Hypoxia causes leukocyte adherence to mesenteric venules in nonacclimatized, but not in acclimatized, rats

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Wood, John G., Leone F. Mattioli, and Norberto C. Gonzalez. Hypoxia causes leukocyte adherence to mesenteric venules in nonacclimatized, but not in acclimatized, rats. J. Appl. Physiol. 87(3): 873–881, 1999.—Although the effects of ischemia-reperfusion have received considerable attention, few studies have directly evaluated the microcirculatory response to systemic hypoxia. The overall objective of this study was to assess the effect of environmental hypoxia on adhesions of circulating leukocytes with rat mesenteric venules by using intravital microscopy. Experiments were designed to 1) characterize the adhesive interactions of circulating leukocytes to venules during acute hypoxia produced by a reduction in inspired PO2, 2) evaluate the role of nitric oxide in these adhesive interactions, 3) determine whether the effect of hypoxia on leukocyte adhesive interactions differs between acclimatized and nonacclimatized rats, and 4) assess whether compensatory changes in nitric oxide formation contribute to this difference. The results showed that acute hypoxia promotes leukocyte-endothelial adherence in mesenteric venules of nonacclimatized rats. The mechanism of this response is consistent with depletion of nitric oxide within the microcirculation. In contrast, no leukocyte-endothelial adherence occurred during hypoxia in rats acclimatized to hypobaric hypoxia. The results are consistent with increased nitric oxide formation due to expression of inducible nitric oxide synthase during the acclimatization period. Further studies are needed to establish the cause of nitric oxide depletion during acute hypoxia as well as to define the compensatory responses that attenuate hypoxia-induced leukocyte-endothelial adherence in the microvasculature of acclimatized rats.

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on leukocyte adhesive interactions differs in acclimatized and nonacclimatized rats, and 4) assess whether compensatory changes in nitric oxide formation play a role in this difference.

METHODS

All surgical and experimental procedures were approved by the Animal Care and Use Committee of the University of Kansas Medical Center. The University of Kansas is accredited by the American Association for the Accreditation of Laboratory Animal Care. Guidelines set by the National Institutes of Health and the Public Health Service Policy on the humane use and care of laboratory animals were followed at all times.

Surgical Preparation

After an overnight fast with free access to water, male Sprague-Dawley rats weighing 250–325 g were anesthetized by an intramuscular injection of urethan (1.5 g/kg). During all procedures, the animal's temperature was maintained at 36–38°C by using a homeothermic blanket system (Harvard Apparatus, Natick, MA) connected to an intraocular temperature probe. Polyethylene cannulas (PE-50) were inserted into a jugular vein and a carotid artery. Lactated Ringer solution was infused via the jugular vein (2 ml/h) while blood pressure was continuously measured by using the carotid artery cannula connected to a digital blood pressure monitor (MicroMed, Louisville, KY). A tracheostomy was performed, and the trachea was intubated by using polyethylene tubing (PE-240).

Intravital Microscopy: Adhesive Interactions of Circulating Leukocytes With Mesenteric Venules

The abdomen was opened along the midline by using a radiocautery (Harvard Apparatus), and the animal was then positioned on a Plexiglas sheet on top of the stage of a Zeiss Axiovert inverted microscope. A section of the small intestine was carefully removed from the abdomen and positioned over a glass coverslip on a Plexiglas sheet to view a mesenteric venule. The mesentery was covered with a piece of Saran wrap to prevent drying of the tissue and to minimize the effect of ambient oxygen on the mesenteric venules. Mesenteric venules were selected for experiments based on the following criteria: 1) straight, unbranched vessels at least 100 µm in length; 2) diameters of 25–40 µm; 3) fewer than three adherent leukocytes observed within a 100-µm segment of the venule during control periods; and 4) no lymphatic vessels adjacent to the venule. The mesentery was superfused (2 ml/min) with phosphate-buffered saline (37°C, pH 7.4) to keep the tissue moist and warm. Images of mesenteric venules (40 objective, 10 eyepiece) were recorded on a videocassette recorder with a time-date generator (Panasonic Video Cassette Recorder with a time-date generator (Panasonic, Elkins-Sinn (Cherry Hill, NJ)). 1,3-Propanediolamine, N,N,N',N'-tetraethylenediamine, N-(1-hydroxy-2-nitroso-5-imino-2,4-pentadiene) was dissolved in phosphate-buffered saline plus 0.02% bovine serum albumin (pH 7.4) on the day of the experiment. Phosphate-buffered saline, bovine serum albumin, l-arginine, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). L-arginine hydrochloride and heparin sodium from porcine intestinal mucosa were purchased from Elkins-Sinn (Cherry Hill, NJ).

Acclimatization to Hypoxia

The animal model of acclimatization to altitude hypoxia has been described previously (7). Briefly, rats were placed for 3 wk in a chamber where air was circulated at a pressure of 370 Torr, which resulted in an inspired PO2 of ~70 Torr. The chamber was opened three times each week for ~30 min to change animal cages and provide food and water. After 3 wk, the animals were removed from the chamber and prepared for intravital microscopy as described above.

Experimental Protocols

Experiments were begun after a stabilization period of ~30 min after surgery. The protocols for the different groups are described in detail below. In all experiments, the animals spontaneously breathed room air or hypoxic gas mixtures through a two-way valve (2384 series, Hans Rudolph, Kansas City, MO), which had been attached to the tracheal tube before the experiment began. Arterial blood samples were collected at the end of each experimental period and analyzed for pH, PO2, and PCO2 with appropriate electrodes at 38°C and corrected to the rat's rectal temperature by using temperature-correction factors for rat blood (7).

Series 1: Effect of Hypoxia on Leukocyte Adherence to Mesenteric Venules of Nonacclimatized and Acclimatized Rats

The protocol consisted of a 10-min period in which the animal breathed room air, followed by a 10-min period of hypoxia, and finally a 10-min recovery period while the animal breathed room air again. Hypoxia was produced by having the animal breathe from a bag containing a mixture of 10% oxygen with the balance consisting of nitrogen. This gas mixture also resulted in an inspired PO2 of ~70 Torr; i.e.,...
approximately the same as that in the hypobaric chamber. The oxygen concentration in the gas mixture was determined with an Applied Electrochemistry oxygen analyzer (7). Adhesive interactions of leukocytes with mesenteric venules were measured during every minute of each experimental period. Experiments were performed in nonacclimatized rats and in rats acclimatized to hypoxia, as described above. Because the initial results showed no increase in leukocyte adherence in acclimatized rats breathing 10% oxygen, all additional experiments in acclimatized rats were performed by using 8.5 and 7.5% oxygen mixtures.

One of the well-known compensations to chronic hypoxia is increased red blood cell formation due to elevated erythropoietin levels. As a result, hematocrit, and therefore blood viscosity, are significantly higher in acclimatized rats compared with nonacclimatized animals. To determine whether the increased hematocrit contributed to the reduced leukocyte adherence during hypoxia in acclimatized rats, the following experiments were performed. After completion of surgical procedures in a group of acclimatized rats, arterial blood was withdrawn and replaced with an equal volume of saline containing 5% bovine serum albumin (3–5.5 ml/rat). Sufficient albumin-saline was given to reduce hematocrit to 47.2 ± 1.9 (n = 4), which was not significantly different from nonacclimatized animals. The effect of systemic hypoxia on leukocyte adherence to mesenteric venules was then examined by using the protocol described above.

Series 2: Effect of Procedures to Increase Tissue Nitric Oxide Levels on Leukocyte Adherence During Hypoxia in Nonacclimatized Rats

The protocol of these experiments was the same as above, except that in separate groups of animals tissue levels of nitric oxide were increased by either superfusing a nitric oxide donor (spermine NONOate, 100 µM) or L-arginine (1 mM) over the mesentery during the hypoxic period. Although spermine NONOate is stable at pH 8.5 in the superfusate, it is highly permeable and diffuses into the tissue, where the compound dissociates in response to the lower pH (~7.4) and releases nitric oxide in this process. In addition to exogenous administration of nitric oxide with spermine NONOate, L-arginine was given to enhance endogenous formation of nitric oxide during hypoxia by increasing the substrate for nitric oxide synthase.

Series 3: Effect of Inducible Nitric Oxide Synthase (iNOS) Inhibition on Leukocyte Adherence During Hypoxia in Acclimatized Rats

The protocol of these experiments consisted of a 10-min control period; a 10-min period in which the mesentery was superfused with 1,4-PBIT (100 µM), an iNOS inhibitor (4), while the rat breathed 8.5% oxygen-91.5% nitrogen mixture; and a 10-min recovery period while the rat breathed room air.

In addition, the ability of 1,4-PBIT to promote leukocyte adherence in nonacclimatized rats was also determined. The animals breathed room air throughout these experiments. After a 10-min control period, 1,4-PBIT (100 µM) was superfused over the mesentery for an additional 10-min period.

Statistical Analysis

Means and standard errors were calculated for all values from each treatment group. The statistical significance of observed differences was evaluated by using a statistical analysis program (Statistix 4.0, Analytical Software, St. Paul, MN). Analysis of variance with Bonferroni’s pairwise comparison of means, Student’s t-test, and paired t-test were used to compare groups. Values of P < 0.05 were considered to be statistically significant.

RESULTS

Series 1: Effect of Hypoxia on Leukocyte Adherence to Mesenteric Venules of Nonacclimatized and Acclimatized Rats

In both nonacclimatized and acclimatized rats, breathing a 10% oxygen-90% nitrogen mixture resulted in a significant decrease in arterial Po2 (PaO2) and an increase in arterial pH, as well as a decrease in arterial PCO2 due to compensatory hyperventilation (Table 1). The acclimatized rats breathing 8.5% oxygen had a PaO2 of 33.5 ± 2.4 Torr (n = 2), and those breathing 7.5% oxygen exhibited a PaO2 of 28.2 ± 1.5 Torr (n = 2). Because no differences in microcirculatory parameters were observed between these three groups of acclimatized rats, the microcirculatory data of all of acclimatized rats were combined.

Figure 1 shows changes in mean arterial blood pressure during a control period of room-air breathing, a 10-min hypoxia period, and a recovery period in which animals returned to room-air breathing. As shown before (26), in all periods arterial pressure was significantly higher in acclimatized rats compared with the nonacclimatized group. Hypoxia caused a rapid and significant decrease in arterial pressure in both groups, whereas the return to room-air breathing resulted in a recovery of arterial pressure to baseline values.

In the nonacclimatized rats, breathing a 10% oxygen-90% nitrogen mixture caused a marked increase in leukocyte adherence to mesenteric venules. Figure 2 shows photomicrographs from a representative experiment. Figure 2, top, represents control conditions during breathing of room air; no leukocytes are seen interacting with the venular endothelium. However, a progressive increase in the number of adherent leukocytes was observed during hypoxia (Fig. 2, bottom). Figure 3 presents the cumulative results for changes in

Table 1. Arterial blood-gas composition in nonacclimatized and acclimatized rats during breathing of room air (control), 10% oxygen-90% nitrogen mixture (hypoxia), and room air again (recovery)

<table>
<thead>
<tr>
<th></th>
<th>Nonacclimatized</th>
<th>Acclimatized to Pa of 370 Torr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PaO2, Torr</td>
<td>PaCO2, Torr</td>
</tr>
<tr>
<td>Control</td>
<td>77.0 ± 3.0</td>
<td>29.8 ± 1.9</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>32.0 ± 3.9</td>
<td>22.8 ± 0.9</td>
</tr>
<tr>
<td>Recovery</td>
<td>81.6 ± 3.3</td>
<td>26.7 ± 2.2</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 rats/group. Pa, barometric pressure; PaO2, arterial Po2; PaCO2, arterial PCO2.
the number of adherent leukocytes as well as shear rate during the control period (breathing room air), hypoxia, and recovery period (breathing room air again). During the control period, the number of adherent leukocytes was not significantly different from zero in either the nonacclimatized or acclimatized rats. Hypoxia resulted in a rapid and progressive increase in the number of adherent leukocytes in the nonacclimatized rats. On removal of hypoxia, no further increase in leukocyte adherence occurred, and a slight decrease was observed; however, the number of adherent leukocytes throughout the recovery period remained significantly greater compared with the control period. In contrast, the number of adherent leukocytes in acclimatized rats remained virtually at zero during both hypoxia and the recovery period.

Hypoxia resulted in a significant decrease in shear rate in both the nonacclimatized and acclimatized rats. Shear rate decreased entirely because of the decrease in blood velocity, as the vessel diameter did not change. Shear rate rapidly returned to baseline values in both groups during the recovery period of room-air breathing. The reduction in leukocyte adherence during hypoxia in acclimatized rats was not due to the increased blood viscosity secondary to the elevated hematocrit. When hematocrit was reduced to the same level as in nonacclimatized rats (47.2 ± 1.9; n = 4), no significant leukocyte adherence was observed during the control, hypoxia, or recovery periods: 0.2 ± 0.1, 0.2 ± 0.1, and 0 ± 0 leukocytes/100 µm, respectively.

Leukocyte rolling velocity significantly decreased during hypoxia compared with control values in nonacclimatized animals, we normalized leukocyte rolling velocity to shear rate of 1,000 s⁻². These normalized values are as follows: control, 140 ± 35; hypoxia, 176 ± 42; and recovery, 79 ± 11 µm·s⁻¹. After normalization, leukocyte rolling velocity was not significantly different during hypoxia compared with control values (P > 0.05). These normalized values for leukocyte rolling do not indicate stronger leukocyte-selectin interactions occurred during hypoxia. Instead, the reduction in
leukocyte rolling velocity shown in Table 2 simply reflects the decrease in shear rate during hypoxia. Leukocyte rolling flux did not significantly change during hypoxia; values for leukocyte flux in nonacclimatized rats in the tenth minute of each period were as follows: control, 22.6 ± 11.9 leukocytes/min; hypoxia, 27.0 ± 10.1 leukocytes/min; and recovery, 16.2 ± 7.6 leukocytes/min. In acclimatized rats, hypoxia caused a slight, nonsignificant decrease in leukocyte rolling velocity (Table 2). In contrast to nonacclimatized rats, leukocyte rolling flux significantly decreased during hypoxia; values for leukocyte flux in acclimatized rats in the tenth minute of each period were as follows: control, 16.8 ± 4.4 leukocytes/min; hypoxia, 8.5 ± 3.9 leukocytes/min (P < 0.05 vs. control); and recovery, 5.7 ± 4.7 leukocytes/min.

Series 2: Effect of Procedures to Increase Tissue Nitric Oxide Levels on Leukocyte Adherence During Hypoxia in Nonacclimatized Rats

Superfusion of the mesentery with either the nitric oxide donor spermine NONOate or with L-arginine did not significantly change the number of adherent leukocytes during room-air breathing (Fig. 4). However, exogenous infusion of the nitric oxide donor completely prevented the increased leukocyte adherence during hypoxia. In contrast, L-arginine only partially attenuated leukocyte adherence during hypoxia, compared with untreated rats. In addition, during the recovery period, there was a tendency for the number of adherent leukocytes to decrease more rapidly in the L-arginine-treated rats, compared with the untreated group. Despite these differences in leukocyte adherence, shear rate during hypoxia decreased to comparable levels in all of these groups. Compared with control values, there were no significant differences in leukocyte rolling velocity during hypoxia in nonacclimatized rats treated with spermine NONOate or L-arginine, although the high variability may have obscured any difference.

Series 3: Effect of iNOS Inhibition on Leukocyte Adherence During Hypoxia in Acclimatized Rats

Figure 5 shows the effect of an inhibitor of iNOS (1,4-PBIT) on leukocyte adherence in acclimatized rats. There was no difference in leukocyte adherence during the control period of room-air breathing between the untreated and 1,4-PBIT-treated groups. Whereas there was no increase in leukocyte adherence during hypoxia in the untreated acclimatized rats, acclimatized rats given 1,4-PBIT showed a significant increase in leukocyte adherence during hypoxia. However, the magnitude of this increase was only 50% of that in nonacclimatized rats during hypoxia. In addition, 1,4-PBIT significantly decreased leukocyte rolling velocity during hypoxia, compared with control values (Table 2).

Table 2. Leukocyte rolling velocity in nonacclimatized and acclimatized rats in control, hypoxia, and recovery

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leukocyte Rolling Velocity, µm/s</th>
<th>Control</th>
<th>Treatment</th>
<th>Hypoxia</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated nonacclimatized</td>
<td></td>
<td>102 ± 25</td>
<td>55.2 ± 13*</td>
<td>69.7 ± 9.8</td>
<td></td>
</tr>
<tr>
<td>SNO-treated nonacclimatized</td>
<td></td>
<td>202 ± 50</td>
<td>231 ± 94</td>
<td>109 ± 14</td>
<td>266 ± 93</td>
</tr>
<tr>
<td>L-arginine-treated nonacclimatized</td>
<td></td>
<td>97.5 ± 12.8</td>
<td>75.0 ± 5.9</td>
<td>52.5 ± 12.2</td>
<td>101.7 ± 7.0</td>
</tr>
<tr>
<td>Untreated acclimatized</td>
<td></td>
<td>140 ± 35</td>
<td>124 ± 40.7</td>
<td>102 ± 24.2</td>
<td></td>
</tr>
<tr>
<td>1,4-PBIT-treated acclimatized</td>
<td></td>
<td>122 ± 29</td>
<td>37.2 ± 7.4*</td>
<td>106 ± 21.7</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE, obtained during 10th minute of each group. Untreated nonacclimatized, n = 5; spermine NONOate (SNO)-treated nonacclimatized, n = 5; L-arginine-treated nonacclimatized, n = 4; untreated acclimatized, n = 8; and 1,4-PBIT-treated acclimatized rats, n = 5. *P < 0.05 vs. control values.
whereas there was no decrease in rolling velocity during hypoxia in untreated acclimatized rats. The specificity of 1,4-PBIT for iNOS is supported by the fact that it had no effect on leukocyte adherence in nonacclimatized rats (Table 3). Under control conditions, no iNOS should be expressed in endothelial cells of the mesenteric microcirculation, which is consistent with these results. Although there were no statistically significant differences in the number of adherent leukocytes between control values and after 1,4-PBIT (Table 3), we cannot rule out the possibility that there is a slight, although nonsignificant, effect of the iNOS inhibitor on leukocyte-endothelial adherence.

Fig. 4. Effect of superfusion of mesentery of nonacclimatized rats with either nitric oxide donor (spermine NONOate; ▲, n = 5) or with L-arginine (▲, n = 4) on hypoxia-induced leukocyte adherence. For comparison, results obtained in untreated nonacclimatized rats shown in Fig. 3 are represented by ○. A: leukocyte adherence; B: shear rate.

Table 3. Effect of 1,4-PBIT on leukocyte adherence to mesenteric venules of nonacclimatized rats

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Control (room air)</th>
<th>Time, min</th>
<th>1,4-PBIT (room air)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3 ± 0.3</td>
<td>11</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>0.3 ± 0.3</td>
<td>12</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>0.3 ± 0.3</td>
<td>13</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>0.2 ± 0.2</td>
<td>14</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>0.2 ± 0.2</td>
<td>15</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>6</td>
<td>0.0 ± 0.0</td>
<td>16</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>7</td>
<td>0.0 ± 0.0</td>
<td>17</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>8</td>
<td>0.0 ± 0.0</td>
<td>18</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>9</td>
<td>0.0 ± 0.0</td>
<td>19</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>10</td>
<td>0.3 ± 0.3</td>
<td>20</td>
<td>0.5 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 animals.
DISCUSSION

The major finding of this study is that hypoxia causes a rapid and progressive increase in leukocyte adherence to mesenteric venules in nonacclimatized rats but does not have this effect in acclimatized rats. In nonacclimatized rats, the time course of adherence during hypoxia was extremely rapid. Compared with baseline values during room-air breathing, we observed a significant increase in the number of adherent leukocytes after only 4 min of hypoxia (P < 0.05). After 10 min of hypoxia, there were 12.4 ± 3.2 adherent leukocytes/100 µm of vessel, which represents a 30-fold increase over control values (0.4 ± 0.2 adherent leukocytes/100 µm). In addition, although the number of adherent leukocytes did not increase further when the animal returned to room-air breathing, adherence decreased only slightly during the recovery period. These results indicate that the hypoxia-induced leukocyte adherence did not reverse rapidly, suggesting that a relatively strong adhesive interaction had developed between the leukocytes and the venular endothelial wall.

Shear rate decreased markedly during hypoxia, compared with values obtained during room-air breathing. The decrease in shear rate was exclusively due to a decrease in blood velocity, since venular diameter remained unchanged. There are two likely causes of the decreased blood velocity during hypoxia: the marked decrease in systemic arterial pressure as well as a sympathetic vasoconstriction of the gastrointestinal circulation that occurs in acute hypoxia and which contributes to lower intestinal blood flow, even in the absence of hypotension (17). Because shear rate is the force at the vessel wall that opposes interactions of leukocytes with the endothelial surface, a decrease in shear rate would favor enhanced leukocyte adherence. However, several lines of evidence suggest that decreased shear rate alone cannot account for the increased leukocyte adherence during hypoxia. First, shear rate decreased to even lower levels during hypoxia in acclimatized rats, compared with nonacclimatized animals, yet no leukocyte adherence was detected in the former (Fig. 3). Second, during the recovery period, shear rate of nonacclimatized rats rapidly increased to baseline levels, yet the number of adherent leukocytes did not decrease significantly (Fig. 3). Finally, the time course of changes in shear rate and leukocyte adherence during hypoxia differ considerably: the maximal decrease in shear rate was observed in the fourth minute of hypoxia, whereas the number of adherent leukocytes continued to increase throughout the hypoxic period and more than doubled between the fourth and tenth minutes (Fig. 3). These results suggest that factors other than shear rate play an important role in the mechanism of hypoxia-induced leukocyte adherence.

Our results differ in several regards from those reported for ischemia-reperfusion. First, the time course of leukocyte adherence during systemic hypoxia is much faster than after ischemia-reperfusion. A significant increase in number of adherent leukocytes was first observed at 30 min of reperfusion after 10 min of total ischemia in the brain (5) and after 1 h of total ischemia in the hamster cheek pouch (24). As noted above, leukocyte adherence was significantly increased after only 4 min of systemic hypoxia in the present study. A critical point is that increased leukocyte adherence during systemic hypoxia is occurring at a time of reduced oxygen delivery to the tissue rather than after reintroduction of oxygen to ischemic tissue (reperfusion). Furthermore, leukocyte adherence did not increase further when oxygen delivery was increased in the normoxic recovery period.

No adherent leukocytes were observed during control or hypoxic conditions in the mesenteric venules of rats that had been acclimatized to hypoxia for 3 wk (Fig. 3). The level of hypoxia to which these animals were acclimatized is comparable to that produced in the nonacclimatized rats. In fact, no significant increase in leukocyte adherence was observed when the acclimatized rats breathed 7.5% oxygen, which resulted in a PaO2 of 28 Torr. This finding suggests that the microvascular response that takes place early in the onset of hypoxia is somehow reversed during the process of acclimatization, such that PaO2 changes do not result in leukocyte adherence after acclimatization. Before attempting to define the adaptive mechanism that results in this lack of hypoxia-induced leukocyte adherence in acclimatized rats, we tried to establish the cause of this microvascular response in nonacclimatized rats.

Because considerable evidence implicates changes in nitric oxide as mediating changes in leukocyte adhesive interactions in various conditions, we tested the hypothesis that decreased nitric oxide levels are responsible for hypoxia-induced leukocyte adherence. Our results are consistent with this hypothesis, as exogenous administration of both a nitric oxide donor spermine NONOate and L-arginine, the precursor for nitric oxide, decreased the number of adherent leukocytes during hypoxia in nonacclimatized rats (Fig. 4). In fact, leukocyte adherence during hypoxia was completely prevented by spermine NONOate, whereas it was significantly attenuated by L-arginine. Our finding of a greater efficacy of the nitric oxide donor compared with L-arginine in preventing this microvascular response is in agreement with observations from studies of ischemia-reperfusion (10, 21). One explanation for this difference is that tissue levels of nitric oxide may be more effectively increased by exogenous delivery of the nitric oxide donor than by increased endogenous nitric oxide formation through L-arginine administration (10), particularly during situations that impair endothelial cell function. In fact, nitric oxide synthase requires oxygen to produce nitric oxide (32), so it is likely that L-arginine may be less effective in restoring nitric oxide formation to normal levels during hypoxia. In addition to attenuating the degree of leukocyte adherence during hypoxia, L-arginine also appeared to decrease the strength of the adhesive interaction. This is supported by the finding that the number of adherent leukocytes rapidly decreased during the recovery period in
L-arginine-treated rats, whereas there was no significant decrease in adherent leukocytes in untreated rats. The differences in leukocyte adherence between untreated and treated groups cannot be attributed to differences in shear rates between these groups during hypoxia or recovery periods.

If decreased nitric oxide levels were responsible for hypoxia-induced leukocyte adherence in nonacclimatized rats, it seemed plausible that the lack of effect of hypoxia in acclimatized rats could be due to compensatory increases in nitric oxide formation in the microcirculation during the process of acclimatization. Under normal conditions, nitric oxide is formed from nitric oxide synthase, an enzyme that is constitutively expressed in endothelial cells. However, an inducible form of this synthase can be induced in various situations involving endothelial cell injury or stress. Recent studies have reported upregulation of iNOS in the pulmonary vasculature after chronic exposure to low PO2 (11, 18, 28). We found that the administration of L-arginine to acclimatized rats increased hypoxia-induced leukocyte adherence (Fig. 5). These results are consistent with upregulation of iNOS during the period of acclimatization. Because the inducible form of the enzyme is known to produce higher amounts of nitric oxide, our results suggest that nitric oxide levels are higher in mesenteric endothelial cells of acclimatized rats. Increased nitric oxide levels could account for the lack of leukocyte adherence in acclimatized rats during breathing of 10% oxygen or gas mixtures that produce lower inspired PO2 than those maintained during acclimatization.

We have obtained evidence that the magnitude of hypoxia-induced leukocyte adherence may be related to decreased nitric oxide levels in nonacclimatized rats and to upregulation of iNOS after acclimatization. In addition to enhanced nitric oxide formation, other factors could also potentially contribute to reduced leukocyte adherence in acclimatized rats. One possible factor contributing to this reduced leukocyte adherence during hypoxia could be the increased hematocrit due to enhanced red blood cell formation during acclimatization. The higher blood viscosity could potentially attenuate leukocyte adherence by increasing the effective shear force on adherent leukocytes at a given shear rate; alternatively, the greater red blood cell mass could result in enhanced oxygen delivery to the venule. However, we found that acute reduction of hematocrit of acclimatized rats to the same level as in nonacclimatized rats did not enhance leukocyte adherence during hypoxia. These results do not support increased viscosity of the blood due to higher hematocrit or greater oxygen content in blood due to higher red blood cell mass as the cause of reduced leukocyte adherence in acclimatized rats.

Whereas our results are consistent with depletion of nitric oxide levels within the microcirculation as the cause of hypoxia-induced leukocyte adherence in nonacclimatized rats, at this time we do not know what decreases this endothelium-derived factor under these conditions. Recent studies have shown that hypoxia reduces nitric oxide formation in endothelial cells in vitro (32) and in an isolated lung preparation (12). One possible explanation is that acute hypoxia promotes the generation of reactive oxidants, which can inactivate nitric oxide. This is supported by the observation that graded hypoxia causes dose-related increases in reactive oxidant generation in isolated cardiomyocytes (3). In addition, antioxidants have been recently shown to improve contractile function of the diaphragm under hypoxic conditions (25). Another contributing factor to lower nitric oxide levels during hypoxia may involve the oxygen dependence of nitric oxide synthase (32). Nitric oxide formation depends on the concentration of both L-arginine and oxygen; a decrease in either may limit its production. However, administration of L-arginine attenuated hypoxia-induced leukocyte adherence, which is consistent with enhanced nitric oxide synthesis. This finding suggests that the reduced oxygen levels during hypoxia were not sufficiently low to completely inhibit nitric oxide formation, as protective effects of L-arginine would not be expected in this situation.

Enhanced reactive oxidant generation has been reported in tissues not only in response to acute hypoxia but during chronic hypoxia as well (20). A recent study showed that oxidative stress upregulated iNOS in the liver (15). Based on these findings, oxidant generation may be involved in the expression of iNOS during chronic hypoxia, as suggested by this study and by others (1, 11, 18, 28). The resulting higher levels of nitric oxide may represent an adaptive response to oxidant stress, since nitric oxide has been proposed to play an important role as an antioxidant in endothelial cells (27). Our results suggest that upregulation of iNOS has occurred after 3 wk of acclimatization to 10% oxygen; we did not examine earlier times to determine when this response first occurred. However, a progressive increase in iNOS expression within the pulmonary vasculature has been demonstrated in rats during 7 days of exposure to a 10%-oxygen environment, whereas a slight but significant increase in pulmonary iNOS level was detected after only 1 day of hypoxia (33).

In summary, these studies showed that acute hypoxia causes leukocyte-endothelial adherence in the mesenteric circulation of nonacclimatized rats. The mechanism of this response appears to involve depletion of nitric oxide within the microcirculation. In contrast, almost no adherent leukocytes were observed during hypoxia in mesenteric venules of rats acclimatized to hypoxia. These results are consistent with increased nitric oxide formation due to expression of iNOS during the acclimatization period. Further studies are needed to establish the cause of nitric oxide depletion during acute hypoxia as well as to define the compensatory responses that attenuate hypoxia-induced leukocyte-endothelial adherence in the microvasculature of acclimatized rats. In addition, whether these responses in the mesenteric circulation represent a general phenomenon to systemic hypoxia in other regional microcirculations remains to be determined.
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