Heat stress attenuates air bubble-induced acute lung injury: a novel mechanism of diving acclimatization

Kun-Lun Huang,[1,2] Chin-Pyng Wu,[2] Yin-Li Chen,[1] Bor-Hwang Kang,[1] and Yu-Chong Lin[3]

[1]*Institute of Undersea and Hyperbaric Medicine, National Defense Medical Center, Taipei 114, Taiwan; and* [2]*Division of Pulmonary Medicine, Department of Internal Medicine, Tri-Service General Hospital, Taipei 114, Taiwan, Republic of China; and* [3]*Department of Physiology, University of Hawaii at Manoa, Manoa, Hawaii 96822*

Huang, Kun-Lun, Chin-Pyng Wu, Yin-Li Chen, Bor-Hwang Kang, and Yu-Chong Lin. Heat stress attenuates air bubble-induced acute lung injury: a novel mechanism of diving acclimatization. *J Appl Physiol* 94: 1485–1490, 2003. First published December 13, 2002; 10.1152/japplphysiol. 00952.2002.—Diving acclimatization refers to a reduced susceptibility to acute decompression sickness (DCS) in individuals undergoing repeated compression-decompression cycles. We postulated that mechanisms responsible for the acclimatization are similar to that of a stress preconditioning. In this study, we investigated the protective effect of prior heat shock treatment on air embolism-induced lung injury and on the incidence of DCS in rats. We exposed rats ($n = 31$) to a pressure cycle that induced signs of severe DCS in 48% of the rats, greater wet-to-dry ratio (W/D) of lung weight compared with the control group (5.48 ± 0.69 vs. 4.70 ± 0.17), and higher protein concentration in bronchoalveolar lavage (BAL) fluid (362 ± 184 vs. 209 ± 78 mg/l) compared with the control group. Rats with DCS expressed more heat shock protein 70 (HSP70) in the lungs than those without signs of disease. Prior heat shock ($n = 12$) increased the expression of HSP70 in the lung and attenuated the elevation of W/D of lung weight (5.03 ± 0.17) after the identical decompression protocol. Prior heat shock reduced the incidence of severe DCS by 23%, but this failed to reach statistical significant ($\chi^2 = 1.94$, $P = 0.163$). Venous air infusion (1.0 ml/40 min) caused profound hypoxemia (54.5 ± 3.8 vs. 83.8 ± 3.2 Torr at baseline; $n = 6$), greater W/D of lung weight (5.98 ± 0.45), and high protein concentration in BAL fluid (595 ± 129 mg/l). Prior heat shock ($n = 6$) did not alter the level of hypoxemia caused by air embolism, but it accelerated the recovery to normoxemia after air infusion was stopped. Prior heat shock also attenuated the elevation of W/D of lung weight (5.19 ± 0.40) and the increase in BAL protein (371 ± 69 mg/l) in air embolism group. Our results showed that the occurrence of DCS after rapid decompression is associated with increased expression of a stress protein (HSP70) and that prior heat shock exposure attenuates the air bubble-induced lung injury. These results suggest that bubble formation in tissues activates a stress response and that stress preconditioning attenuates lung injury on subsequent stress, which may be the mechanism responsible for diving acclimatization.

diving acclimatization; decompression sickness; air embolism; heat shock protein

DIVING ACCLIMATIZATION IS a phenomenon that occurs when individuals undergoing repeated compression-decompression cycles are able to reduce their susceptibility to acute decompression sickness (DCS). Postulated mechanisms for the acclimatization include depletion of gas micronuclei (34), desensitization (30), and decomplementation (29). These theories hypothesize that the acclimatization is due to consumption of offensive factors induced by "silent bubbles," which exist in tissues after decompression but do not lead to acute symptoms of DCS. However, attractive as this theory may be, rigorous studies are lacking to support the explanation of this phenomenon. Repetitive pressure exposures did not consume the plasma complement proteins (10, 26). Broome et al. (3) reported in an animal model of DCS that pretreatment with a soluble complement receptor failed to prevent DCS. In additon, the complement proteins of human divers were found to remain within normal ranges when they were in a regular diving schedule (12). Therefore, the "consumption theory" should be reexamined.

Preconditioning is a protective mechanism that occurs when prior sublethal stresses increase the ability of tissues to withstand subsequent insults, such as heat, ischemia, hypoxia, hypoglycemia, drugs, and inflammation. Ischemic preconditioning has been shown to protect the heart against myocardial infarction in several animal species (5, 22). Recovery from septic shock makes the animal more resistant to ischemia-reperfusion injury to the heart (25). Hyperthermic preconditioning profoundly attenuates cellular damage induced by a subsequent oxidative challenge in cultured endothelial cells (8). Furthermore, pretreatment with heat produces a "cross-tolerance" to various types of insults (9, 20). Evidence supports the involvement of heat shock proteins in many of these protective effects (2, 4, 15). Specific overexpression of heat shock protein 70 (HSP70) by gene transfer into pulmonary epithelium protected the rats from sepsis-induced lung injury and increased the animal survival rate (31). Although the mechanism of protection remains unknown, it might be associated with induction of protective cytokines (16).

Diving acclimatization protects divers from acute DCS in a pattern similar to the protective preconditioning. Silent bubbles occur during daily pressure exposures (12) and can be considered as a subsymptomatic stress. Repeated stress responses induced by silent bubbles is a form of preconditioning. We hereby propose an "induction theory" hypothesizing that repetitive daily diving is a form of preconditioning that reduces the severity of acute tissue injury caused by subsequent exposure to intravascular bubbles. The purpose of this study was to test this induction theory by using the animal models of DCS or air embolism-induced acute lung injury.

MATERIALS AND METHODS

Thermal preconditioning. All of the experimental procedures were in accordance with the *Guiding Principle in the Care and Use of Animals* approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing 300–350 g were lightly anesthetized by an intraperitoneal injection of pentobarbital sodium (25 mg/kg). Each animal was placed on the heating pad of a temperature control device (Homeothermic Pt-100, Dr Instruments, Taiwan, Republic of China), and the body temperature was measured via a rectal probe. A light bulb (100 W) was used to accelerate heating and quick adjustment of body temperature. The heat shock was induced by increasing the core temperature to 41°C for 15 min. The animals were then killed by an overdose of pentobarbital sodium at 4, 6, 16, and 24 h after heat shock treatment. The right upper lobe of the lungs was excised for the determination of heat shock protein.

Decompression sickness. The rats were placed in an acrylic hyperbaric chamber and were pressurized with air to 6 atmospheres absolute (ATA) for 2 h. The chamber was ventilated with compressed air at 15 l/min to maintain a low-CO_2 environment. Chamber temperature was maintained constant at 27°C. The animals were then decompressed at a rate of 2 ATA/min. After completing the decompression procedure, rats were examined for signs of DCS for 2 h. The symptoms observed included dragging of a hind leg(s), dyspnea, agitation, collapsing into unconsciousness, and death. The rats that died during the 2-h observation period were immediately evaluated for lung injury, and the lungs were excised for the analysis of HSP70. The surviving rats were evaluated at 4 h after the decompression. In the heat shock pretreatment group, rats were subjected to a compression-decompression cycle 4 h after the heat shock exposure. The control group received no hyperbaric exposure or heat shock pretreatment.

Pulmonary air embolism. Under general anesthesia with pentobarbital sodium (50 mg/kg ip), the animals underwent tracheotomy and cannulation to aid spontaneous breathing and to facilitate bronchoalveolar lavage (BAL) at the end of an experiment. The femoral vein was catheterized for infusing air. The femoral artery was catheterized for monitoring blood pressure and for blood sampling. For inducing pulmonary air embolism, we infused nitrogen gas via the femoral vein catheter at a rate of 25 μl/min for 20 or 40 min by using a Harvard infusion pump (Millis, MA). The total amount of air infused was 0.50 or 1.00 ml, respectively. We did not make an attempt to determine the size of the air bubbles in circulation in this study. However, air infusion to an isolated lung model generated air bubbles ranging from 0.4 to 0.5 mm in diameter (13, 14). Arterial blood was collected from the

femoral artery catheter in ice-chilled syringes for blood-gas analysis (IL-1610, Instrumentation Laboratory, Milano, Italy) before and during venous air infusion as well as at the end of each experiment. The animals were subjected to lung injury evaluation and HSP70 determination 40 min after the completion of air infusion. Rats in the control group ($n = 6$) received no air infusion or heat shock exposure but did receive anesthesia and arterial catheterization. In the other groups of rats ($n = 6$ in each group), the air embolism was induced 4 or 16 h after the heat shock treatment.

Evaluation of lung injury. At the end of each experiment, the rats were killed by an overdose of pentobarbital sodium and midline thoracotomy. The right lung was excised as the right hilum was clamped. The upper lobe was excised and stored at −20°C for heat shock protein determination. The remaining right lung was weighed and dried in a 60°C oven for 48 h. The dry weight was then measured to obtain the wet-to-dry ratio (W/D) of lung weight, an indicator of pulmonary edema (11, 19). BAL was performed to the left lung with 5 ml PBS in 2.5-ml aliquots after cannulation of the left bronchus. The recovered BAL fluid was centrifuged at 250 *g* for 10 min. The protein concentration of the supernatant was determined by using bicinchoninic acid protein assay reagents (Pierce, Rockford, IL).

Determination of heat shock protein. The expression of HSP70 was determined via the Western immunoblotting (4, 15). The harvested lung tissue was homogenized in cold lysis buffer (1 ml) and centrifuged at 12,000 *g* for 5 min at 4°C. The protein concentration in supernatant was quantified by using a Coomassie protein assay reagent (Pierce) and was diluted to a final concentration of 40 μg/20 μl. The protein was denatured in boiling water for 5 min, and the aliquots containing equal amounts of protein were suspended in SDS-glycerol loading buffer containing 12.5% Tris, 3% SDS, 20% glycerol, 5% mercaptoethanol, and 0.05% bromophenol blue. The proteins were separated by SDS-polyacrylamide gel electrophoresis (Mini-PROTEAN II, Bio-Rad, Milano, Italy) with 40 μg total protein loaded per lane. Proteins were then transferred to a polyvinylidene difluoride transfer membrane (Amersham Pharmacia Biotech, Taipei, Taiwan, Republic of China). Nonspecific binding to the membrane was blocked by 5% nonfat dry milk in PBS-Tween 20 overnight at 4°C. The blots were incubated with a primary monoclonal antibody (mouse anti-human IgG$_1$) specific for HSP70 (Jackson ImmunoResearch, West Groves, PA). The membrane was then subjected to five washes with PBS-Tween 20 and incubated with the secondary antibody (goat anti-mouse IgG, conjugated with horseradish peroxidase, dilution 1:1,000; Jackson ImmunoResearch) for 1 h at room temperature. The membranes were then developed with a 10-ml solution of the enhanced chemiluminescence detection system for 1 min and exposed to a film.

Statistical analysis. Data are expressed as means ± SD. The incidence of DCS after decompression was evaluated by using χ^2 test. The differences of W/D of lungs and BAL fluid analysis between groups were evaluated by using one-way ANOVA. The changes of arterial Po_2 (Pa_{O_2}) were evaluated by using ANOVA with repeated measures. When the variables were found different, a multiple-comparison test (Fisher's paired least significant difference) was performed. A value of $P < 0.05$ was accepted as significant.

RESULTS

Evidence of thermal preconditioning. Heat shock increased the expression of HSP70 in the lung tissue. The significant expression of HSP70 appeared as early
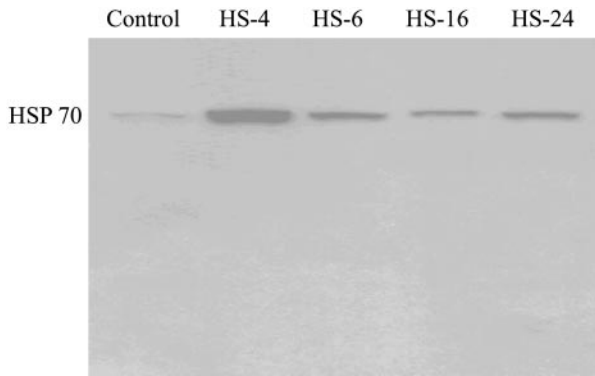
Fig. 1. Western immunoblotting for expression of heat shock protein 70 (HSP70) in the lung of rats exposed to hyperthermia. Lanes represent time course (-hours) of HSP70 expression after heat shock (HS).
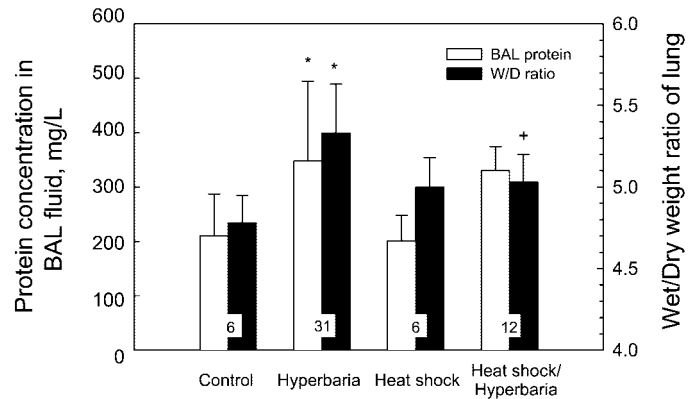

Fig. 2. Effect of prior heat shock on acute lung injury induced by rapid decompression after hyperbaric (6 atmospheres absolute) exposure for 2 h. Values are means ± SD. Nos. in bars are group sizes. BAL, bronchoalveolar lavage; W/D, wet-to-dry ratio. *$P < 0.05$ compared with the control group. +$P < 0.05$ compared with the hyperbaria group.

as 4 h after heat stress and was sustained for another 20 h (Fig. 1). On the basis of this result, the protection effect of prior heat shock in this study was tested 4 and/or 16 h after heat stress.

Effects of thermal preconditioning on DCS. Experiencing a compression-decompression cycle, 48% of rats (15 of 31) presented significant signs of DCS, including severe dyspnea, paralysis, and death (Table 1). In the rats that died within 2 h after the decompression, the chest wall was opened, revealing numerous air bubbles occupying the inferior vena cava. This incidence of DCS was not statistically different from that of 12 rats that received prior heat shock 4 h before the compression-decompression experiment, in which 25% of the animals ($n = 3$) showed severe DCS ($\chi^2 = 1.94$, $P = 0.163$).

Pressure exposure caused significantly higher W/D of lung weight (5.48 ± 0.69) and protein concentration in the BAL fluid (362 ± 184 mg/l) compared with the control group (4.70 ± 0.17 and 209 ± 78 mg/l, respectively). Prior heat shock attenuated the elevation in W/D of lung weight ($P < 0.05$) but not the increase in BAL fluid protein (Fig. 2). Heat shock by itself did not affect the W/D of lung or BAL protein concentration.

DCS and HSP70 expression. Four hours after decompression, the expression of HSP70 in the lung was higher in rats with severe signs of DCS than those without DCS (Fig. 3). The pressure cycle by itself, if no DCS occurred, increased the HSP70 expression only slightly compared with the control group. The HSP70 expression was similarly increased in rats with signs of DCS, either with or without prior heat shock treatment.

Effects of thermal preconditioning on pulmonary air embolism. Venous air infusion for 20 min decreased Pa_{O_2} by 39% from the baseline. The Pa_{O_2} returned gradually after air infusion was stopped, but it remained lower than the baseline at 40 min (Fig. 4). Doubling the infusion duration (40 min) did not cause further decrease in Pa_{O_2}, but it delayed the recovery of arterial oxygenation after cessation of air infusion. Prior heat shock did not alter the level of hypoxemia during ve-

Table 1. *Occurrence of signs of DCS in rats after rapid decompression from hyperbaric exposure of 6 ATA for 2 h*

	Without Heat Shock ($n = 31$)		Heat Shock Pretreatment ($n = 12$)	
Signs of DCS	No. of rats	Time, min	No. of rats	Time, min
Dyspnea	15(48%)	19 ± 8	3(25%)	22 ± 6
Dragging of hindlimbs	10(32%)	27 ± 10	1(8%)	25
Collapsing into unconsciousness	4(13%)	26 ± 13	1(8%)	30
Death	4(13%)	36 ± 16	1(8%)	45
Agitation and rolling	2(6%)	20 ± 7	0	
Total*	15(48%)		3(25%)	

Values of time are means ± SD; n, no. of rats/group. DCS, decompression sickness; ATA, atmospheres absolute. *Rats presenting ≥1 sign of DCS.
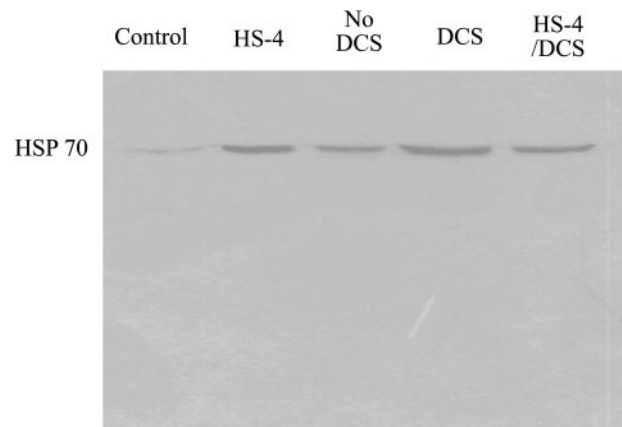

Fig. 3. Western immunoblotting for expression of HSP70 in the lung of rats after rapid decompression from 6 atmospheres absolute. HS-4, prior heat shock 4 h before HSP70 determination; No DCS, rats did not present signs of decompression sickness; DCS, rats presented signs of decompression sickness; HS-4/DCS, rats received prior heat shock 4 h before hyperbaric exposure and presented signs of DCS.
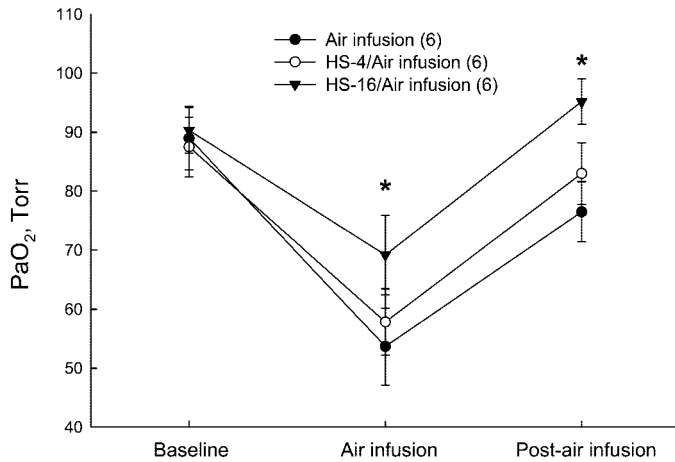
Fig. 4. Arterial hypoxemia induced by venous air infusion at rate of 25 μl/min for 20 min. HS-4/Air infusion and HS-16/Air infusion, prior heat shock 4 and 16 h, respectively, before air infusion. Pa_{O_2}, arterial P_{O_2}. Values are means ± SD. Nos. in parentheses are group sizes. *$P < 0.05$ compared with the group that received only air infusion.
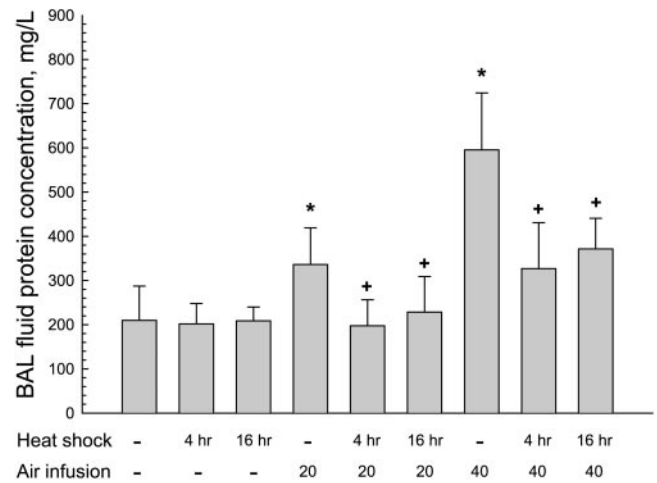

Fig. 6. BAL protein concentration in rats after venous air infusion. Values are means ± SD. *$P < 0.05$ compared with the control group that received no air infusion or prior heat shock treatment. +$P < 0.05$ compared with the groups that received only air infusion for respective durations.

nous air infusion, but it accelerated the recovery of oxygenation.

Air infusion for 20 and 40 min significantly increased the W/D of lung weight by 21 and 25%, respectively (Fig. 5). Prior heat shock significantly attenuated the increase of W/D of lung weight caused by air infusion for 40 min, but the attenuation was not statistically significant in the other group that received air infusion for 20 min. The protein concentration in BAL fluid was increased by 61 and 190% after air infusion for 20 and 40 min, respectively (Fig. 6). The increase in BAL protein concentration was significantly attenuated by prior heat shock.

DISCUSSION

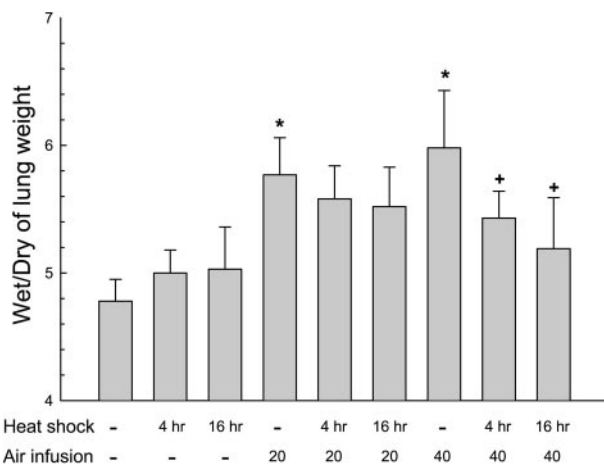Diving acclimatization has been described as an adaptive mechanism to decompression stress after re-

Fig. 5. W/D of lung weight in rats after venous air infusion. Values are means ± SD. -, Treatment not done; 20, 20 min; 40, 40 min. *$P < 0.05$ compared with the control group that received no air infusion or prior heat shock treatment. +$P < 0.05$ compared with the group that received only air infusion.

petitive pressure exposures. This adaptation reduces a diver's susceptibility to or severity of DCS. The mechanism contributing to the diving acclimatization, however, remains obscure. We propose an induction theory hypothesizing that repetitive compression-decompression is a form of preconditioning that generates protective factors and reduces the severity of acute tissue injury during the subsequent bubble formation. To test this hypothesis, we must obtain evidence showing, in an animal model, that 1) exposure to pressure cycles causes severe DCS and lung injury, 2) heat shock treatment induces expression of bioprotective factor such as HSP70, 3) prior induction of expressing such protective factors reduces the incidence or severity of DCS and lung injury, and 4) repeated pressure exposures should have the similar effects as prior heat shock.

In this study, we found that exposure to pressure cycle slightly increased the expression of HSP70 in the lungs. The occurrence of DCS enhanced the HSP70 expression to a level similar to that induced by heat shock exposure. The HSP70 is one of the main stress proteins induced by heat shock in mammals (32). Detection of HSP70 expression has become a standard to evaluate stress response and thermal preconditioning (21). HSP70 expression may be induced by a variety of stresses, including heat, ischemia, hypoglycemia, and drugs (2). The slightly increased HSP70 expression after pressure exposure in this study may be nonspecific. However, stress from the pressure exposure itself is one of the possibilities, leading to the silent bubbles that emerged after decompression. In contrast to this nonspecific increase, expression of HSP70 in the rats with severe DCS was disease related. Our results showed that rats with severe signs of DCS expressed much higher levels of HSP70 compared with those without DCS. This indicates that DCS by itself is able to induce a stress response.

Although prior heat shock significantly enhanced the expression of HSP70, it did not show a protective effect to the occurrence of DCS in our study. Forty-eight percent of the normal rats presented signs of severe DCS after pressure exposure, as opposed to 25% in the group with prior heat shock. Although the incidence of DCS was only one-half that of control rats, the difference was statistically insignificant. This suggested that induction of stress response could not reduce the decompression risk in rats. The insignificant protection against DCS may be due to an inadequate induction of HSP70 expression by heat shock, an indistinguishable high severity of DCS, or an unrelated mechanism between HSP70 expression and diving acclimatization. The protocol of heat shock used in this study is a well-documented method of inducing heat stress (2, 27). It has been shown that this heat pretreatment significantly attenuates tissue injuries induced by a variety of insults, such as cardiac surgery (18), sepsis (9, 28), and ischemia-reperfusion (15). The Western blot analysis showed that heat shock protocol used in this study sufficed to induce significant HSP70 expression. This indicates that the heat shock protocol did cause significant amount of HSP70 expression. It appears that the protocol of pressure exposure (6 ATA for 2 h) may induce symptoms of DCS that are too severe to be evaluated for the protection effects of heat shock pretreatment. By examining the index of pulmonary edema, we found that prior heat shock did attenuate the increase in W/D of lung weight induced by DCS. This suggested that there might be a protective effect of heat shock against the DCS-induced lung injury that could not be differentiated by the gross symptomatic indications. To detect the protective effects of prior heat shock, we need an animal model to induce a similar quantity of bubbles in the body and more quantitative tissue injury analysis.

Decompression sickness is an air bubble-induced tissue injury. Our result showed that air bubbles existed in the circulation of rats with severe signs of DCS. This further ensured the role of air bubble formation in causing tissue injury after decompression. Our laboratory has investigated the air bubble-induced lung injury in animals in vivo (11) as well as in an isolated and perfused lung model (13, 14). It was found that venous air embolism is a feasible animal model for evaluating lung injury caused by DCS. We, therefore, tested our hypothesis in an animal model of pulmonary air embolism. We found that venous air infusion caused an acute lung injury as shown in profound hypoxemia, increased protein concentration in BAL fluid, and elevated W/D of lung weight. Pretreatment with heat shock 4 h before air infusion significantly reduced the increase in BAL protein, although the reduction in W/D of lung weight was not statistically significant. The protective effects of thermal preconditioning became statistically significant as the air infusion was done 16 h after heat shock. These results suggested that thermal preconditioning protects the rats from air embolism-induced lung injury. However, the mecha-

nism of protection continues to require further investigation.

Air bubbles produce their effects by mechanical obstruction, altering the biochemical environment, or both. Bubble formation interrupts blood flow and compresses against or disrupts tissues. Air bubbles can also initiate an air-liquid interface reaction in tissues, which activates plasma proteins, including clotting factors, enzymes, and immunoglobulins (17). In addition, the complement system, polymorphonuclear leukocytes, and oxygen metabolites have been proven as factors that mediate the air bubble-induced tissue injury (1, 7, 13, 14, 29). Protection from air bubble-induced tissue injury may result from a smaller number (or size) of bubble formations or from less tissue reaction to air bubbles. Wisloff and Brubakk (33) reported that endurance exercise reduced bubble formation and increased survival in rats exposed to hyperbaric pressure. Although the mechanism has not been discussed, it may be due to a stress response induced by exercise. Endurance exercise is a stressor that increases the expression of HSP70 and may represent a powerful prevention agent against tissue injury in several models (6, 23, 24). These reports suggest that stresses such as endurance exercise can activate bioprotective mechanisms and may have a protective effect against DCS. It is not known whether heat shock pretreatment can reduce the bubble formation after rapid decompression from a hyperbaric environment. Nevertheless, we demonstrated that prior heat shock protects the lung from air bubble-induced injury in rats that received a constant amount of air infusion. This suggests that the protection involves mechanisms more than a reduction in bubble formation.

In summary, our results showed that DCS induced a stress response as evidenced by the expression of heat shock protein. Although prior heat shock did not reduce the incidence of acute DCS after hyperbaric exposure, it attenuated decompression-related lung injury. Prior heat shock prevented the animals from acute lung injury induced by pulmonary air embolism, in which the pathophysiology is similar to acute DCS. Therefore, we conclude that bubble formation in tissues after decompression can activate a stress response and that adaptation to repeated stress may be the mechanism responsible for the phenomenon of diving acclimatization.

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