Interaction between reactive oxygen species and nitric oxide in the microvascular response to systemic hypoxia

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Steiner, Dawn R. S., Norberto C. Gonzalez, and John G. Wood. Interaction between reactive oxygen species and nitric oxide in the microvascular response to systemic hypoxia. J Appl Physiol 93: 1411–1418, 2002.—Systemic hypoxia results in oxidative stress due to a change in the reactive oxygen species (ROS)-nitric oxide (NO) balance. The mechanism responsible for these hypoxia-induced inflammatory responses is still not completely known, but accumulating evidence supports a role for reactive O2 species (ROS) in this phenomenon. A series of in vitro studies demonstrated that a reduction in PO2 leads to increased ROS generation within cardiomyocytes (9), hepatocytes (6), and endothelial cells (1). These studies provided compelling evidence that mitochondria are the major site of hypoxia-induced ROS formation, specifically via ubisemiquinone within the electron transport chain (6, 9).

The concept of ROS generation as a critical event in the pathogenesis of hypoxia-induced microvascular inflammatory responses is appealing. Oxidative stress has clearly been established as an underlying cause of microvascular injury in other settings, including ischemia/reperfusion, hemorrhagic shock/resuscitation, diabetes, and hypercholesterolemia (5). We have recently obtained evidence that supports a major role for ROS in microvascular responses to hypoxia in intact animals. The fluorescence intensity of an oxidant-sensitive probe [dihydorhodamine 123 (DHR)] within mesenteric venules was markedly increased during systemic hypoxia (32). Administration of antioxidants attenuated the hypoxia-induced increase in DHR fluorescence (32), the increase in leukocyte adherence/emigration, and the increase in vascular permeability (33). Collectively, these results indicate that systemic hypoxia promotes oxidative stress within the mesenteric microcirculation and are consistent with the concept that ROS act at the onset of the microvascular inflammatory response to hypoxia.

ROS are known to promote endothelial dysfunction through several mechanisms, including direct action on the vascular endothelium, promoting formation of

Although considerable effort has been made to define microvascular responses to ischemia/reperfusion (5, 13), relatively little information exists regarding the effect of systemic hypoxia, i.e., a reduction in blood oxygen (O2) levels without an interruption in flow. Results from in vitro systems have shown that a reduction in PO2 of the perfusate increases leukocyte adherence to cultured endothelial cells (2, 21) and to umbilical vein segments (4, 22). However, opposing results have also been obtained, as decreased adhesive interactions between granulocytes and human umbilical vein endothelial cells were observed during hypoxia (24). Recent in vivo studies have demonstrated microvascular inflammatory responses after a reduction in inspired PO2 because systemic hypoxia resulted in rapid leukocyte adherence to postcapillary venules of the cremasteric (3) and mesenteric microcirculations (34). Furthermore, exposure of conscious animals to hypoxia for 4 h enhanced leukocyte emigration into the perivascular space and increased vascular permeability to albumin (33).

The mechanism responsible for these hypoxia-induced inflammatory responses is still not completely known, but accumulating evidence supports a role for reactive O2 species (ROS) in this phenomenon. A series of in vitro studies demonstrated that a reduction in PO2 leads to increased ROS generation within cardiomyocytes (9), hepatocytes (6), and endothelial cells (1). These studies provided compelling evidence that mitochondria are the major site of hypoxia-induced ROS formation, specifically via ubisemiquinone within the electron transport chain (6, 9).

The concept of ROS generation as a critical event in the pathogenesis of hypoxia-induced microvascular inflammatory responses is appealing. Oxidative stress has clearly been established as an underlying cause of microvascular injury in other settings, including ischemia/reperfusion, hemorrhagic shock/resuscitation, diabetes, and hypercholesterolemia (5). We have recently obtained evidence that supports a major role for ROS in microvascular responses to hypoxia in intact animals. The fluorescence intensity of an oxidant-sensitive probe [dihydorhodamine 123 (DHR)] within mesenteric venules was markedly increased during systemic hypoxia (32). Administration of antioxidants attenuated the hypoxia-induced increase in DHR fluorescence (32), the increase in leukocyte adherence/emigration, and the increase in vascular permeability (33). Collectively, these results indicate that systemic hypoxia promotes oxidative stress within the mesenteric microcirculation and are consistent with the concept that ROS act at the onset of the microvascular inflammatory response to hypoxia.

ROS are known to promote endothelial dysfunction through several mechanisms, including direct action on the vascular endothelium, promoting formation of
lipid inflammatory mediators, and/or depleting the cellular levels of nitric oxide (NO), an endogenous antioxidant (12). In support of the latter mechanism operating in hypoxia, we have demonstrated that administration of an NO donor attenuates hypoxia-induced leukocyte adherence (34) and increased vascular permeability (33). However, it is not known whether these effects of NO were due to its antioxidant action, i.e., combination with superoxide (26), thus limiting the increase in ROS during hypoxia. Alternatively, the effect of the NO donor could be the result of restoring NO levels that had been depleted during hypoxia after a reduction in NO synthesis, since O2 is a substrate for NO (19).

The present experiments were designed to continue our studies of the potential significance of changes in the ROS-NO balance in hypoxia-induced microvascular injury. Specifically, experiments were designed to: 1) assess the magnitude of ROS generation and changes in leukocyte-endothelial adhesive interactions during systemic hypoxia produced by breathing gas mixtures containing 15, 10, or 7.5% O2 (if an increase in ROS and/or a change in the ROS-NO balance is a necessary event in the microvascular response to hypoxia, ROS levels would be expected to increase in proportion to the severity of hypoxia); 2) determine whether the beneficial effects of an NO donor on hypoxia-induced microvascular responses are the result of the antioxidant action of NO (if this is the case, administration of an NO donor would be expected to lower ROS levels during hypoxia); and 3) compare the effect of administration of exogenous NO with that of the NO precursor L-arginine. NO levels could be reduced during hypoxia due to a decrease in NO generation; this could be due to either a lack of O2 substrate or decreased NOS activity and would eventually result in increased ROS levels. Alternatively, hypoxia could promote increased ROS production that would lead to enhanced NO degradation. To distinguish between these two alternatives, we reasoned that comparison between the effects of administration of L-arginine and NO would provide insights into the mechanism of ROS-NO interactions in the microvascular response to hypoxia. If decreased NO levels are the result of a limitation in NO production, L-arginine administration would not be expected to reduce ROS or decrease leukocyte adherence during hypoxia. If, on the other hand, NO levels are increased in hypoxia due to enhanced NO degradation via ROS, it would be expected that L-arginine administration would have an effect comparable to that of exogenous NO.

METHODS

All surgical and experimental procedures involving animals received prior approval from the Animal Care and Use Committee at the University of Kansas Medical Center. The University of Kansas is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. Guidelines established 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. Male Sprague-Dawley rats (Sasco, Omaha, NE) weighing 200–300 g were fasted overnight and anesthetized with urethane (1.5 g/kg im). A polyethylene catheter (PE-50) was then inserted into the right common carotid artery to collect blood samples and measure systemic arterial blood pressure (Micro-Med, Louisville, KY). Animals were used in experiments only if mean arterial pressure was >85 mmHg during the normoxic control period. A catheter (PE-50) was also inserted into the right jugular vein to infuse lactated Ringer solution (2 ml/h) and administer drugs. A tracheotomy was performed with the use of polyethylene tubing (PE-240).

The animal’s temperature was maintained at 36–37°C during all procedures by using a homeothermic blanket system connected to an intrarectal temperature probe (Harvard Apparatus, Natick, MA). The abdomen was opened along the midline by using a radiocautery (Harvard Apparatus). The animal was then positioned on a Plexiglas sheet on top of the stage of a Zeiss Axiosvert inverted microscope. A section of the small intestine was carefully exteriorized and positioned over a glass coverslip on the microscope slide to view the mesenteric venules. The mesentery was covered with Saran wrap to prevent drying of the tissue and to minimize the effect of ambient O2 on the mesenteric venules.

Adhesive interactions of circulating leukocytes in mesenteric venules. Mesenteric venules were selected for experiments by using the following criteria: 1) straight, unbranched vessels at least 100 μm in length, 2) vessel diameters of 20–40 μm, 3) no adjacent vessels within 100 μm of the venule, and 4) fewer than two adherent leukocytes within a 100-μm segment of the venule. Images of the mesenteric venules (×40 objective) were recorded on a S-VHS videocassette recorder (JVC, Elmwood Park, NJ) with a timedate generator (Panasonic, Osaka, Japan) using a video camera (Panasonic). Venuolar diameter was measured by using a video caliper (Microcirculation Research Institute, College Station, TX). An optical Doppler velocimeter (Microcirculation Research Institute) was used to measure centerline red blood cell velocity in venules. Average red blood cell velocity was calculated as centerline velocity/1.6 (8). Venuolar wall shear rate, which represents the physical force generated at the vessel wall due to movement of blood, was calculated as 8 × (average red blood cell velocity/venular diameter) (15). The extent of leukocyte adherence within mesenteric venules was assessed off-line during playback of the videotapes. The total number of adherent leukocytes was determined in each minute of the experiment by counting the number of leukocytes that remained stationary for more than 30 s (34).

As previously described (32), the oxidant-sensitive probe DHR was used to quantitate ROS levels within mesenteric venules. DHR fluoresces by forming rhodamine 123 when oxidized, principally by hydrogen peroxide-dependent reactions. The fluorescence produced by rhodamine 123 was determined by using an intensified charge-coupled device camera (Hamamatsu Photonics, Shizouka, Japan). Recordings of the DHR fluorescence were made during brief intervals (15 s) to avoid light-induced activation of the probe. The fluorescence intensity was later measured during playback of videotapes by using image analysis software (National Institutes of Health Image, 1.61). The intensity of the fluorescent signal was measured in five adjacent areas (5-μm-diameter circles) along the vessel and averaged to obtain a single estimate of the fluorescent signal during each experimental period. The same field of view was maintained throughout the experiment to ensure that measurements of DHR fluorescence were obtained in the same section of the venule under each experimental condition. Values for fluorescence
during hypoxia and normoxic recovery were expressed relative to the value observed during the normoxic control period, which was defined as 100%.

Drugs and chemicals. All solutions were prepared on the day of the experiment. (Z)-1-[[aminopropyl]-N,4-[[aminopropyl]ammonio]-butyl]amino[diazen]-1-ium-1,2-diolate [ spermine NONOate (SNO), Cayman Chemical, Ann Arbor, MI] was dissolved in PBS (Sigma Chemical, St. Louis, MO) at pH 8.5. The rate of decomposition of this NO donor is both pH and temperature dependent (20) and is relatively stable at pH 8.5 (33). At pH 7.4, approximately the pH of tissue, SNO then spontaneously dissociated into NO, L-Arginine (Sigma Chemical) was prepared in PBS (pH 7.4). DHR was obtained from Molecular Probes (Eugene, OR), and urethane was purchased from Sigma Chemical.

Experimental protocols. Experiments began after a 30-min postsurgery stabilization period. In all experiments, animals spontaneously breathed through a two-way valve (2384 series, Hans Rudolph, Kansas City, MO) that was 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 adjusted to the rat’s rectal temperature by using temperature-correction factors for rat blood (11).

Series 1: systemic hypoxia and ROS levels. The protocol consisted of an initial 10-min normoxic control period in which the animals breathed room air (21% O2-79% N2), a 20-min equilibration period after intravenous injection of DHR (10 μg/kg), a 10-min hypoxic period in which the animals breathed either 15% O2-85% N2, 10% O2-90% N2, or 7.5% O2-92.5% N2, followed by a 10-min normoxic recovery period in which the animals breathed room air again. Recordings of DHR fluorescence were made at the end of the normoxic, hypoxic, and normoxic recovery periods.

Series 2: hypoxia and leukocyte-endothelial interactions. This experimental protocol consisted of a 10-min normoxic period, a 10-min hypoxic period in which the animals breathed gas mixtures containing either 15% O2, 10% O2, or 7.5% O2 in nitrogen, followed by a 10-min normoxic recovery period. The number of adherent leukocytes and venular shear rate were determined in these experiments as previously described.

Series 3: tissue NO levels, ROS levels, and leukocyte-endothelial adhesive interactions. DHR fluorescence in the mesenteric microcirculation was determined as described previously. After the normoxic period, either SNO or L-arginine was administered as indicated above. Rats breathed either 10 or 7.5% O2 for 10 min, and then room air during a 10-min normoxic recovery period. Recordings of DHR fluorescence were made at the end of each experimental period. In the experiments designed to examine the effect of SNO and L-arginine on hypoxia-induced adhesive interactions, after a 10-min normoxic period, the mesentery was superfused with SNO (100 μM) throughout the remainder of the experiment. Twenty minutes after SNO administration was started, animals breathed either 10 or 7.5% O2 for 10 min and then breathed room air for 10 min (normoxic recovery period). Leukocyte adherence and venular shear rate were measured in these experiments as previously described. The protocol for the next experiments was the same as described above except that L-arginine (1 mM, 2 ml/h) was infused intravenously in place of SNO superfusion.

Statistical analysis. Data are presented as means ± SE. Analysis of variance with Bonferroni’s pair-wise comparison of means was used to compare differences between groups (Statistix 4.0, Analytical Software, St. Paul, MN). Values of P < 0.05 were considered to be statistically significant.

RESULTS

Table 1 shows the effect of changes in the inspired PO2 on arterial PO2 (Pao2), Pco2 (Paco2), and pH. As expected, graded reduction of the inspired O2 increased the severity of systemic hypoxia, as indicated by the progressive decrease in Pao2. Hypoxia did not result in a significant change in Paco2 in rats breathing 15 or 10% O2, whereas Paco2 was significantly decreased in rats breathing 7.5% O2 compared with normoxic values. Arterial pH significantly increased during hypoxia in each group of animals.

Table 1. Arterial blood PO2, Pco2, and pH during systemic hypoxia

<table>
<thead>
<tr>
<th></th>
<th>15% O2 (n = 5)</th>
<th>10% O2 (n = 6)</th>
<th>7.5% O2 (n = 5)</th>
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<tr>
<td></td>
<td>NX</td>
<td>HX</td>
<td>NX Rec</td>
</tr>
<tr>
<td>PO2</td>
<td>92 ± 4</td>
<td>64 ± 3*</td>
<td>94 ± 5*</td>
</tr>
<tr>
<td>Pco2</td>
<td>32 ± 4</td>
<td>33 ± 2</td>
<td>32 ± 5</td>
</tr>
<tr>
<td>pH</td>
<td>7.33 ± 0.01</td>
<td>7.39 ± 0.01*</td>
<td>7.30 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE obtained during minute 10 of the normoxia (NX), hypoxia (HX), and normoxic recovery (NX Rec) periods. *P < 0.05 HX vs. NX, †P < 0.05 NX Rec vs. HX, §P < 0.05 HX 10% O2 and HX 7.5% O2 vs. HX 15% O2.

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different animals. Adherent leukocytes are indicated by the solid triangles. In all rats, no adherent leukocytes were observed during normoxia, whereas the number progressively increased as the severity of hypoxia became greater. The cumulative results from these experiments are shown in Fig. 4. During normoxia, the number of adherent leukocytes was not significantly different from zero in any group. There was no significant increase in leukocyte adherence in rats breathing 15% O₂, whereas larger reductions in inspired O₂ resulted in rapid, statistically significant increases in the number of adherent leukocytes. Furthermore, the extent of leukocyte adherence was dependent on the severity of hypoxia: leukocyte adherence was significantly higher in animals breathing 7.5% O₂ (10.2 ± 0.7 leukocytes/100 μm) compared with 10% O₂ (10.7 ± 0.7 leukocytes/100 μm) or 15% O₂ (1 ± 0.4 leukocytes/100 μm). During the normoxic recovery period, a progressive decrease was observed. The rapid increase in leukocyte adherence during hypoxia and the decrease in normoxic recovery agree with our previous observations (28, 34) and differ from the pattern observed in ischemia/reperfusion (5, 13) in which leukocyte adherence occurs as a result of en...

Fig. 1. Effect of hypoxia on dihydrorhodamine 123 (DHR) fluorescence intensity of the mesenteric microcirculation. Photographs correspond to 3 different rats breathing room air (normoxia) and after 10 min of breathing either 15% O₂ (top right), 10% O₂ (middle right), and 7.5% O₂ (bottom right). DHR fluorescence intensity increases with the severity of hypoxia, indicating increased microvascular reactive O₂ species (ROS) levels.

Fig. 2. Effect of increasing severity of hypoxia on DHR fluorescence intensity levels in the mesenteric microcirculation. Recordings of DHR fluorescence were made during brief intervals (~15 s) to avoid light-induced inactivation of the probe. Values of fluorescence intensity during hypoxia and normoxic recovery in each animal were expressed as percent of the value observed during the normoxic control period. Values are means ± SE; NX, normoxic control period; HX, hypoxic period; NX Rec, normoxic recovery period. Hypoxia was produced by breathing either 15, 10, or 7.5% O₂. Each period lasted 10 min. *P < 0.05 NX vs. HX; †P < 0.05 NX Rec vs. corresponding HX; ‡P < 0.05 HX 10% O₂ vs. HX 15% O₂; §P < 0.05 HX 7.5% O₂ vs. HX 10% O₂.

Fig. 3. Adherence of leukocytes to mesenteric venular endothelium during hypoxia. Photographs correspond to 3 different rats breathing room air (normoxia) and after 10 min of breathing 15% O₂ (top right), 10% O₂ (middle right), and 7.5% O₂ (bottom right). Adherent leukocytes (a, v) were defined as leukocytes that remained stationary for >30 s.
Enhanced ROS generation associated with elevated tissue O2 levels on reperfusion.

Venular shear rate decreased significantly during hypoxia (Fig. 4, bottom). Compared with normoxic control values, the decrease in shear rate was ~30% in animals breathing 15% O2 and ~70% in animals breathing either 10 or 7.5% O2. Each animal was exposed to only one hypoxic gas mixture. Bars represent ±1 SE on either side of the mean.

Fig. 4. Effects of systemic hypoxia on leukocyte-endothelial adherence (top) and venular shear rate (bottom) in mesenteric microcirculation. Number of adherent leukocytes and shear rate were determined every minute for 10 min during the normoxic control period, hypoxic period, and normoxic recovery. Hypoxia was induced by breathing 15, 10, or 7.5% O2. Each animal was exposed to only one hypoxic gas mixture. Bars represent ±1 SE on either side of the mean.

VENULAR SHEAR RATE

Venular shear rate decreased significantly during hypoxia (Fig. 4, bottom). Compared with normoxic control values, the decrease in shear rate was ~30% in animals breathing 15% O2 and ~70% in animals breathing either 10 or 7.5% O2. There was no significant difference in venular shear rate during hypoxia produced by 10 or 7.5% O2, however, hypoxia-induced leukocyte adherence was significantly higher with 7.5% O2. These results add further support to the view that the decrease in shear rate alone cannot account for hypoxia-induced leukocyte adherence (34).

Fig. 5. Effect of the NO donor spermine NONOate (SNO) on DHR fluorescence intensity of the microcirculation of 2 rats during room air breathing (normoxia) and after 10 min of breathing 10% O2 (top right) or 7.5% O2 (bottom right). After a normoxic control period (left), superfusion of the microcirculation with SNO (100 μM) was initiated during normoxia and maintained throughout the experiment. DHR fluorescence was determined 20 min later (middle) after which the animals breathed 10% (top right) or 7.5% O2 (bottom right) for an additional 10 min.

Series 3: Interventions to Increase Tissue NO Levels

Figure 5 shows a representative photograph of the effect of SNO on DHR fluorescence in mesenteric venules. The fluorescence intensity within the venular wall was reduced when SNO was administered during normoxia, and little increase was observed when SNO-treated animals were made hypoxic by either 10 or 7.5% O2. Figure 6 shows the results for DHR fluorescence from this series of experiments. Treatment with SNO attenuated the increase in DHR fluorescence intensity during 10 and 7.5% O2 breathing (105 ± 6 and 188 ± 3%, respectively). Administration of L-arginine...
Leukocyte adherence, hypoxia between untreated and SNO- or L-arginine-treated rats. There were no significant differences in shear rate during leukocyte-endothelial adhesive interactions. There were no significant differences in shear rate during hypoxia between untreated and SNO- or L-arginine-treated rats. Accordingly, the ability of L-arginine and SNO to attenuate hypoxia-induced leukocyte adherence cannot be attributed to an increased shear rate opposing leukocyte-endothelial adhesive interactions. There were no significant differences in shear rate during hypoxia between untreated and SNO- or L-arginine-treated rats.

**DISCUSSION**

The major findings of this study are 1) the magnitude of the increases in ROS levels and the extent of leukocyte adherence in mesenteric venules during systemic hypoxia is inversely related to the PO2, 2) administration of an NO donor attenuates the hypoxia-induced increase in ROS levels as well as the number of adherent leukocytes, and 3) L-arginine, a precursor of NO, was as effective as the NO donor in preventing leukocyte-adhesive interactions during hypoxia.

The observed increase in ROS during hypoxia confirms previous findings from our laboratory (28, 32) and others (6, 9). Furthermore, the dependence of ROS levels on the PO2 observed in the present studies agrees with in vitro observations of a similar relationship in isolated cardiomyocytes (9) and hepatocytes (7). To our knowledge, the present study provides the first evidence of such an inverse relationship between microvascular ROS levels and PO2 during graded systemic hypoxia in intact animals.

The extent of leukocyte adherence during hypoxia was proportional to the increase in ROS levels (Fig. 7). Interestingly, although breathing 15% O2 significantly increased venular ROS, there was no detectable change in leukocyte adherence at this degree of systemic hypoxia (Fig. 4). This implies that ROS levels must increase above a threshold before microvascular inflammatory responses are initiated. Once this threshold was reached, the extent of leukocyte adherence was proportional to ROS levels (Fig. 7). In fact, when responses to all degrees of hypoxia were examined, we observed a highly significant correlation (r^2 = 0.93) between venular ROS and the number of adherent leukocytes.

Interventions designed to increase NO levels by either administration of exogenous NO or of L-arginine were equally effective in preventing hypoxia-induced increases in ROS levels as well as leukocyte adherence to mesenteric venules (Fig. 7), thereby suggesting that hypoxia is associated with an increase in ROS and a

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### Table 2. Effect of SNO on leukocyte adherence and shear rate during systemic hypoxia

<table>
<thead>
<tr>
<th></th>
<th>NX</th>
<th>NX + SNO</th>
<th>HX + SNO</th>
<th>NX Rec + SNO</th>
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<tr>
<td></td>
<td>5 min</td>
<td>10 min</td>
<td>10 min</td>
<td>20 min</td>
</tr>
<tr>
<td>Leukocyte adherence, no. per 100 μm venule</td>
<td>0 ± 0</td>
<td>0.2 ± 0.2</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Shear rate, s⁻¹</td>
<td>526 ± 93</td>
<td>505 ± 82</td>
<td>504 ± 51</td>
<td>505 ± 54</td>
</tr>
<tr>
<td></td>
<td>7.5% O₂ + SNO (n = 5)</td>
<td>7.5% O₂ + SNO (n = 5)</td>
<td>7.5% O₂ + SNO (n = 5)</td>
<td>7.5% O₂ + SNO (n = 5)</td>
</tr>
<tr>
<td>Leukocyte adherence, no. per 100 μm venule</td>
<td>0.2 ± 0.2</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Shear rate, s⁻¹</td>
<td>584 ± 128</td>
<td>590 ± 103</td>
<td>624 ± 99</td>
<td>673 ± 123</td>
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Values are means ± SE. SNO, spermine NONOate. *P < 0.05 HX + SNO vs. NX + SNO; †P < 0.05 NX Rec + SNO vs. HX + SNO.

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### Table 3. Effect of L-Arg on leukocyte adherence and shear rate during systemic hypoxia

<table>
<thead>
<tr>
<th></th>
<th>NX</th>
<th>NX + L-Arg</th>
<th>HX + L-Arg</th>
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<tr>
<td></td>
<td>5 min</td>
<td>10 min</td>
<td>10 min</td>
<td>20 min</td>
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<tr>
<td>Leukocyte adherence, no. per 100 μm venule</td>
<td>0.6 ± 0.3</td>
<td>0.3 ± 0.3</td>
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<tr>
<td>Shear rate, s⁻¹</td>
<td>756 ± 6</td>
<td>679 ± 11</td>
<td>749 ± 16</td>
<td>722 ± 36</td>
</tr>
<tr>
<td></td>
<td>7.5% O₂ + L-Arg (n = 5)</td>
<td>7.5% O₂ + L-Arg (n = 5)</td>
<td>7.5% O₂ + L-Arg (n = 5)</td>
<td>7.5% O₂ + L-Arg (n = 5)</td>
</tr>
<tr>
<td>Leukocyte adherence, no. per 100 μm venule</td>
<td>0.2 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>0.25 ± 0.25</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Shear rate, s⁻¹</td>
<td>606 ± 77</td>
<td>661 ± 138</td>
<td>581 ± 75</td>
<td>598 ± 86</td>
</tr>
</tbody>
</table>

Values are means ± SE. L-Arg, L-arginine. *P < 0.05 HX + L-Arg vs. NX + L-Arg; †P < 0.05 NX Rec + L-Arg vs. HX + L-Arg.

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decrease in NO levels. However, the mechanism underlying these changes is not known. Two alternative explanations are possible: 1) excess ROS generated during hypoxia consume NO or 2) NO generation is decreased during hypoxia, allowing ROS levels to rise.

Superoxide and NO are continuously produced within endothelial cells under normal conditions, without apparent adverse effects. Approximately 2–4% of molecular O₂ is normally converted to superoxide; on a molar basis, the rate of NO synthesis is far greater than that of superoxide. Superoxide avidly interacts with NO (26) at a rate approximately threefold higher than with the endogenous inactivating enzyme superoxide dismutase. The difference in these reaction rates has led to the proposal that the most important physiological role of NO is its antioxidant action (5, 14). In basal conditions, inactivation of superoxide by NO maintains low ROS levels within cells. Although NO is consumed by superoxide in this process, the higher basal rate of NO generation ensures NO levels sufficient for its anti-inflammatory actions.

A marked increase in the rate of superoxide generation would result in NO depletion. As NO levels decrease, superoxide interactions with superoxide dismutase would progressively increase, thereby resulting in oxidative stress through hydrogen peroxide formation. An increase in superoxide (30) and hydrogen peroxide generation (9) has been demonstrated in isolated cells during hypoxia. Thus a possible scenario for hypoxia-induced microvascular injury could be 1) hypoxia increases ROS generation and 2) higher superoxide levels result in NO depletion even without a change in the rate of NO synthesis. Our observation that the NO donor almost completely prevented the hypoxia-induced increase in ROS levels as well as the increase in leukocyte-endothelial adherence (34) is consistent with this hypothesis. Surprisingly, although the ability of NO donors and L-arginine to reduce microvascular dysfunction is often attributed to the antioxidant action of NO, we are not aware of studies supporting this interpretation by examining the effect of exogenous NO administration on microvascular ROS levels.

An alternative mechanism could also explain the effect of the NO donor in hypoxia; namely, that NO levels decrease as a result of limited O₂ substrate availability and that the reduction in NO leads to increased ROS. Low O₂ levels have been proposed to directly decrease the rate of NO synthesis and thereby reduce tissue NO (19). Rengasmy and Johns (25) first suggested that O₂ substrate limitation may regulate NO synthesis on the basis of their observation that the activity of purified NOS was dependent on O₂ concentration. Reductions in PO₂ were reported to impair NO production in cultured endothelial cells (31) and in isolated, perfused lung preparations (16, 18). Dweik et al. (10) observed progressive decreases in the levels of exhaled NO in normal individuals when inspired PO₂ was decreased from 21 to 5% O₂.

If NO synthesis were impaired during systemic hypoxia, the lower levels of the antioxidant NO could result in elevated ROS levels since superoxide dismutase interactions would predominate over superoxide-NO interactions. This is supported by the observation that inhibition of NOS leads to increased ROS levels and increased leukocyte-endothelial interactions (23, 29).

Our results with L-arginine do not support the possibility that a reduction in NO levels during hypoxia is due primarily to a limitation in O₂ substrate availability. The effects of L-arginine were qualitatively and quantitatively similar to those observed with the NO donor: both interventions nearly completely attenuated the hypoxia-induced increases in ROS levels and in leukocyte adherence. If NO levels decreased primarily due to reduced NOS activity, L-arginine administration could not enhance endogenous NO formation and produce the effects that we observed. These results are in agreement with a recent study showing that L-arginine administration increased NO formation in human subjects at high altitude (27). Although there is abundant evidence indicating that the activity of purified NOS is dependent on in vitro PO₂, it is possible that the enzyme activity may be modified by other factors in vivo. In this respect, it has been shown that cytosolic calcium, which is known to stimulate eNOS activity, is increased in endothelial cells during hypoxia (17).

In summary, the present study provides further support for a role of ROS in the microvascular response to systemic hypoxia by demonstrating a proportional change in ROS levels and the magnitude of leukocyte-endothelial interactions. Second, the results provide evidence of the interaction between ROS and NO in the hypoxia-induced microvascular response. The data support the notion that systemic hypoxia results in NO depletion secondary to inactivation by ROS rather than decreased generation of NO as a consequence of insufficient O₂ substrate.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-39443 (to N. C. Gonzalez) and HL-64195 (to J. G. Wood).

Fig. 7. Number of adherent leukocytes plotted as a function of DHR fluorescence intensity. Each symbol represents an experimental group. Bars represent ± 1 SE on either side of the mean.
REFERENCES


