European Review for Medical and Pharmacological Sciences 2018; 22: 1071-1077

The role of AMPK/mTOR signal pathway in brain injury following chronic intermittent hypoxia in growing rats

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Abstract. – OBJECTIVE: To investigate the role of AMPK-mTOR signal pathway in brain injury induced by chronic intermittent hypoxia (CIH) in young rats.

MATERIALS AND METHODS: Forty healthy male Sprague-Dawley rats (3-4 weeks old, weighing 80-100 g) were randomly assigned to four groups: 2-week-CIH group (2IH), 4-week-CIH group (4IH), 2-week-simulated air control group (2AC) and 4-week-simulated air control group (4AC). TUNEL staining was used to detect the cell apoptosis in the hippocampus and pre-frontal cortexes, respectively. The Western blot was conducted to analysis the P-AMPK (Phospho-AMP-activated protein kinase) and P-mTOR (phosphorylated mammalian target of rapamycin) protein expression.

RESULTS: The neurons apoptosis in the hippocampus and pre-frontal cortex in 2IH and 4IH groups increased significantly, compared with that of in 2AC and 4AC groups, ($p<0.05$, respectively). Moreover, 4IH group exhibited significantly increased apoptosis rates than 2IH group ($p<0.05$). 2IH and 4IH groups exhibited increased protein expression levels of P-AMPK in the hippocampus and prefrontal cortexes compared with 2AC and 4AC groups ($p<0.05$, respectively), whereas the protein expression of P-mTOR decreased after CIH treatment ($p<0.05$, respectively). Higher expression levels of P-AMPK and lower levels of P-mTOR were observed in 4IH group compared to 2IH group. No difference of apoptotic cells and protein expression of P-AMPK and P-mTOR was exhibited between 2AC and 4AC groups.

CONCLUSIONS: CIH induces neural apoptosis in a time-dependent manner by activating AMPK and inhibiting mTOR phosphorylation in young rats.

Key Words

Chronic intermittent hypoxia, Obstructive sleep apnea-hypopnea syndrome, Hippocampus, Prefrontal cortexes, AMPK/mTOR, Apoptosis.

List of Abbreviations

CIH: Chronic intermittent hypoxia; OSAHS: Obstructive sleep apnea-hypopnea syndrome; AI: Apoptosis index; P-AMPK: Phospho-AMP-activated protein kinase; P-mTOR: phosphorylated mammalian target of rapamycin. AMPK: Adenosine 5'-monophosphate (AMP)-activated protein kinase. mTOR: mammalian target of rapamycin. TBS: Tris-buffered saline.

Introduction

Obstructive sleep apnea-hypopnea syndrome (OSAHS) is characterized by repetitive episodes of partial or complete collapse of the upper airway during sleep, resulting in recurrent nocturnal apnea, chronic intermittent hypoxia (CIH), transitory hypercapnia and sleep fragmentation[1]. This clinical syndrome could impair neuronal cells function, which could cause cognitive decline in OSAHS children[2]. Cognitive impairment in OSAHS individuals is involved with various cognitive domains, such as attention/vigilance, memory, and global cognitive function as well as executive function[3,4]. Oxidative stress and apoptosis play significant roles in OSAHS[1,5-7]. OSA can represent a risk factor for stroke and death, mainly related to the endothelial dysfunction, with the formation of atherosclerosis caused by hypoxia through oxidative stress[8]. AMPK is a key protein among energy regulator family, which is widely distributed in the nervous system, and it is closely related to the survival of neurons. In the condition of ischemic and hypoxia, AMPK is activated to phosphorylated AMPK (P-AMPK) by AMP/ATP ratio changes[9]. P-AMPK content in neurons was significantly increased after ischemia in the cerebral ischemia-reperfusion mice model[10]. Meanwhile, human neuroblastoma cells apoptosis could be induced by AMPK agonist *in vitro*, suggesting that AMPK could regulate the apoptosis of neuronal cell[11]. However, little data has been found about the role of AMPK/mTOR pathway in neurons apoptosis induced by CIH. Here we established the CIH rat model to observe the neurons apoptosis of the related functional areas, and detected the changes of P-AMPK and P-mTOR protein expression after CIH treatment.

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Materials and Methods

Animals and Grouping

Forty healthy male Sprague-Dawley (SD) rats (3-4 weeks old, weighing 80-100 g) were provided by the Experimental Animal Center of Wenzhou Medical College with the certificate No. [SYXK (Zhejiang) 2005-0061]. All procedures performed in studies involving animals were in accordance with the Ethical Committee of Wenzhou Medical College, China. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The rats were housed five per cage with *ad libitum* access to food and water at specific pathogen free condition. All rats were randomly divided into four groups with 10 rats per group: 2-week-CIH group (2IH), 4-week-CIH group (4IH), 2-week-simulated air control group (2AC) and 4-week-simulated air control group (4AC).

Model Establishment

To establish the OSAHS animal model, rats in 2IH and 4IH groups were placed in atmospheric intermittent hypoxia cabin for 2 weeks and 4 weeks, respectively. Nitrogen was ventilated at pressure of 0.3 kPa for 30 s and paused for 30 s, and oxygen was ventilated at flow rate of 25 L/min for 12 s and paused for 18 s. The above process was considered as a cycle. The oxygen concentration was 9.0% ± 1.5% in hypoxia chamber and oxygen concentration 21.0% ± 0.5% when reoxygenation, and the CO_2 concentration was less than 0.01%. The hypoxia and reoxygenation were performed for 7.5 h per day (from 8:50 to 16:20) for 7 days. During the period of suspension of ventilation, the highest oxygen concentration, the lowest oxygen concentration and CO_2 concentration in intermittent hypoxic cabin were measured. The rats in control group were placed into the control air chamber under the same conditions, compressed air was ventilated and oxygen concentration remained at 21.0% ± 0.5%. The experiments procedure was controlled by a single-chip program. During the experiment, the cabins were covered with thick cloth during the day to simulate night, so the cabin remained dark and young rats were kept sleeping.

Tissue Extraction

The rats were sacrificed immediately after the end of the experiment. The left and the right hippocampus, and the prefrontal cortex were harvested on the ice after the skulls were stripped. The prefrontal cortex was also divided into left and right two parts according to the sagittal position. Twenty hippocampus and prefrontal cortex samples were collected per group and rinsed in saline buffer. The two parts of hippocampus and cortex in each group were fixed with 4% paraformaldehyde for 12 h. TUNEL staining was conducted on paraffin embedded tissues. The rest of the tissues was stored in liquid nitrogen.

Detection of Neuronal Apoptosis in the Hippocampus and Prefrontal Cortex

TUNEL staining was used to detect the neuronal apoptosis according to the instruction provided by the TUNEL kit (Roche, Basel, Switzerland). Microscopic examination of nuclei with brown granules was considered as apoptotic cells. The number of apoptotic cells under high power field (× 400) was calculated randomly for at least five visions. Apoptosis index (AI) was expressed as apoptotic cells per 100 cells (%).

Western Blotting Analysis of p-AMPK and p-mTOR Protein in Hippocampus and Prefrontal Cortex

Western blotting analyses were performed as described previously to detect the expression of p-AMPK and p-mTOR in the right hippocampus and cortex from No. 1-3 rats in each group and the left hippocampus and cortex from NO.7-10 rats in each group[12]. Briefly, all tissues were homogenized in lysis buffer to extract total protein. The protein concentration was determined by bicinchoninic acid (BCA) protein concentration assay kit (Pierce, Appleton, WI, USA). 100 μg of protein extract from all samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Beijing, China) and transferred to 0.2 μm nitrocellulose membrane (GE Healthcare, Life Sciences, Shanghai, China). After protein transfer, the membrane was blocked in 5% skim milk solution in TBS (Tris-buffered saline) with Tween 20 for 2 h at room temperature and incubated overnight at 4°C with AMPK and mTOR primary antibody (Cell Signal Technology, Danvers, MA, USA), GAPDH antibody (Danvers, MA, USA) and β-tubulin antibody (Danvers, MA, USA) at 1:1000 dilutions. The membrane was developed with goat anti-rabbit secondary antibody labeled with horseradish peroxidase (HRP) (Thermo Fisher Scientific Inc., Waltham, MA, USA) incubated for 2 h in blocking buffer at 1:5000 dilutions, and scanned on Gel-Pro gel analysis

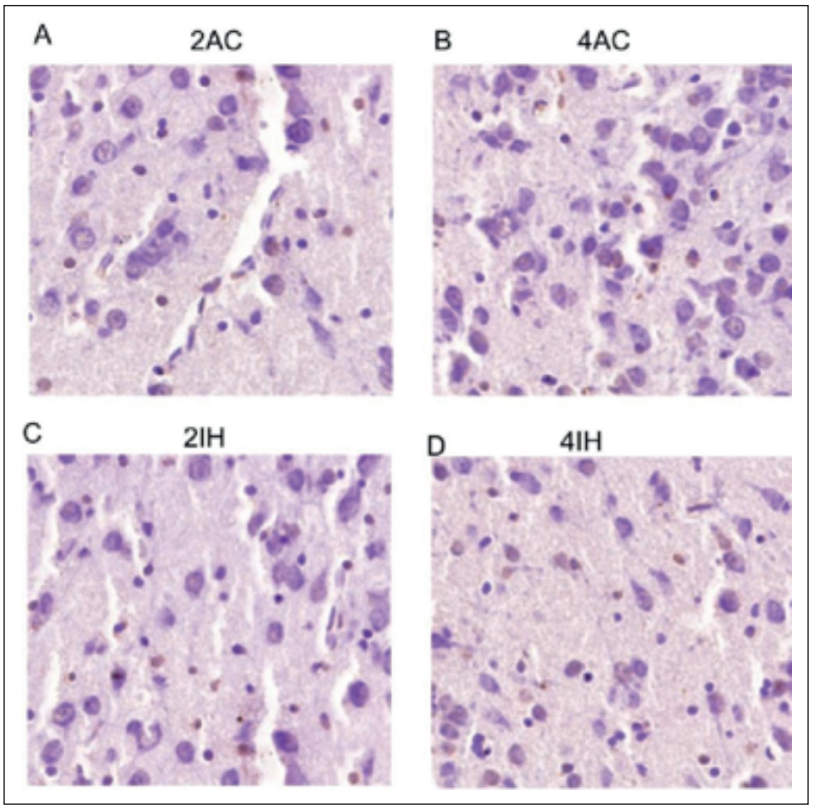

Figure 1. Comparison of cell apoptosis among groups, 2AC (A), 4AC (B), 2IH (D) and 4IH (D), in the area of prefrontal cortex by TUNEL staining. (DAB*400).

software, which was used to analyze the results. Relative expression level of protein was calculated as cumulative optical density of the target protein band/cumulative optical density (IOD).

Statistical Analysis

The statistical analysis of our study data was performed using SPSS for Windows version 19.0 software (IBM SPSS Inc., Armonk, NY, USA). Normal distribution data were expressed as mean ± standard deviation. Normal distribution data were analyzed using one-way ANOVA analysis. For comparison between groups, LSD test was used if the variance was homogeneous, otherwise Tamhane's T2 test was used. $p<0.05$ was considered statistically significant.

Results

Detection of Neuronal Apoptosis in the Hippocampus and Prefrontal Cortex

TUNEL assay was applied to efficiently detect the apoptosis level of cortical neurons (Figure 1) and hippocampal (Figure 2) in each group. The differences between chronic intermittent hypoxia group and control group were significant ($F = 69.7$, $p<0.05$; $F = 16.4$, $p<0.05$) in cortical neurons and hippocampus both at week 2 and week 4 (Table I).

Western Blotting Analysis of p-AMPK and p-mTOR Protein in Hippocampus and Prefrontal Cortex

The phosphorylation level of AMPK and mTOR was examined by Western blot in both chronic intermittent hypoxia group and the control group at week 2 (Figure 3) and week 4 (Figure 4). The phosphorylation level of AMPK was significantly higher in the chronic intermittent hypoxia group compared with the control group ($F = 584.0$, $p<0.05$; $F = 296$, $p<0.05$, Table II). A similar change was also found for the activation of mTOR ($F = 125.37$, $p<0.05$; $F = 238.24$, $p<0.05$, Table III).

Discussion

To detect the molecules related to cognitive neuron apoptosis, we established a chronic intermittent hypoxic model to mimic the pathophysiology of OSAHS. The associations between chronic intermittent hypoxia and the expression

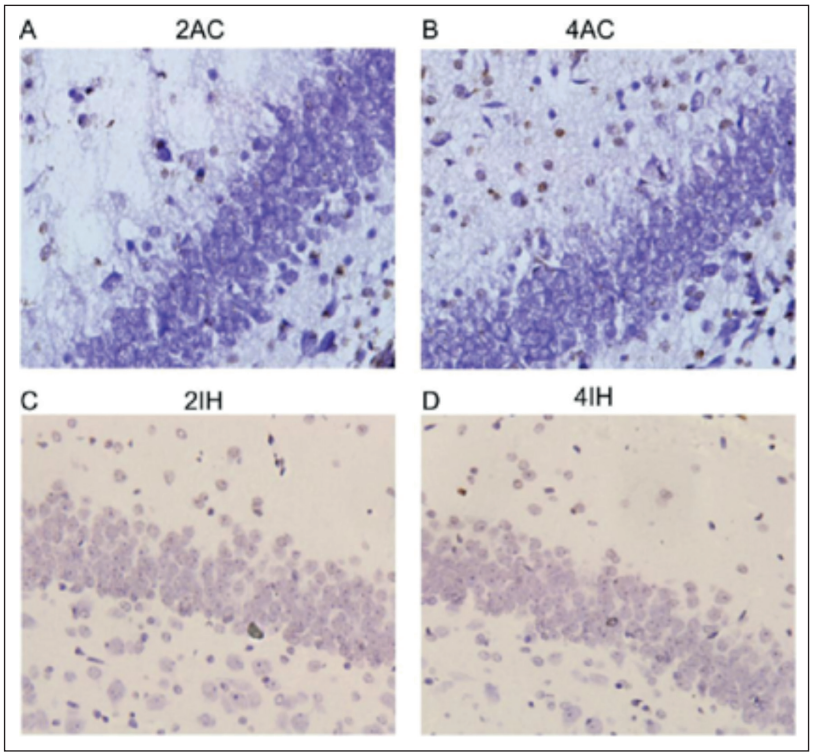

Figure 2. Comparison of cell apoptosis among groups, 2AC (A), 4AC (B), 2IH (D) and 4IH (D), in the area of hippocampus by TUNEL staining. (DAB*400).

of AMPK have, for the first time, been demonstrated: the expression of AMPK in hippocampus and cortex was increased, while the level of phosphorylated mTOR was decreased in 2IH and 4IH groups. The decrease of mTOR phosphorylation level was more obvious in 4IH group, indicating that the increasing of AMPK and the decreasing of mTOR would lead to neuronal apoptosis.

It was reported that untreated pediatric OSAHS may also result in serious morbidity in cardiovascular, neurobehavioral, and somatic growth and development, in addition of direct damage to the normal structure and function of the respiratory system[8]. Among those non-respiratory system complications of OSAHS, cognitive impairment is one of the most common.

Table I. Comparison of apoptosis index among groups (%).

Groups	Hippocampus	Prefrontal cortex
2AC	2.68±0.26	2.74±0.38
2IH	14.94±0.25#	12.42±0.43#
4AC	3.12±0.36	2.89±0.32
4IH	20.78±0.36*▲	17.02±0.34*▲
F-value	69.72	16.42
***p*-value**	<0.05	<0.05

#p<0.05 2IH *vs*. 2AC group; *p<0.05 4IH *vs*. 4AC group; ▲p<0.05 4IH *vs*. 2IH group.

Cognitive impairment in children with OSAHS may result in such as attention deficits, impaired learning ability, week memory, attention deficit, low judgment ability and execution ability[13]. Then, it will lead to the decline of children's learning, memory and social adaptability, which is a huge burden for the community and the family. However, the pathophysiology of cognitive impairment in OSAHS children is still unclear. OSAHS is characterized by intermittent hypoxemia during sleep, which is different from

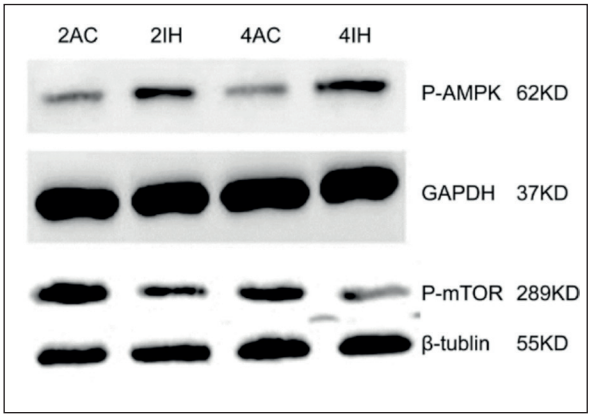

Figure 3. Comparison of P-AMPK and P-mTOR protein expression among groups in the area of prefrontal cortex by Western blot.

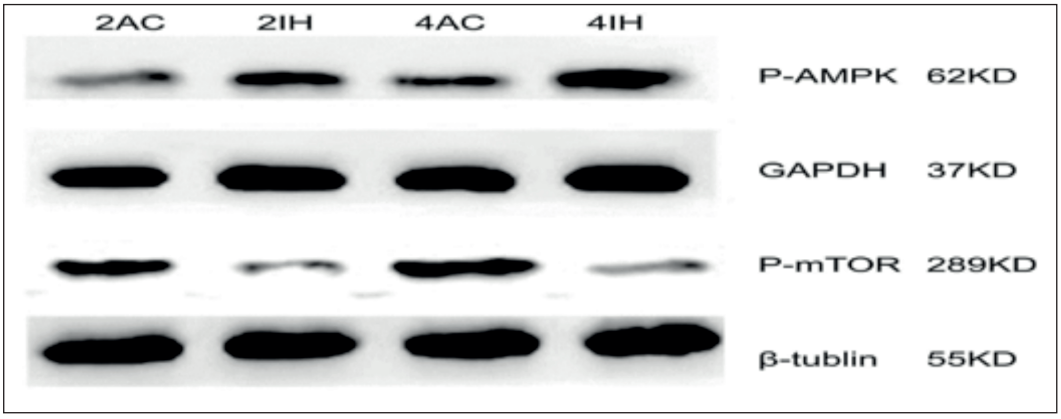

Figure 4. Comparison of P-AMPK and P-mTOR protein expression among groups in the area of hippocampus by Western blot.

persistent hypoxia[14,15]. Compared with persistent hypoxia, intermittent hypoxia is more difficult to tolerate, and it will result more severe damaged. In addition, extensive researches focused on adult OSAHS and confirmed that it can lead to a series of diseases, including cardiovascular and cerebrovascular disease and even sudden death. In recent years, OSAHS in children is emerging as a new concern. It has been found that OSAHS has a high morbidity in children and is the major cause of sleep disorders in children. In some researches, the occurrence rate of OSAHS disease in children was reported as high as 3%[16,17]. Pediatric OSAHS has become widely recognized as likely cause of significant morbidity among children. This study will provide theoretical basis for the prevention and treatment of pediatric OSAHS with cognitive impairment. Moreover, AMPK, as a regulator of cellular energy in adenosine-induced apoptosis, has attracted much attention[18]. AMPK (AMP-activated protein kinase) is a conserved protein kinase, mainly coordinating cell metabolism and energy supply. It was found that the phosphorylation level of AMPK will increase in the hypoxic conditions, leading to cell energy metabolism imbalance and ultimately inducing apoptosis[19]. Lucchi et al[20] found that AMPK can be activated and apoptosis of cancer cells can be induced by the use of 8-cl-adenosine. In the β-cell apoptosis induced by high doses of glucosamine, AMPK were also activated as a result of the inhibition of cell glucose uptake. Reducing the activation of AMPK by glucagon Peptide (GLP-1), pancreatic β-cell apoptosis could be decreased significantly[21]. The research suggested that AMPK activation plays a key role in pancreatic apoptosis. In our study, the phosphorylation level of AMPK in IH group was significantly higher than that in the control group both at hippocampus and prefrontal cortex in a time-dependent way. The finding confirmed the increasing of p-AMPK is related with apoptosis, which was consistent with previous studies[18]. Meanwhile, mTOR (mammalian target of

Table II. Comparison of P-AMPK protein expression among groups (P-AMPK/GAPDH).

Groups	Hippocampus	Prefrontal cortex
2AC	0.3291±0.00860	0.3382±0.01124
2IH	0.5696±0.01913#	0.6294±0.01910#
4AC	0.3474±0.01208	0.3231±0.00246
4IH	0.9247±0.00817*▲	0.9339±0.00479*▲
F-value	583.983	<0.05
***p*-value**	<0.05	<0.05

#p<0.05 2IH *vs*. 2AC group; *p<0.05 4IH *vs*. 4AC group; ▲p<0.05 4IH *vs*. 2IH group.

Table III. Comparison of P-mTOR protein expression among groups (P-mTOR/β-tublin).

Groups	Hippocampus	Prefrontal cortex
2AC	0.6903±0.0301	0.7725±0.0617
2IH	0.3852±0.0423#	0.4523±0.0185#
4AC	0.7506±0.0564	0.8548±0.0175
4IH	0.2015±0.0107*▲	0.2651±0.0203*▲
F-value	125.37	238.24
***p*-value**	<0.05	<0.05

#p<0.05 2IH *vs*. 2AC group; *p<0.05 4IH *vs*. 4AC group; ▲p<0.05 4IH *vs*. 2IH group.

rapamycin) is an important downstream pathway of AMPK, a conserved serine/threonine protein kinase that regulates cell proliferation and apoptosis together with AMPK. The mTOR signaling pathway acts as a molecular system integrator to support organism and cellular interactions with the environment[22]. mTOR would manipulate the cell biofunction under undesirable environment including hypoxia, decreased sugar content, and induced anabolic reduction[23]. In hypoxic conditions, protein synthesis was decreased by the activation of AMPK and the inhibition of mTOR. The stress signal was passed through various ways; more hypoxia inducible factors, such as 4E-BP1, S6K and S6, were transcripted and dephosphorylated to adapt hypoxic environment. Hypoxia could damage hippocampal neural stem cells through the mTOR pathway, affecting the hippocampus neural stem cell proliferation and development, and inhibiting the cognition of young rats[24]. Therefore, decreasing of mTOR would provide a marker neuronal apoptosis after chronic intermittent hypoxia.

Conclusions

We observed the influence of chronic intermittent hypoxic on rat brain neuronal apoptosis, and investigated the phosphorylation of AMPK, mTOR. Our result indicated that chronic intermittent hypoxia can induce hippocampal, cortical cell apoptosis, by activating AMPK and inhibiting mTOR. Therefore, our result shows that activation of AMPK-mTOR pathway may involve in chronic intermittent hypoxia apoptosis process and play an important role in neuronal damage.

Conflict of Interest

All authors declared they have no conflict of interest.

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