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Original Article

Effect of hyperbaric oxygen therapy on Akt signaling pathway in secondary injury associated with brain trauma

Xiang'en Meng, Yu Zhang, Na Li, Danfeng Fan, Chen Yang, Hang Li, Dazhi Guo, Shuyi Pan

Department of Hyperbaric Oxygen, Navy General Hospital, Beijing, P. R. China

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Abstract: Background: The aim of this study was to investigate the effect of hyperbaric oxygen therapy on the Akt signaling pathway in the brain tissue around the injured area following traumatic brain injury in rats. Methods: The modified Feeney freefall method was applied to establish a rat model of traumatic brain injury. Sixty male SD rats were randomly divided into three groups: a sham operation group, a traumatic brain injury group and a hyperbaric oxygen therapy after traumatic brain injury group. At 12 and 24 hours after the injury was introduced, the neurological function score was evaluated. Western blot and immunohistochemistry assays were carried out to detect the expression of pAkt, cleaved caspase-3 and Bcl-2 in the cortex around the injured area 24 hours after brain injury, and immunohistochemistry assays were performed to detect the expression of pAkt and cleaved caspase-3 in the brain tissue around the injured area. The TUNEL assay was conducted to detect apoptosis of neuronal cells 24 hours after the trauma. Results: Hyperbaric oxygen therapy significantly increased the expression of pAkt ($P<0.001$), inhibited the expression of cleaved caspase-3 in the brain tissue around the injured area after traumatic brain injury ($P<0.001$), enhanced the expression of the anti-apoptotic protein Bcl-2 ($P<0.001$), and improved the neurological function score ($P<0.001$). Conclusion: Hyperbaric oxygen can exert a neuroprotective effect on a traumatic brain injury by activating the Akt signaling pathway.

Keywords: Traumatic brain injury, hyperbaric oxygen, Akt

Introduction

With the rapid social and economic development of China, the incidence of traumatic brain injury in the country is showing a rising trend, with high mortality and high morbidity, resulting in a great economic and personal burden on the patients, their families and society [1]. Studies have shown that the mechanisms responsible for pathological damage in traumatic brain injury include primary brain injury and secondary brain injury. Primary brain injury occurs at the time of the external violence, representing a stage apart from any clinical intervention, while secondary brain injury occurs in a few hours to a few days after the traumatic brain injury and is the focus of clinical interventions. Currently, the treatment of traumatic brain injury mainly consists of comprehensive clinical treatment involving surgery, low temperature treatment and drugs. A recent study found that hyperbaric oxygen therapy showed positive efficacy in patients with traumatic brain injury [2]. Laboratory and clinical studies have shown that hyperbaric oxygen treatment can increase oxygen contents, thereby increasing the cerebral oxygen metabolic rate and the oxygen content in blood. These changes exert a neuroprotective effect by improving energy metabolism and reducing the generation of oxygen free radicals, decreasing intracranial pressure and alleviating cerebral edema, and blocking the vicious cycle of brain hypoxia-cerebral edema. However, the specific molecular mechanism underlying the neuroprotective effect of hyperbaric oxygen in traumatic brain injury has not yet been fully elucidated. To better apply hyperbaric oxygen therapy in traumatic brain injury, understanding the therapeutic effect and the mechanism of action of hyperbaric oxygen in traumatic brain injury at the molecular level has important implications. Previous stud-

ies have found that the activation of the PI3K/Akt signaling pathway after traumatic brain injury can exert a significant neuroprotective effect [3]. Therefore, this study focused on the influence of hyperbaric oxygen on the PI3K/Akt signaling pathway after traumatic brain injury.

Materials and methods

Experimental animals and groups

A total of 60 healthy adult male SD rats were randomly divided into three groups: a sham operation group, a traumatic brain injury group, and a hyperbaric oxygen therapy group. The traumatic brain injury model was established in the rats using the Feeney method. Following anesthetization using chloral hydrate (4 mg/kg, intraperitoneal injection), the rats were fixed on a stereotactic frame. After disinfection of the affected skin, a bone window with a diameter of 5 mm was created with an orthopedic drill, at 3 mm to the right of the coronary seam line and 3 mm behind the sagittal suture in the rats, with an intact dura. A 40-g weight vertically impacted the right side dura pad as a free falling body from a height of 15 cm, with an injury depth of 3 mm and a diameter of 4 mm. Only fenestration surgery was performed on the rats in the sham operation group. This study was approved by the ethics committee on experimental animals at our hospital.

Hyperbaric oxygen therapy

Hyperbaric oxygen therapy was provided following the method described in a previous report in the literature [4]. Hyperbaric oxygen treatment was performed within 2 h after the traumatic brain injury model was established, the rats in the hyperbaric oxygen therapy group were placed in an animal cabin, which was then washed with pure oxygen for 10 min, and the final concentration of oxygen in the cabin was >95%. Subsequently, the pressure was evenly increased to 0.12 Mpa (gauge pressure), where it remained for 60 min. Next, the pressure was evenly decreased for 20 min to atmospheric pressure. A total of two cycles were conducted, with an interval of 10 hours. During the experiment, the behavior of the rats in the cabin was closely observed through the observation window. The rats in the normal control group and the traumatic brain injury group were also placed in the pressurized cabins, to simulate all of the environmental conditions of the experimental procedures except for the pressure and oxygen concentration.

Evaluation of the neurological function in the rats after traumatic brain injury

The neurological function scores of the rats after traumatic brain injury were determined according to neurological severity scores (NSS) [5], mainly for the motor function test, sensory test, balance test, physiological response defects and abnormal movement. The highest score was 18. If the rat could not perform the task or show the appropriate response, a score of 1 was assigned. A score of 13-18 was considered to represent severe injury, 7-12, moderate injury; and 1-6, mild injury. The neurological function scores were evaluated at three time points: before the generation of the model and at 12 h and 24 h after the traumatic brain injury.

Immunoblot assay

At 24 hours after introduction of the traumatic brain injury, the rats were anesthetized, followed by transapical infusion of 100 ml of saline, and the brain tissue around the injury was then collected and stored in a -80°C refrigerator until needed. First, 100 mg of brain tissue from around the injury was mixed with lysis buffer (product of Beyotime Biotechnology Co., Nantong, Jiangsu, China) at 1:10, after which the sample was fully homogenized using a glass homogenizer for complete lysis and then centrifuged at 4°C (14,000 rpm, 10 min). The supernatant was collected, and the protein concentration was determined using the Bradford reagent (product of Beyotime Biotechnology, China). This total protein sample was mixed with 5× loading buffer at the ratio of 1:4 and denatured in boiling water for 10 min. After 35 μg of protein was loaded into each well of an SDS-PAGE gel, electrophoresis was performed at 80 V for 30 min and 100 V for 60 min, followed by electrophoretic transfer of the proteins from the SDS-PAGE gel to a membrane (250 mA, 90 min). The membrane containing the proteins was blocked with 5% skim milk for 1 hour. Diluted primary antibodies against P-Akt, Bcl-2, cleaved caspase-3, and β-actin (1:1000, purchased from Cell Signaling Co., USA) were added, followed by incubation at 4°C overnight with shaking. After the membrane

Table 1. Neurological function scores at 12 and 24 hours in the rats in the three groups

Group	Number of cases	Before treatment	12 h	24 h
Sham operation group	20	0	1.3±0.01	1.1±0.01
Traumatic brain injury group	20	0	14.98±3.36	15.39±2.12*
Hyperbaric oxygen therapy group	20	0	13.68±2.05	13.01±1.53#
F value			220.8	514.6
P value			<0.0001	<0.0001

*P<0.05 compared with the sham operation group, #P<0.05 compared with the traumatic brain injury group.

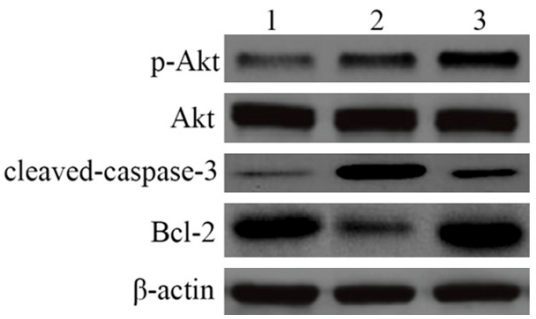

Figure 1. Detection of p-Akt, Bcl-2 and cleaved caspase-3 protein expression in each group (a representative image is shown, 1: the sham operation group; 2: the traumatic brain injury group; 3: the hyperbaric oxygen therapy group).

was washed, the secondary antibody was added, followed by incubation at room temperature for 1 h. The membrane was washed again, and incubated with ECL reagent. The grayscale intensity was analyzed with Image J software.

Immunohistochemical detection

Ten rats in each group were subjected to perfusion with paraformaldehyde, and their brains were collected to obtain paraffin-embedded sections. The brain tissue sections were conventionally deparaffinized and dehydrated, followed by antigen restoration with citrate buffer and blocking with normal goat serum. Cleaved caspase-3 and p-Akt antibodies at a dilution of 1:50 were incubated with the tissue slices for 24 hours in a moisturizing chamber at 4°C overnight. The slices were subsequently washed with PBS for 3×15 min, and incubated with horseradish peroxidase-labeled goat anti-rabbit IgG. After development with 3,3'-diaminobenzidine, the slices were mounted, and imaged under a light microscopy.

Statistical analysis

The results were processed using SPSS 15.0 software. The data were presented as mean ± standard deviation (SD). The neurological function scores were compared using the Kruskal-Wallis test, while other comparisons among groups were carried out through ANOVA analysis. A difference between two groups showing P<0.05 was considered statistically significant.

Results

Effect of hyperbaric oxygen therapy on the neurological function scores of rats with traumatic brain injury

In this study, a total of 60 rats were used. No death occurred among the rats during the experiment. Compared with the sham operation group, the neurological function scores of the rats in the traumatic brain injury group were significantly downregulated (**Table 1**, P<0.05); Compared with the traumatic brain injury group, hyperbaric oxygen therapy significantly improved the neurological deficits of the rats with traumatic brain injury (**Table 1**, P<0.05).

Effect of hyperbaric oxygen on Akt expression

Compared with the sham operation group (**Figure 1**; **Table 2**), the expression of pAkt protein in the brain tissue around the traumatic brain injury was increased (P<0.05), while the expression of Bcl-2 was significantly decreased. Hyperbaric oxygen therapy significantly up-regulated the expression of pAkt and Bcl-2 and significantly inhibited the expression of cleaved caspase-3 protein (P<0.05).

Effect of hyperbaric oxygen therapy on the expression of the apoptosis proteins Bcl-2 and cleaved caspase-3

Western blot analysis showed that compared with the sham operation group, the expression of the anti-apoptotic protein Bcl-2 in the traumatic brain injury group was significantly reduced, while the expression of the cleaved cas-

Table 2. Analysis of the western blot results for P-Akt, cleaved caspase-3 and Bcl-2 in the three groups

Group	Number of cases	P-Akt	Akt	Cleavage caspase-3	Bcl-2	P-Akt positive rate	Cleavage caspase-3 positive rate
Sham operation group	10	0.12±0.02	1.01±0.02	0.02±0.01	1.06±0.21	28.3±0.1	2.3±0.01
Traumatic brain injury group	10	0.19±0.06	0.98±0.05	0.62±0.23*	0.15±0.04*	33.5±5.6	69.2±6.8*
Hyperbaric oxygen therapy group	10	0.73±0.18#	1.02±0.11	0.24±0.12#	1.21±0.35#	72.1±4.5#	27.3±2.3#
F		91.84	0.8667	41.01	58.69	332.8	665.3
P		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

*P<0.05 compared with the sham operation group, #P<0.05 compared with the traumatic brain injury group.

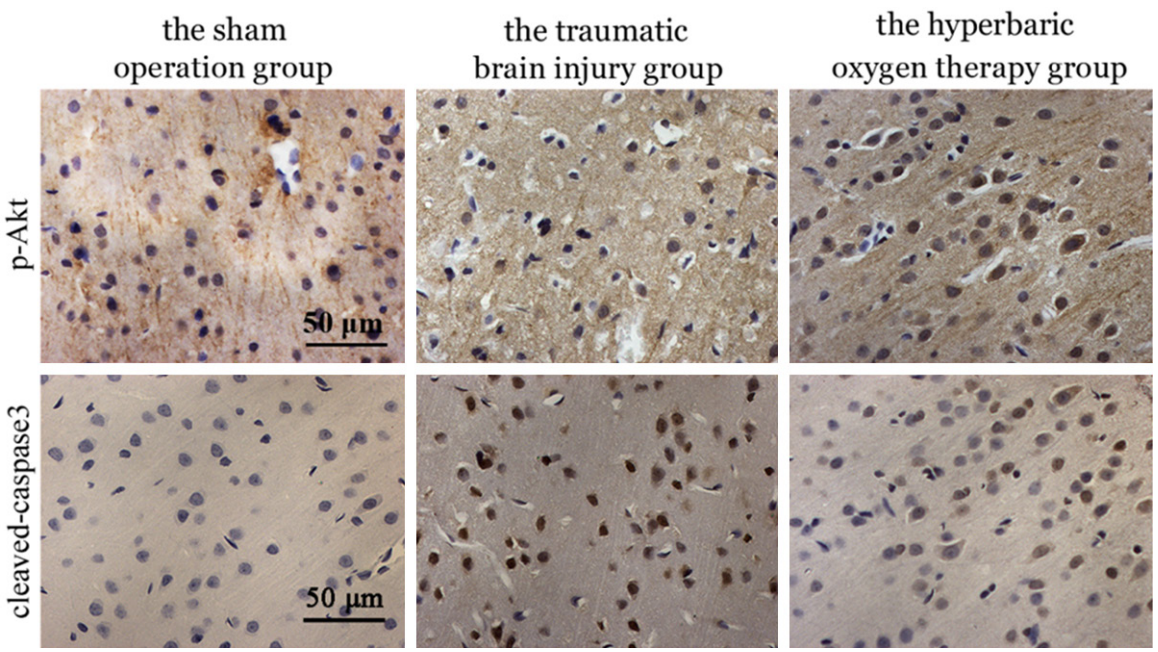

Figure 2. Immunohistochemistry results for the p-Akt (upper panel) and cleaved caspase-3 (lower panel) proteins (representative images are shown, magnification ×400).

pase-3 protein was significantly increased (P<0.05). Compared with the traumatic brain injury group, hyperbaric oxygen therapy significantly enhanced the expression of the anti-apoptotic protein Bcl-2 and inhibited the expression of the cleaved caspase-3 protein (P<0.05).

Immunohistochemical staining results

The immunohistochemical staining results showed that compared with the sham operation group, the number of cells positive with pAkt did not differ significantly in the traumatic brain injury group, whereas hyperbaric oxygen therapy significantly increased the number of cells with positive pAkt expression. Additionally, the number of cells exhibiting positive expression of the cleaved caspase-3 protein in the traumatic brain injury group was significantly increased (**Figure 2**; **Table 2**, P<0.05), while hyperbaric oxygen therapy significantly suppressed the number of cleaved caspase-3-positive cells (**Figure 2**; **Table 2**, P<0.05). These results are consistent with the results of the immunoblotting assays performed in this study.

Discussion

Akt is a serine/threonine protein kinase that is widely expressed in various tissues and cells. The molecular structure of Akt consists of three different functional domains: a pleckstrin homology domain (PH) at the amino terminus, a catalytic domain in the middle, and a C-terminal regulatory domain in which the phosphorylation of serine/threonine residues is required for complete activation of Akt. In the resting state,

Akt is mostly located in the cytoplasm. When the cell is stimulated by an external factor, the intracellular upstream phosphatidylinositol 3-kinase (PI3K) is activated to produce the secondary messenger phosphatidylinositol 3,4,5-triphosphate (PIP3) at the plasma membrane and PIP3 binds to the PH domain in Akt protein. Then, Akt is transported from the cytoplasm to the cell membrane. Thereafter, Ser473 and Thr308 in Akt are simultaneously activated by phosphorylation, and the activated Akt is translocated into the cytoplasm or nucleus to regulate a variety of cellular activities and biological effects via the phosphorylation of a series of downstream target proteins [6]. The activated Akt can control the survival of neurons by regulating multiple downstream signaling pathways, whose specific mechanisms of action include inhibiting cell apoptosis after binding to glycogen synthase kinase-3; exerting an anti-apoptotic effect by phosphorylating the members of the Bcl-2 family and inhibiting the expression of cleaved caspase-3, a key protein in apoptosis execution; and preventing the release of the apoptotic factor cytochrome c in the mitochondria to inhibit or down-regulate the mitochondrial apoptotic pathways [7].

Cleaved caspase-3 is a critical protein in the execution of neuronal apoptosis after traumatic brain injury. Previous studies have shown that the expression of cleaved caspase-3 protein in brain tissue specimens from rats and clinical patients with traumatic brain injury is significantly increased and that the use of a drug that down-regulates the expression of cleaved caspase-3 can significantly reduce the number of apoptotic neurons following traumatic brain injury and significantly improve the neurological function score. The most important substrate for caspase-3 is poly (ADP-ribose) polymerase (PARP), which is mainly related to DNA repair and the monitoring of gene integrity. When apoptosis begins, PARP (116 kD) is cleaved into two fragments of 31 kD and 85 kD at Asp216-Gly217 by caspase-3 so that the two zinc finger structures involved in DNA binding in PARP are separated from the catalytic domain in the carboxyl terminus and lose their normal function. As a result, the activity of the Ca2+/Mg2+-dependent endonuclease that is negatively regulated by PARP is further increased to cleave DNA in the nucleosomes, ultimately leading to neuronal apoptosis.

Previous studies have shown that Akt plays an important role in neuronal apoptosis following cerebral ischemia-reperfusion injury [8], subarachnoid hemorrhage [9], traumatic brain injury [10] and spinal cord injury [11]. Inhibiting Akt expression can significantly increase the number of apoptotic neurons and aggravate the injury to nerve function, whereas promoting its activity may reduce neuronal damage and improve the prognosis of neurological function in rats with brain injury. A study on traumatic brain injury in an animal model found that the expression of P-Akt could be detected 2 h after the brain trauma, reaching a peak value at 6 h and then gradually decreasing. Various neurotrophic factors can play a neuroprotective role after brain trauma by activating the PI3K/Akt signaling pathway. Exogenous neuroprotective factors that up-regulate the activity of the Akt signaling pathway can also reduce the extent of the nerve damage caused by traumatic brain injury. The use of a PI3K inhibitor can significantly increase the damage to neurons after brain trauma, suggesting that the up-regulated PI3K/Akt signaling pathway is significant for the treatment of brain trauma [12]. The results of this study showed that compared with the model group, at 24 h after hyperbaric oxygen therapy, the expression of P-Akt in rat brain tissue was significantly increased, which is consistent with the findings of previous studies. Further investigation showed that the expression level of cleaved caspase-3 in the brain tissue of the rats subjected to hyperbaric oxygen treatment was significantly lower, while the expression of the anti-apoptotic protein Bcl-2 was significantly increased, indicating that up-regulating the Akt signaling pathway may be one of the functional mechanisms underlying the neuroprotective effect of hyperbaric oxygen.

In summary, hyperbaric oxygen therapy can significantly increase the activity of PI3K/Akt in the brain tissue surrounding a traumatic brain injury in rats. This study provides a better understanding of the molecular mechanism of hyperbaric oxygen therapy in traumatic brain injury. The results of this study are significant for guiding the clinical application of hyperbaric oxygen therapy in traumatic brain injury.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Shuyi Pan, Department of Hyperbaric Oxygen, Navy General Hospital, 6 Fucheng Road, Haidian District, Beijing 100048, P. R. China. Tel: +86-10-66957590; Fax: +86-10-66957590; E-mail: shuyipan_oooo@126.com

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