ORIGINAL RESEARCH

Inhibitors of Hypoxic Pulmonary Vasoconstriction Prevent High-Altitude Pulmonary Edema in Rats

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Objective.—Rapid ascent to high altitude causes hypoxic pulmonary vasoconstriction (HPV) and leads to high-altitude pulmonary edema (HAPE) in susceptible humans. Vasodilating agents lessen HAPE (as evidenced by radiographic and gas exchange measurements), but data establishing their effectiveness on alveolar protein content and hemorrhage are lacking. This study was designed to assess whether preventing HPV reduces the alveolar-capillary barrier leak characteristic of HAPE.

Methods.—Rats were pretreated with saline (control group) or acetazolamide (20 mg/kg) or nickel chloride (60 mg/kg) (experimental groups) to prevent HPV and were exposed to high altitude (0.5 atm for 24 hours) in a hypobaric chamber. High-altitude pulmonary edema was then assessed by gravimetric analysis of heart and lung tissue, a visual score of lung hemorrhage, and measurement of protein content in bronchoalveolar lavage fluid.

Results.—Saline-treated rats developed a mild protein leak indicative of early HAPE that was prevented by inhibition of HPV: protein in bronchoalveolar lavage fluid of saline-treated rats, 21.6 ± 3.2 mg/dL (mean $\pm$ SEM); HPV-inhibited rats, 12.6 ± 0.7 mg/dL; air-exposed rats, 13.4 ± 1.4 mg/dL ($P < .05$ saline vs other groups, analysis of variance [ANOVA]). The lungs of saline-treated rats were also mildly hemorrhagic (3.4 ± 0.9 on a scale of 1–9, where 1 is normal), and the lungs of HPV-inhibited rats appeared normal (1.2 ± 0.1) ($P = .032$). Finally, right ventricle weight (adjusted for initial body weight) increased in saline-treated rats: saline-treated, 0.64 ± 0.02 ; HPV-inhibited, 0.56 ± 0.02 ; air-exposed, 0.59 ± 0.02 ($P < .05$, saline vs HPV-inhibited group).

Conclusion.—The results demonstrate that treatment with NiCl_2 or acetazolamide prevents HAPE in rats and are consistent with a role for elevated pulmonary artery pressure in the pathogenesis of HAPE.

Key words: acetazolamide, high-altitude pulmonary edema, hypoxia, pulmonary hypertension, rat, stress failure

Introduction

High-altitude pulmonary edema (HAPE) develops in susceptible humans after rapid ascent to elevations higher than 8000 feet,^{1,2} and the injury is similar in rats.^{3–6} Pathogenic hemodynamic changes in the pulmonary circulation preceding HAPE in humans include increases in pressure in pulmonary arteries, arterioles, and capillaries (and possibly small pulmonary veins).^{7–9} In humans, various antagonists of hypoxic pulmonary vaso-

constriction (HPV) (calcium channel blockers, beta-2 agonists, alpha blockers, and nitric oxide [NO]) prevent HAPE or hasten its resolution^{10–14} as assessed by clinical history, physical examination, gas exchange, and radiological measurements.

The “stress failure” hypothesis of HAPE proposes that disruption of the alveolar-capillary barrier develops during rapid ascent because HPV is strong but unevenly distributed; therefore, capillaries downstream of relatively weakly constricted arteries are overperfused and leak.^{4,15} The leak, as assessed by bronchoalveolar lavage fluid (BALF) analysis, is one of increased protein content and mild alveolar hemorrhage but no initial inflammatory characteristics.² The presumption of the stress-failure hypothesis is that pressure elevation occurs rap-

Presented in part at the American Physiological Society Intersociety Meeting, San Diego, CA, August 24–28, 2002.

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idly and the capillary walls do not have sufficient time to remodel and strengthen.

In order to study the issues of HPV, HAPE, and capillary integrity, we conducted studies in a rat model of HAPE to determine the fundamental importance of vascular pressure increase in causing the pathognomic alveolar leak characteristics of HAPE. Rats were pretreated with NiCl_2 (an inducer of heme oxygenase 1)¹⁶ or acetazolamide (a widely used drug to prevent and treat acute mountain sickness)¹⁷ to prevent pulmonary artery hypertension during rapid high-altitude exposure. These drugs were chosen because they inhibit HPV but do not have significant systemic vasodilating properties as do calcium channel blockers, which are the standard drugs used to prevent HAPE in humans. High-altitude pulmonary edema was then assessed by gravimetric and bronchoalveolar lavage techniques after a 24-hour exposure to half atmospheric pressure (380 mm Hg) in a hypobaric chamber.

Methods

EXPERIMENTAL DESIGN

A total of 27 male pathogen-free Sprague Dawley rats (300–400 g) (Harlan Sprague Dawley, Indianapolis, IN) were used in this study. Eleven rats (HPV-inhibited group) received HPV-inhibitors before high-altitude exposure (5 received NiCl_2 and 6 received acetazolamide). Ten rats (saline-treated control group) received saline before exposure, and 6 rats (air control group) received no treatment. All agents were administered by intraperitoneal (i.p.) injection, and all procedures were performed at sea level. In addition, all protocols were approved by The Animal Care Committee of the University of Hawaii and conformed to National Institutes of Health guidelines for animal research.

EXPERIMENTAL PROCEDURES

To begin an experiment, paired rats (1 HPV-inhibited rat and 1 saline-treated control rat) were weighed and body temperatures were recorded with a rapidly responding cloacal thermometer (Miller and Weber Inc, Queens, NY). The rats were then injected (under isofluorane anesthesia) with saline (0.9% NaCl, 1 mL volume, i.p.) or HPV-inhibitor: a single injection of NiCl_2 (60 mg/kg, i.p.) before exposure or 2 injections of acetazolamide (20 mg/kg, i.p.) 12 hours apart. Rats were given 50 minutes in room air to allow distribution of injected chemicals and were then exposed to moderate altitude (570 mm Hg pressure) for 10 minutes in a hypobaric chamber. After the 10-minute adjustment period (1 hour after in-

jection), the vacuum was increased to lower the pressure to 380 mm Hg for 24 hours with food and water provided ad libitum. At completion of the 24-hour exposure period, rats were removed from the chamber (1 rat was processed immediately for data collection and the other rat was placed in 10% O_2 for later use). The order of rats for initial data collection was alternated between experiments to ensure similar overall exposure conditions for all treatment groups.

The procedure for postexposure data collection was as follows. First, body weight and body temperature were recorded as before. Rats were then anesthetized (sodium pentobarbital, 50 mg/kg body weight, i.p.), and the abdominal cavity was opened by midline incision. Rats were euthanized by exsanguination of the abdominal aorta and the chest was opened. Lung appearance (unblinded assessment) was then recorded by assigning values within a range of 1 to 9 to describe hemorrhage on the lung surface (1 indicated normal appearance with an absence of hemorrhage and 9 indicated hemorrhage of the entire visible lung surface).¹⁸ The left main bronchus was then ligated and the trachea cannulated for bronchoalveolar lavage of the right lung with 5 mL 0.9% NaCl. After lavage, the middle left lung lobe was removed, trimmed, and blotted for gravimetric determination. Finally, the heart was removed and the right ventricle trimmed and blotted for determination of wet weight. Lung lobes were placed in an incubator at 55°C for up to 1 week to determine stable dry tissue weight.

Protein concentration in BALF (supernatant used for measurement after centrifugation) was determined by the Microprotein-PR kit (Sigma Chemical Co, St Louis, MO). This assay is based on the method of Fujita et al¹⁹ and measures the increase in absorbance (600 nm) that occurs when the pyrogallol red-molybdate complex binds basic amino acid groups in protein. The absorbance change is directly proportional to protein concentration in the sample.

HIGH-ALTITUDE EXPOSURE

The hypobaric chamber (1 cubic-foot volume) was constructed from a vacuum desiccator (Labconco, Kansas City, MO). A high-volume low-pressure rotary vane oil-free vacuum pump (Model 0523 series; Gast Manufacturing Inc, Benton Harbor, MI) was used to produce simulated high altitude. This pump was designed for continuous use over long periods of time and does not generate oil-associated fumes that could interfere with experimental results. Pressure in the chamber was adjusted by balancing suction from the vacuum pump with an air leak into the chamber. Rats were placed inside a Plexiglas cage during high-altitude exposure. The floor

of the cage was layered with wood chips and color-indicating CO₂ absorbent (Sodasorb; 34 WR Grace and Co, Atlanta, GA), and additional containers of absorbent were placed on top of the cage. Holes were drilled in the sides of the cage to allow free flow of air through the cage and prevent possible CO₂ accumulation. The rate of airflow through the chamber was measured at 5 L/min. This rate of flow provided 1 exchange of chamber air every 5.7 minutes, which was sufficient to prevent color change in Sodasorb during exposure.

Statistical analysis of data was performed by a Student's *t* test and 1-way ANOVA (Primer of Biostatistics: The Program, McGraw-Hill, New York, NY). The *t* test was applied when 2 groups of data points were analyzed for significance, and 1-way ANOVA was used when data from 3 groups were tested.

Results

All rats survived the 24-hour period of high-altitude exposure. The lungs of saline-treated control rats were hemorrhagic with visible signs of lung injury on autopsy (lung score = 3.4 ± 0.9). In contrast, the lungs of HPV-inhibited rats appeared normal (lung score = 1.2 ± 0.1). The differences in lung appearance values for saline-treated rats vs HPV-inhibited rats were significant ($P = .032$ by *t* test).

Bronchoalveolar lavage was performed on all rats after exposure to high altitude. Bronchoalveolar lavage fluid protein concentration in the lungs of saline-treated control rats ($n = 10$) was 21.6 ± 3.2 mg/dL and in air-exposed control rats ($n = 6$) was 13.4 ± 1.4 mg/dL. Bronchoalveolar lavage fluid protein concentrations in HPV-inhibited rats were similar to those in air-exposed controls: NiCl₂-treated ($n = 5$) was 11.6 ± 0.7 and acetazolamide-treated ($n = 6$) was 13.5 ± 1.1 . The values for NiCl₂ and acetazolamide were lower than the values for saline-treated rats ($P = .048$ and $.075$, respectively, vs saline treated). When values for NiCl₂-treated and acetazolamide-treated rats ($n = 11$) were combined (HPV-inhibited group), the protein concentration value was 12.6 ± 0.7 . This value was significantly different from BALF protein concentration in saline-treated rats ($P = .009$). Values for HPV-inhibited rats and air controls were similar and significantly less than values for saline-treated rats exposed to high altitude ($P < .05$ saline vs other groups by ANOVA) (Table 1).

The right ventricles were also heavier in saline-treated rats after 24-hours exposure to high altitude when adjusted for initial body weight (Table 2). The right ventricle/initial body weight in saline-treated rats ($n = 10$) was 0.64 ± 0.02 g; HPV-inhibited ($n = 10$), 0.56 ± 0.02 g; and air controls ($n = 6$), 0.59 ± 0.02 g. The

Table 1. Protein concentration (mg/dL) in bronchoalveolar lavage fluid from rats exposed to high altitude (0.5 atm/24 hours) or sea level (air control)

<i>Group†</i>		
<i>HPV inhibited (n)</i>	<i>Saline treated (n)</i>	<i>Air control (n)</i>
12.6 ± 0.7 (11)	$21.6 \pm 3.2^*$ (10)	13.4 ± 1.4 (6)

†Hypoxic pulmonary vasoconstriction (HPV)-inhibited rats received a single intraperitoneal (i.p.) injection of NiCl₂ (60 mg/kg body weight) 1 hour before high-altitude exposure or 2 injections of acetazolamide (20 mg/kg, i.p.) 1 hour before exposure and 11 hours after initiation of exposure. Saline-treated rats received 1 mL saline instead of HPV inhibitor. Air-control rats were untreated. Values are mean $\pm$ SEM.

* $P < .05$ vs other groups by analysis of variance.

difference in values for saline-treated rats vs the HPV-inhibited group is significant at $P < .05$.

Gravimetric measurements were also taken on lung tissues from all rats. Hypoxic pulmonary vasoconstriction inhibition did not produce significant differences in any of these measurements during high-altitude exposure, although values for HPV-inhibited rats were typically closer to those of air-exposed rats than to saline-treated rats (Table 2).

Hematocrit, body weight, and body temperature were also measured in rats during high-altitude exposure. Hemococoncentration occurred in all high-altitude-exposed rats, but the response was not altered by HPV-inhibition: hematocrit values for HPV-inhibited rats ($n = 11$) were $50.4\% \pm 0.5\%$; for saline-treated rats ($n = 10$), $50.6\% \pm 0.8\%$; and for air controls ($n = 5$), $43.7\% \pm 1.0\%$ ($P < .05$ air vs other groups). Both groups of rats exhibited similar body-weight losses during high-altitude exposure (HPV-inhibited rats [$n = 11$] lost $9.87\% \pm 0.75\%$ and saline-treated rats [$n = 10$] lost $8.34\% \pm 0.3\%$ [$P = .084$]). Finally, the loss in body temperature was also similar between groups: HPV-inhibited rats lost $1.4 \pm 0.2^\circ\text{C}$ and saline-treated rats lost $1.9 \pm 0.2^\circ\text{C}$ ($P = .094$).

Discussion

In this study, we found that pretreatment with NiCl₂ or acetazolamide prevented HAPE in rats exposed to 0.5 atm pressure ($\sim 18\,000$ feet) for 24 hours. Saline-treated rats developed hemorrhagic lungs with a mild but significant protein leak, as shown by an increase in protein content of BALF (Table 1). An increase in right ventricular weight that is suggestive of pulmonary hypertension was also observed (Table 2). These changes were

Table 2. Summary of gravimetric data from rats exposed to high altitude (0.5 atm/24 hours) or sea level (air control)

	Group†		
	HPV inhibited (n)	Saline treated (n)	Air control (n)
Wet LL ± SEM	0.4974 ± 0.02 (11)	0.5249 ± 0.02 (10)	0.4605 ± 0.03 (6)
Dry LL ± SEM	0.1039 ± 0.001 (11)	0.1088 ± 0.001 (10)	0.0966 ± 0.01 (6)
Wet/dry ± SEM	4.79 ± 0.09 (11)	4.82 ± 0.06 (10)	4.78 ± 0.06 (6)
Wet LL/BW _i ± SEM	1.23 ± 0.03 (11)	1.29 ± 0.04 (10)	1.21 ± 0.05 (6)
RV/BW _i ± SEM	0.56* ± 0.02 (10)	0.64 ± 0.02 (10)	0.59 ± 0.02 (6)

†Hypoxic pulmonary vasoconstriction (HPV)-inhibited rats received a single intraperitoneal (i.p.) injection of NiCl₂ (60 mg/kg body weight) 1 hour before high-altitude exposure or 2 injections of acetazolamide (20 mg/kg, i.p.) 1 hour before exposure and 11 hours after initiation of exposure. Saline-treated rats received 1 mL saline instead of HPV inhibitor. Air-control rats were untreated. Values are mean ± SEM. All weights are in grams except for the middle left lung lobe (LL) and right ventricle (RV) body-weight ratios, which are expressed in g/kg initial body weight (BW_i).

**P* < .05 vs saline group by analysis of variance.

not seen in rats treated with NiCl₂ or acetazolamide during high-altitude exposure. Because NiCl₂ and acetazolamide inhibit HPV in animal studies,^{16,17} these findings support a causative role for HPV in an animal model of HAPE.

Our results with inhibition of HPV are consistent with those of Omura et al.,⁵ the only other study to inhibit HPV in rats during high-altitude exposure. In their study, Omura and colleagues added NO (83 parts per million) to room air and observed 6% mortality compared with 40% mortality in the control group. Although we and others^{3,6,20–24} have not observed similar mortality in rats under approximately similar exposure conditions, the study by Omura et al does show that inhibition of HPV extends survival in rats. Although NO is a potent inhibitor of HPV at these concentrations, it may have other effects that might alter the development of HAPE apart from reducing pulmonary artery pressure. These include possible stimulation of active alveolar epithelial salt and water reabsorption or a general tightening of the alveolar-capillary barrier.²⁵ The agents we used, NiCl₂ and acetazolamide, have neither of these actions and make a stronger case for the primary role of pulmonary artery hypertension in HAPE.

The mechanism of HPV inhibition by acetazolamide is unknown but may involve changes in intracellular pH of pulmonary vascular smooth muscle that lead to diminished vasoconstrictor responses.¹⁷ NiCl₂ is a potent inducer of heme-oxygenase, which generates carbon monoxide (CO) from the metabolism of heme.¹⁶ Carbon monoxide is a vasodilator, acting in much the same fash-

ion as NO acts to increase smooth muscle cGMP.^{26,27} Both NiCl₂ and acetazolamide have little effect on systemic blood pressure, unlike standard calcium channel blockers such as nifedipine. These agents should therefore prove useful in clarifying the role of HPV in HAPE.

We did not measure pulmonary artery pressures or arterial oxygenation in these experiments because of the technical and invasive aspects of these measurements. Therefore, it is possible that other potential actions of NiCl₂ and acetazolamide apart from reduction in pulmonary artery pressure may have reduced lung edema. With respect to NiCl₂, induction of bilirubin and CO (both having immune-modulating effects) could have had anti-inflammatory effects. However, the vast majority of work in rats exposed to similar magnitudes and duration of hypoxia show little to no evidence of inflammation in the alveolar space in the absence of pretreatment with agents known to induce an inflammatory response.^{3–6,22,28} Thus, we do not believe that NiCl₂ prevented lung edema by suppression of inflammation. In the case of acetazolamide, it is conceivable that by virtue of its ventilatory stimulation and diuretic effects, these could have prevented lung edema. Regarding the drug-induced diuresis, however, we did not see any difference in body-weight changes across the groups; all animals experienced roughly a 10% drop in body weight. Although acetazolamide may have generated greater ventilation and thus increased alveolar PO₂ for the given ambient hypoxia and reduced the stimulus for HPV, this mode of action is ultimately an effective depressant of

HPV. There are no reported data of anti-inflammatory effects of any carbonic anhydrase inhibitor.

There has been much effort to develop an animal model of HAPE that shares the same features of the human disease. In this regard, the rat has been used most often, but it nevertheless still differs from humans. In general, the injury appears to be milder. We observed only a 2-fold rise in alveolar protein concentration compared with the 5- to 10-fold rise in human BALF.²⁹ Our results (Table 2), like those of others, show that wet- to dry-weight ratios of noninfected rat lungs at 24 hours of hypoxic exposure do not rise (or only barely rise) as one might expect with edema.^{3,5,6,22,28} One explanation for the lack of lung weight gain, despite alveolar hemorrhage and increased alveolar protein concentration, is the significant diuresis and antidipsogenic effect of acute hypoxia that lead to about a 10% loss of body weight. Another possible difference is the greater magnitude of mean pulmonary artery pressure elevation in humans with HAPE compared with rats at an equivalent altitude (37 mm Hg in humans vs 24 mm Hg in rats).^{6,8} Last, human HAPE typically occurs in the setting of strenuous physical activity, such as climbing, which cannot be recreated in the small confines of an animal hypobaric chamber.

One hypothesis to explain the pathogenesis of HAPE is that HPV in pulmonary arteries and arterioles is uneven. This leads to some capillaries becoming overperfused and either developing greater permeability to large molecular weight compounds or becoming physically disrupted. Hultgren³⁰ initially proposed the overperfusion hypothesis and concluded that increases in flow-related shear stress in overperfused capillaries causes the capillary walls to leak. A number of studies support regional heterogeneity of HPV. For example, Dawson and colleagues³¹ showed that alveolar hypoxia nearly doubled the dispersion of transit times through the pulmonary circulation in a lobe of dog lung. The distribution of India-ink particles injected into the pulmonary circulation during alveolar hypoxia is also more uneven than during normoxia.³² In addition, high-resolution methods such as the deposition of injected microspheres demonstrate uneven regional HPV with acute hypoxia.^{33,34}

West et al³⁵ modified Hultgren's hypothesis and suggested that pulmonary capillaries undergo pure pressure-related stress failure during hypoxia-induced overperfusion. They demonstrated that capillary breaks occur in pulmonary capillaries during high-altitude exposure and proposed that stress failure causes HAPE.^{4,15} In relation to mechanisms of injury, Tsukimoto and coworkers³⁶ further suggested that white cells become activated after contact with exposed basement membranes and cause

additional injury. A recent study by Swenson et al²⁹ supports this possible sequence of events. Bronchoalveolar lavage fluid, collected at the onset of HAPE, had elevated protein concentration and red cells, but neutrophils were absent and there were no elevations above normal in a host of proinflammatory cytokines, unlike in studies of more prolonged HAPE.^{37,38}

We have shown that 2 different inhibitors of HPV (NiCl₂ and acetazolamide) prevent HAPE in a rat model of this disease. Thus, as in humans, HPV is pathogenic in rats. Our findings suggest that acetazolamide, a drug already used commonly and safely at high altitude to prevent or reduce acute mountain sickness, may also be of benefit in preventing HAPE by inhibition of HPV.

Acknowledgments

This study was funded by The Hawaii Community Foundation (Geist Award # HCF 20010643) and NIH NHLBI # HL24163.

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