間歇性低氧適應對於熱中風保護機制之探討

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摘 要

熱中風爲一種在世界各地都會發生之臨床急症，其臨床症狀有體溫過高，並且 50% 以上的遇難者伴有中樞神經系統損傷以及多重器官衰竭。在動物模式下發現，熱中風動物出現動脈血壓下降及顱內壓上升、腦缺血及腦神經細胞損傷，而腦內組織也釋放出大量的多巴胺 (dopamine)、 血清張力素 (serotonin)、 致熱性細胞介質素(cytokines)及麩胺酸 (glutamate)。 上述的熱中風症狀都能因事先於大鼠腦中誘發熱休克蛋白 72 (HSP72)而減緩。於是我們假設大鼠經過數週間歇低氧訓練之後，可能會經由誘發體內重要器官（如腦組織、心臟、主動脈、腎上腺等）熱休克蛋白 72 型之表現，而改善在熱中風過程中之腦心血管功能，進一步對熱中風所致的循環性休克及腦缺血性神經細胞損傷產生保護作用。將 SD 雄鼠隨機分爲間歇性低氧適應訓練組及對照組，間歇性低氧適應訓練組動物在低壓艙（壓力設定 0.45 ATA）每天 4 小時，每星期訓練五天，如此持續訓練兩週。熱中風之誘發乃將大鼠持續曝露於高溫 43 ℃下，當平均動脈壓與局部腦血流量自其高點迅速下降之時，視爲熱中風生成。我們將比較有無運動訓練之大鼠在熱中風生成後，其血壓、心跳、腦血流及腦神經細胞損傷指數之差異。並測量在熱中風生成過程中，心血管動力參數的變化。另外在大鼠腦中植入顱內微透析探針來收集並偵測腦中一氧化氮之濃度與神經細胞損傷及缺血標記含量之變化，包括麩胺酸、甘油、乳酸、焦葡萄酸塩、一氧化氮及氧化自由基。腦皮質組織中之 HSP72 表現含量之分析則使用 HSP72 單株抗體，利用聚丙烯胺膠體電泳與西方墨漬技術偵測。結果顯示間歇性低氧適應訓練可以經由增加熱休克蛋白質 72 的表現並改善熱中風所引起之神經細胞損傷。

關鍵詞：熱中風，間歇性低氧適應，循環性休克，大腦缺血，心輸出量

Effects of Intermittent Hypoxia Adaptation on Experimental Heatstroke Rat Model

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Abstract

Heatstroke is a serious clinical problem in many parts of world. The symptoms of heatstroke include hyperthermia, central nervous system disorders and multiple organ failures in more than 50 % of the victims. In the animal model heatstroke, rats display arterial hypotension, intracranial hypertension, cerebral ischemia, cerebral dopamine, serotonin, cytokines and glutamate overload and cerebral neuronal damage. However, the above-mentioned heatstroke syndromes are attenuated by induction of heat shock protein 72 (HSP72) in rat brain. Other evidences have also demonstrated that HSP 72 can be detected in heart from rats with endurance exercise or hypoxia training. This raises the possibility that pretreatment of rats with physical exercise protects against the heatstroke-induced arterial hypotension, cerebral ischemia and cerebral neuronal damage via inducing HSP 72 and improves cardiovascular function of rats during heatstroke. Male SD rats are randomly assigned to either a heatstroke control group or to an intermittent hypoxia adaptation group. Trained animals were put in a hypoxia chamber 5 days/week, 4 hours/day with intensity 0.45 ATA for 2 weeks. Heatstroke is induced by exposing the

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animals to a high temperature (43℃); the moment at which mean arterial pressure (MAP) and cerebral blood flow (CBF) decreased from their peak values is taken as the time of heatstroke onset. To address the question properly, we will compare the temporal profiles of MAP, CBF, heart rate and cerebral neuronal damage score after heatstroke in rats with or without physical exercise training and detect the changes of cardiovascular parameters (cardiac output and heart stroke volume) through the heatstroke period. Implanting an intracranial microdialysis probe will help assess the NO, free radicals release and markers of cerebral ischemia and cellular injury (including glycerol, glutamate and lactate/pyruvate ratio) in brain. The HSP 72 in brain cortex tissue was analyzed by SDS-PAGE and Western blotting with monoclonal anti-HSP 72 antibody. The data indicate that intermittent hypoxia adaptation increased HSP72 expression and attenuated heatstroke induced neuronal damage.

Keywords: Heatstroke, Intermittent Hypoxia Adaptation, Circulatory Shock, Cerebral Ischemia, Cardiac Output.

I. Introduction

Heatstroke is characterized by hyperpyrexia and multiorgan dysfunction. Animal heatstroke models fulfill the empirical triad used for the diagnosis of classic human heatstroke [1]. Multiorgan dysfunctions ensue from severe heatstroke, including hepatic and renal dysfunction, pulmonary and brain edema, hypotension, cerebral ischemia and injury, and activated inflammation. Despite considerable focus on the goal of regenerating damaged brain pathways following heatstroke, only limited progress has been made. Enhancing endogenous mechanisms of plasticity in spared neural pathways may be a more achievable goal to promote functional recovery. Chronic hypoxia preconditioning is known to induce local and systemic adaptive responses such as the increased blood haemoglobulin concentration and tissue oxygen delivery [2-3]. Chronic hypoxia preconditioning also improves brain tissue oxygenation by increasing brain capillary density [4]. Mild hypoxic/ ischemia preconditioning increases the neuronal resistance to subsequent severe hypoxia/ischemia [5-6]. Additionally, the most popular preventive approach for high altitude pulmonary edema is gradual ascending which is actually a kind of hypoxic preconditioning [7]. To our knowledge, the mechanisms underlying the protective effects exerted by mild hypoxic preconditioning remain unclear. To deal with the question, the present study was first attempt to assess whether cardiovascular, and ischemic and oxidative damage to brain can be induced by heatstroke in rats. Second, to assess whether mild hypobaric hypoxia preconditioning (0.44-0.88 ATA or 334-668 Torr or a simulated ~4000~8000 m altitude exposure, 5h/day, 5 days a week for consecutive 2 weeks) is able to prevent the occurrence of the proposed heatstroke-induced above-mentioned disorders.

II. Materials and methods

1. Animals

Adult Sprague-Dawley rats (weight 253-274 g) were obtained from the Animal Resource Center of the National Science Council of the Republic of China (Taipei, Taiwan). The animals were housed 4 in a group at an ambient temperature of 22±1^{o}C, with a 12-h light/dark cycle. Pellet rat chow and tap water were available *ad libitum*. All protocols were approved by the Animal Ethics Committee of the Chi Mei Medical center (Tainan, Taiwan) in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act. Adequate anesthesia was maintained to abolish the corneal reflex and pain reflexes induced by tail pinching throughout all experiments (approximately 8 h) by a single intraperitoneal dose of urethane (1.4 g/kg body weight). At the end of the experiments, control rats and any rats that had survived heatstroke were killed with an overdose of urethane. Animals were assigned randomly

to one of the following 4 groups: (1) the normothermia group, (2) the intermittent hypoxia adaptation (IHA) group, (3) the heatstroke (HS) group, (4) the intermittent hypoxia adaptation with heatstroke (IHA+HS) group.

2. Surgery and physiological parameter monitoring

The right femoral artery was cannulated with polyethylene tubing (PE50) for blood pressure monitoring. Physioloical parameters monitoring included core temperature (Tco), mean arterial pressure (MAP), cardiac output (CO), total peripheral resistance (TPR), stroke volume (SV), and heart rate (HR).

3. Induction of heatstroke

Before the initiation of heatstroke, the Tco of urethane-anesthetized rats was maintained at about 36oC by means of a folded heating pad, except during heat stresses at a room temperature of 24oC. Heatstroke was induced by increasing folded heating pad temperature to 43oC by means of circulating hot water. The time point at which the MAP dropped to 25 mmHg from the peak level was considered as the onset of heatstroke. At this time point, the average Tco was found to be 43.2 ± 0.2oC. Immediately after the onset of heatstroke, the heating pad was removed and the animals were allowed to recover at room temperature (24oC). Our pilot study showed that the latency for the onset of heatstroke (time interval between the start of heat stress and the onset of heatstroke) was found to be 70 ± 3 mins for the heatstroke groups. Therefore, in the present study, the time point of 70 mins denoted the onset of heatstroke, whereas the time point of 85 mins denoted the 15th minute after the onset of heatstroke.

4. Intermittent hypoxia adaptation (IHA)

Rats were randomly assigned to one of the following three groups: the IHA (18.3% O2 at 0.45 ATA for 5 hours daily for consecutive 5 days per week) group, and the non-IHA or normobaric air (NBA) (21% O2 at 1.0 ATA) group. Two weeks after the start of IHA, the IHA rats were subjected to simulated heatstroke.

5. Measurement of cardiac output

The animal's trachea was intubated, and the animal was artificially respired at 50 breaths/min, with tidal volume of 20 mL and inspiration-to-expiration ratio at 1:2. A 3S transonic flow probe (Transonic System, Taconic, NY) was implanted around the ascending aorta as described by smith [7]. Briefly, the chest was opened at the third intercostal space to expose the heart. A small section (1 cm long) of the ascending aorta was freed from connective tissue. The flow probe was then implanted around the root of the ascending aorta. The chest incision was closed, and a negative intra- thoracic pressure was restored. The values of total peripheral resistance (TPR) were obtained by dividing MAP by cardiac output (CO). The values of stroke volume (SV) were obtained by dividing CO by HR.

6. Extracellular levels of glutamate, lactate-pyruvate ratio, nitric oxide, glycerol, and 2,3-dihydroxybenzoic acid in cortex

The brain (cortex) samples were prepared for determination of extracellular levels of glutamate, lactate-pyruvate ratio, glycerol, nitric oxide, and 2,3-dihydroxybenzoic acid. The dialysates were injected onto a CMA600 microdialysis analyzer (Carnegie Medicine, Stockholm, Sweden) for measuring lactate, pyruvate, glycerol, and glutamate. The nitric oxide (NO) concentrations in the dialysates were measured with the Eicom ENO-20 NOx- analysis system (Eicom Kyoto, Japan). The concentrations of 2,3-dihydroxybenzoic acid (2,3-DHBA) were measured by a modified procedure based on the hydroxylation of sodium salicylate by hydroxyl radicals, leading to the production of 2,3-DHBA .

7. Protein extraction and Western blot analysis

The brain cortex tissue was ground under liquid nitrogen using a mortar and pestle, then mixed with RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 0.1 M NaCl pH 7.5, 0.25% Na-deoxycholate, 1% IGEPAL-630, proteinase inhibitor cocktail tablet (Roche), phosphatase inhibitors with 1mM Na3VO4, 1 mM NaF) and spun down at 12,000 rpm for 5 min. The supernatant was collected and stored at -70oC Proteins were quantified with the DC protein assay kit (Bio-Rad) and 2X sample buffer (1 M Tris-HCl pH 6.8, 10% SDS, 0.01% β-ME, 0.01% Bromophenol blue, 20% Glycerol) and then separated in 10% ~ 12% gradient sodiumdodecyl sulfate-polyacrylamide gel electrophoresis at 150 V for 3.5 ~ 4 h. Electrophoresed proteins were transferred to PVDF membranes (Bio-Rad semi-dry transfer system) at 1.2 mA per 1cm2 for 1.5 h. The PVDF membranes were incubated at room temperature for 1 h in blocking buffer (PBST, 5 % (v/v) defat milk). Primary antibodies of anti-HSP72 (Stressgen) were diluted in PBST buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 2 mM KH2PO4 pH 7.4, 0.1% (v/v) Tween 20). The membranes were incubated at room temperature for 1 h. The immunoblots were washed four times in PBST buffer for 15 min, immersed in the secondary antibody solution containing anti-rabbit (anti-HSP72) antibodies for 1 h, and then diluted 15000-fold in PBST buffer. The membranes were then washed in PBST buffer for 15 min four times. Detect in ECL (PerkinElmer).

8. Statistical analyses

Statistical comparisons between groups were done with analysis of variance (ANOVA) and Duncan's multiple-range test was used for post hoc multiple comparisons among means. All data are reported as means ± S.E.M. A P value of less than 0.05 was considered to be significant.

III. Results

1. IHA attenuates hypotension, intracranial hypertension, cerebral hypoperfusion, ischemia, and hypoxia, and hyperpyrexia during heatstroke

Figure 1 depicts the effects of heat exposure (43oC for 70 min) on physiologic variables in heatstroke rats pretreated with or without IHA. In heatstroke rats without WBC treatment, the ICP and Tbr were both significantly higher at 85 min after the start of heat exposure than they were for normothermic controls. In contrast, the values for MAP, CPP, CBF, and brain PO2 were significantly lower than those of normothermic controls. Precondition with IHA significantly attenuated the heat stress-induced arterial hypotension, intracranial hypertension, cerebral hypoperfusion, cerebral ischemia and hypoxia, and hyperpyrexia.

2. IHA reduced cortex ischemia, hypoxia and oxidative stress in heatstroke

Figure 1 showed that the hypothalamic levels of CBF and PO2 for HS rats were significantly lower at 85 mins after the start of heat stress than they were for normothermia control rats. On the other hand, the hypothalamic levels of glycerol, 2,3-DHBA, and NOx- for HS rats were significantly higher at 85 mins after the start of heat stress than they were for normothermic controls (Fig. 2). Heat-induced decreased cortex levels of CBF and PO2 as well as the increased cortex levels of glycerol, 2,3-DHBA, and NOx- were all significantly reduced by IHA (Fig. 1 and Fig. 2).

3. IHA attenuates heatstroke induced cardiac dysfunction

Table 1 summarizes the effects of heatstroke (43oC for 70 mins) on several cardiovascular parameters in normothermic controls, untreated heatstroke rats, and heatstroke rats treated with whole body cooling. In untreated heatstroke rats, the values of Tco were significantly higher at 70-85 mins after initiation of heatstroke compared with those of normothermic controls. In contrast, the values of MAP, HR, CO, SV, and TPR were all significantly lower than those of the normothermic controls. The heatstroke–induced hyperthermia and cardiac dysfunction were significantly reduced by intermittent hypoxia adaptation (IHA).

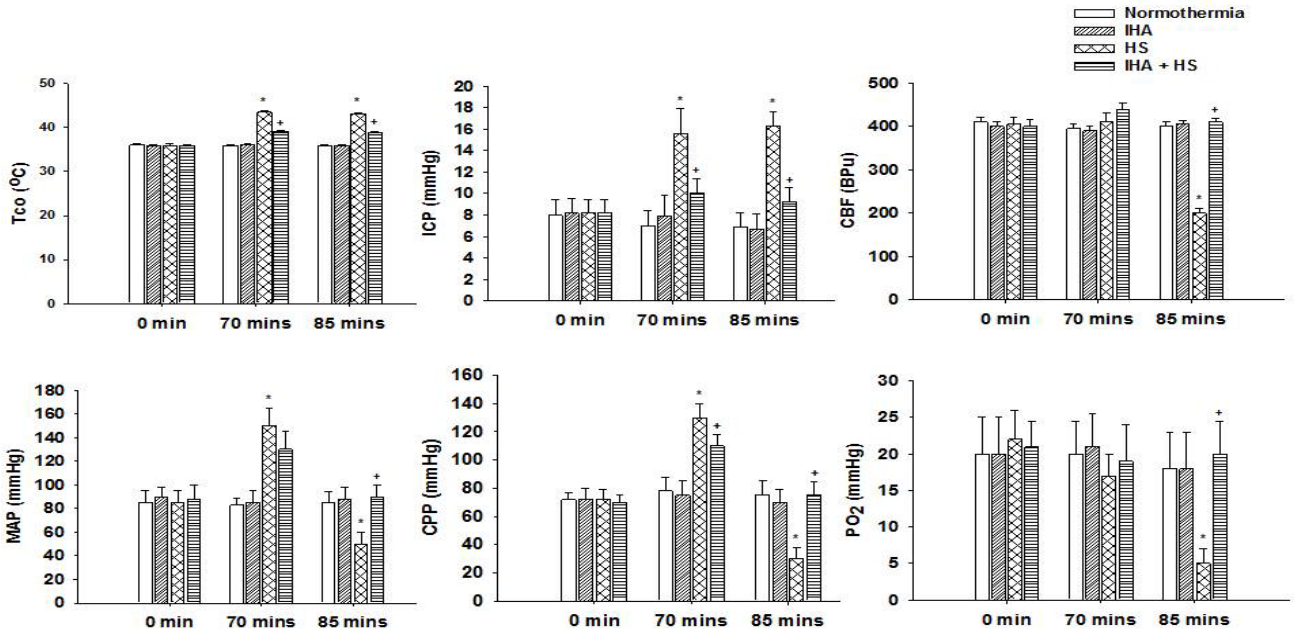

Figure 1 IHA attenuates hypotension, intracranial hypertension, cerebral hypoperfusion, ischemia, and hypoxia, and hyperpyrexia during heatstroke.

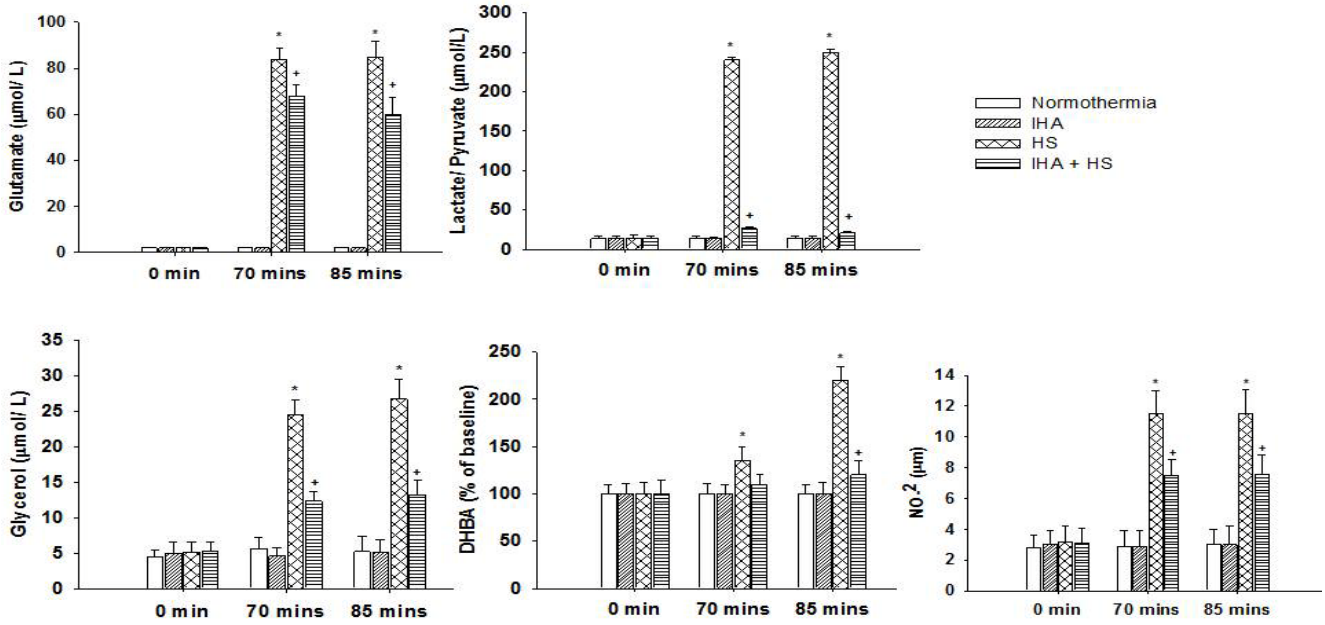

Figure 2 IHA reduced cortex ischemia, hypoxia and oxidative stress in heatstroke.

4. IHA attenuates heatstroke induced cardiac dysfunction

Table 1 summarizes the effects of heatstroke (43^{O}C for 70 mins) on several cardiovascular parameters in normothermic controls, untreated heatstroke rats, and heatstroke rats treated with whole body cooling. In untreated heatstroke rats, the values of Tco were significantly higher at 70-85 mins after initiation of heatstroke compared with those of normothermic controls. In contrast, the values of MAP, HR, CO, SV, and TPR were all significantly lower than those of the normothermic controls. The heatstroke–induced hyperthermia and cardiac dysfunction were significantly reduced by intermittent hypoxia adaptation (IHA).

Table 1 IHA attenuates heatstroke induced cardiac dysfunction

Groups/Time course	Tco (^{O}C)	MAP (mmHg)	HR (heat/min)	CO (ml/min)	SV (ml/heat)	TPR (mmHg/min ml^{-1})
NC						
0 min	36.1 ± 0.4	88 ± 2	360 ± 12	40 ± 5	0.11 ± 0.03	2.2 ± 0.2
70 min	36.2 ± 0.3	92 ± 3	364 ± 14	46 ± 5	0.15 ± 0.02	2.3 ± 0.4
85 min	36.3 ± 0.3	90 ± 3	358 ± 11	45 ± 3	0.14 ± 0.03	2.2 ± 0.3
IHA						
0 min	36.3 ± 0.4	90 ± 3	368 ± 13	43 ± 5	0.11 ± 0.04	2.1 ± 0.2
70 min	36.2 ± 0.3	85 ± 3	355 ± 12	47 ± 5	0.11 ± 0.08	2.3 ± 0.4
85 min	36.1 ± 0.3	91 ± 2	365 ± 16	47 ± 4	0.12 ± 0.05	2.5 ± 0.5
HS						
0 min	36.5 ± 0.2	90 ± 4	389 ± 13	46 ± 4	0.11 ± 0.02	2.1 ± 0.2
70 min	43.5 ± 0.6*	150 ± 5*	450 ± 17*	35 ± 5*	0.09 ± 0.02*	1.3 ± 0.1$^{※}$
85 min	43.1 ± 0.5*	50 ± 6*	120 ± 15*	8 ± 2*	0.03 ± 0.02*	0.8 ± 0.2$^{※}$
IHA+HS						
0 min	36.2 ± 0.3	80 ± 3	380 ± 15	45 ± 3	0.11 ± 0.03	2.1 ± 0.2
70 min	39.5 ± 0.5*	130 ± 4*	299 ± 14*	39 ± 5*	0.09 ± 0.01*	1.1 ± 0.2$^{※}$
85 min	38.7 ± 0.3†	90 ± 3†	346 ± 18†	46 ± 4†	0.13 ± 0.02†	2.3 ± 0.3†

Values are means ± SEM of 8 rats per group. HS groups, exposed to 43^{O}C, had heat exposure withdrawn at 70 minutes and then were allowed to recover at 24^{O}C. In NC group, the animal were kept at 24^{O}C throughout the whole course of experiments. In HS+C group, whole body cooling was adopted at time “70 min” for 15 mins. * P<0.05 compared with corresponding control values in NC group; † P<0.05 compared with HS group (ANOVA followed by Duncan's test).

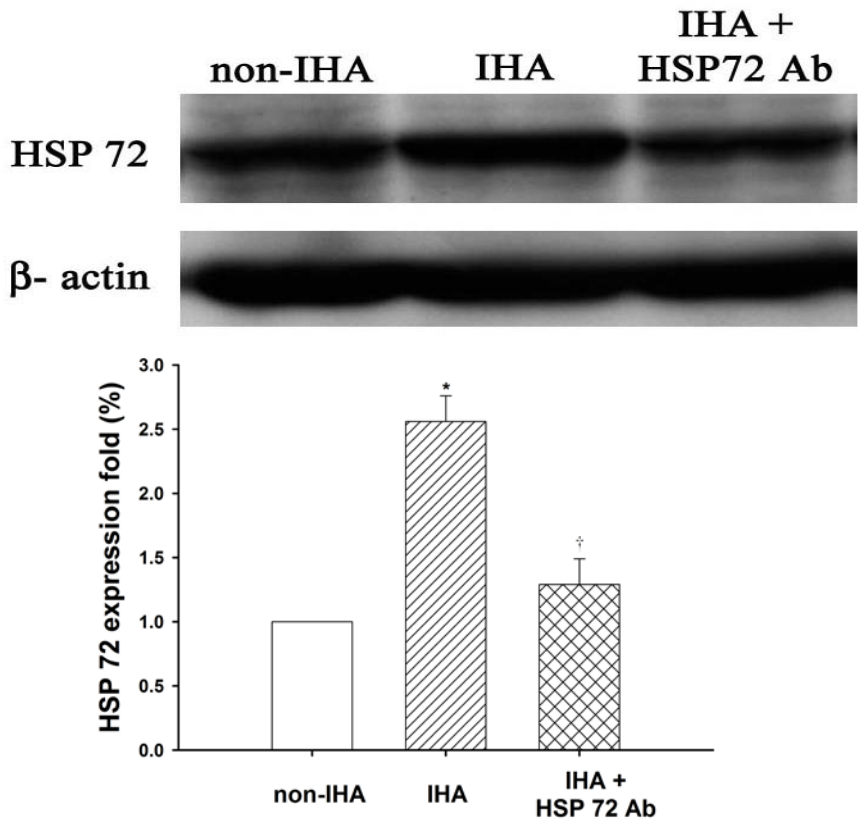

Figure 3 IHA caused HSP-72 overexpression in the cortex

5. IHA caused HSP-72 overexpression in the cortex

The O.D. values of protein assay by the densitometer for cortex tissues obtained from different groups were summarized in Figure 3. Brain tissues were obtained from the animals killed 2 weeks after the start of IHA with or without HSP-72 Ab, or the equivalent time for the non-IHA group. Western analyses revealed that IHA rats had higher cortex expression of HSP-72 than these of non-IHA rats ($P<0.001$).

IV. Discussion and conclusion

It is well documented that glutamate and lactate-to-pyruvate ratio are two markers of cellular ischemia, whereas glycerol is a marker of how severely cells are affected by the ongoing pathology [8]. Our previous studies also demonstrated that animals with traumatic brain injury had higher values of extracellular levels of glutamate, lactate-to-pyruvate, and glycerol in ischemic cortex [9-10]. Our current findings showed that increased levels of glutamate, lactate-to-pyruvate ratio, and glycerol in the brain cortex of non-IHA rats after a simulated HS were observed. The HS-induced increased cellular ischemia and damage markers were associated with an increased ICP, which could be significantly reduced by IHA. The current results further showed HSP-72 Ab preconditioning significantly abolished the IHA-induced beneficial effects in reducing brain ischemia and damage. Intermittent hypoxia adaptation affects the expression of a variety of genes as cells adapt to altered metabolic status or induce programmed cell death. HIF-1 is a heterodimeric basichelix–loop– helix transcription factor that is sensitive to oxygen. HIF-1 transactivates various genes including those encoding erythropoietin, glucose transporters, glycolytic enzymes, and vascular endothelial growth factor [11]. By regulating these signaling cascades, HIF-1 may mediate cellular adaptation, and, thereby protecting cells from hypoxic insult. Although intermittent hypoxia adaptation increases expression of HSP72, we found that the increase in HSP72

expression was greater than that of HIF-1 (data not shown) under the conditions tested, indicating that HSP72 is more susceptible than HIF-1 to O2 regulation. Therefore, it is not surprising that periodic oxygen supplements more effectively reduced inducible HIF-1 levels than HSP72 levels. While the relationship between HIF-1 and HSP72 expression in response to reduction of intracellular oxygen tension is not well understood, recent studies suggest that HSPs, specifically HSP72 and HSP90, may delay degradation of HIF-1 by directly interacting with the Per-ARNT-Sim domain to compete with HIF-1 /ARNT for the binding site following activation of a PI3K/Akt pathway [12-13].

In this study, we demonstrated evidence concerning the potential of intermittent hypoxia to induce functional recovery of brain capacity following HS in rodent models. In important respects, rats and humans share a common organization of the thermoregulation control system, suggesting that rats are useful as a model to investigate basic mechanisms of brain plasticity following injury and/or for developing novel therapeutic strategies in humans. Repetitive intermittent hypoxia could be easily applied in human HS patients since it is a non-invasive, safe, and easy- to- use strategy. Since we also have evidence that repetitive intermittent hypoxia increases HSP72 expression in brain cortex, this treatment may also be an effective strategy to treat non-HS impairments. Preliminary attempts to utilize repetitive intermittent hypoxia to improve motor cortex neuron function in paraplegic patients have shown promising results in this regard [14]. Collectively, available evidence suggests that further investigation of intermittent hypoxia to treat brain ischemia and damage deficits following HS are warranted.

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