Whole-body hypoxic preconditioning protects mice against acute hypoxia by improving lung function

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Zhang, Shelley XL., James J. Miller, David Gozal, and Yang Wang. Whole-body hypoxic preconditioning protects mice against acute hypoxia by improving lung function. *J Appl Physiol* 96: 392–397, 2004; 10.1152/japplphysiol.00829.2003.—Survival in severe hypoxia such as occurs in high altitude requires previous acclimatization, which is acquired over a period of days to weeks. It was unknown whether intrinsic mechanisms existed that could be rapidly induced and could exert immediate protection on unacclimatized individuals against acute hypoxia. We found that mice pretreated with whole-body hypoxic preconditioning (WHPC, 6 cycles of 10-min hypoxia-10-min normoxia) survived significantly longer than control animals when exposed to lethal hypoxia (5% O2, survival time of 33.2 ± 6.1 min vs. controls at 13.8 ± 2.1 min, n = 10, P < 0.005). This protective mechanism became operative shortly after WHPC and remained effective for at least 8 h. Accordingly, mice subjected to WHPC demonstrated improved gas exchange when exposed to sublethal hypoxia (7% O2, arterial blood Po2 of 49.9 ± 4.2 vs. controls at 39.7 ± 3.6 Torr, n = 6, P < 0.05), reduced formation of pulmonary edema (increase in lung water of 0.491 ± 0.111 vs. controls at 0.894 ± 0.113 mg/mg dry tissue, n = 10, P < 0.02), and decreased pulmonary vascular permeability (lung lavage albumin of 7.63 ± 0.63 vs. controls at 18.24 ± 3.39 mg/dl, n = 6–10, P < 0.025). In addition, the severity of cerebral edema caused by exposure to sublethal hypoxia was also reduced after WHPC (increase in brain water of 0.254 ± 0.052 vs. controls at 0.491 ± 0.034 mg/mg dry tissue, n = 10, P < 0.01). Thus WHPC protects unacclimatized mice against acute and otherwise lethal hypoxia, and this protection involves preservation of vital organ functions.

Pulmonary edema; vascular permeability; acclimatization

*HIGHLIGHTED TOPIC* | *Oxygen Sensing in Health and Disease*

HYPOXIA IS A FREQUENT CONDITION that not only develops during high-altitude sojourns but also occurs in many medical disorders. The major consequences of hypoxia are related to the limitation in cellular energy supply that it imposes through the arrest of glucose oxidation/phosphorylation and the resulting ATP depletion (13). Depending on the severity of the hypoxic insult, progressive intracellular acidosis and edema and subcellular organelle damage may eventually lead to cell loss, organ failure, and ultimately death of the entire organism (16, 34). The ubiquitous occurrence of hypoxia and its potential consequences have led to the development of intrinsic protective mechanisms. For example, individuals remaining at high altitudes for days or weeks become more tolerant to hypoxia. This phenomenon, known as acclimatization, is acquired via adaptive changes at biochemical, cellular, and molecular levels. During acclimatization, oxygen exchange in the lung is enhanced by increased pulmonary ventilation through increased sensitivity and stimulation of peripheral chemoreceptors (1, 18) and increased diffusing capacity due to increased exchange surface area (36). In parallel, oxygen delivery and utilization in peripheral tissues are improved by increased numbers of red blood cells through hypoxic upregulation of erythropoietin (9), decreased affinity of hemoglobin for oxygen due to accumulation of phosphate compounds inside red blood cells (27), increased vascularity in peripheral tissues through hypoxic angiogenesis (33), and increased oxygen utilization through upregulation of mitochondrial enzymes (17, 30). Furthermore, recent studies on Andean and Himalayan native populations have uncovered that some of the mechanistic components leading to hypoxic acclimatization have been incorporated into the genome of these highlander populations through selective transmission of beneficial genetic variants (5, 28). However, if the initial hypoxic insult is very severe or potentially lethal, the relatively slow processes of acclimatization would be ineffective in preserving survival. Thus the existence of rapidly recruitable defense mechanisms would be of great value in the preservation of organismal homeostasis. Indeed, it is now well established that mammalian organs become more resistant to a lethal ischemic insult after exposure to brief episodes of sublethal ischemia. This ubiquitous phenomenon was first described in the heart and has been termed ischemic preconditioning (23). Compared with the acclimatization processes described above, preconditioning is a fast-acting mechanism, being effective shortly after induction. Of note, similar levels of organ protection can be induced by hypoxic rather than ischemic preconditioning (8, 19, 32). Although the exact mechanisms underlying ischemic/hypoxic preconditioning are still being delineated, it is now recognized that they involve recruitment of complex cellular signaling cascades and downstream gene and protein regulatory processes. For example, the late phase of hypoxic/ischemic preconditioning in cardiac tissue involves activation of protein kinase C (24), Srf protein tyrosine kinase (25), and Janus kinase (40) signaling pathways; nuclear translocation of transcription factors such as NF-κB (41) and STATs (40); and, eventually, upregulation of inducible nitric oxide synthase (12, 37), neuronal nitric oxide synthase (38), and cylooxygenase 2 (31, 38).
The powerful beneficial effects afforded by acclimatization and preconditioning not only have been exploited in recent years by athletes and mountain climbers for improved exercise performance training (20, 26) but also have found interesting applications in the treatment of human diseases (4, 30) and the preservation of donor organs (7, 35). However, both acclimatization and preconditioning mechanisms have limitations. On the one hand, although acclimatization protects the entire organism against systemic hypoxia, the protection is acquired in a limited manner and can be insufficient to protect individual organs. Thus, if intrinsic mechanisms exist that can be rapidly induced and can, in turn, exert immediate and generalized protection to the whole organism against acute hypoxia, development of paradigms aimed at initiating and optimizing the occurrence of such phenomena could have far-reaching implications in health and disease.

The present study was designed to investigate whether whole-body hypoxic preconditioning (WHPC) of an intact animal conferred rapid protection against acute hypoxia and, if so, what might be the physiological basis for such protection. For this purpose, we preconditioned conscious mice with a brief course of intermittent hypoxia and tested whether this treatment improved their survival under lethal hypoxia. We also examined the efficacy of this treatment in maintaining normal function of vital organs under severe hypoxia. We report that mice preconditioned 2 h earlier survived significantly longer when exposed to lethal hypoxia compared with controls and that this protection was associated with reduced pulmonary vascular permeability, reduced pulmonary and cerebral edema, and improved gas exchange. These results demonstrate for the first time that an intrinsic protective mechanism can be rapidly induced to protect an unacclimatized organism against lethal systemic hypoxia.

METHODOLOGY

Animals. C57BL/6 (Taconic Farms, Germantown, NY) and ICR (Harlan, Indianapolis, IN) mice of 10–12 wk of age were used in this study. To avoid the confounding influence of sex hormones on multiple aspects of hypoxia biology, including psychological/behavioral and metabolic, only male animals were included in the study. All procedures were approved by the Animal Care and Use Committee of the University of Louisville.

Hypoxic exposure. Conscious mice were exposed to various hypoxic conditions in environmental chambers (OxyCycler, BioSpherix, Redfield, NY). The ambient O2 concentration within the chambers was continuously measured by an O2 analyzer and adjusted according to computerized profiles set for the experiments with the use of a computerized servo-controlled system as described previously (10). Ambient CO2 in the chambers was maintained at <0.03%, humidity at 40–50%, and temperature at 22–24°C. Mice were acclimatized in the chambers in each experiment and analyzed with a blood-gas analyzer (ABL 520, Radiometer America, Westlake, OH) to ensure accuracy of oxygen concentrations (Table 1). During all experiments, mice were given standard breeding rodent chow and water ad libitum.

Survival. Survival under lethal hypoxia was examined in control mice and mice pretreated with WHPC (groups 1 and 2, Fig. 1A). WHPC was administered by subjecting mice to six cycles of 10 min of hypoxia (8% O2)-10 min of normoxia (21% O2), after which animals were allowed to breathe room air for 2 h. Control mice were placed in an adjacent chamber for the same duration but breathed room air. Both groups were then exposed to lethal hypoxia (5% O2) for up to 1 h, and the time of death was defined as the time of the last gasping by three investigators (two of the investigators were blinded). The effect of WHPC on survival was also examined at 4, 8, and 24 h after the WHPC pretreatment (groups 3–5, Fig. 1A).

Blood gases. Blood gases were measured in control mice and mice pretreated with WHPC (groups 6 and 7, Fig. 1B) during both normoxic and hypoxic ventilation. For this purpose, control mice and mice subjected to WHPC 2 h earlier were anesthetized with intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg), tracheo-cannulated, and ventilated with room air at 110 breaths/min with a stroke volume of 0.01 ml/g, a peak inspiratory pressure of 12 cmH2O, and periodic sigh breaths via a rodent ventilator (ASV, Harvard Apparatus, Holliston, MA). A PE-10 catheter was placed into the left carotid artery for blood sampling and for recording systemic blood pressure. After a 10-min equilibrium, a blood sample was drawn for measurement of blood gases (ABL 520 gas analyzer, Radiometer). A hypoxic gas mixture containing 7% O2-93% N2 was then used to ventilate the animals for 4 h. At the end of the experiment, blood gases were measured again. This anesthetized, ventilated model had to be used because blood sampling from mice placed inside the environmental chambers was not feasible. Systolic arterial blood pressure was maintained at >75 mmHg during the experiment with fluid supplementation and blood transfusion.

Lung and brain water content. Wet-to-dry weight ratio was used as an index of tissue water content (2). Lung and brain tissues were collected from the following groups of mice (Fig. 1C): (1) control mice (group 8), (2) control mice exposed to sublethal hypoxia for 6 h (group 9), (3) mice subjected to WHPC 2 h earlier (group 10), and (4) mice subjected to WHPC 2 h earlier and then exposed to sublethal hypoxia for 6 h (group 11). Mice were killed and thoroughly exsanguinated before their lungs and brains were excised en bloc. Tissue samples were blot dried and placed in preweighed aluminum foil trays. The wet weight of the tissue was registered immediately with an electronic balance to an accuracy of 0.1 mg. The tray with the tissue was then baked in an oven at 65°C for 40 h when a constant weight was achieved. After the dry weight of the tissue was registered, the water content of the tissue was calculated as wet weight minus dry weight and expressed as milligrams of water per milligrams of dry tissue.

Albumin in bronchoalveolar lavage. Another set of four groups of mice undergoing identical exposures as delineated above were used for bronchoalveolar lavage (BAL) (groups 12–15, Fig. 1D). Mice were killed, and tracheas were cannulated with a 19-gauge Leur adapter. BAL was performed by infusing and withdrawing 1 ml of

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<th>Table 1. Oxygen concentrations in environmental chambers during various exposures</th>
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<td>Actual mean ± SE*</td>
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* n = 15.
Bonferroni correction. Comparisons between control and WHPC mice one-way ANOVA followed by unpaired Student t-analyzer (Roche Diagnostics, Indianapolis, IN). Scientific, Lincoln Park, MI) and a Cobas Mira Plus automated faxia-sensitive control mice (P<0.005).

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RESULTS

Survival. When exposed to 5% O2, all control C57BL/6 mice died within a short period of time (Fig. 2A, average survival time of 13.8 ± 1.2 min, n = 10), indicating that this level of hypoxia was lethal to this strain. In contrast, C57BL/6 mice that were subjected to WHPC 2 h earlier survived significantly longer under the same lethal hypoxic conditions (Fig. 2A, average survival time of 33.2 ± 6.1 min, n = 10, P < 0.005 vs. controls). Intriguingly, 3 of the 10 preconditioned mice survived for more than 1 h in this otherwise unconditionally lethal environment (observations were terminated at 1 h). Subsequent studies on the time course of the protective effect afforded by WHPC demonstrated that the protection against lethal hypoxia persisted for at least 8 h in these mice (Fig. 3). Thus a potent, intrinsic protective mechanism can be acutely induced and renders the entire organism more resistant to hypoxia. The procedures used to elicit WHPC did not appear to cause severe organ injury, as indicated by the absence of any deterioration in blood-gas measurements and the absence of pulmonary and cerebral edema in these animals (see below).

To assess the effectiveness of WHPC in a mouse strain that approximated a genetically diverse population, we conducted the same experiment in outbred ICR mice. We found that the majority (9 of 10) of the control ICR mice was sensitive to hypoxia and died quickly when exposed to 5% O2, whereas one of the mice was naturally resistant to hypoxia and survived for more than 1 h (Fig. 2B). Consequently, although the average survival time of ICR mice with WHPC pretreatment 2 h earlier was similar to that of C57BL/6 mice with WHPC (Fig. 3, 28.3 ± 7.1 min, n = 10, P > 0.1 vs. C57BL/6), it was not significantly different from that of control ICR mice. Interestingly, the average survival time of ICR mice with WHPC pretreatment was significantly longer than that of control ICR mice when the mouse with intrinsic hypoxic resistance was excluded from the control group (P < 0.005). The lack of statistical difference between the two groups was, therefore, likely caused by a β error. However, because a sample size of 78 animals per group would be required to refute the probability of a β error, we investigated inbred strain mice only in subsequent experiments.

Blood gases. To investigate mechanisms underlying the generalized protection afforded by this novel phenomenon of

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A group 1  norm  lethal hyp
2 WHPC norm 2 h lethal hyp
3 WHPC norm 4 h lethal hyp
4 WHPC norm 8 h lethal hyp
5 WHPC norm 24 h lethal hyp

B 6 norm sublethal hyp 4 h
7 WHPC norm 2 h sublethal hyp 4 h

C 8 norm
9 norm sublethal hyp 6 h
10 WHPC norm 2 h
11 WHPC norm 2 h sublethal hyp 6 h

D 12 norm
13 norm sublethal hyp 6 h
14 WHPC norm 2 h
15 WHPC norm 2 h sublethal hyp 6 h

Fig. 1. Experimental groups. WHPC: whole-body hypoxic preconditioning; norm, normoxia; hyp, hypoxia. A: groups for the survival study. B: groups for the measurement of blood gases. C: groups for the measurement of tissue water content. D: groups for the measurement of albumin in lavage. See the text for details.

ice-cold, sterile normal saline three times. The BAL fluid was centrifuged immediately at 1,000 g for 10 min at 4°C. The cell-free supernatant was collected and stored at 4°C for measurement within 24 h. Albumin concentration in the BAL was quantitated with the use of a sensitive immunoassay kit (range of 0.5–30 mg/dl; Pointe Scientific, Lincoln Park, MI) and a Cobas Mira Plus automated analyzer (Roche Diagnostics, Indianapolis, IN).

Statistical analyses. Comparisons among groups were made with a one-way ANOVA followed by unpaired Student’s t-tests with the Bonferroni correction. Comparisons between control and WHPC mice or normoxic and hypoxic mice were made with unpaired Student’s t-test. A P value of <0.05 was considered significant.

RESULTS

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WHPC, mice were exposed to sublethal hypoxia. We found that anesthetized control C57BL/6 mice ventilated with 7% O\textsubscript{2} for 4 h developed severe acidosis and hypoxemia as determined by arterial blood-gas analysis (Table 2). These arterial blood-gas disturbances, however, were significantly milder in mice that were subjected to WHPC 2 h earlier (Table 2). These results are consistent with the prolonged survival induced by WHPC in C57BL/6 mice exposed to lethal hypoxia.

**Water content in lung and brain tissues.** With the understanding that the central nervous system and the lung are highly sensitive to acute hypoxia and tissue edema is one of the main causes for hypoxic fatality, we examined whether WHPC acted to reduce the severity of hypoxic pulmonary edema and therefore resulted in improved gas exchange. We found that conscious C57BL/6 mice developed measurable pulmonary edema after exposure to 7% O\textsubscript{2} for 6 h in environmental chambers (Fig. 4A). The severity of pulmonary edema, however, was significantly reduced in mice pretreated with WHPC 2 h earlier, as evidenced by the significantly smaller net increase in lung water content between the hypoxic and normoxic groups (Fig. 4B). Similarly, pretreatment of WHPC also significantly reduced the severity of cerebral edema caused by exposure to sublethal hypoxia (Fig. 4, C and D). Furthermore, a substantial leakage of plasma albumin into the air space of the lung was detected in control mice after 6 h of hypoxia (Fig. 5), indicating the presence of increased pulmonary vascular permeability. In contrast, albumin leakage was almost completely prevented by pretreatment of mice with WHPC (Fig. 5). These results demonstrate that WHPC alleviates hypoxic brain and pulmonary edema and the latter, in turn, leads to improved gas-exchange function in the lung.

**DISCUSSION**

This study shows that WHPC prolongs the survival time of unacclimatized mice under lethal hypoxia. To our knowledge, this observation constitutes the first description on the existence of an intrinsic protective mechanism, in unacclimatized mammals, that can be acutely induced to protect against lethal hypoxia. Furthermore, the protective effect of WHPC is associated with improved metabolic function in peripheral tissues (as shown by improved blood pH) and improved gas-exchange function in the lung (as shown by improved blood P\textsubscript{O\textsubscript{2}}), the latter involving the ability of WHPC in the prevention of the development of protein leakage into the pulmonary alveolar space and pulmonary edema. Thus both the protection afforded by WHPC and the mechanisms underlying this protection involve unique characteristics that are not known for hypoxic acclimatization.

The lung and the brain are the two vital organs that exhibit the greatest susceptibility to hypoxic injury (13, 14). Indeed, pulmonary hypertension and pulmonary edema rapidly develop in both humans and experimental animals subjected to severe hypoxia (3, 13). Among the many detrimental consequences of pulmonary edema, the increased thickness of the respiratory membrane (alveolocapillary block) and the decreased oxygen diffusing capacity are probably the most important in further determining survival during severe hypoxic conditions, since oxygen supply to peripheral tissues will be further compromised, which may eventually cause multiple organ failure (3, 13). Therefore, the lung can be viewed as one of the “rate-limiting” organs in the ability of the organism to survive severe hypoxia. Accordingly, pulmonary edema is one of the two major causes of death in humans and animals exposed to acute high-altitude hypoxia (3, 13, 14). On the basis of our present findings, it appears conceivable that the protection against lethal hypoxia afforded by WHPC is mediated, at least in part, through improved organ function in the lung, particularly through decreased pulmonary vascular permeability, decreased pulmonary edema, and improved gas exchange. On the basis of this principle, it was predicted that the brain might be another target organ of the protective effect of WHPC. Indeed, hypoxic cerebral edema was reduced in mice pretreated with WHPC compared with controls. This latter observation further supports the notion that WHPC acts to improve the function of “rate-limiting” organs under hypoxia.

Although the exact mechanisms by which hypoxia causes pulmonary edema are not fully understood, increased capillary pressure and increased vascular permeability along with impaired alveolar fluid clearance have been postulated to be the major determinants of hypoxia-induced pulmonary injury (3, 21, 22). Our finding that WHPC almost completely prevented leakage of albumin into alveolar space leading to partial prevention of pulmonary edema supports the notion that both increased capillary pressure and increased vascular permeability contribute to the development of hypoxic pulmonary edema. Moreover, this finding suggests that the action of

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<td>Room air</td>
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<td>pH</td>
<td>7.409±0.009</td>
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<td>P\textsubscript{CO\textsubscript{2}}, Torr</td>
<td>30.8±1.2</td>
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<td>P\textsubscript{O\textsubscript{2}}, Torr</td>
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Values are means ± SE. *P < 0.025 vs. control mice at 7% O\textsubscript{2}; †P < 0.05 vs. control mice at 7% O\textsubscript{2}. 

![Fig. 3. Time courses of the protection against lethal hypoxia afforded by WHPC. The protection against lethal hypoxia remained effective 8 h after the induction of WHPC in C57BL/6 mice. The potential protection in ICR mice appeared to be less lasting, as the survival time of the WHPC group at 8 h was not different from the control group, even when compared with only the hypoxia-sensitive subgroup. Data are means ± SE; n = 10 in each group.](image-url)
WHPC is more effective and efficient in maintaining normal permeability in the pulmonary vascular bed under severe hypoxia but less so in maintaining a normal filtration pressure in the pulmonary microcirculation. Confirmation of the latter point will require measurements of pulmonary hemodynamics in future studies.

An interesting observation in the present study was the heterogeneity in both the susceptibility to acute hypoxia and the response to WHPC among individual animals. Individual variation was anticipated among the outbred ICR mice due to their genetic diversity; therefore, this strain would be expected to behave similarly to various rat strains and human populations in which different genetic backgrounds are present (6, 15, 29). However, significant variation was also evident among the inbred C57BL/6 mice who shared the same genotype (see Fig. 2A). Epigenetic modification of the genome and environmental influences might be responsible for this phenomenon (11, 39), although the precise mechanism for different phenotypes among individual animals with the same genotype is presently unknown. Of note, variable responses to hypoxic acclimatization have been observed in human twins in studies carried out in the former Soviet Union (30), suggesting that the variability in WHPC responses in inbred mouse strains might be extended to humans as well. This possibility raises an important issue because the heterogeneity in susceptibility to hypoxia and the functional responses documented following WHPC could be related. Indeed, it could be argued that those mice that survived lethal hypoxia for more than 1 h after WHPC were in fact hypoxia-resistant mice, such that their prolonged survival was unrelated to any protective effect conferred by WHPC. However, this scenario appears unlikely considering the consistently reproducible responses of the inbred C57BL/6 mice to WHPC and the absence of naturally hypoxia-resistant mice in this strain. The overall magnitude of protection afforded by WHPC is also likely to display significant variability, especially in outbred animals. As previously shown for hypoxic acclimatization (30), the increment in hypoxic tolerance is expected to be greater in more susceptible animals compared with those who exhibit increased intrinsic resistance to lethal hypoxia. On the basis of discrepancies in the protective effect size of WHPC and of acclimatization among animals of the same inbred strain, it is reasonable to assume that the mechanisms of intrinsic tolerance to hypoxia and those rapidly induced by WHPC may have some molecular and cellular pathways in common.

In conclusion, an intrinsic protective mechanism against hypoxia can be rapidly induced in unacclimatized mice by WHPC. This novel mechanism exerts immediate and potent protection on the animals, prolonging their survival time under otherwise lethal hypoxic conditions. The protection at the organism level is likely mediated by improved function in vital organs, such as the lung and the brain. Compared with previously known intrinsic protective mechanisms against hypoxia-
ischemia, the protection induced by WHPC demonstrates unique features, especially in the time course and the scope of the protection it affords. It is thus likely that unique cellular and molecular mechanisms are involved in the induction and in the mediation of this novel phenomenon. A thorough understanding of these mechanisms may enable us to mimic the powerful protection afforded by WHPC and ultimately to develop potential therapeutic interventions in the future.

GRANTS

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