Alveolar macrophages are necessary for the systemic inflammation of acute alveolar hypoxia

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First published July 26, 2007; doi:10.1152/japplphysiol.00312.2007.—Alveolar hypoxia (FIO2 0.10) rapidly produces inflammation in the microcirculation of skeletal muscle, brain, and mesentery of rats. Dissociation between tissue PO2 values and inflammation, plus the observation that plasma from hypoxic rats activates mast cells and elicits inflammation in normoxic tissues, suggest that the response to hypoxia is initiated when mast cells are activated by an agent released from a distant site and carried by the circulation. These experiments tested the hypothesis that this agent originates in alveolar macrophages (AM). Male rats were depleted of AM by tracheal instillation of clodronate-containing liposomes. Four days after treatment, AM recovered by bronchoalveolar lavage were <10% of control. Control rats received buffer-containing liposomes. As expected, alveolar hypoxia (FIO2 0.10) in control rats increased leukocyte-endothelial adherence, produced degranulation of perivascular mast cells, and increased fluorescent albumin extravasation in the cremaster microcirculation. None of these effects was seen when AM-depleted rats were exposed to hypoxia. Plasma obtained from control rats after 5 min of breathing 10% O2 elicited inflammation when applied to normoxic cremasters. In contrast, normoxic cremasters did not develop inflammation after application of plasma from hypoxic AM-depleted rats. Supernatant from AM cultured in 10% O2 produced increased leukocyte-endothelial adherence, vasoconstriction, and albumin extravasation when applied to normoxic cremasters. Normoxic AM supernatant did not produce any of these responses. The effects of hypoxic supernatant were attenuated by pretreatment of the cremaster with the mast cell stabilizer cromolyn. These data support the hypothesis that AM are the source of the agent that initiates hypoxia-induced systemic inflammation by activating mast cells.

microcirculation; leukocyte-endothelial interactions; reduced inspired oxygen

ALVEOLAR HYPOXIA induced by a reduction in inspired PO2 results in a rapid and widespread inflammatory response in mesentery (7, 37–39) skeletal muscle (9, 28), and brain (23) of rats. This response is characterized by increased leukocyte-endothelial interactions (9, 14, 28, 39), generation of reactive oxygen species (ROS) (14, 37) and depletion of nitric oxide (NO) (31), mast cell activation (9, 32), and increased vascular permeability (14, 38). If hypoxia is maintained for several days, the inflammation subsides and reduction of PO2 to even lower levels does not elicit inflammation, indicating an acclimatization of the vascular endothelium to prolonged hypoxia (39).

Recent studies in the cremaster microcirculation suggest that the systemic inflammation of hypoxia is not initiated by the reduced peripheral tissue PO2 (28); rather, inflammation appears to be triggered by a mediator released from a distant site and transported by the systemic circulation. Selective reduction of cremaster microvascular PO2 does not elicit mast cell activation or increased leukocyte-endothelial interactions if systemic arterial PO2 is normal; on the other hand, reduction of systemic arterial PO2 by breathing 10% O2 increases leukocyte-endothelial interactions and activates mast cells in the cremaster, even when cremaster microvascular PO2 is normal (9, 28). Further support for the idea of a trigger of inflammation transported by the circulation was provided by the observation that plasma obtained from conscious rats breathing 10% O2 for 5 min produced mast cell degranulation and increased leukocyte-endothelial interactions in the normoxic cremaster microcirculation (27). These responses were not due to a nonspecific effect of plasma and were not the result of inflammatory mediators released by mast cells or adherent leukocytes of the hypoxic donor rat. The lack of effect of plasma obtained from blood equilibrated in vitro with low-PO2 gas mixtures indicated that the substances responsible for the inflammation in normoxic cremaster were not generated by blood cells (27).

The inflammation produced by plasma from hypoxic rats is blocked by both angiotensin II (ANG II) receptor antagonists and inhibitors of angiotensin-converting enzyme (ACE) (13). Since the effects of an ACE inhibitor, applied locally, are essentially the same as those produced by ANG II receptor blockade, it would appear that the inflammatory response is a result of local generation of ANG II. Furthermore, while stabilization of mast cells with cromolyn blocks the inflammation produced by plasma from hypoxic rats, as well as the inflammation of alveolar hypoxia, cromolyn does not modify the inflammatory response to topical application of ANG II (13). This suggests that activation of the renin-angiotensin system (RAS) occurs downstream from mast cell activation. Interactions between mast cells and the RAS, including renin release from activated cardiac mast cells, have been documented previously (21, 30).

A possible interpretation of the results discussed above is that reduction of alveolar PO2 leads to the release of a substance that is transported from a distant site by the systemic circulation. Mast cells activated by this substance stimulate the RAS

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and elicit systemic inflammation. Neither the nature nor the source of the putative trigger of the inflammation is known. The widespread and rapid nature of the response, as well as the carriage of the mediator by the circulation, suggest a substance released or rapidly synthesized by cells in an organ with a high blood flow rate. These features make lung alveolar macrophages (AM) an attractive possibility as a source of the mediator. AM are among the first cells to sense changes in alveolar Po$_2$. Macrophages synthesize and store a number of substances that could trigger inflammation, and these substances could rapidly reach the blood supply and distribute throughout the systemic circulation. Although most known effects of AM stimulation take place within the lung itself, systemic effects of AM activation have been documented (19, 33, 34). The present experiments were designed to test the hypothesis that AM are the source of the putative mediator of the systemic inflammation of alveolar hypoxia. We reasoned that if this is the case, depletion of AM would attenuate the systemic inflammatory response to acute alveolar hypoxia, and that supernatant of isolated AM cultured in hypoxic conditions would initiate an inflammatory response in normoxic tissue. The results obtained support this hypothesis.

**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 Accreditation of Laboratory Animal Care.

**Depletion of AM.** Airway instillation of clodronate-containing liposomes was used to deplete AM. Liposomes were composed of phosphatidylcholine and cholesterol in a 6:1 molar ratio and contained either clodronate or phosphate-buffered saline (PBS). Clodronate was a gift of Roche Diagnostics (Mannheim, Germany). It was encapsulated in liposomes as described previously (36).

Rats were anesthetized with pentobarbital sodium (35 mg/kg ip), and the trachea was exposed with a midline incision. The animals were randomly assigned to either an AM depletion group, which received liposomes containing clodronate, or a control group, in which the rats were administered PBS-containing liposomes. The animals were positioned at an angle of 30° head up, and 350 µL of a solution containing 250 mg clodronate liposomes/mL was injected between the tracheal cartilages with a 28-gauge needle. The skin incision was sutured, and the animals remained in the tilted position for ~30 min, after which they were returned to their cages. The animals recovered uneventfully, and experiments were carried out 4 days after liposome administration.

The effectiveness of clodronate-containing liposomes was estimated by measuring the number of AM recovered in bronchoalveolar lavage (BAL). Four days after liposome administration, four rats that had received clodronate-containing liposomes and four that had received PBS liposomes were anesthetized with pentobarbital sodium (35 mg/kg ip). After placement of a PE-50 catheter in the jugular vein, a tracheotomy was performed and a PE-240 catheter was inserted in the trachea. An overdose of pentobarbital sodium (150 mg/kg iv) was injected to euthanize the animals, and BAL was carried out. Saline (10 mL) was injected slowly into the lungs; the saline was then removed and transferred into a plastic tube. This operation was repeated 10 times. The saline recovered was centrifuged, and the cell pellets were pooled and resuspended in 1 mL of saline. AM were counted in a hemocytometer.

**Intravital microscopy of the cremaster muscle.** Male Sprague-Dawley rats (175–225 g) were anesthetized with urethane (1.5 g/kg im) after an overnight fast with free access to water. Body temperature was maintained at 36–38°C with a homeothermic blanket system connected to a rectal probe. PE-50 catheters were inserted in the jugular vein and in the carotid artery. Lactated Ringer solution was infused continuously via the jugular vein at a rate of 2 ml/h. A carotid artery catheter was used to monitor blood pressure continuously with a digital monitor. A tracheotomy was performed, and a PE-240 catheter was connected to a rodent nonrebreathing two-way valve. The animals breathed spontaneously throughout the experiment.

The right cremaster muscle was prepared for intravital microscopy as described previously (2). 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 the muscle temperature at 35°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 (>40 objective) were recorded on a videocassette recorder with a time-date generator. Straight, unbranched venules of 100-µm length, 20- to 40-µm diameter, and no adjacent lymphatics were selected for microscopic observation. A laser Doppler velocimeter, which was used to measure venular center line red blood cell velocity. Average red blood cell velocity was calculated as center line velocity/1.6 (8). Venule diameter was measured with the analySIS Software Image System. A section of venule of ~50-µm length was selected. This section was within the marking dots used to align the velocimeter. The distance between opposing endothelial layers was measured at five evenly spaced points (~10 µm distant from one another) within the selected area of the vessel. The average of the five values was used as the venule diameter. The same points were selected in all measurements in a given muscle. Venule shear rate ($s^{-1}$) was calculated as: $8 \times$ [average red blood cell velocity (mm/s)/venular diameter (mm)] (18). Shear rate is the velocity gradient between layers of a fluid circulating with laminar flow, and it provides an estimate of the forces that oppose leukocyte adherence to the endothelium. Venular diameter remains relatively unchanged since postcapillary venules are devoid of vascular smooth muscle; therefore, shear rate is proportional to blood velocity and, ultimately, blood flow. Like blood flow, shear rate is a function of perfusion pressure, vessel diameter, and blood viscosity. Adherent leukocytes were defined as those that remained stationary for ≥30 s. Leukocyte adherence was expressed as the number of adherent leukocytes per 100 µm of vessel length.

Second- or third-order arterioles (15–30 µm diameter), not adjacent to the venules chosen for observation, were also studied. Arterioles and venules were observed alternatively every 2 min. Arteriolar red blood cell velocity was measured with an optical Doppler velocimeter. Arteriolar diameter was measured with the analySIS Software Image System by the same approach described for venule diameter. Changes in arteriolar diameter were expressed as the ratio experimental/control for individual experiments. Control was the average diameter of all the measurements obtained in the 10-min control period.

Ruthenium red (1 g/100 mL) was applied topically before the experiment to document mast cell degranulation in vivo (29).

**Measurement of extravasation of fluorescent albumin after 4 h of exposure to hypoxia.** Conscious macrophage-depleted and control rats were placed in a chamber in which 10% O$_2$/90% N$_2$ was circulating. Four hours later, the animals were anesthetized with urethane (1.5 mg/kg im). Arterial and venous catheters were placed under anesthesia, and the cremaster was dissected as described above while the animals breathed 10% O$_2$. After a suitable venule was found under bright-field microscopy, fluorescein isothiocyanate (FITC)-labeled bovine albumin was injected intravenously (50 mg/kg) and images were obtained every 5 min under fluorescence microscopy. To minimize photobleaching, fluorescence recording was ~15 s in a given area.

Fluorescence from the FITC-labeled albumin (excitation wavelength 420–490 nm, emission wavelength 520 nm) was detected with an F-View Software 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, with the analySIS Software Program. Extravascular fluorescence intensity was measured on both sides of the 100-μm venule segment while avoiding areas with underlying vessels. The same section of the microcirculation was analyzed in all the images of a given experiment. The magnitude of albumin extravasation was estimated by the ratio of extra- to intravascular fluorescence intensity.

Exposure of conscious plasma donor rats to hypoxia. Male Sprague-Dawley rats (250–300 g) were anesthetized with pentobarbital sodium (35 mg/kg ip). PE-50 catheters were placed in the carotid artery and external jugular vein, tunneled subcutaneously, exteriorized at the back of the neck, and flame sealed. Two to three hours after complete recovery from anesthesia, the mast cell stabilizer cromolyn was injected intravenously (35 mg/kg). Cromolyn was administered to prevent the mast cell degranulation and increased leukocyte-endothelial adherence induced by alveolar hypoxia in the donor rat (27).

Thirty minutes after cromolyn administration, the rats were placed into a Lucite chamber in which 10% O2-90% N2 was circulated. At 5 min of exposure to hypoxia a 3-ml blood sample was obtained in a syringe coated with heparin (1,000 USP units/ml), and the blood was centrifuged at 1,500 rpm for 10 min. The cell pellets were pooled per rat was 0.45 and 0.07 × 10⁶ in the clodronate group (n = 4) and 6.7 ± 0.3 × 10⁶ in the PBS group (n = 4; P < 0.001).

RESULTS

Depletion of AM with clodronate liposomes. Four days after liposome administration, the number of AM recovered in BAL per rat was 0.45 ± 0.07 × 10⁶ in the clodronate group (n = 4) and 6.7 ± 0.3 × 10⁶ in the PBS group (n = 4; P < 0.001).

Effects of alveolar hypoxia on cremaster microcirculation.

Alveolar hypoxia was induced in six macrophage-depleted rats and six control rats. Table 1 shows that depletion of AM did not significantly modify the response to alveolar hypoxia. Both groups showed a decrease in mean arterial blood pressure and a small, not significant increase in heart rate. Reduction in inspired PO2 resulted in similar values of alveolar gas-arterial blood PO2 difference, arterial PO2, and arterial PCO2 in both clodronate- and PBS-treated animals, indicating that macrophage depletion does not affect efficacy of pulmonary gas exchange and the ventilatory response to hypoxia.

Table 1. Mean arterial blood pressure, heart rate, and arterial blood gases in anesthetized rats exposed to alveolar hypoxia (10% O2 breathing)

<table>
<thead>
<tr>
<th></th>
<th>MABP, mmHg</th>
<th>HR, beats/min</th>
<th>A-aPO2, Torr</th>
<th>PacO2, Torr</th>
<th>PaCO2, Torr</th>
<th>pH,</th>
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<tr>
<td>Clodronate</td>
<td>110±9</td>
<td>358±14</td>
<td>11±5</td>
<td>90±4</td>
<td>38±2</td>
<td>7.36±0.03</td>
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<tr>
<td>PBS</td>
<td>112±6</td>
<td>350±15</td>
<td>14±3</td>
<td>89±6</td>
<td>37±3</td>
<td>7.37±0.03</td>
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<td>Alveolar hypoxia</td>
<td></td>
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<tr>
<td>Clodronate</td>
<td>72±3*</td>
<td>398±12</td>
<td>7±3</td>
<td>35±2*</td>
<td>26±2*</td>
<td>7.50±0.04*</td>
</tr>
<tr>
<td>PBS</td>
<td>74±5*</td>
<td>401±14</td>
<td>8±3</td>
<td>33±3*</td>
<td>24±4*</td>
<td>7.51±0.02*</td>
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<td></td>
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<tr>
<td>Clodronate</td>
<td>107±4</td>
<td>366±11</td>
<td>11±6</td>
<td>93±4</td>
<td>36±4</td>
<td>7.34±0.05</td>
</tr>
<tr>
<td>PBS</td>
<td>110±6</td>
<td>374±15</td>
<td>8±3</td>
<td>96±5</td>
<td>36±3</td>
<td>7.36±0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE for 6 rats in each group [clodronate and phosphate-buffered saline (PBS)]. MABP, mean arterial blood pressure; HR, heart rate; A-aPO2, alveolar gas-arterial blood PO2 difference (alveolar PO2 calculated with alveolar gas equation, assuming that arterial and alveolar PCO2 were the same); PacO2, arterial blood PO2; PaCO2, arterial blood PCO2; pH, arterial plasma pH. *P < 0.05 vs. corresponding control.
receiving PBS liposomes showed a transitory arteriolar vasoconstriction followed by vasodilation, as observed previously (7). AM depletion attenuated the transitory vasoconstriction, without modifying the vasodilatory response (Fig. 1).

Figure 2, top, is a representative microphotograph of the cremaster microcirculation illustrating the characteristic hypoxia-induced degranulation of mast cells surrounding an arteriole in the control rats (9, 13, 32). In contrast, degranulation of mast cells was not observed in the rats pretreated with clodronate-containing liposomes (Fig. 2, bottom).

Exposure of the PBS liposome-treated rats to hypoxia in the conscious state resulted in a marked increase in extravasation of FITC-albumin, as shown by the high extravascular-to-intravascular fluorescence ratio. Albumin extravasation in this group became apparent almost immediately after the intravenous administration of FITC albumin and continued throughout the 30 min of observation (Fig. 3). In contrast, the group pretreated with clodronate liposomes showed a significantly lower extravasation of FITC-albumin compared to the PBS group (Fig. 3).

Fig. 1. Effect of alveolar hypoxia in macrophage-depleted rats (clodronate) and in control rats [phosphate-buffered saline (PBS)]. Hypoxia was induced by breathing 10% O2. Values are means ± SE for 6 rats in each group. *P < 0.05 vs. corresponding average control; §P < 0.05 clodronate vs. PBS at equal time points. Exp, experimental.

Fig. 2. Representative photomicrographs of the cremaster microcirculation after 10 min of alveolar hypoxia showing mast cells (arrows) surrounding arterioles. Top: control rat. Bottom: macrophage-depleted rat. Uptake of ruthenium red denotes mast cell degranulation.

Fig. 3. Extravasation of fluorescein isothiocyanate (FITC)-albumin expressed as extravascular-to-intravascular fluorescence intensity. Values are means ± SE of 6 rats in each group. §P < 0.05 clodronate vs. PBS at equal time points.
lower extra-to-intravascular fluorescence ratio, which remained at levels <0.1 throughout the observation period.

Effects of topical application of ANG II and of compound 48/80. Topical application of ANG II, an inflammatory agent that participates in the inflammation of alveolar hypoxia (10), induced an increase in leukocyte-endothelial adherence in the normoxic cremasters of both PBS (n = 6)- and clodronate (n = 6)-treated rats (Fig. 4, top). Similarly, the mast cell secretogogue compound 48/80 increased leukocyte-endothelial adherence to similar extents in both groups (Fig. 4, bottom). Each group consisted of six rats.

Effects of application of plasma from hypoxic rats on normoxic cremaster. As expected from our previous observations (27), plasma obtained from the rats treated with PBS liposomes (n = 6) produced an inflammatory response in the cremaster microcirculation of normoxic rats. This response was characterized by increased leukocyte-endothelial adherence, decreased venular shear rate, and arteriolar vasoconstriction (Fig. 5). In contrast, the response of the normoxic cremaster to plasma from macrophage-depleted hypoxic rats (n = 6) was significantly smaller. Although leukocyte-endothelial adherence showed a significant, albeit small, increase after the second application of plasma, the values were always substantially lower than those observed after application of plasma from hypoxic rats treated with PBS liposomes. Venular shear rate was always significantly lower than control in the rats receiving plasma from PBS liposome-treated rats, while plasma from the macrophage-depleted rats produced only a small decrease in shear rate that did not reach statistical significance. The decrease in shear rate in the rats receiving control hypoxic plasma is most likely due to upstream arteriolar vasoconstriction (Fig. 5, bottom). This is consistent with the observation that arteriolar vasoconstriction in the rats receiving plasma from AM-depleted animals was significantly attenuated.
Effects of application of AM supernatant to the normoxic cremaster. Figure 6 summarizes the effects of topical application of \(0.75 \text{ ml}\) of AM supernatant onto the normoxic cremaster. Variables depicted are leukocyte-endothelial adherence (Fig. 6A), venular shear rate (Fig. 6B), arteriole diameter (Fig. 6C), and FITC-albumin extravasation (Fig. 6D). The data presented in Fig. 6, A–C, represent the average of six rats per group. Six additional animals per group were used in the experiments summarized in Fig. 6D. Supernatant obtained from AM cultured in normoxia did not have a significant effect on any of the variables investigated. On the other hand, supernatant obtained from AM cultured in hypoxia produced a rapid increase in leukocyte adherence (Fig. 6A), transitory decreases in venular shear rate (Fig. 6B) and arteriole diameter (Fig. 6C), and a significant extravasation of FITC-albumin shortly after intravenous administration (Fig. 6D). These responses are qualitatively similar to those elicited by alveolar hypoxia (Figs. 1 and 3) and by plasma obtained from hypoxic rats (Fig. 5; Refs 13 and 27). Pretreatment with the mast cell stabilizer cromolyn (0.11 mg/ml applied topically onto the cremaster; Fig. 6) abolished all the responses. Continuous intravenous infusion of the ANG II type 1 and type 2 receptor blocker \([\text{Sar}^1,\text{Thr}^8] \text{ANG II} (30 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1})\); Fig. 6) also blocked the microvascular response to supernatant of AM cultured in hypoxia. Figure 7 shows a representative photomicrograph of the cremaster microcirculation before and \(\sim 20 \text{ min}\) after application of hypoxic AM supernatant. The uptake of ruthenium red after supernatant application indicates activation of perivascular mast cells.

**DISCUSSION**

The results of this study indicate that AM play a key role in the systemic inflammation of alveolar hypoxia. This is based on three lines of evidence: 1) depletion of AM substantially attenuates the systemic inflammatory response that follows acute alveolar hypoxia; 2) topical application of plasma from AM-depleted hypoxic rats fails to promote inflammation in normoxic cremaster muscles; and 3) supernatant from AM cultured in hypoxia induces an inflammatory response in normoxic skeletal muscle similar to that induced by alveolar hypoxia and by plasma from hypoxic rats with normal AM content. These observations provide further support for the hypothesis that the systemic inflammation of alveolar hypoxia is initiated by a substance released from a distant site and carried by the circulation and point to AM as the source of this substance.

In the present experiments, alveolar hypoxia was induced by breathing 10% \(\text{O}_2\). This is a severe level of hypoxia resulting in an inspired \(\text{PO}_2\) equivalent to that encountered at altitudes close to 5,500 m. Nonacclimatized humans acutely exposed to altitude hypoxia may develop illnesses that vary in severity from acute mountain sickness (AMS) to high-altitude cerebral edema or high-altitude pulmonary edema. An inflammatory component has been demonstrated in all these conditions (4, 15, 17), although the role of inflammation as a causal agent is still unclear. While the incidence of altitude illnesses increases in proportion to the severity of hypoxia, the rate of ascent is also important: while only 50% of trekkers that take \(\geq 5 \text{ days}\) to ascend above 4,000 m develop AMS, 84% of individuals flying to a site at 3,680 m develop AMS (4, 16). There are many examples of individuals rapidly exposed to levels of hypoxia similar to those used in our studies: for instance, South American miners commute to altitudes close to 6,000 m, and large numbers of military personnel are rapidly deployed to altitude areas. Similarly, many altitude sites can be reached rapidly by air or ground transportation. Thus the animal model used in these studies is a useful tool to investi-
gate pathological conditions associated with acute hypoxic exposure, a condition that occurs with increasing frequency.

AM were depleted by airway instillation of liposomes containing clodronate. This is a well-tested procedure that produces a marked temporary decrease in the number of alveolar macrophages (5, 20). In the present experiments, the number of AM recovered by BAL 4 days after treatment was <10% of the number recovered in the controls. Liposomes introduced in the alveolar space are phagocytosed by AM; the clodronate contained in the liposomes is released and induces macrophage apoptosis and necrosis (20, 36). Since AM are the principal phagocytic cell type in the alveoli of naive lungs, they constitute a preferential target for liposomes. Clodronate released by necrotic AM could reach the systemic circulation; however, it has virtually no effect thanks to its short circulatory half-life (36). For instance, while intravenous administration of clodronate encapsulated in liposomes depletes spleen and liver macrophages, an intravenous clodronate solution has no effect, most likely because of the rapid disappearance of clodronate from plasma (11).

In the present experiments, rats receiving PBS-containing liposomes were used as controls. This should account for nonspecific effects of instillation of a small volume of liposome-containing fluid in the lungs. These effects, nevertheless, appear to be negligible: previous findings show that administration of PBS or clodronate liposomes does not alter alveolar architecture (5). Furthermore, only a small number of leukocytes were recovered in BAL fluid in rats treated either with clodronate liposomes or with PBS liposomes (11), indicating that neither treatment results in overt pulmonary damage. This agrees with our observation that cardiovascular and pulmonary gas exchange functions of animals treated with PBS liposomes during normoxia and hypoxia (Table 1) were similar to those observed previously in untreated hypoxic rats (39). Furthermore, the similar effects of hypoxia on gas exchange variables of both clodronate and PBS liposome groups indicate that the ventilatory response to short-term hypoxia as well as the efficacy of pulmonary gas exchange were not influenced by AM depletion (Table 1).

All the markers characteristic of alveolar hypoxia-induced inflammation in skeletal muscle and other microvascular beds (9, 13, 37–39), increased leukocyte adherence, mast cell degranulation, and albumin extravasation, which were present in the control rats, were significantly attenuated in the rats depleted of AM. The lack of effect of alveolar hypoxia was not due to an inability of the cremaster microcirculation of AM-depleted rats to mount an inflammatory response: topical application of either ANG II or the mast cell secretagogue compound 48/80 produced similar increases in leukocyte-endothelial interactions in AM-depleted and control animals. These inflammatory agents were selected because of the known participation of the RAS (13) and mast cell activation (9, 13, 23, 32) in alveolar hypoxia-induced inflammation.

Together, these results indicate that AM are necessary for the systemic inflammatory response to alveolar hypoxia. Our previous data (9, 27, 28) suggest that the systemic inflammation of alveolar hypoxia is triggered by a substance carried by the circulation from a distant site. The lack of effect of plasma from hypoxic AM-depleted rats on the normoxic cremaster suggests that AM are the source of this substance(s). The effects of hypoxic AM supernatant on the normoxic cremaster microcirculation provide strong additional support for this possibility. Normoxic AM supernatant had no effect on any of the inflammatory markers investigated, indicating that exposure of AM to reduced PO2 is necessary to elicit an inflammatory response. The effects of hypoxic AM supernatant were blocked by pretreatment of the muscle with the mast cell stabilizer cromolyn, as well as with a nonspecific ANG II receptor antagonist. Thus the inflammation produced by topical application of hypoxic AM supernatant is blocked by agents that also block the inflammatory responses induced by application of plasma from hypoxic rats (13, 27) as well as alveolar hypoxia. This suggests that the agents contained in hypoxic rat plasma and in hypoxic AM supernatant set in motion similar inflammatory mecha-

Fig. 7. Photomicrograph of the normoxic cremaster microcirculation before (top) and 20 min after (bottom) application of hypoxic AM supernatant. Uptake with ruthenium red denotes degranulation of perivascular mast cells.
nisms that involve mast cell activation and stimulation of the RAS. Most known effects of AM stimulation occur within the lung; however, there is also evidence of systemic inflammatory effects resulting from substances released by AM. Examples are the bone marrow effects of cytokines released by AM in idiopathic pulmonary fibrosis (33) or in response to exposure to particulate matter (19). Cytokines released from AM after particle deposition in the lung are thought to act on the bone marrow to produce the release of platelets and leukocytes into the systemic circulation and stimulate the release of acute-phase proteins leading to systemic inflammation (34). The data obtained in the present experiments illustrate another case in which AM stimulation produces effects in the systemic microcirculation.

While the present results point to AM as the source of the substance that initiates the systemic inflammation of alveolar hypoxia, the nature of this putative mediator remains unknown. Alveolar hypoxia results in increased expression of mRNA for TNF-α, macrophage inflammatory protein-1β, and ICAM-1 in the lung, all of which are reduced by depletion of AM (22, 35). However, in the present experiments, AM supernatant induced an inflammatory response only after 30 min of hypoxic incubation. This suggests that the agent responsible for the inflammation is either preformed or rapidly synthesized by AM in response to hypoxia. AM release a number of inflammatory agents that, alone or in combination, could initiate inflammation. Preformed cytokines may be released during hypoxia and rapidly increase leukocyte-endothelial adhesive interactions and initiate inflammation in the systemic circulation. The observation that the inflammation elicited either by hypoxia or by plasma obtained from hypoxic rats is abrogated by stabilization of mast cells (9, 13, 27, 32) suggests that the mediator agent is a mast cell secretagogue stored or synthesized in AM. Mast cell stimulation by plasma from hypoxic rats activates the local RAS (13). According to this scenario, a mast cell secretagogue released by macrophages in response to reduced alveolar PO2 would be transported by the circulation and act on perivascular mast cells. Mast cell stimulation would activate the RAS to initiate inflammation. Examples of interactions between mast cells and the RAS are the presence of chymase, an ACE, in the mast cells of some species (3, 25) and of renin, which enter into the systemic circulation. Preformed cytokines may be released during hypoxia and rapidly increase leukocyte-endothelial adhesive interactions and initiate inflammation in the systemic circulation. The observation that the inflammation elicited either by hypoxia or by plasma obtained from hypoxic rats is abrogated by stabilization of mast cells (9, 13, 27, 32) suggests that the mediator agent is a mast cell secretagogue stored or synthesized in AM. Mast cell stimulation by plasma from hypoxic rats activates the local RAS (13). According to this scenario, a mast cell secretagogue released by macrophages in response to reduced alveolar PO2 would be transported by the circulation and act on perivascular mast cells. Mast cell stimulation would activate the RAS to initiate inflammation. Examples of interactions between mast cells and the RAS are the presence of chymase, an ACE, in the mast cells of some species (3, 25) and of renin, recently discovered in human cardiac mast cells (21, 30). One group of mast cell secretagogues released by AM is neuropeptides such as adrenomedullin, CGRP, or substance P (12). These are proinflammatory agents with a vast array of physiological functions (6) that are known to participate in inflammatory processes (40), including pulmonary inflammatory responses to hypoxia and sepsis (1, 41).

It should be recognized that hypoxia-induced inflammation is a complex phenomenon in which mechanisms with different time courses may participate. For instance, leukocyte-endothelial interactions do develop in response to hypoxia in vitro (10, 24, 26), in conditions in which the mechanism described by us obviously does not participate. However, the vastly different time course of the response, hours versus minutes, suggests that the mechanisms underlying the in vivo response described in the present experiments are different from those responsible for the effects documented in vitro. Regardless of additional mechanisms that may develop at later stages, the results obtained in the present study support the idea of an AM-initiated systemic inflammatory response in intact animals exposed to reduced environmental Po2. The nature of the agents involved, as well as the relative contribution of this versus other possible mechanisms to the response of the intact animal to alveolar hypoxia should be the subject of future research.

GRANTS
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