Exercise training prevents the inflammatory response to hypoxia in cremaster venules

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Systemic hypoxia produces microvascular inflammation in several tissues, including skeletal muscle. Exercise training (ET) has been shown to reduce the inflammatory component of several diseases. Alternatively, ET could influence hypoxia-induced inflammation by improving tissue oxygenation or increasing mechanical antiadhesive forces at the leukocyte-endothelial interface. The effect of 5 wk of treadmill ET on hypoxia-induced microvascular inflammation was studied in the cremaster microcirculation of rats using intravital microscopy. In untrained rats, hypoxia (arterial PO2 = 32.3 ± 2.1 Torr) increased leukocyte-endothelial adherence from 2.3 ± 0.4 to 10.2 ± 0.3 leukocytes per 100 µm of venule (P < 0.05) and was accompanied by extravasation of FITC-labeled albumin after 4 h of hypoxia (extra-/intravascular fluorescence intensity ratio = 0.50 ± 0.07). These responses were attenuated in ET (leukocyte adherence was 1.5 ± 0.4 during normoxia and 1.8 ± 0.7 leukocytes per 100 µm venule after 10 min of hypoxia; extra-/intravascular fluorescence intensity ratio = 0.11 ± 0.02; P < 0.05 vs. untrained) despite similar reductions of arterial (32.4 ± 1.8 Torr) and microvascular PO2 (measured with an oxyphor-quenching method) in both groups. Shear rate decreased during hypoxia to similar extents in ET and untrained rats. In addition, circulating blood leukocyte count was similar in ET and untrained rats. The effects of ET on hypoxia-induced leukocyte-endothelial adherence remained up to 4 wk after discontinuing training. Thus ET attenuated hypoxia-induced inflammation despite similar effects of hypoxia on tissue PO2, venular shear rate, and circulating leukocyte count.

leukocyte-endothelial adherence; vascular permeability, microvascular PO2; venular shear rate

Acute altitude illnesses occur in nonacclimatized subjects in the early stages of hypoxic exposure. These range from the relatively mild acute mountain sickness (AMS) to the potentially lethal high-altitude pulmonary edema and high-altitude cerebral edema. These diseases are characterized by increased vascular permeability and body fluid retention (10, 11). The pathogenesis of AMS, high-altitude cerebral edema, and high-altitude pulmonary edema is unclear. The role of inflammation is currently the subject of intense debate. Although an inflammatory component has been demonstrated in all three altitude illnesses (2, 3, 14, 30), it is unclear whether inflammation is the cause or an epiphenomenon secondary to mechanical or hemodynamic changes (19, 38). Regardless of whether inflammation plays a role as a causative agent, it may negatively influence the development and clinical outcome of these conditions.

Exercise training has been shown to have a beneficial effect on diseases that have an inflammatory component (5, 13, 23, 33). Whether exercise training influences altitude illness has not been studied systematically. No correlation between maximal O2 consumption and scores of AMS severity was observed, suggesting that athletic fitness does not play a role in adaptation to altitude (22). On the other hand, training for 8 wk before exposure to altitude was shown to reduce AMS scores compared with an untrained control group (31).

Exercise training could influence hypoxia-induced inflammation in several ways. Exercise training results in increased NO availability (13, 17, 24, 39), increased levels of endogenous antioxidants (26, 28), and induction of heat shock proteins (29, 34), all of which have an anti-inflammatory effect. We have shown that administration of exogenous NO and antioxidants attenuates hypoxia-induced inflammation in rats (36, 40). Furthermore, preliminary evidence suggests that expression of inducible NO synthase and upregulation of the heat shock protein heme oxygenase-1 participate in the increased resistance to hypoxia-induced inflammation of acclimatized rats (4). Alternatively, exercise training could improve systemic O2 delivery such that the reduction in microvascular PO2 (PmO2) during hypoxia may be attenuated compared with that of untrained rats. In addition, mechanical antiadhesive forces at the leukocyte-endothelial interface could be increased as a result of hemodynamic changes brought about by exercise training.

The objective of this study was to determine whether an exercise training regime demonstrated to significantly increase aerobic capacity in rats (7) would influence the microvascular inflammatory response to hypoxia, as assessed by leukocyte-endothelial adherence and vascular permeability changes. The pattern of response to hypoxia was studied immediately after...
termination of the exercise program and for 4 wk after training cessation.

METHODS

All procedures were approved by the Animal Care and Use Committee of the University of Kansas Medical Center, an institution accredited by the American Association for the Accreditation of Laboratory Animal Care.

Training protocol. Seven-week-old male Sprague-Dawley rats, \( \sim 150 \) g, were randomly assigned to untrained and trained groups. Both groups were housed in the same room of the animal care facility. Training lasted 5 wk and was performed on an eight-lane treadmill. Work rate increased gradually over 1 wk until it reached 30 m/min on a 10° incline, 1 h/day, 5 days/wk. This work rate was maintained for the last 4 wk of the training protocol. Rats were rested at least 24 h before each experiment. Two additional groups of rats were tested at the last 4 wk of the training protocol. Rats were rested at least 24 h before each experiment. Two additional groups of rats were tested at 2 or 4 wk after completion of the training program to determine the persistence of the effects of exercise training. The exercise training protocol employed in this study has been shown to increase maximal \( O_2 \) consumption by \( \sim 20\% \) and skeletal muscle citrate synthase activity by more than twofold (7).

Measurement of leukocyte-endothelial adhesive interactions. At the end of the training period, rats were anesthetized with urethane (1.5 g/kg) after an overnight fast with free access to water. Body temperature was maintained at 36–38°C by using a homeothermic blanket system connected to an intratracheal temperature probe. PE-50 catheters were inserted in the jugular vein and carotid artery. Saline was infused continuously via the jugular vein at a rate of 3 ml/h. Arterial blood pressure was measured with a digital blood pressure monitor connected to the carotid artery catheter. A tracheotomy was performed, and PE-240 tubing was connected to a rodent nonrebreathing two-way valve. The animals breathed spontaneously throughout the experiment.

Intravital microscopy. The right cremaster muscle was prepared for intravital microscopy as described previously (1). The rat was placed on the platform of a Nikon E600 FN microscope, and the cremaster was spread over a hollow Lucite cylinder, the top of which was sealed with a glass slide. Water was circulated through the cylinder to maintain muscle temperature at 37°C. Temperature was monitored continuously via a thermistor placed underneath the muscle. The cremaster was covered with Saran wrap throughout the experiment.

Images of the cremaster microcirculation (x40 objective) were recorded on a videocassette recorder connected to a time-date generator. Straight, unbranched venules at least \( 100 \mu m \) in length and 20–40 \( \mu m \) in diameter, with fewer than three adherent leukocytes in a 100-\( \mu m \) segment and no adjacent lymphatics, were selected for microscopic observation. An optical Doppler velocimeter was used to measure venular centerline red blood cell velocity. Average red blood cell velocity was calculated as centerline velocity/1.6 (6). Wall shear rate, which represents the force generated at the vessel wall by the movement of blood, was calculated as \( 8 \) × (average red blood cell velocity/venular diameter) (12). Adherent leukocytes were defined as leukocytes that remained stationary for \( \geq 30 \) s. Leukocyte adherence was expressed as the number of adherent leukocytes per 100 \( \mu m \) of vessel.

Measurement of \( P_mO_2 \). A method based on the \( P_O_2 \) dependence of phosphorescence lifetime was used to determine \( P_mO_2 \) (27). The measurement of \( P_mO_2 \) was carried out in separate experiments in which the cremaster was prepared as described above, but the microcirculation was not visualized with intravital microscopy. The oxyphor Pd-porphyrin dendrimer (R2) was injected intravenously (16 mg/kg). At this concentration, R2 binds completely to albumin (18); in addition, R2 has a negative net charge, which further restricts the probe to the vascular space. Phosphorescence was measured using a phosphorimeter (PMOD 5000; Oxygen Enterprises, Philadelphia, PA) with a bifurcated light guide positioned 2–4 mm above the cremaster. The excitation light emitted from the light guide illuminated a cylinder within the cremaster of \( \sim 1 \) mm in diameter and \( \sim 500 \mu m \) deep. The phosphorescence signal was averaged over 200 ms, and measurements were obtained every 2 min. An excellent correlation was observed between \( P_O_2 \) values obtained using a \( P_O_2 \) electrode and the phosphorescence quenching method in the same blood sample (32).

Experimental protocol for intravital microscopy and \( P_mO_2 \). Approximately 30 min were allowed for animals to recover from surgery. These experiments consisted of a 10-min normoxic control period, a 10-min hypoxic period in which the animals spontaneously breathed 10% \( O_2 \)-90% \( N_2 \), and a 10-min recovery period. Arterial blood samples for measurement of \( \rho H \), \( P_O_2 \), and \( P_CO_2 \) were obtained at the end of each experimental period. In some of the experiments, circulating white and red blood cell count and \( Hb \) concentration were also determined. Approximately one-half of the experiments were directed to study leukocyte-endothelial adhesive interactions via intravital microscopy; in the remaining experiments, \( P_mO_2 \) was measured as described above.

Fig. 1. Time course of leukocyte-endothelial adherence during systemic hypoxia. Systemic hypoxia was induced by breathing 10% \( O_2 \). Untrained animals, \( n = 6 \); exercise trained animals, \( n = 6 \); trained animals rested for 2 wk after last exercise session, \( n = 3 \); trained animals rested for 4 wk after last exercise session, \( n = 3 \).

Fig. 2. Time course of microvascular \( P_O_2 \) during systemic hypoxia. Untrained animals, \( n = 6 \); trained animals, \( n = 6 \). The numerical inset indicates mean arterial \( P_O_2 \) values (Torr) in each period.
Measurement of albumin extravasation in rats exposed to hypoxia for 4 h. On the day before the experiment, rats were anesthetized with pentobarbital sodium (45–50 mg/kg). Camillas (PE-50) were inserted into a jugular vein and a carotid artery. The catheters were tunneled subcutaneously to the back of the neck, exteriorized, cut at a length of 2 in., and flame-sealed. Once these surgical procedures were completed, the animals were placed on a heating pad to maintain their body temperature during recovery from the anesthetic. The rats were placed into individual cages and fasted overnight with free access to water.

The following day, the conscious rats were placed into a Plexiglas chamber where 10% O2-90% N2 was circulated for a period of 4 h. After 4 h, the animals were anesthetized with urethane (1.5 g/kg) and prepared for intravital microscopy. The animals breathed 10% O2 throughout surgery and for the remainder of the experiment. After a vessel was found, FITC-labeled bovine albumin was injected intravenously (50 mg/kg), and images were obtained every 5 min for a total of 30 min.

Fluorescence from the FITC-labeled albumin (excitation wavelength, 420–490 nm; emission wavelength, 520 nm) was detected with the F-View Soft Imaging System camera. Images were analyzed for intravascular fluorescence intensity in an area including the full width of the vessel and 100 μm in length, using the analySIS Software Program. Extravascular fluorescence intensity was measured on both sides of the 100-μm venule segment while avoiding areas with underlying vessels.

Statistics. Data are presented as means ± SE. Data after a given treatment were compared with the corresponding pretreatment values by using a t-test for paired samples. A P value of ≤0.05 was considered to indicate a significant difference. Intergroup comparisons were made by one-way analysis of variance using the Bonferroni test for multiple comparisons.

RESULTS

Trained rats weighed 318 ± 8 g, which is not significantly different from their untrained littermates (334 ± 10 g; P > 0.05) at the end of the training protocol. As expected from our previous studies (8, 32), systemic hypoxia produced a reversible increase in leukocyte-endothelial adherence in untrained rats (Fig. 1). Trained rats, on the other hand, showed no increase in leukocyte-endothelial adherence, either when studied immediately after cessation of training or 2 or 4 wk after training (Fig. 1). Reduction in arterial Po2 from 86.2 ± 5 to 32.3 ± 2 Torr was accompanied by a decrease in PmO2 from 34.3 ± 2 to 5.5 ± 0.3 Torr at the end of hypoxia in the untrained group. In the trained group, a similar reduction in arterial Po2 from 83.0 ± 5 to 33.4 ± 3 was accompanied by a decrease in PmO2 from 34.4 ± 2 to 5.8 ± 1 Torr (Fig. 2). Thus, despite similar decreases in arterial Po2 and PmO2 during hypoxia, trained rats did not show increased leukocyte-endothelial adhesive interactions. The absence of leukocyte adherence in trained animals was not due to lower levels of white blood cells in the trained rats than in their untrained littermates (Table 1). Leukocyte count did not change significantly after training. Hypoxia, however, was accompanied by a decrease in the circulating white blood cell count in both groups of rats (Table 1). No differences in red blood cell or hemoglobin concentrations were detected among groups, either in normoxia or in hypoxia (Table 1).

Breathing low O2 caused a reversible decrease in the shear rate within cremaster venules of untrained rats from 243 ± 40 s⁻¹ before the onset of hypoxia to 72 ± 26 s⁻¹ after 10 min of hypoxia (Fig. 3). Trained rats also showed a decrease in shear rate during hypoxia beginning at 232 ± 21 s⁻¹ before hypoxia and dropping to 85 ± 16 s⁻¹ at the end of the hypoxic period. These values are not significantly different from the corresponding values in the untrained group. Similar changes in shear rate were observed after 2 and 4 wk of cessation of exercise training (Fig. 3). Because venular diameter did not change during hypoxia, the decreases in shear rate are dependent only on changes in red blood cell velocity, which is proportional to changes in blood flow. Blood flow decreases to the cremaster are most likely due to the drop in blood pressure during hypoxia in anesthetized animals. Mean arterial blood

### Table 1. Circulating blood leukocyte, red blood cell, and hemoglobin concentrations in sedentary, trained, and posttraining animals

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<td>WBC cells, 10⁶/μl</td>
<td>14.5±2.9</td>
<td>14.0±1.0</td>
<td>15.1±2.8</td>
<td>10.8±2.9</td>
<td>11.6±0.8</td>
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<td>14.8±3.5</td>
<td>16.5±1.4</td>
<td>16.3±2.4</td>
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<tr>
<td>RBC cells, 10⁹/μl</td>
<td>8.8±0.5</td>
<td>8.4±0.1</td>
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<td>7.9±0.4</td>
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<td>8.6±0.5</td>
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<td>[Hb], g/dl</td>
<td>16.0±0.6</td>
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<td>16.8±0.8</td>
<td>15.8±0.5</td>
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Values are means ± SE. Blood cell count of untrained (UT; n = 6), trained (T; n = 6), and posttraining (Off T; n = 5) animals breathing room air (normoxia), 10% O2 (hypoxia), and room air again (recovery). WBC, leukocytes; RBC, red blood cells; [Hb] blood hemoglobin concentration. *P < 0.05 vs. respective normoxia value.
The major finding of this study is that exercise training prevents hypoxia-induced increases in leukocyte-endothelial adherence and in vascular permeability in skeletal muscle. The cremaster is not a locomotor muscle; accordingly, the effects observed are likely to be the result of systemic effects of exercise training rather than effects secondary to the increased mechanical activity of the muscle. Effects of training on muscle groups that did not participate in exercise have been demonstrated before (13, 25). The effects of exercise training on leukocyte-endothelial adherence persisted for up to 1 mo after exercise training was discontinued.

Increased leukocyte-endothelial adhesive interactions in postcapillary venules are the initial manifestation of microvascular inflammation. This coordinated process is regulated by glycoproteins expressed in endothelial cells and leukocytes (9) and involves rolling of leukocytes, followed by firm adhesion to venular endothelial cells, and emigration to the extravascular space following a chemotactic gradient (9), which, in the case of hypoxia-induced inflammation, appears to be the result of mast cell activation (37). Adherent leukocytes release proteolytic enzymes and reactive O2 species that injure the venular wall and increase vascular permeability (9). Exercise training may have influenced one or more of these steps in the inflammatory cascade. Although the precise mechanism responsible for the effect of exercise training cannot be ascertained from the present data, several possible factors that could influence the inflammatory response to hypoxia can be ruled out.

Reduction of inspired PO2 resulted in similar decreases in PmO2 in both trained and untrained groups, suggesting that the effect of exercise training was not due to trained rats experiencing a less severe degree of tissue hypoxia. Phosphorescence quenching was used to provide a measurement of PmO2 in the microcirculation within the physiological context of the intact animal. The probe binds to albumin and is therefore localized to the intravascular compartment. The estimate of PmO2 obtained in these experiments is the result of a signal originating in the blood contained in the tissue sampled; accordingly, it is a weighted average determined by the relative proportion of the blood contained in the arterioles, capillaries, and venules. Although the method as used in these experiments does not provide an exact value of PO2 in each of these vascular segments, it provides a reliable estimate of the state of oxygenation of the tissue under study, since PmO2 reflects the balance between the rates of O2 delivery and consumption by the tissue. One possible factor that could have influenced the value of PmO2 in the untrained rats is the effect of albumin extravasation due to the increase in vascular permeability.
Extravasation of albumin will determine that the phosphorescence signal will originate from the intravascular as well as the interstitial space of the muscle. Although we observed increased vascular permeability to FITC-albumin in untrained rats, extravasation in trained rats was minimal (Figs. 4 and 5). Despite this, measured PmO2 values were essentially the same in both groups. This suggests that the PmO2 values of the untrained rats were not affected by albumin leakage to the extravascular space. It is difficult to envision a situation in which a given reduction of arterial P02 would result in essentially the same value of PmO2, generated from distribution of the phosphor throughout different compartments with different P02 values. It is more likely that after 10 min of hypoxia, when PmO2 values were determined, the magnitude of albumin extravasation in the untrained rats was negligible. This possibility is supported by an earlier observation that 20 min of more severe hypoxia did not result in increased permeability to albumin in skeletal muscle microcirculation (21).

The fact that circulating levels of leukocytes were similar in trained and untrained groups indicates that the lack of leukocyte adherence during hypoxia in trained rats, both immediately as well as up to 1 mo after cessation of training, cannot be explained by a reduced number of circulating leukocytes in the trained animals. Interestingly, there were reversible decreases in circulating leukocyte concentration during hypoxia in both groups. There were no consistent changes in either red blood cell or blood hemoglobin concentration with hypoxia, suggesting that the decrease in leukocyte concentration was not due to intra- to extravascular water shifts. It is likely that the transitory decrease in circulating leukocytes was the result of leukocyte sequestration in the microcirculation. Leukocyte velocity is markedly reduced during blood transit through the lungs (15, 16). The pulmonary vasoconstriction of hypoxia may exaggerate this process. Furthermore, leukocyte adherence to endothelial cells should contribute to decrease the concentration of circulating leukocytes; however, the extent to which this phenomenon influences circulating leukocyte levels is difficult to estimate. We have observed that leukocyte-endothelial adherence is widespread in untrained rats (8, 20, 32, 42); however, it is not clear whether the protective effect of exercise training extends to microvascular beds other than skeletal muscle. Although the role of adherence of leukocytes to the endothelium on circulating leukocyte levels during hypoxia is unresolved, it is clear that the lack of leukocyte-endothelial adherence in trained rats is not due to an insufficient number of leukocytes.

Hypoxia was accompanied by a decrease in shear rate in both groups. Shear rate is the force generated by the flow of blood along the venular wall that opposes adherence of leukocytes to the endothelium (12). Thus it was important to determine whether exercise training attenuated the decrease in shear rate during hypoxia in anesthetized animals. Our measurements of shear rate show no difference between trained and untrained rats. Thus a higher shear rate during hypoxia is not the cause of the prevention of leukocyte adherence in trained animals.

The data obtained in these experiments indicate that the protective effect of exercise training on an early marker of microvascular inflammation, i.e., increased leukocyte-endothelial adhesive interactions, cannot be explained on the basis of a less severe level of hypoxia, a smaller number of circulating leukocytes, or a smaller shear rate in the trained vs. untrained animals.

The effects of hypoxia and exercise training were assessed on another marker of inflammation, namely microvascular permeability. Changes in microvascular permeability were assessed from the distribution of fluorescently labeled albumin. A longer time of exposure to hypoxia (4 h) was chosen because previous experiments by others (21) and unpublished results from our laboratory suggest that the time course of the increase in vascular permeability during hypoxia is relatively slow compared with that of leukocyte-endothelial adhesive interactions. Because of the necessity to maintain the animals in hypoxia for a relatively long period of time, we elected to maintain the animals under the more physiological conscious state for most of the duration of the experiment. The data represented in Figs. 5 and 6 show that extravasation of FITC-albumin is significantly lower in the trained animals after over 4 h of hypoxia, indicating that the hypoxia-induced increase in vascular permeability is markedly attenuated by exercise training.

In summary, exercise training protects against hypoxia-induced increases in leukocyte adherence and vascular permeability in the nonlocomotory cremaster despite decreases in arterial P02 and PmO2 to similar levels in trained and untrained rats during hypoxia. The effects are not due to lower leukocyte levels or a higher shear rate in the trained animals, suggesting that exercise training may intervene at other steps in the inflammatory cascade. The effects on a nonlocomotory muscle suggest that exercise training attenuates hypoxia-induced inflammation through systemic effects. The mechanisms involved, as well as possible effects on other microcirculatory beds, remain to be elucidated. These results support previous observations of a beneficial role of exercise training on inflammatory diseases and may lead to better understanding of the role of inflammation in altitude illnesses.

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