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ORIGINAL ARTICLE

Attenuating systemic inflammatory markers in simulated high-altitude exposure by heat shock protein 70-mediated hypobaric hypoxia preconditioning in rats



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Background/Purpose: The primary goal of this study was to test whether high-altitude exposure (HAE: 0.9% O₂ at 0.47 ATA for 24 hours) was capable of increasing the systemic inflammatory markers as well as the toxic organ injury indicators in rats, with a secondary goal to test whether preinduction of heat shock protein (HSP) 70 by hypobaric hypoxia preconditioning (HHP: 18.3% O₂ at 0.66 ATA for 5 h/day on 5 days consecutively for 2 weeks) attenuated the proposed increased serum levels of both the systemic inflammatory markers and the toxic organ injury indicators.

Methods: Rats were assigned to: (1) non-HHP (21% O₂ at 1.0 ATA)+non-HAE (21% O₂ at 1.0 ATA) group; (2) non-HHP+HAE group; (3) HHP+non-HAE group; (4) HHP+HAE group; and (5) HHP+HSP70 antibodies (Ab)+HAE group. For the HSP70Ab group, a neutralizing HSP70Ab was injected intravenously at 24 hours prior to HAE. All the physiological and biochemical parameters were obtained at the end of HAE or the equivalent time period of non-HAE. Blood samples were obtained for determination of both the systemic inflammatory markers (e.g., serum tumor necrosis factor- α , interleukin-1 β , E-selectin, intercellular adhesion molecule-1, and liver myeloperoxidase activity) and the toxic organ injury indicators (e.g., nitric oxide metabolites, 2,3-dihydroxybenzoic acid, and lactate dehydrogenase).

Conflicts of interest: The authors have no conflicts of interest relevant to this article.

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Results: HHP, in addition to inducing overexpression of tissue HSP70, significantly attenuated the HAE-induced hypotension, bradycardia, hypoxia, acidosis, and increased tissue levels of both the systemic inflammatory markers and the toxic organ injury indicators. The beneficial effects of HHP in inducing tissue overexpression of HSP70 as well as in preventing the HAE-induced increased levels of the systemic inflammatory markers and the toxic organ injury indicators could be significantly reduced by HSP70Ab preconditioning.

Conclusion: These results suggest that HHP may downgrade both the systemic inflammatory markers and the toxic organ injury indicators in HAE by upregulating tissue HSP70.

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Introduction

Hypoxia encountered at high-altitude exposure (HAE) is associated with acute mountain sickness, high-altitude pulmonary edema (HAPE), and high-altitude cerebral edema.¹ People who travel to high altitude are commonly confronted with problems such as mental dysfunction and memory deficit,² insomnia, dizziness, nausea,³ hypophagia⁴ and motor impairment.⁵ The HAE-induced acute pulmonary edema is thought to be related to increased sympathetic tone, exaggerated hypoxic pulmonary vasoconstriction, decreased hypoxic ventilator drive, increased pulmonary capillary pressure, failure of pulmonary capillaries, and alveolar fluid leak across capillary endothelium.⁶ In addition, high-altitude pulmonary hypertension is associated with a free radical-mediated reduction in pulmonary nitric oxide bioavailability.⁷ In addition to the hydrostatic stress, increased alveolar–capillary permeability caused by inflammation may be necessary or causal. For example, Schoene and colleagues^{8,9} found that neutrophils and elevated concentrations of plasma proteins, thromboxane metabolites, and proinflammatory cytokines in bronchoalveolar lavage fluid were observed in patients with well-established HAE. In animals, severe hypoxia (0–3% oxygen) stimulates vascular endothelial cells, leukocytes and macrophages *in vitro* to release proinflammatory cytokines.^{10–13} In rats, a simulated HAE (0.9% O₂ at 0.47 ATA for 24 hours) caused pulmonary edema, inflammation, and hemorrhage^{14,15} as well as brain edema, hippocampal oxidative stress, and cognitive dysfunction.¹⁶

It has been well documented that the pathogenesis of multiple organ dysfunction syndrome (MODS) in septic shock is related to tissue production and release of the systemic inflammatory markers including proinflammatory cytokines interleukin-6 (IL-6), interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and myeloperoxidase and the toxic organ injury indicators nitric oxide metabolites (NO_x⁻), dihydroxybenzoic acid (DHBA), and lactate dehydrogenase (LDH) in rats.^{17,18} It is not known whether HAE, like toxic septic shock, is able to cause elevation of both the systemic inflammatory markers and the organ injury indicators.

Hypobaric hypoxia preconditioning (HHP) is known to increase blood hemoglobin and tissue oxygen delivery^{19,20} and to increase the neuronal resistance to subsequent severe hypoxia.^{21,22} More recently, we observed that HHP [18.3% O₂ at 0.66 ATA (atmosphere absolute) for 5 h/day on 5 days consecutively for 2 weeks] in rats, in addition to inducing lung overexpression of HSP70, significantly

attenuated the HAE-induced pulmonary edema, inflammation, and ischemic and oxidative damage in rats.¹⁵ Again, it is unknown whether the proposed HAE-induced increased systemic inflammatory markers and the toxic organ injury indicator can be affected by HSP70-mediated HHP.

Therefore, the present study was first to assess the changes of the tissue levels of the systemic inflammatory markers, the organ injury indicators, the cardiovascular parameters, and the blood gas and acid–base parameters during HAE in rats without or with HHP. Then, the secondary aim was to determine whether the proposed beneficial effect of HHP in reducing the systemic inflammatory markers and the toxic organ injury indicators during HAE is caused by the preinduction of HSP70 prior to the onset of HAE.

Materials and methods

Animals

Adult Sprague–Dawley rats (weight 254 \pm 12 g) were obtained from the Animal Resource Center of the National Science Council of the Republic of China (Taipei, Taiwan). The animals were housed four in a group at an ambient temperature of 22 \pm 1°C, with a 12-hour light/dark cycle. Pellet rat chow and tap water were available *ad libitum*. All protocols were approved by the Animals 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 (IACUC Approval No: 100052305). At the end of the experiments, control rats and any rats that had survived HAE were killed with an overdose of sodium pentobarbital.

Experimental groups and hypobaric hypoxia preconditioning (HHP)

Rats were randomly assigned to one of the following five groups: (a) the (non-HHP+normobaric air (NBA)) group: animals were treated with non-HHP or NBA (21% O₂ at 1.0 ATA) for 2 weeks plus NBA; (b) the (non-HHP+HAE) group: animals were treated with non-HHP or NBA for 2 weeks plus HAE (9.7% O₂ at 0.47 ATA); (c) the (HHP+NBA) group: animals were treated with HHP [13.9% O₂ at 0.66 ATA (atmosphere absolute) for 5 h/day on 5 days consecutively for 2 weeks] plus NBA; (d) the (HHP+HAE) group: animals were treated with HHP plus HAE; and (e) the

(HHP+HSP70Ab+HAE) group: animals were treated with HHP plus HSP70Ab and HAE. The (non+HHP+HAE) group, the (HHP+HAE) group, and the (HHP+HSP70Ab+HAE) group rats were subjected to simulated HAE at 2 weeks after the start of non-HHP or HHP (Fig. 1).

A simulated HAE model

HHP rats or non-HHP rat were randomly exposed to a simulated HAE (9.7% O₂ at 0.47 ATA) of 6000 m (or 19,685 feet) in a hypobaric chamber (Institute of Aviation Medicine, Beijing, China) for 24 hours.^{14,15} The temperature of the hypobaric chamber was maintained at 25 ± 1°C and humidity at 40–50% with an air flow rate of 4 L/h and a barometric pressure of 355 mmHg the animals were provided with adequate quantities of food and water during hypoxic exposure.

Inhibition of HSP70 activity

The protective role of HSP70 in the rat brain^{7,23} or in the rat lung¹⁵ has been studied previously by using anti-HSP70 antibody (Ab). A neutralizing polyclonal rabbit anti-mouse HSP70Ab (0.2 mg/kg of body weight; SPA-812 E, Assay Designs) dissolved in nonpyrogenic sterile saline was injected intravenously at 24 hours before simulated HAE.

Experimental protocols

In Experiment 1, non-HHP rats (*n* = 8), HHP rats (*n* = 8), and (HHP+HSP70Ab) rats (*n* = 8) were killed by decapitation and their livers were removed for determination of protein expression of HSP70. (Fig. 1)

In Experiment 2, all the non-HHP rats (*n* = 8), HHP rats (*n* = 8), and HHP+HSP70Ab rats (*n* = 8) were subjected to HAE for 24 hours and their effects on values of mean arterial pressure (MAP), heart rates (HR), and blood levels of PaCO₂, SO₂, HCO₃⁻, and pH were determined in these groups of rats under general anesthesia of an intraperitoneal (i.p.) dose of sodium pentobarbital (60 mg/kg of body

weight). Both (non-HHP+NBA) group and (HHP+NBA) group rats were used as controls.

In Experiment 3, 24 hours after HAE, all the non-HHP rats (*n* = 8), HHP rats (*n* = 8), and HHP+HSP Ab rats (*n* = 8) were killed by decapitation and their bloods were obtained for determination of serum levels of glutamate, lactate-to-pyruvate ratio, glycerol, NO_x, 2,3-DHBA, IL-1β, TNF-α, IL-10, E-selectin, soluble intercellular adhesion molecule-1 (ICAM-1) and LDH. In addition, their liver tissues were obtained for determination of myeloperoxidase (MPO) activity. Both the (non-HHP+NBA) group rats and the (HHP+NBA) rats were used as controls.

Physiologic variable monitoring

At the end of HAE for the HAE group or the equivalent time period for the NBA group, the animals were anesthetized and their bilateral femoral arteries were cannulated using polyethylene tubes. One tube was used for continuous monitoring of MAP and heart rate (HR) via a pressure transducer, whereas the other was used for collecting blood samples to determine the animal's arterial pH, PaCO₂, SO₂, and HCO₃⁻ levels. Both the MAP and HR were recorded using a polygraph. Core temperature was monitored by a thermocouple inserted into the rectum. The core temperature was maintained at a constant level of ~37°C with a hot water circulating heating pad. After stabilization, all physiologic variables were obtained for data presentation and then the blood samples were obtained for biochemical determination.

Protein preparation

Peripheral liver tissues were frozen in liquid nitrogen and then stored at -80°C until analyzed. The tissue was homogenized in ice-cold isolation solution (250 mmol/L sucrose, 10 mmol/L triethanolamine, 1 μg/mL leupeptin and 0.1 mg/mL phenylmethyl sulfonyl fluoride (PMSF)). Homogenates were centrifuged at 12000 rpm for 10 minutes at 4°C to separate incompletely homogenized tissue. The supernatants were obtained and the protein concentrations

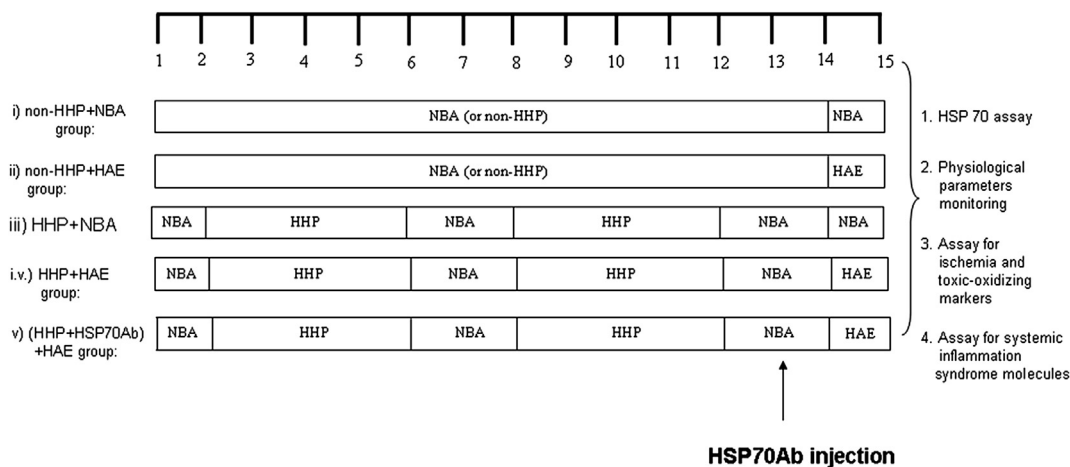


Figure 1 Experimental design. The HHP group received 18.3% O₂ at 0.66 ATA for 5 hours daily for 5 days consecutively for 2 weeks. These three groups of rats were subjected to HAE (9.8% O₂ at 0.47 ATA) for 24 hours. HAE = high-altitude exposure; HHP = hypobaric hypoxia preconditioning; HSP70Ab = heat shock protein 70 antibody; NBA = normobaric air.

were measured using a protein assay kit (Sunbio, Taipei, Taiwan). For deglycosylation of proteins, an *N*-glycosidase F Deglycosylation kit (Roche) was used.

Western blot analysis

Total proteins (50 µg/sample) were diluted in 5× loading buffer [0.25 mol/L Tris/HCl (pH 6.8), 10% sodium dodecyl sulfate (SDS), 0.5% bromophenol blue, 50% glycerol and 0.5 mol/L dithiothreitol and then boiled for 5 minutes. SDS/polyacrylamide gel electrophoresis (PAGE) was carried out on 12% gradient gels. The proteins were electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes treated previously with methanol and blocked for 1 hour at room temperature (24°C) in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) containing 5% nonfat dried milk. Membranes were incubated overnight at 4°C with an anti-HSP70 antibody (1:40000 dilution; Santa Cruz Biotechnology, CA, USA) in TBS-T containing 5% nonfat dried milk. After washing in TBS-T, the membranes were incubated with horseradish peroxidase-labeled anti-rabbit antibody (Santa Cruz, 1:3000) for 2–3 hours at room temperature. Blots were developed with enhanced chemiluminescence agents (ECL plus; Sunbio) before exposure to X-ray film. To confirm equivalent loading of samples, the same membranes were incubated with an anti-β-actin antibody (1:1000 dilution; Santa Cruz Biotechnology) and was visualized via enhanced chemiluminescence as described above. For quantification, Western blots were scanned using a Minolta scanner and Adobe Photoshop software. The labeling density was quantified using Lab-Works software (UVP). The relative density of the HSP70 band was normalized to the density of the β-actin band to represent the amount of HSP70 protein. The ratio of non-HHP group was regarded as 100%, and the results for HHP and HHP+HSP70Ab group are expressed as a percentage of the value from the non-HHP group.

Determination of proinflammatory cytokines, glutamate, glycerol, lactate-to-pyruvate ratio, 2,3-dihydroxybenzoic acid (2,3-DHBA), nitric oxide metabolites (NO_x⁻), LDH and ICAM-1

At the end of the experiments, the animals were killed and blood samples were collected. After centrifugation, the serum was frozen at -80°C for biochemical determination. The concentration of TNF-α, IL-1β, IL-10, and ICAM-1 were determined using double-antibody sandwich ELISA (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instruction. Optical densities were read on a plate reader set at 450 nm for TNF-α, IL-1β, IL-10, and ICAM-1. The concentration of cytokines in the samples were calculated from the standard curve multiplied by the dilution factor and were expressed as pg/mL. For determination of the glutamate and glycerol levels, and the lactate-to-pyruvate ratio, aliquots of samples were injected into a CMA600 microdialysis analyzer (Carnegie Medicine, Stockholm, Sweden).²⁴

Nitric oxide is an unstable molecule that is easily degraded into nitrite (NO₂⁻) and nitrate (NO₃⁻) ions.²⁵ NO₂⁻ and NO₃⁻ levels were measured using the HPLC-NO detector

system (ENO-10; Eicom, Kyoto, Japan), as previously reported.²⁶ In brief, NO₂⁻ and NO₃⁻ were separated on a reverse phase column (NO-PAK, 4.6 × 50 mm; Eicom, Kyoto, Japan) and NO₃⁻ was reduced to NO₂⁻ by passage through a reduction column (NO-RED; Eicom, Kyoto, Japan). NO₂⁻ was determined as the azo dye compound by the Griess reaction using a spectrophotometer. These oxidative NO products were also evaluated as NO_x⁻ (NO₂⁻ plus NO₃⁻).

The concentrations of hydroxyl radicals were measured by a modified procedure based on the hydroxylation of sodium salicylates by hydroxyl radicals, leading to the production of 2,3-DHBA and 2,5-DHBA.^{27,28} A Ringer's solution containing 0.5 mM sodium salicylates was perfused through the microdialysis probe at a constant flow rate (1.2 µL/min). An Alltrina reverse-phase C18 column (BAS, 150 × 1 mm ID, particle size 5 µm) was used to separate the DHBAs, and the mobile phase consisted of a mixture of 0.1M chloroacetic acid, 26.87 nM disodium EDTA, 688.16 nM sodium octyl sulfate, and 10% acetonitrile (pH3.0). The retention time of 2,3-DHBA and 2,5-DHBA were 8.1 and 6.0 minutes, respectively. In addition, LDH was measured to evaluate the extent of organ injury by Fuji DRI-CHEM 3030 (Fuji Photo film Co., Tokyo, Japan).

Measurement of E-selectin

Rat peripheral polymorphonuclear (PMN) cells were isolated from the whole blood of rats and treated with heparin (100 units/mL). Erythrocytes were allowed to sediment for 30 minutes after the addition of 1 mL of 6% dextran (weight/volume in PBS) to 10 mL blood. After sedimentation, the plasma containing leukocytes was centrifuged twice at 300g for 5 minutes each. The precipitates were mixed with 70% osmolality-adjusted Percoll and centrifuged at 30,000g for 30 minutes at 26°C. The PMN-riched layer was fractionated. Each fraction was washed twice with Hank's balanced salt solution, and the cell number was counted. The purity of the PMNs was determined to exceed 95% by Giemsa staining cells (1 × 10⁶ cells/tube) were incubated with a rabbit polyclonal antibody to CD62E (ab1898; Abcam PIC332, Cambridge, UK) or control. After washing, the cells were stained with a secondary-antibody (goat polyclonal to rabbit immunoglobulin G (IgG)-HCL [FITC]. [ab6717]; Abcam PIC). Cells were incubated for 1 hour at 4°C and washed. The cells were mixed with oligosaccharide dyes and incubated for 20 minutes, and then co-incubated with KM93 for 60 minutes. The fluorescence intensity of cells was analyzed with a FACStar (Becton Dickinson, Franklin Lakes, NJ, USA).

Determination of myeloperoxidase activity

A spectrophotometric method was used to determine MPO activity in the liver.²⁹ A 100-µL aliquot of serum was mixed with 900 µL of 50 mmol/L phosphate buffer (pH 6.0) containing 0.167 mg/mL of *O*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. One unit of peroxidase activity (i.e., the amount of enzyme decomposing 1 µmol of hydrogen peroxide) was calculated from the oxidation of *O*-dianisidine using an absorption coefficient of 11.3/mM/cm at 460 nm.

Statistical analysis

All quantitative data were evaluated for Gaussian (normal) distribution. Values are expressed as means \pm standard error (SE) and were analyzed with one-way analysis of variance (ANOVA), followed by the Newmann–Keuls *post hoc* test if $p < 0.05$. For all statistical analyses, SPSS software version 10.0 (SPSS Inc., Chicago, IL, USA) was used.

Results

HHP increased HSP70 protein in liver tissues

Western blot analysis revealed that expression of HSP70 protein in liver tissues from the HHP group was significantly higher than those from the non-HHP group ($p < 0.01$; Fig. 2). However, the expression of HSP70 protein in liver tissues from the HHP+HSP70Ab group was significantly lower than those from the HHP group ($p < 0.01$; Fig. 2).

HHP reduced HAE-induced decrease in MAP, HR, SO_2 , $PaCO_2$, HCO_3^- and pH values after HAE

The values of MAP, HR, and blood levels of $PaCO_2$, SO_2 , HCO_3^- and pH in the non-HHP rats were significantly lower

24 hours after HAE than for the non-HAE controls (non-HHP+NBA group or HHP+NBA group; Fig. 3). However, HHP significantly reduced the HAE-induced decrease of MAP, HR, and blood levels of $PaCO_2$, HCO_3^- , SO_2 , and pH. Additionally, HSP70Ab preconditioning significantly reduced the beneficial effects of HHP in HAE (Fig. 3).

HHP reduced HAE-induced the increase in toxic oxidizing radicals and toxic organ damage indicator

The blood levels of toxic oxidizing agents like NO_x^- and 2,3-DHBA and the toxic organ damage indicator like LDH in the non-HHP rats were significantly higher 24 hours after HAE than for the non-HAE controls (non-HHP+NBA group or HHP+NBA group) (Fig. 4). The HAE-induced increased blood levels of the toxic oxidizing radicals and the toxic organ damage indicator were all significantly reduced by HHP (Fig. 4). Again, the beneficial effects of HHP in HAE were significantly attenuated by HSP70Abs preconditioning (Fig. 4).

HHP attenuated HAE-induced increased serum levels of systemic inflammatory response molecules

The blood levels of systemic inflammatory response molecules including IL-1 β , TNF- α , E-selectin, ICAM-1, and liver levels of MPO activity in the non-HHP+HAE group rats were all significantly higher 24 hours after HAE than for the non-HAE controls (non-HHP+NBA group or HHP+NBA group) (Fig. 5). HHP, in addition to increasing blood levels of an antiinflammatory cytokine like IL-10, significantly reduced HAE-induced increased blood levels of IL-1 β , TNF- α , E-selectin and ICAM-1 and increased liver levels of MPO activity 24 hours after HAE. Again, the beneficial effects of HHP in inhibiting activated inflammation that occurred 24 hours after HAE were all significantly attenuated by HSP70Ab preconditioning (Fig. 5).

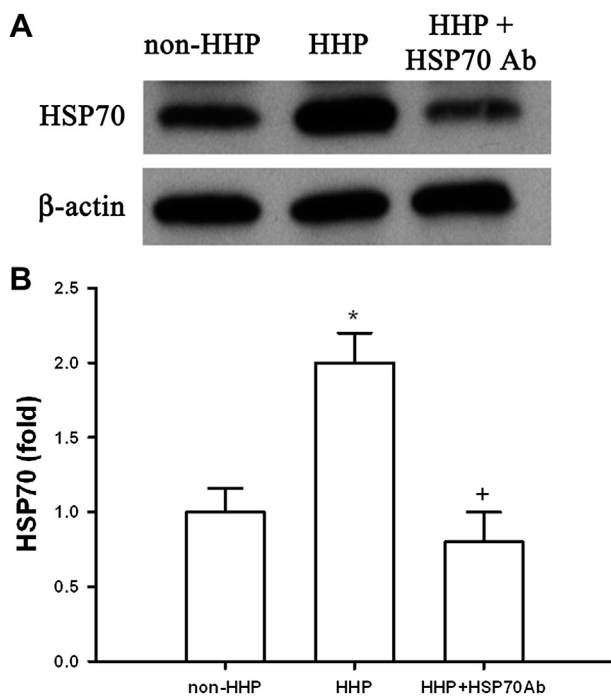


Figure 2 (A) Immunoblot of HSP70. (B) Western blot analysis of HSP70 in the liver of non-HHP rats ($n = 8$), HHP rats ($n = 8$), and HHP+HSP70Ab rats ($n = 8$). Fold-change values represent a mean of eight samples ($n = 8$) divided by the mean of the eight controls ($n = 8$). Data are presented as means \pm SD. * $p < 0.01$ for the HHP group versus the non-HHP group. + $p < 0.01$ for the HHP+HSP70Ab group versus the HHP group. HHP = hypobaric hypoxia preconditioning; HSP70 = heat shock protein 70; HSP70Ab = heat shock protein 70 antibody.

Discussion

The reduction in barometric pressure and the consequent fall in the PaO_2 at higher altitudes lead to hypobaric hypoxia. If the hypoxia is severe or sustained, as shown in the present results, a drop of the oxygen saturation in multiple organs is inevitable leading to MODS. Indeed, as demonstrated in the current study, 24 hours after the start of HAE, the non-HHP animals displayed hypoxia, hypotension, bradycardia, acidosis, and tissue upregulation of both the systemic inflammatory markers including TNF- α , soluble intercellular adhesion molecule-1, E-selectin, IL-1 β , IL-6, and myeloperoxidase, and the toxic organ injury indicators including NO_x , DHBA, and LDH. In addition, our previous finding showed that directly after being taken out to the ambient from exposure to simulated HAE of 6000 m in a hypobaric chamber for 24 hours, the non-HHP animals had higher scores of acute lung injury, acute pleurisy, increased proinflammatory cytokines, and increased cellular ischemia and oxidative damage markers in the bronchoalveolar fluid.¹⁵ Furthermore, HAE caused brain

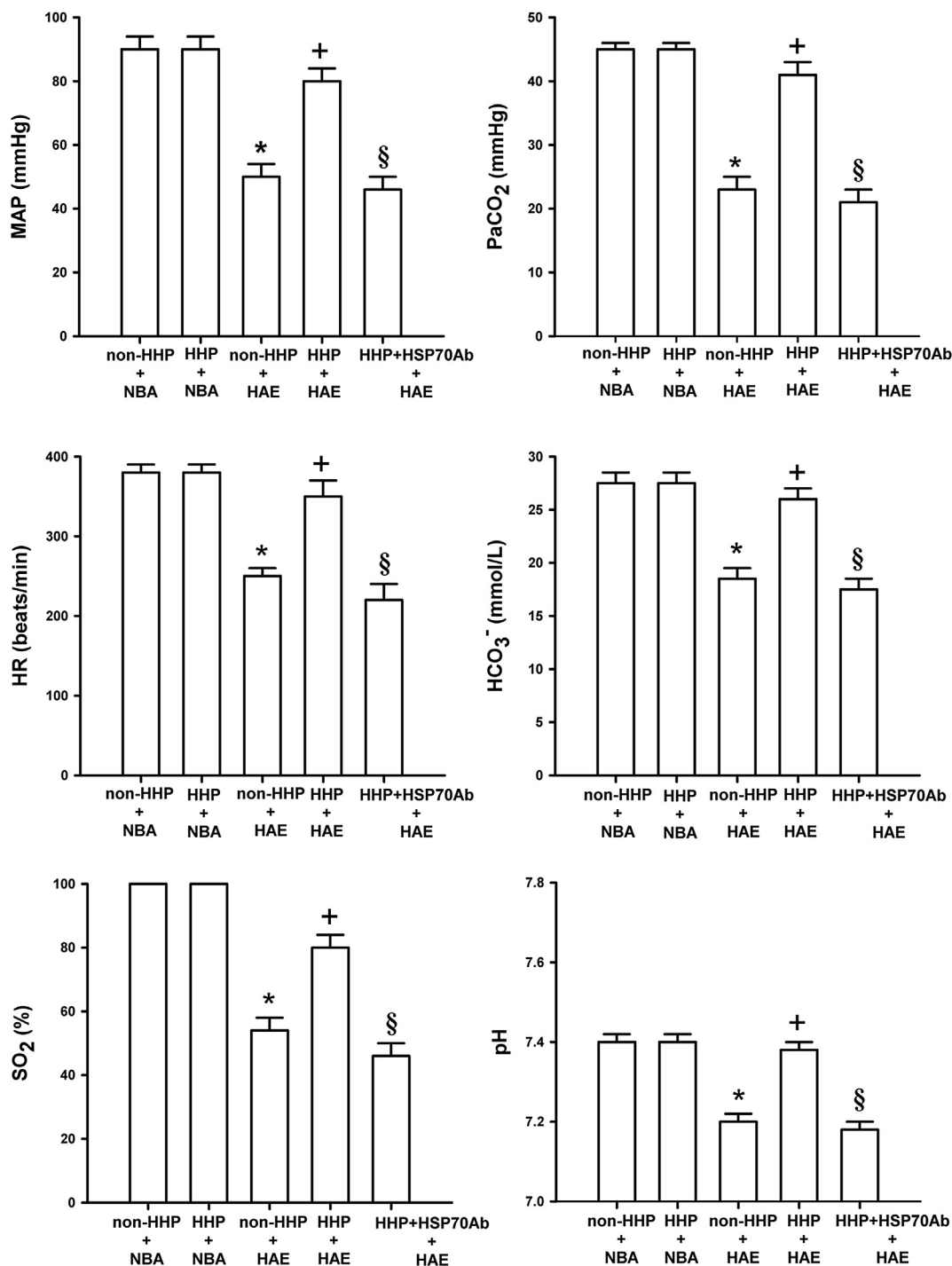


Figure 3 Values of mean arterial pressure (MAP), heart rate (HR), and the percentage of oxygen saturation (SO₂%), PaCO₂, HCO₃⁻, and pH in the blood for the (non-HHP+NBA) group, (HHP+NBA) group, (non-HHP+HAE) group, (HHP+HAE) group, and (HHP+HSP70Ab+HAE) group obtained at the end of HAE or the equivalent time period for the NBA group. Data are presented as means ± SD of *n* = 8 each group. * *p* < 0.05 for the (non-HHP+HAE) group versus the (non-HHP+NBA) group. + *p* < 0.05 for the (HHP+HAE) group versus (non-HHP+HAE) group; § *p* < 0.05 for (HHP+HSP70Ab+HAE) group. HAE = high-altitude exposure; HHP = hypobaric hypoxia preconditioning; HSP70Ab = heat shock protein 70 antibody; NBA = normobaric air.

edema, hippocampal oxidative damage and cognitive dysfunction in rats.¹⁶ Putting these observations together, it can be inferred that MODS can be induced by HAE in rats. This statement is at least in part consistent with the current concept that the acute adult syndromes of high altitude are

acute mountain sickness, HAPE and high-altitude cerebral oedema.³⁰ The present data also support the idea that human responses to the hypobaric hypoxia of high altitude may be used as a means of exploring elements of the pathophysiology of critical illness.³⁰

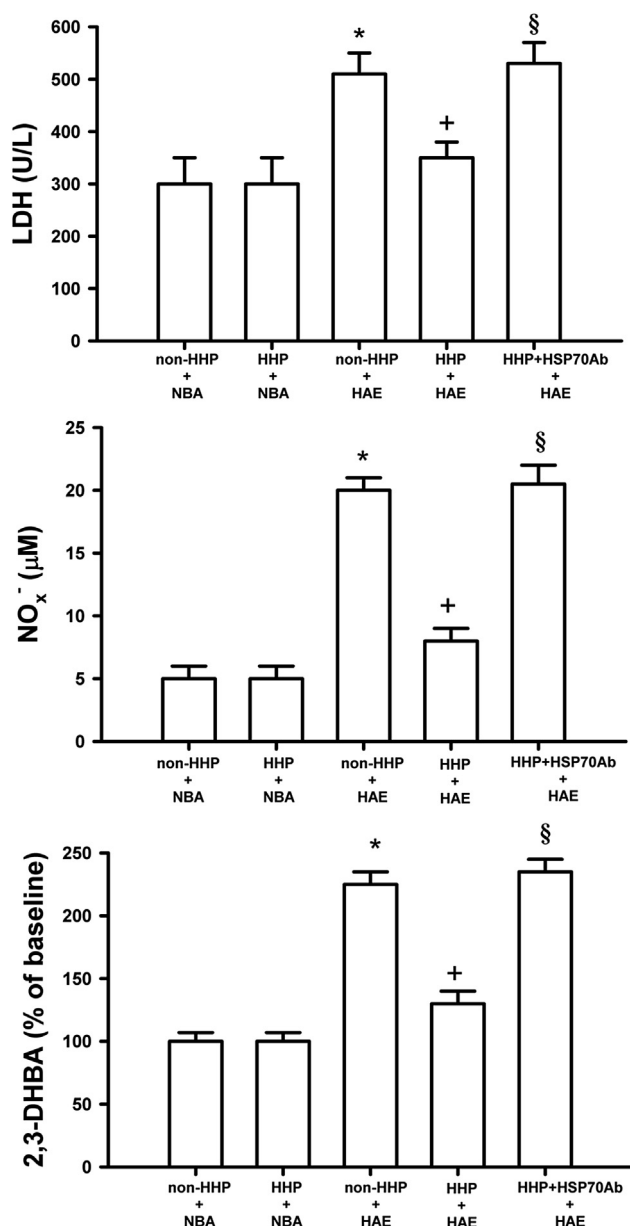


Figure 4 Values of serum LDH, NO_x, and 2,3-DHBA for the (non-HHP+NBA) group, (HHP+NBA) group, (non-HHP+HAE) group, (HHP+HAE) group, and (HHP+HSP70Ab+HAE) group obtained at the end of HAE or the equivalent time period for the NBA group. Data are presented as means \pm SD of $n = 8$ for each group. * $p < 0.05$ for the (non-HHP+HAE) group versus the (non-HHP+NBA) group. + $p < 0.05$ for the (HHP+HAE) group versus the (non-HHP+HAE) group. § $p < 0.05$ for the (HHP+HSP70Ab+HAE) group versus the (HHP+HAE) group. DHBA = dihydroxybenzoic acid; HAE = high-altitude exposure; HHP = hypobaric hypoxia preconditioning; HSP70Ab = heat shock protein 70 antibody; LDH = lactate dehydrogenase; NBA = normobaric air; NO_x = nitric oxide metabolites.

It is well documented that HSP70 induced by physiologic and pathological stress protects against subsequent damage via increasing the tolerance of affected tissues.^{31,32} Chronic hypoxia improved acute myocardial stress,³³ severe

sepsis,³⁴ ischemic penumbra,^{35,36} and heatstroke^{37,38} by upregulating HSP70. Indeed, as shown in the present results, the (HHP+NBA) group rats shared with the (non-HHP+NBA) group rats the same levels of the physiological parameters, the systemic inflammatory markers and the toxic organ injury indicators. However, compared with those of the (non-HHP+NBA) or the (HHP+NBA) group rats, the (HHP+HAE) group rats had significantly higher levels of both the systemic inflammatory markers and the toxic organ injury indicators. HHP, in addition to inducing HSP70 in multiple organs significantly attenuated the HAE-induced increased tissue levels of both the systemic inflammatory markers and the toxic organ injury indicators. Our results showed that preinduction of HSP70 protein with a sublethal dose of HAE (e.g., HHP: 18.3% O₂ at 0.66 ATA) were able to protect against a subsequent lethal damage exerted by a lethal dose of HAE (9.7% O₂ at 0.47 ATA).

The beneficial effects of HHP in preventing the occurrence of the increased systemic inflammatory markers and the toxic organ injury indicators could be significantly attenuated by HSP70Ab preconditioning. Thus, it appears that HHP may attenuate HAE-induced systemic inflammation and MODS by upregulating HSP70 in multiple organs. Again, our current results are supported by the findings of Zhang et al.³⁹ Their results suggested that administration of geranylgeranylacetone prevented acute hypoxic damage to the brain and that the underlying mechanism involved induction of HSP70.³⁹ Recently, it has been shown that intravenously administered HSP70 neutralizing Abs bind to the intracellular pool of HSP70 antigen in the neurons and significantly attenuate the expression of HSP70 in brain tissue.⁸ In the present study, we followed the same regimen as those of Liebelt et al.⁸ and observed that HHP induced a 100% higher expression of HSP70. Inhibition of this HSP70 expression with Ab, which might affect the intracellular pool of HSP70 protein, blocked HAE-induced increased levels of the systemic inflammatory markers as well as the toxic organ injury indicators. In future studies, it would be worthwhile assessing what is the line between HHP and HAE. That is, to bring the protective effect of HHP on HAE, to what extent is this strategy effective rather than also detrimental?

Various serum molecules like TNF- α , IL-1 β , ICAM-1 and E-selectin were shown to be related to the pathophysiology of systemic inflammatory response syndrome.^{40,41} As shown in the present study, the full spectrum of the systemic inflammatory response syndrome molecules could be induced by HAE. HHP, in addition to inducing both HSP70 and IL-10, significantly ameliorated the HAE-induced increased serum systemic inflammatory syndrome molecules. It should be stressed that the serum levels of IL-10 are believed to be an antiinflammatory cytokine.⁴² It is possible that HHP-induced overexpression of HSP70 may downregulate the production or release of these systemic inflammatory markers but upregulate the production or release of serum IL-10. Inhibition of HSP70 activity by HSP70Ab preconditioning abolished the beneficial effects of HHP in HAE. Although the aforementioned observations tended to support the causal role played by activated inflammation in the pathogenesis of HAE-induced MODS, prospective human studies measuring proinflammatory cytokines in plasma and other related parameters found no

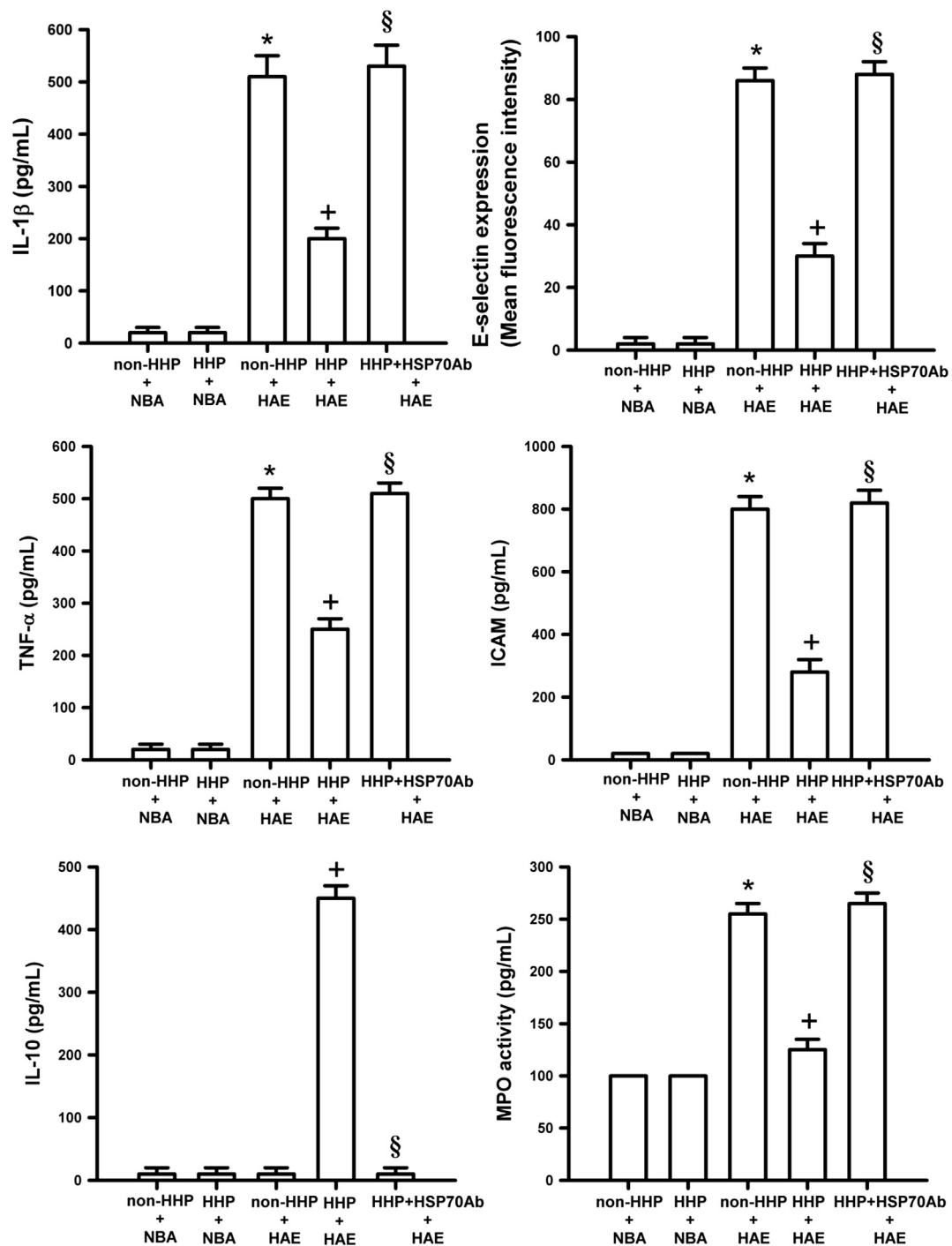


Figure 5 Values of serum IL-1 β , TNF- α , IL-10, E-selectin, ICAM-1, and liver MPO activity for the (non-HHP+NBA) group, (HHP+NBA) group, (non-HHP+HAE) group, (HHP+HAE) group, and (HHP+HSP70Ab+HAE) group obtained at the end of HAE or the equivalent time period for the NBA group. Data are presented as mean \pm SD of $n = 8$ for each group. * $p < 0.05$ for the (non-HHP+HAE) group versus the (non-HHP+NBA) group. + $p < 0.05$ for the (HHP+HAE) group versus the (non-HHP+HAE) group. § $p < 0.05$ for the (HHP+HSP70Ab+HAE) group versus the (HHP+HAE) group. HAE = high-altitude exposure; HHP = hypobaric hypoxia preconditioning; HSP70Ab = heat shock protein 70 antibody; ICAM-1 = intercellular adhesion molecule-1; IL = interleukin; MPO = myeloperoxidase; NBA = normobaric air; TNF = tumor necrosis factor.

evidence for inflammation before or with the onset of HAPE.^{43,44} Swenson et al.⁵ further demonstrated that early HAPE was characterized by pulmonary hypertension that led to pulmonary edema, with normal levels of leukocytes,

cytokines and eicosanoids. Besides inflammation, other parameters like hypoxia, hypotension, bradycardia, acidosis, and overproduction of toxic-oxidizing radicals may be related to the occurrence of HAPE.

It was reported that hypoxia caused excitotoxicity of cells by activating nitric oxide synthase and generating NO_x .⁴³ Generation of NO_x and 2,3-DHBA after hypoxia could cause protein oxidation,⁴⁴ lipid oxidation,⁴⁵ or cell death. Maiti and colleagues⁴⁶ showed that an increase in 2,3-DHBA and NO_x levels was noted in the rat brain which was exposed to simulated high altitude equivalent to 6100 m in animal decompression chamber for 3–7 days. Our present results also showed that the non-HHP animals had increased 2,3-DHBA and NO_x levels in serum after simulated HAE of 6000 m for 24 hours. 2,3-DHBA and NO_x are two well-known markers of cellular oxidative damage.^{47,48} The organ injury indicator like LDH^{17,18} in serum was also increased in our animals 24 hours after HAE. Additionally, the beneficial effects of HHP in reducing cellular oxidative damage to multiple organs could be significantly attenuated by HSP70Ab preconditioning.

The results obtained here show that HHP in addition to inducing overproduction of HSP70 and IL-10, protects against the MODS that occurred during simulated HAE of 6000 m for 24 hours in rats. The beneficial effects of HHP may be attributed to the inhibition of: (1) production or release of systemic inflammatory response markers including serum TNF- α , IL-1 β , ICAM-1, and E-selectin and liver myeloperoxidase activity; (2) production or release of the toxic organ injury indicators including NO_x , 2,3-DHBA and LDH; and (3) hypoxia, hypotension, bradycardia, and acidosis, and thus, leading to an improvement in rats with HAE-associated MODS. The beneficial effects of HHP in preventing the occurrence of HAE-associated MODS could be significantly reduced by HSP70Ab preconditioning. It is inferred that HHP may attenuate the occurrence of the MODS caused by HAE by upregulating HSP70 in rats.

Evidence has accumulated to indicate that hypoxic preconditioning protects against ischemic brain injury in animals.^{49–52} Our previous¹⁵ and present results also show that hypoxic preconditioning may be beneficial for preventing overproduction of both the systemic inflammatory markers and the toxic organ injury indicators in HAE. In fact, the most popular preventive approach for HAPE is gradual ascending,⁵³ which is actually a type of hypoxia preconditioning. However, it has also been found that hyperbaric oxygen preconditioning (HBO_2P) elicits similar preconditioning efficacy in reducing ischemic brain damage.⁵⁴ Although HHP possesses significant protection in preventing tissue damage, it has not been used clinically because of safety concerns. In contrast, HBO_2P is more attractive and easily accepted ethically because it has been used safely for various disorders.^{55–57} Recently, we have demonstrated reducing pulmonary injury by HBO_2P ¹⁴ or HHP¹⁵ during simulated HAE in rats. Preinduction of HSP70 in various organs including brain¹⁶ and lung,¹⁵ respectively, by HBO_2P and HHP is found to be beneficial in improving the outcome of HAE in rats. Our present results further show that attenuating systemic inflammatory markers in simulated HAE by HSP70-mediated HHP in rats. Putting these observations together, it appears that HHP or HBO_2P attenuates HAE-induced tissue injury by preinduction of HSP70 in multiple organs. HBO_2P increased tissue oxygen levels by giving 100% oxygen at higher pressure. After HBO_2P therapy, the body experiences relative hypoxia because the oxygen level was returned to a normal level of

21%. Therefore, repeating HBO_2P therapy may produce a cycle of hyperoxia and then hypoxia, and lead to hypoxia-inducing factor-1 α (HIF-1 α) accumulation. Several studies reported that HBO_2 therapy induced hypoxic tolerance by upregulating HIF-1 α and its downstream genes.^{58–60} Thus, it appears that both HBO_2P and HHP improve the outcome of HAE by preinduction of both HSP70 and HIF-1 α in various organs at least.

This experiment has several limitations. First, our study used very low numbers of animals. Second, the rat model of HAE resembles the physiologic response of a primate only to a certain point. It cannot predict exactly the human response by extrapolation of data from our rat model. Our approach is promising and deserves further preclinical and clinical research to explore elements of the pathophysiology of critical illness.

It should be mentioned that we have previously demonstrated that a sublethal dose of heat shock (42 °C for only 15 minutes) induced HSP70 protein overexpression in the tissue that was detected 4 hours after treatment.⁶¹ This heat treatment did not cause heatstroke syndromes (e.g., hypotension and reduced survival). In contrast, a lethal dose of heat shock (43 °C for 60 minutes) did not induce HSP70 overexpression but caused heatstroke syndromes. Heat shock preconditioning (42 °C for 15 minutes) induced HSP70 which was correlated well with anatomical histochemical, and hemodynamic protection in heatstroke. It is likely that HHP, but not HAE, induced overexpression of tissue HSP70 which was correlated with protection in high mountain sickness.

In summary, the current study demonstrates that preinduction of HSP70 in various organs with a sublethal dose of HAE (e.g., HHP: 18.3% O_2 at 0.66 ATA for 5 h/day on 5 days consecutively for 2 weeks) protects against a subsequent lethal damage (e.g., increased levels of both the systemic inflammatory markers and the toxic organ injury indicators) exerted by a lethal dose of HAE (9.7% O_2 at 0.47 ATA for 24 hours).

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References

1. Sartori C, Rimoldi SF, Scherrer U. Lung fluid movement in hypoxia. *Prog Cardiovasc Dis* 2010;**52**:493–6.
2. Bärtsch P, Shaw S, Franciolli M, Gnädinger MP, Weidmann P. Atrial natriuretic peptide in acute mountain sickness. *J Appl Physiol* 1988;**65**:1929–37.
3. Scherrer U, Vollenweider L, Delabays A, Bärtsch P. Inhaled nitric oxide for high-altitude pulmonary edema. *N Engl J Med* 1996;**334**:624–9.
4. Busch T, Bärtsch P, Pappert D, Grünig E, Hildebrandt W, Elser H, et al. Hypoxia decreases exhaled nitric oxide in mountaineers susceptible to high-altitude pulmonary edema. *Am J Respir Crit Care Med* 2001;**163**:368–73.
5. Swenson ER, Maggiorini M, Mongovin S, Gibbs JS, Greve I, Mairbäurl H, et al. Pathogenesis of high-altitude pulmonary

- edema: inflammation is not an etiologic factor. *J Am Med Assoc* 2002;**287**:2228–35.
6. Stream JO, Grissom CK. Update on high-altitude pulmonary edema: pathogenesis, prevention, and treatment. *Wilderness Environ Med* 2008;**19**:293–303.
 7. Bailey DM, Dehnert C, Luks AM, Menold E, Castell C, Schendler G, et al. High-altitude pulmonary hypertension is associated with a free radical-mediated reduction in pulmonary nitric oxide bioavailability. *J Physiol* 2010;**588**:4837–47.
 8. Schoene RB, Hackett PH, Henderson WR, Sage EH, Chow M, Roach RC, et al. High-altitude pulmonary edema. Characteristics of lung lavage fluid. *JAMA* 1986;**256**:63–9.
 9. Schoene RB, Hackett PH, Henderson WR, Sage EH, Chow M, Roach RC, et al. The lung at high altitude: bronchoalveolar lavage in acute mountain sickness and pulmonary edema. *J Appl Physiol* 1988;**64**:2605–13.
 10. Naldini A, Carraro F, Silvestri S, Bocci V. Hypoxia affects cytokine production and proliferative responses by human peripheral mononuclear cells. *J Cell Physiol* 1997;**173**:335–42.
 11. Leeper-Woodford SK, Detmer K. Acute hypoxia increases alveolar macrophage tumor necrosis factor activity and alters NF-kappaB expression. *Am J Physiol* 1999;**276**:L909–16.
 12. Ali MH, Schlidt SA, Chandel NS, Hynes KL, Schumacker PT, Gewertz BL. Endothelial permeability and IL-6 production during hypoxia: role of ROS in signal transduction. *Am J Physiol* 1999;**277**:L1057–65.
 13. Yan SF, Tritto I, Pinsky D, Liao H, Huang J, Fuller G, et al. Induction of interleukin 6 (IL-6) by hypoxia in vascular cells. Central role of the binding site for nuclear factor-IL-6. *J Biol Chem* 1995;**270**:11463–71.
 14. Li Z, Gao C, Wang Y, Liu F, Ma L, Deng C, et al. Reducing pulmonary injury by hyperbaric oxygen preconditioning during simulated high altitude exposure in rats. *J Trauma* 2011;**71**:673–9.
 15. Lin HJ, Wang CT, Niu KC, Gao C, Li Z, Lin MT, et al. Hypobaric hypoxia preconditioning attenuates acute lung injury during high altitude exposure in rats via up-regulating heat shock protein-70. *Clin Sci* 2011;**121**:223–31.
 16. Lin H, Chang CP, Lin HJ, Lin MT, Tsai CC. Attenuating brain edema, hippocampal oxidative stress, and cognitive dysfunction in rats using hyperbaric oxygen preconditioning during simulated high altitude exposure. *J Trauma* 2012;**72**:1220–7.
 17. Wu JY, Tsou MY, Chen TH, Chen SJ, Tsao CM, Wu CC. Therapeutic effects of melatonin on peritonitis-induced septic shock with multiple organ dysfunction syndrome in rats. *J Pineal Res* 2008;**45**:106–16.
 18. Srinivasan V, Spence DW, Moscovitch A, Pandi-Perumal SR, Trakht I, Brown GM, et al. Malaria: therapeutic implications of melatonin. *J Pineal Res* 2010;**48**:1–8.
 19. Cervós-Navarro J, Diemer NH. Selective vulnerability in brain hypoxia. *Crit Rev Neurobiol* 1991;**6**:149–82.
 20. Monge C, León-Velarde F. Physiological adaptation to high altitude: oxygen transport in mammals and birds. *Physiol Rev* 1991;**71**:1135–72.
 21. Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, et al. 'Ischemic tolerance' phenomenon found in the brain. *Brain Res* 1991;**528**:21–4.
 22. Samoilov MO, Lazarevich EV, Semenov DG, Mokrushin AA, Tyul'kova EI, Romanovskii DY, et al. The adaptive effects of hypoxic preconditioning of brain neurons. *Neurosci Behav Physiol* 2003;**33**:1–11.
 23. Chang C, Chang AY, Chan SH. Neuroprotective role of heat shock protein 70 in the rostral ventrolateral medulla during acute mevinphos intoxication in the rat. *J Biomed Sci* 2004;**11**:748–55.
 24. Chou YT, Lai ST, Lee CC, Lin MT. Hypothermia attenuates circulatory shock and cerebral ischemia in experimental heatstroke. *Shock* 2003;**19**:388–93.
 25. Yamada K, Nabeshima T. Simultaneous measurement of nitrite and nitrate levels as indices of nitric oxide release in the cerebellum of conscious rats. *J Neurochem* 1997;**68**:1234–43.
 26. Togashi H, Mori K, Ueno K, Matsumoto M, Suda N, Saito H, et al. Consecutive evaluation of nitric oxide production after transient cerebral ischemia in the rat hippocampus using in vivo brain microdialysis. *Neurosci Lett* 1998;**240**:53–7.
 27. Obata T. Use of microdialysis for in-vivo monitoring of hydroxyl free-radical generation in the rat. *J Pharm Pharmacol* 1997;**49**:724–30.
 28. Yang CY, Lin MT. Oxidative stress in rats with heatstroke-induced cerebral ischemia. *Stroke* 2002;**33**:790–4.
 29. Mullane KM, Kraemer R, Smith B. Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. *J Pharmacol Methods* 1985;**14**:157–67.
 30. Grocott M, Montgomery H, Vercueil A. High-altitude physiology and pathophysiology: implications and relevance for intensive care medicine. *Crit Care* 2007;**11**:203. review.
 31. Giffard RG, Yenari MA. Many mechanisms for hsp70 protection from cerebral ischemia. *J Neurosurg Anesthesiol* 2004;**16**:53–61. review.
 32. Matsumori Y, Hong SM, Aoyama K, Fan Y, Kayama T, Sheldon RA, et al. Hsp70 overexpression sequesters AIF and reduces neonatal hypoxic/ischemic brain injury. *J Cereb Blood Flow Metab* 2005;**25**:899–910.
 33. Das DK, Maulik N, Moraru II. Gene expression in acute myocardial stress. Induction by hypoxia, ischemia, reperfusion, hyperthermia and oxidative stress. *J Mol Cell Cardiol* 1995;**27**:181–93. review.
 34. Bruemmer-Smith S, Stüber F, Schroeder S. Protective functions of intracellular heat-shock protein (HSP) 70-expression in patients with severe sepsis. *Intensive Care Med* 2001;**27**:1835–41.
 35. Weinstein PR, Hong S, Sharp FR. Molecular identification of the ischemic penumbra. *Stroke* 2004;**35**:2666–70.
 36. Fei G, Guo C, Sun HS, Feng ZP. Chronic hypoxia stress-induced differential modulation of heat-shock protein 70 and presynaptic proteins. *J Neurochem* 2007;**100**:50–61.
 37. Wen HC, Lee CC, Lee WC, Huang KS, Lin MT. Chronic hypoxia preconditioning increases survival in rats suffering from heatstroke. *Clin Exp Pharmacol Physiol* 2002;**29**:435–40.
 38. Wang LC, Chio CC, Wu MH, Wu MH, Lee YS, Huang CY, et al. Hypobaric hypoxia preconditioning attenuates experimental heatstroke syndromes via preinduction of heat shock protein 70. *Am J Med Sci* 2012;**344**:383–90.
 39. Zhang K, Zhao T, Huang X, Liu ZH, Xiong L, Li MM, et al. Preinduction of HSP70 promotes hypoxic tolerance and facilitates acclimatization to acute hypobaric hypoxia in mouse brain. *Cell Stress Chaperones* 2009;**14**:407–15.
 40. Morecroft JA, Spitz L. The role of inflammatory mediators in necrotizing enterocolitis. *Semin Neonatol* 1997;**2**:273–80.
 41. Boldt J, Wollbrück M, Kuhn D, Linke LC, Hempelmann G. Do plasma levels of circulating soluble adhesion molecules differ between surviving and nonsurviving critically ill patients? *Chest* 1995;**107**:787–92.
 42. Standiford TJ, Strieter RM, Lukacs NW, Kunkel SL. Neutralization of IL-10 increases lethality in endotoxemia. Cooperative effects of macrophage inflammatory protein-2 and tumor necrosis factor. *J Immunol* 1995;**155**:2222–9.
 43. Kleger GR, Bärtsch P, Vock P, Heilig B, Roberts 2nd LJ, Ballmer PE. Evidence against an increase in capillary permeability in subjects exposed to high altitude. *J Appl Physiol* 1996;**81**:1917–23.
 44. Bärtsch P, Eichenberger U, Ballmer PE, Gibbs JS, Schirilo C, Oelz O, et al. Urinary leukotriene E(4) levels are not increased prior to high-altitude pulmonary edema. *Chest* 2000;**117**:1393–8.

45. Butterfield DA, Lauderback CM. Lipid peroxidation and protein oxidation in Alzheimer's disease brain: potential causes and consequences involving amyloid beta-peptide-associated free radical oxidative stress. *Free Radic Biol Med* 2002;**32**:1050–60.
46. Maiti P, Singh SB, Sharma AK, Muthuraju S, Banerjee PK, Ilavazhagan G. Hypobaric hypoxia induces oxidative stress in rat brain. *Neurochem Int* 2006;**49**:709–16.
47. Hsiao SH, Chang CP, Chiu TH, Lin MT. Resuscitation from experimental heatstroke by brain cooling therapy. *Resuscitation* 2007;**73**:437–45.
48. Hsu SF, Niu KC, Lin CL, Lin MT. Brain cooling causes attenuation of cerebral oxidative stress, systemic inflammation, activated coagulation, and tissue ischemia/injury during heatstroke. *Shock* 2006;**26**:210–20.
49. Gidday JM, Fitzgibbons JC, Shah AR, Park TS. Neuroprotection from ischemic brain injury by hypoxic preconditioning in the neonatal rat. *Neurosci Lett* 1994;**168**:221–4.
50. Matsushima K, Hakim AM. Transient forebrain ischemia protects against subsequent focal cerebral ischemia without changing cerebral perfusion. *Stroke* 1995;**26**:1047–52.
51. Vannucci RC, Towfighi J, Vannucci SJ. Hypoxic preconditioning and hypoxic-ischemic brain damage in the immature rat: pathologic and metabolic correlates. *J Neurochem* 1998;**71**:1215–20.
52. Wagner BP, Nedelcu J, Martin E. Delayed postischemic hypothermia improves long-term behavioral outcome after cerebral hypoxia-ischemia in neonatal rats. *Pediatr Res* 2002;**51**:354–60.
53. Maggiorini M. High altitude-induced pulmonary oedema. *Cardiovasc Res* 2006;**72**:41–50.
54. Towne JE, Harrod KS, Krane CM, Menon AG. Decreased expression of aquaporin (AQP)1 and AQP5 in mouse lung after acute viral infection. *Am J Respir Cell Mol Biol* 2000;**22**:34–44.
55. Hu SL, Hu R, Li F, Liu Z, Xia YZ, Cui GY, et al. Hyperbaric oxygen preconditioning protects against traumatic brain injury at high altitude. *Acta Neurochir Suppl* 2008;**105**:191–6.
56. Niu KC, Lin MT, Chang CP. Hyperbaric oxygen improves survival in heatstroke rats by reducing multiorgan dysfunction and brain oxidative stress. *Eur J Pharmacol* 2007;**569**:94–102.
57. Ostrowski RP, Graupner G, Titova E, Zhang J, Chiu J, Dach N, et al. The hyperbaric oxygen preconditioning-induced brain protection is mediated by a reduction of early apoptosis after transient global cerebral ischemia. *Neurobiol Dis* 2008;**29**:1–13.
58. Gu GJ, Li YP, Peng ZY, Xu JJ, Kang ZM, Xu WG, et al. Mechanism of ischemic tolerance induced by hyperbaric oxygen preconditioning involves upregulation of hypoxia-inducible factor-1alpha and erythropoietin in rats. *J Appl Physiol* 2008;**104**:1185–91.
59. Peng Z, Ren P, Kang Z, Du J, Lian Q, Liu Y, et al. Up-regulated HIF-1alpha is involved in the hypoxic tolerance induced by hyperbaric oxygen preconditioning. *Brain Res* 2008;**1212**:71–8.
60. Salhanick SD, Belikoff B, Orlow D, Holt D, Reenstra W, Buras JA. Hyperbaric oxygen reduces acetaminophen toxicity and increases HIF-1alpha expression. *Acad Emerg Med* 2006;**13**:707–14.
61. Yang YL, Lin MT. Heat shock protein expression protects against cerebral ischemia and monoamine overload in rat heatstroke. *Am J Physiol Heart Circ Physiol* 1999;**276**:H1961–7.