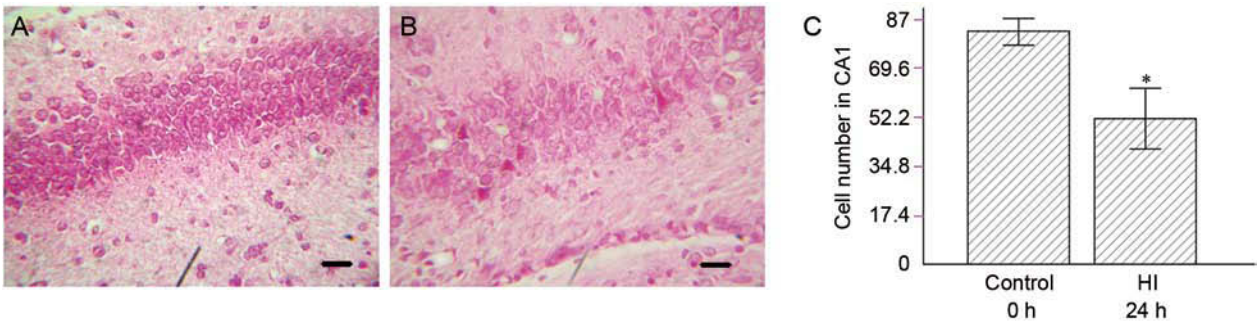

Fig. 1. Histological staining of hippocampal sections. CA1 pyramidal neurons in control (A) and hypoxic-ischemic (HI) brain (B) stained with hematoxylin and eosin. Scale bars: 20 μm. C: Stereology measurements of CA1 pyramidal neurons. ****P* **<0.05 *vs* control.

brain damage in neonatal rats.

Immunostaining of GLUTs in Hypoxic-Ischemic Rat Brain

GLUT1 and GLUT3 immunostaining was performed throughout the hippocampus from pups 24 h after hypoxic-ischemic injury. Strong staining for GLUT1 was detected in the walls of blood vessels (Fig. 2), and GLUT3 immunoreactivity was found in hippocampal neurons (Fig. 3). Control pups showed very weak immunolabeling of GLUT1 and GLUT3 (Figs. 2A, 3A). When the pups were subjected to the stress of hypoxic-ischemic injury, extensive positive immunostaining for both GLUT1 and GLUT3 was detected in the hippocampus (Figs. 2C, 3C). GLUT1 increased ~2.2-fold and GLUT3 ~2-fold (Figs. 2E, 3E). There was also a marked enhancement of GLUT1 and GLUT3 immunoreactivity in the hippocampal sections from pups treated with proges-

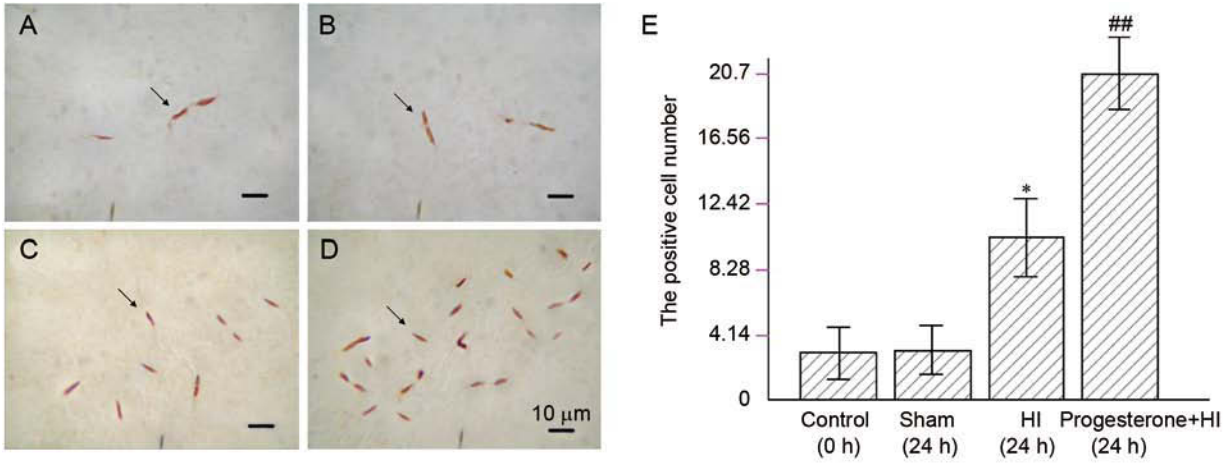

Fig. 2. Expression of GLUT1 protein in blood vessels. A: Control pups. B: Sham-operated pups. C: Hypoxic-ischemic pups. D: Progesterone-treated hypoxic-ischemic pups. Arrows indicate blood vessel walls with GLUT1 immunoreactivity Scale bars: 10 μm. E: Stereological measurement of cells labeled with GLUT1 antibody. *P <0.05 *vs* control. $^{\#\#}P$ <0.05 compared with control and hypoxia-ischemia. HI, hypoxic-ischemia.

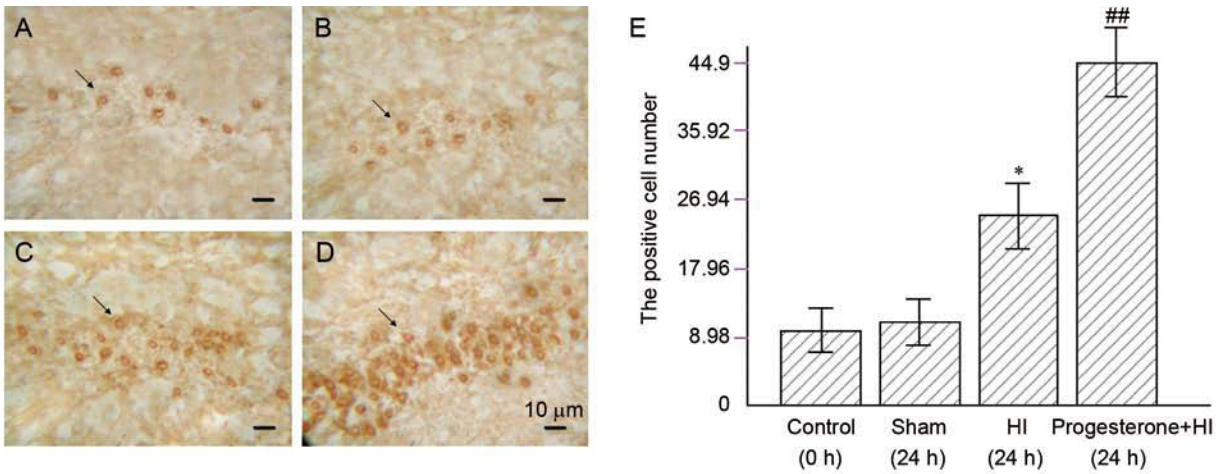

Fig. 3. Expression of GLUT3 protein in hippocampal CA region. A: Control pups. B: Sham-operated pups. C: Hypoxic-ischemic pups. D: Progesterone-pertreated hypoxic-ischemic pups. Arrows indicate cells with GLUT3 immunoreactivity. Scale bars: 10 μm. E: Stereological measurement of cells labeled with GLUT3 antibody. *P <0.05 vs control. $^{\#\#}P$ <0.05 compared with control and hypoxia-ischemia. HI, hypoxic-ischemia.

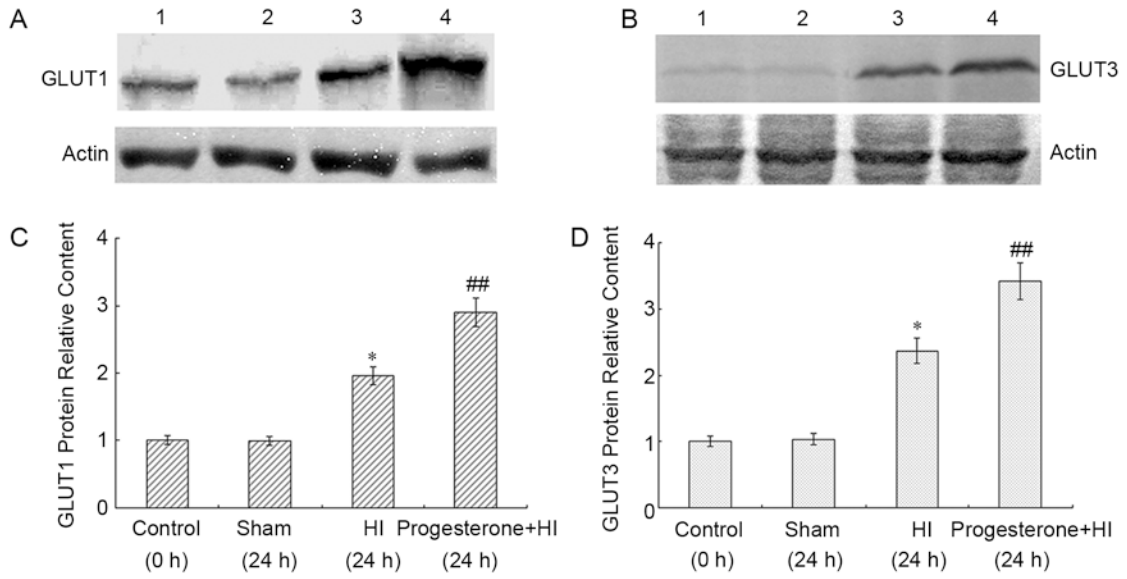

Fig. 4. Detection of GLUT1 and GLUT3 proteins in hippocampus by Western blot assay. A and B: Western blots of GLUT1 (A) and GLUT3 (B) in the hippocampus. Lane 1, control pups; lane 2, sham-operated pups; lane 3, hypoxic-ischemic pups; lane 4, progesterone-pretreated hypoxic-ischemic pups. β-actin served as the internal loading control. C and D: Relative protein levels. Mean ± SEM. **P* <0.05 *vs* control. ##*P* <0.05 compared with control and hypoxia-ischemia. HI, hypoxic-ischemia.

terone (Figs. 2D, 3D). The ratios of increase were ~5-fold in both GLUT1- and GLUT3-positive cells (Figs. 2E, 3E).

Progesterone Upregulates both GLUT1 and GLUT3 Protein Expression in Hippocampus During Hypoxic-Ischemic Injury

GLUT proteins were detected by Western blot and both GLUT1 and GLUT3 had low expression levels in the control brain. Hypoxia-ischemia increased GLUT1 and GLUT3 protein levels in the hippocampus, and pre-treatment with 8 mg/kg progesterone further enhanced the accumulation of these glucose transporters in hippocampus until 24 h after injury (Fig. 4A, B). The protein levels of GLUT1 increased ~2-fold and GLUT3 ~2.5-fold in pups subjected to hypoxic-ischemic injury. And the expression of GLUT1 increased 2.9-fold and GLUT3 3.5-fold in the hippocampus in pups with a single dose of progesterone.

Progesterone Enhances GLUT1 and GLUT3 mRNA Expression in Hippocampus after Hypoxic-Ischemic Injury

To investigate the effect of progesterone on the transcription of the glucose transporters, electrophoresis was used to check the quality of the purified total RNA and RT-PCR products (Fig. 5). RT-PCR results demonstrated that ischemia-hypoxia induced the expression of GLUT1 and GLUT 3 mRNA within 24 h after injury. Progesterone treatment also enhanced the accumulation of these transcripts (Fig. 5A, B). The relative contents of GLUT1 and GLUT3 transcripts are shown in Fig. 5D, E. Compared with control pups, a ~1.3-fold increase in both GLUT1 and GLUT3 mRNA content was detected in hypoxic-ischemic pups. Moreover, the mRNA content of GLUT1 increased 1.5-fold and GLUT3 1.7-fold in progesterone-pretreated pups.

DISCUSSION

We report here that hypoxia-ischemia induced the accumulation of GLUT1 and GLUT3 mRNA and protein in neonatal rat hippocampus at 24 h after hypoxic-ischemic injury. Progesterone pretreatment increased both the mRNA and protein levels of GLUT1 and GLUT3, suggesting that progesterone improves glucose transport in the hypoxia-ischemia-injured brain. The glucose transporters GLUT1 and GLUT3 may be downstream targets in the progesterone neuroprotective pathway.

Stroke is an important clinical problem worldwide, but

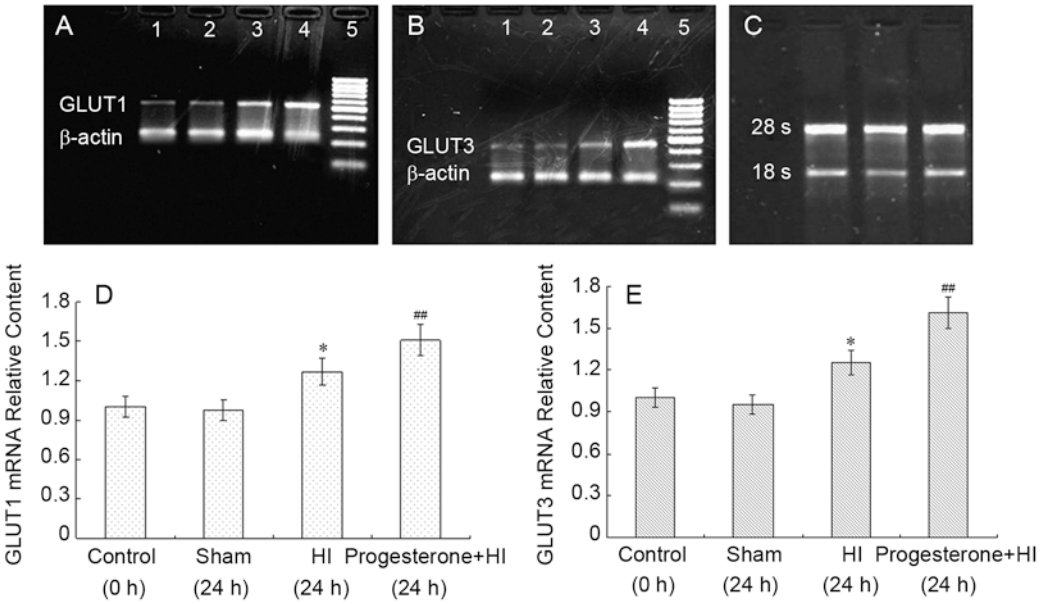

Fig. 5. RT-PCR measurement of GLUT1 and GLUT3 mRNA expression. A and B: Electrophoresis was used to check the PCR products of GLUT1, GLUT3 and β-actin. Lane 1, control pups; lane 2, sham-operated pups; lane 3, hypoxic-ischemic pups; lane 4, progesterone-treated hypoxic-ischemic pups; lane 5, 100-bp DNA ladder. C: Purified total RNA. D and E: Relative GLUT1 and GLUT3 mRNA content. Mean ± SEM. ****P* **<0.05 *vs* control. ##*P* <0.05 compared with control and hypoxia-ischemia. HI: hypoxia-ischemia.**

there is no clinically effective treatment to reduce mortality and improve functional outcome[12]. Fortunately, considerable experimental evidence has shown that progesterone is an efficient candidate to treat the systemic diseases such as stroke and traumatic brain damage by decreasing the development of cerebral edema and reducing the inflammatory cascade while providing trophic support to damaged neurons[11-13, 15]. Progesterone has a very broad and substantial treatment window for the treatment of CNS injury and beneficial effects on the outcome of stroke. Furthermore, hormone replacement therapy in human phase I/II clinical trials highlights the critical role of progesterone in the development and repair of the CNS after injury[12, 14]. In this work, we evaluated the regulation of glucose transporters by progesterone in neonatal rats subjected to hypoxic-ischemic injury to further elucidate its neuroprotective benefits on the energy-transfer pathway.

Glucose is the primary energy source for the brain and is essential for the maintenance of neuronal functions. During stroke, the membrane potential drops to ~0 mV. Because of the rapid depletion of cellular glucose and oxygen, the changes in neuronal excitability induce the release of ions such as potassium and calcium, and also cause mitochondrial dysfunction as well as the over-accumulation of neurotransmitters. These finally result in neuronal damage and abnormal brain function[2-4]. Thus, the regulation of brain glucose transport plays a key role during the initiation phase of stroke and other pathophysiological conditions. Glucose is known to be transported across the BBB by GLUT1 and distributed to neurons by GLUT3, coinciding with our evidence that GLUT1 is predominantly expressed in endothelial cells and GLUT3 is primarily located on neurons.

We also found that hypoxic-ischemic stress induced rapid increases in GLUT1 and GLUT3 mRNA and protein expression in neonatal rats at 24 h after injury, consistent with a previous report that the expression of GLUT1 is induced from 12 h to 7 days of recovery after transient global ischemia[8]. Furthermore, studies of middle cerebral arterial ischemia in adult rats also show an ~11-fold increase in GLUT1 mRNA transcript expressed in vascular endothelial cells at 12 h of reperfusion, and an 8-fold increase in brain GLUT3 mRNA expression at 48 h of reperfusion[10]. Conversely, a loss of GLUT3 expression has been reported at four days, but not at 24 h, after ischemia[8]. The significant decrease in GLUT3 protein *in vivo* may originate from the

apoptosis of neurons following ischemia. Experiments using cultured cells further indicate that both GLUT1 and GLUT3 protein expression increase when astrocytes are exposed to ischemic stress[6]. Moreover, overexpression of GLUT1 protects striatal neurons against stroke[16]. The above evidence suggests that inducible GLUT1 and GLUT3 may partially contribute to the enhanced storage of intracellular glycogen during reperfusion after ischemia. More importantly, the induction of GLUT expression is critical to the energy supply of the brain and GLUTs may function as antagonists against the stress of the temporary reduction of blood flow during ischemic injury, suggesting that they are potential therapeutic targets for the clinical treatment of ischemic brain injury.

Various factors such as ketogenic diets, glycogen, ethanol, oxygen tension, mitochondrial function, neurotransmitters and peptide hormones are potential candidates involved in the regulation of GLUT transporters at both the cellular and organ levels[17-21]. Studies show that estrogen rescues brain capillary endothelial cells and increases GLUT transporter levels in the BBB in penumbral ischemic areas, causing a decrease in the size of the focal ischemic lesion[17] . Research has also shown that estrogen augments glucose transporters in the cerebral cortex and modulates the expression of GLUT1 in the BBB[22,23]. Furthermore, estrogen and progesterone improve the sensitivity to insulin[24,25], and insulin treatment of both cell cultures and animals dramatically changes neuronal glucose uptake by promoting the translocation of GLUT1 and GLUT3[26-28]. Our findings emphasized that progesterone treatment increased GLUT1 and GLUT3 transporter expression in the hypoxic-ischemic neonatal brain and GLUTs may also be downstream targets in the hormone therapy pathway. The regulation of GLUT1 and GLUT3 by progesterone and estrogen may be partially attributed to the effect of progesterone on insulin sensitivity. Increases in GLUT1 and GLUT3 gene expression in blood vessels and the hippocampus may represent a compensatory mechanism for the increase of glucose utilization during reperfusion. Such an effect could contribute to the neuroprotection by progesterone during cerebral ischemic injury. Further research is directed to investigating the effect of progesterone on glucose uptake in cultured neurons subjected to oxygen–glucose deprivation and to reveal the possible transduction involved in the progesterone regulation pathways.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (81100912), the Science Foundation of Xinxiang Medical University, China (CL and ZD 2009-63) and the Science and Technology Project of Henan Province, China (092102310098).

Received date: 2012-07-30; Accepted date: 2012-08-20

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