

# The Systemic Inflammation of Alveolar Hypoxia Is Initiated by Alveolar Macrophage-Borne Mediator(s)

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Alveolar hypoxia produces widespread systemic inflammation in rats. The inflammation appears to be triggered by activation of mast cells by a mediator released from alveolar macrophages, not by the reduced systemic partial pressure of oxygen (PO<sub>2</sub>). If this is correct, the following should apply: (1) neither mast cells nor tissue macrophages should be directly activated by hypoxia; and (2) mast cells should be activated when in contact with hypoxic alveolar macrophages, but not with hypoxic tissue macrophages. We sought here to determine whether hypoxia activates isolated alveolar macrophages, peritoneal macrophages, and peritoneal mast cells, and to study the response of the microcirculation to supernatants of these cultures. Rat mesenteric microcirculation intravital microscopy was combined with primary cultures of alveolar macrophages, peritoneal macrophages, and peritoneal mast cells. Supernatant of hypoxic alveolar macrophages, but not of hypoxic peritoneal macrophages, produced inflammation in mesentery. Hypoxia induced a respiratory burst in alveolar, but not peritoneal macrophages. Cultured peritoneal mast cells did not degranulate with hypoxia. Immersion of mast cells in supernatant of hypoxic alveolar macrophages, but not in supernatant of hypoxic peritoneal macrophages, induced mast cell degranulation. Hypoxia induced release of monocyte chemoattractant protein-1, a mast cell secretagogue, from alveolar, but not peritoneal macrophages or mast cells. We conclude that a mediator released by hypoxic alveolar macrophages activates mast cells and triggers systemic inflammation. Reduced systemic PO<sub>2</sub> and activation of tissue macrophages do not play a role in this phenomenon. The inflammation could contribute to systemic effects of diseases featuring alveolar hypoxia.

**Keywords:** hypoxia; systemic inflammation; alveolar macrophages; mast cells; monocyte chemoattractant protein

Alveolar hypoxia, induced by reduction of inspired PO<sub>2</sub>, initiates a rapid and widespread inflammatory response in mesentery (1), skeletal muscle (2, 3), and brain (4) of rats. The inflammation is characterized by increased microvascular levels of reactive oxygen species (ROS) (5), perivascular mast cell degranulation (2, 3, 6), increased leukocyte-endothelial adhesive interactions (1), and extravasation of albumin (7).

Studies in the cremaster microcirculation suggest that the inflammation elicited by alveolar hypoxia is not triggered by the reduction of cremaster PO<sub>2</sub>, but rather by a mediator released from a distant site and transported by the circulation. This idea is supported by two lines of evidence: first, selective reduction of cremaster PO<sub>2</sub> does not produce mast cell degranulation and

## CLINICAL RELEVANCE

This study demonstrates a direct link, via a circulating mediator, between activation of alveolar macrophages by low alveolar PO<sub>2</sub>, and degranulation of systemic mast cells, the first step in the systemic inflammatory cascade. A candidate mediator, monocyte chemoattractant protein-1, was identified. Roles of low tissue PO<sub>2</sub> and activation of tissue macrophages were ruled out. The phenomenon described could participate in the inflammation underlying the systemic effects seen in conditions associated with low alveolar PO<sub>2</sub>.

inflammation in the cremaster microcirculation unless alveolar PO<sub>2</sub> is also reduced (2, 3); second, plasma obtained from hypoxic rats applied to the normoxic cremaster produces an inflammatory response similar to that elicited by alveolar hypoxia (8). The response to hypoxic rat plasma is not due to inflammatory mediators released into plasma by activated mast cells or adherent leukocytes of the donor rat; furthermore, the agent(s) responsible for the inflammation is not generated by blood cells (8). Further investigation has shown that the putative mediator activates mast cells and initiates an inflammatory cascade that includes activation of the renin-angiotensin (Ang) system (RAS) (9).

A role for alveolar macrophages as a source of the putative mediator is supported by findings that alveolar macrophage depletion *in vivo* attenuates the inflammatory response to alveolar hypoxia, and that supernatant of alveolar macrophages cultured in hypoxia induces mast cell degranulation and inflammation in normoxic cremaster muscle (10). Similar to the inflammation elicited by alveolar hypoxia (9), the response to alveolar macrophage supernatant was abrogated by pretreatment with cromolyn, a mast cell stabilizer, and with AngII receptor antagonists (10).

The present experiments were designed to provide evidence of a direct link between the activation of alveolar macrophages by the reduced alveolar PO<sub>2</sub> and the degranulation of tissue mast cells, which initiates the inflammation of hypoxia. We reasoned that, if alveolar macrophage-borne mediator(s) triggers the inflammation by activating mast cells, reduced PO<sub>2</sub> would not directly activate primary mast cell cultures; on the other hand, these cells would degranulate when placed in contact with supernatant of hypoxic alveolar macrophages. Furthermore, we hypothesized that hypoxia would not directly activate isolated resident tissue macrophages, and that mast cells exposed to supernatant of resident tissue macrophages cultured in hypoxia would not undergo degranulation. The results confirm our hypothesis that the inflammation of alveolar hypoxia is triggered by mediator(s) released by activated alveolar macrophages, and rule out possible contributions of local hypoxia and of resident tissue macrophages in the initiation of the inflammation. A mast cell secretagogue, monocyte chemoattractant protein (MCP)-1, was identified as a possible candidate for the putative mediator of hypoxia-induced systemic inflammation.

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The phenomenon described here highlights a systemic effect of alveolar macrophage activation, and could provide a possible pathogenic mechanism to explain the systemic consequences of conditions associated with reduced alveolar PO<sub>2</sub>.

## MATERIALS AND 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.

The experiments described here included *in vivo* studies in which supernatant of primary cell cultures, as well as pharmacological agents, were applied topically to the mesentery of normoxic rats, and *in vitro* studies performed in primary cell cultures.

Alveolar macrophages in an intact animal are normally exposed to a higher PO<sub>2</sub> than are peritoneal macrophages or peritoneal mast cells, and this difference subsists in conditions of hypoxia. Accordingly, humidified gas mixtures for the cell culture experiments were used with nominal concentrations of 21, 10, and 0% O<sub>2</sub>. All gas mixtures contained 5% CO<sub>2</sub>, with the balance made up with N<sub>2</sub>. These gas mixtures provided PO<sub>2</sub> values that encompass the range observed *in vivo*, from normoxic to hypoxic conditions, in the cell types studied here. The PO<sub>2</sub> attained in the liquid phase during gaseous equilibration in an open system depends on the efficacy of the equilibration system. In the present experiments, the cell cultures, placed in an incubator at 37°C, were gassed via a needle inserted in the cap of the culture dish and connected to the gas source. Care was taken to place the tip of the needle a few millimeters above the surface of the culture medium so as to not disturb the culture. To directly determine the efficacy of the equilibrating system, PO<sub>2</sub> of the medium was measured directly in some of the experiments using a phosphorescence decay method (11). This technique is currently employed in our laboratory to measure microvascular PO<sub>2</sub> of intact animals (2, 3). The actual supernatant PO<sub>2</sub> values were (mean ± SEM): 0% O<sub>2</sub>: 4.8 ± 0.8 mm Hg; and 10% O<sub>2</sub>: 65.3 ± 0.9 mm Hg. These values were attained within 2–3 minutes of equilibration. Exposure to 21% O<sub>2</sub> produced PO<sub>2</sub> values outside the range of the method (>100 mm Hg). Alveolar macrophages may be exposed *in vivo* to PO<sub>2</sub> of approximately 65 mm Hg in moderate cases of hypoxia. In contrast, peritoneal macrophages and mast cells exposed to 10% O<sub>2</sub> will be in an environment which is at the high end of the PO<sub>2</sub> values observed *in vivo*. On the other hand, PO<sub>2</sub> of approximately 5 mm Hg is the value to which peritoneal macrophages, mast cells, and other systemic tissue cells would be exposed in a rat breathing 10% O<sub>2</sub> (2, 3).

### Intravital Microscopy

The procedures for intravital microscopy of the mesentery have been described in detail before (1). Briefly, male Sprague-Dawley rats (220–300 g) were anesthetized with urethane (1.5 g/kg body weight intramuscularly). PE-50 catheters (Becton-Dickinson, Sparks, MD) were placed in the jugular vein and carotid artery for injection of solutions and measurement of arterial blood pressure. The abdomen was opened via a midline incision, and the ileo-cecal portion of the intestine was gently drawn out, exteriorized, and mounted on a transparent plastic stage. Single, unbranched, postcapillary venules, with a diameter of 20–40 μm, were selected for microscopic observation. Adherent leukocytes were defined as those remaining stationary for at least 30 seconds. Leukocyte–endothelial adherence (LEA) was expressed as number of adherent leukocytes per 100 μm of venule length. Ruthenium red (5 mg/100 ml) (12) was used to estimate *in vivo* mast cell degranulation intensity with the AnaliSYS Software System (Soft Imaging Systems Corp., Lakewood, CO). At least five mast cells were analyzed in each field of observation. At the end of the experiment, the rats were killed with an intravenous overdose of 150 mg/kg sodium pentobarbital.

### Culture of Isolated Alveolar Macrophages

Male Sprague-Dawley rats (300–350 g) were anesthetized with pentobarbital sodium (35 mg/kg, intraperitoneal). After placement of a PE-50 catheter in the jugular vein, a tracheotomy was performed, and a PE-240 catheter placed in the trachea. An overdose of pentobarbital

sodium (150 mg/kg) was injected intravenously and bronchoalveolar lavage was performed, as previously described in detail (10). The cells were plated in a T-25 sterile flask at 37°C and equilibrated with 5% CO<sub>2</sub> in air for 45 minutes until the macrophages were firmly adhered to the flask. The supernatant was replaced and, depending on the experimental protocol, the cell culture was equilibrated with humidified gas mixtures with nominal concentrations of 0, 10, or 21% O<sub>2</sub> for 30 or 60 minutes. The supernatant was then removed and frozen at –70°C until use, or the cultures were used on the same day.

### Culture of Peritoneal Mast Cells and Peritoneal Macrophages

Peritoneal macrophages and peritoneal mast cells were harvested by lavage of the peritoneal cavity in rats anesthetized with urethane (1.5 g/kg, intramuscularly). The macrophages were separated from mast cells by differential centrifugation using a Percoll solution (13). Mast cells isolated by this procedure exceed 95% purity. Macrophages isolated had essentially no contamination with mast cells.

The separated mast cells and peritoneal macrophages were resuspended in 2 ml of Dulbecco's modified Eagles medium with 10% serum and plated in a T-25 sterile flask at 37°C in 10% O<sub>2</sub>–5% CO<sub>2</sub>–85% N<sub>2</sub> for 45 minutes. The supernatant was discarded with a pipette and replaced with 2 ml of serum-free Dulbecco's modified Eagles medium. The cell cultures were equilibrated with humidified mixtures with nominal O<sub>2</sub> concentrations of 10% (normoxia) or 0% (hypoxia). Depending on the experimental protocol, the supernatant was removed and frozen at –70°C until use, or the cultures were used on the same day.

### Measurement of H<sub>2</sub>O<sub>2</sub> in Supernatant of Alveolar and Peritoneal Macrophages

An electrochemical detection system (Apollo 4000; World Precision Instruments, Sarasota, FL) was used to determine H<sub>2</sub>O<sub>2</sub> concentration in 0.3 ml aliquots of supernatant of alveolar and peritoneal macrophages isolated and cultured on the day of measurement. Measurements were performed in samples removed at 15-minute intervals using an H<sub>2</sub>O<sub>2</sub>-sensitive electrode at 37°C. After measurement, the sample was returned to the culture dish. When the experiments were finished, 2 ml of a solution of 0.4% Trypan blue was added to the culture and mixed for 2 minutes. Photographs of five different areas of the culture, containing approximately 200 cells each, were obtained within 30 minutes of Trypan blue addition. Cell viability was expressed as the percentage of cells excluding Trypan blue.

### Measurement of Inflammatory Mediators

The effect of hypoxia on supernatant levels of several cytokines and chemokines was investigated by initially screening cytokine and chemokine levels with a multianalyte ELISA Array (SABiosciences Corp., Frederick, MD). Agents investigated in the initial screen included IL-1β, -4, -6, -10, -12, and -17α, INF-γ, TNF-α, transforming growth factor-B1, MCP-1, and macrophage inflammatory protein (MIP)-1α and -1β. The initial screen was followed by individual assay (Single Analyte ELISA; SABiosciences Corp.) to determine levels of MCP-1 in supernatant of alveolar macrophages, peritoneal macrophages, and peritoneal mast cells.

### Statistical Analysis

Data are presented as means (±SEM). In both the *in vivo* and *in vitro* experiments, each preparation served as its own control, with the data obtained after a given experimental treatment compared with the average of the values obtained during the control period in the same experiment. Significance was established using a *t* test for paired values. Intergroup comparisons were made with a one-way ANOVA with the Bonferroni correction for multiple comparisons.

## RESULTS

### Experiments in the Mesenteric Microcirculation

All experiments of this series had the same format: after a 30-minute control period, approximately 0.5 ml of supernatant or of a pharmacological agent was distributed evenly over the mesentery. The microcirculation was observed for an additional

30 minutes. The supernatants used had been frozen, and were thawed at 37°C immediately before use.

**Effect of alveolar or peritoneal macrophage supernatant applied on the mesentery of normoxic rats.** Supernatant of alveolar macrophages, which had been equilibrated with 10% O<sub>2</sub>, produced mast cell degranulation and LEA (Figure 1A) (*n* = 5 rats studied). In contrast, neither supernatant from alveolar macrophages equilibrated with 21% O<sub>2</sub> (Figure 1B, *n* = 5) or from peritoneal macrophages equilibrated with 0% O<sub>2</sub>, (Figure 1C, *n* = 5) produced mast cell degranulation or LEA.

**Role of the RAS on the response of the microcirculation to hypoxic alveolar macrophage supernatant.** Topical application of AngII (100 nM) produced an increase in LEA without producing mast cell degranulation (Figure 2A, *n* = 5). This indicates that the effect of AngII occurs at the leukocyte–endothelial interface, and is not mediated by mast cell activation.

Application of supernatant of alveolar macrophages equilibrated with 10% O<sub>2</sub> to the mesentery of rats pretreated with the nonspecific angiotensin II (ANGII) receptor antagonist Sar<sup>1</sup>, Thr<sup>8</sup>, AngII, continuously infused intravenously at a rate of 30 μg<sup>-1</sup> · kg<sup>-1</sup> · min<sup>-1</sup> (Figure 2B, *n* = 5) or with the nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase inhibitor, apocynin (1 mM applied topically; Figure 2C, *n* = 5), essentially produced the same results: although mast cell degranulation occurred in response to the application of supernatant, no increase in LEA was observed.

Figure 3 shows the effects of AngII receptor blockade (Figure 3A, *n* = 5) and of apocynin (Figure 3B, *n* = 5) on the response of the microcirculation to the basic mast cell secretagogue C4880 (14). When applied to the mesentery or cremaster microcirculation, C4880 induces mast cell degranulation and LEA (2, 6). In the present experiments, pretreatment with

AngII receptor blocker (Figure 3A) or with apocynin (Figure 3B) did not prevent the mast cell degranulation, but inhibited the LEA induced by C4880.

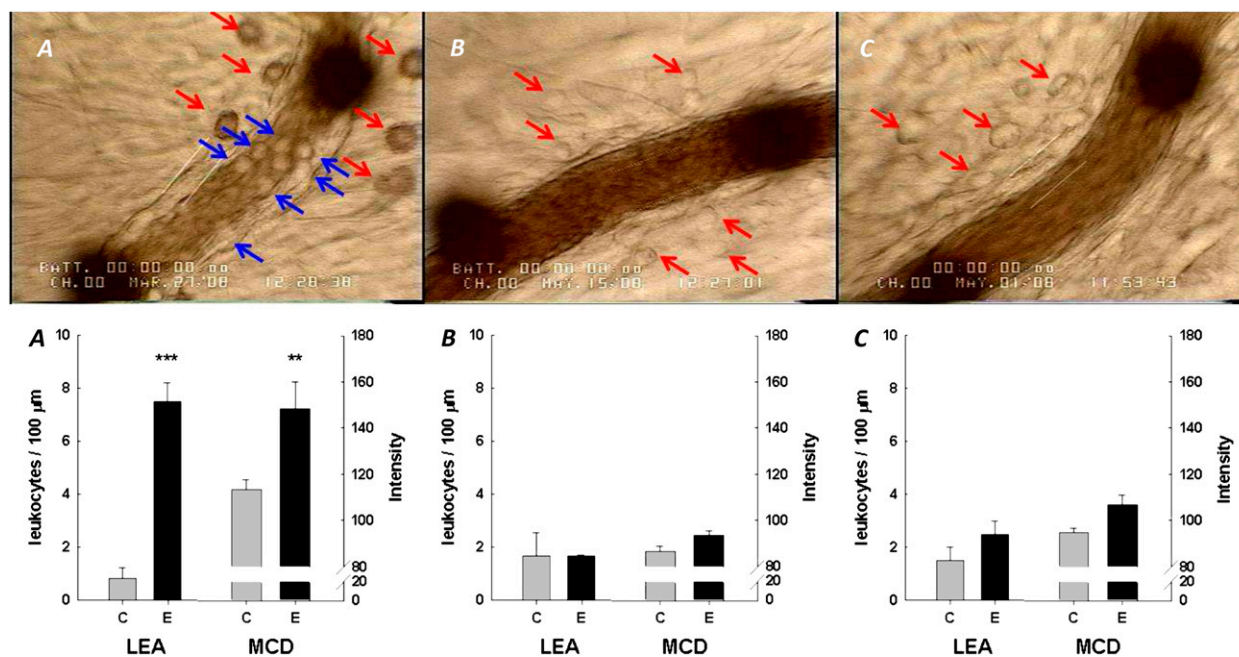
These results indicate that, as seen in the cremaster (9, 10), the increased LEA produced in the mesentery by hypoxic alveolar macrophage supernatant is mediated by activation of the RAS; the RAS is activated by mast cell degranulation induced, in turn, by an agent released into the supernatant by hypoxic alveolar macrophages. The effectiveness of the NADPH oxidase antagonist, apocynin, on the inflammatory response further suggests that the effects of RAS activation are mediated by NADPH oxidase assembly.

### Experiments in Primary Cell Cultures

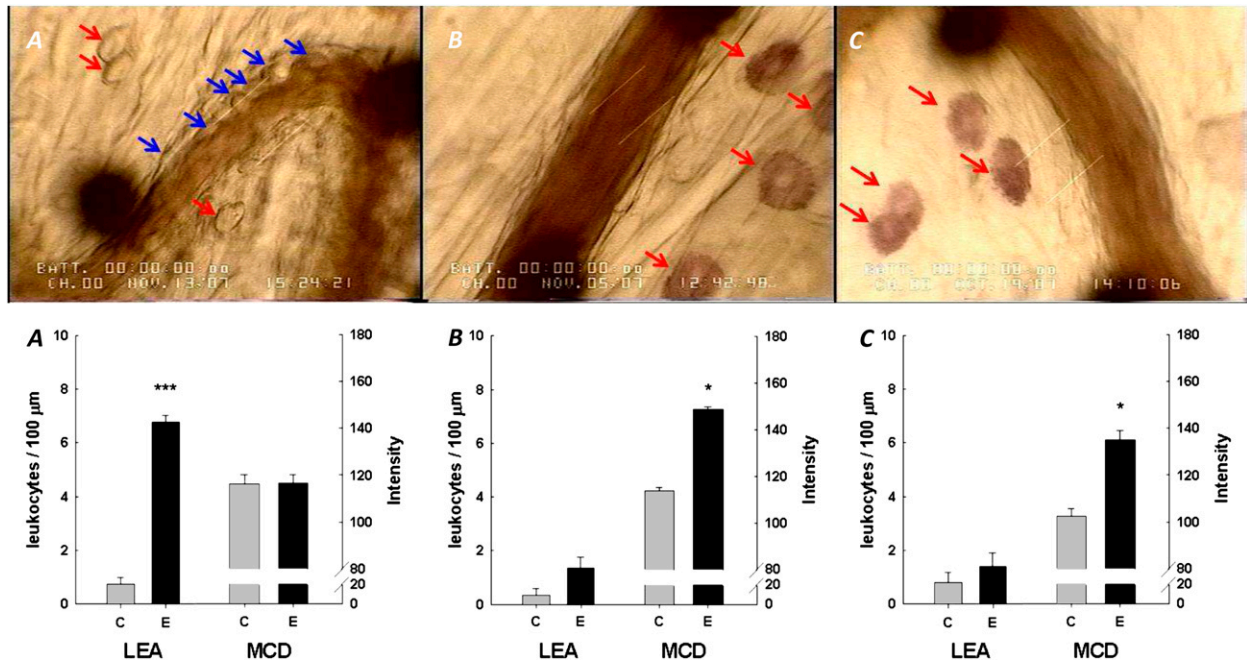
#### H<sub>2</sub>O<sub>2</sub> release by isolated alveolar and peritoneal macrophages.

All the experiments in this series had the same format: after a 45-minute period of equilibration with 21% O<sub>2</sub> for alveolar and 10% O<sub>2</sub> for peritoneal macrophages, the cultures were equilibrated for 60 minutes with the experimental gas mixture. Samples for supernatant H<sub>2</sub>O<sub>2</sub> concentration measurement were obtained at the end of the normoxic control period and every 15 minutes thereafter.

Table 1 shows the changes in supernatant H<sub>2</sub>O<sub>2</sub> concentration with respect to their respective control samples in the different groups. Under control conditions (21% O<sub>2</sub>), alveolar macrophage supernatant H<sub>2</sub>O<sub>2</sub> concentration averaged 0.23 (±0.05) nM/liter/10<sup>6</sup> cells, without significant differences among groups. Continuation of exposure to 21% O<sub>2</sub> for 1 additional hour resulted in a gradual decrease in H<sub>2</sub>O<sub>2</sub> concentration of the supernatant. Hypoxia (nominal 10% O<sub>2</sub> and 0% O<sub>2</sub>) produced a significant net increase in alveolar macrophage supernatant H<sub>2</sub>O<sub>2</sub> concentration. The increase was significantly higher when the nominal O<sub>2</sub> concentration of the equilibrating



**Figure 1.** Effect of topical application of alveolar macrophages and peritoneal macrophages supernatant on the mesentery. *Top:* representative microphotographs of the mesenteric microcirculation after topical application of supernatant hypoxic alveolar macrophages (A), normoxic alveolar macrophages (B), and hypoxic peritoneal macrophages (C). The *large dots* are used to align the optical Doppler velocimeter, and, occasionally, they are moved to obtain a better image of the leukocyte–endothelial interface for photographs. The *red arrows* point to the mast cells; the *blue arrows* identify adherent leukocytes. *Bottom:* average values of leukocyte–endothelial adherence (LEA), leukocytes/100 μm, and intensity of mast cell degranulation (MCD) in arbitrary units. Presented are mean (±SEM) values at the end of the control (C) and experimental (E) periods, respectively; *n* = 5 rats studied in each group. \*\**P* < 0.01; \*\*\**P* < 0.001.

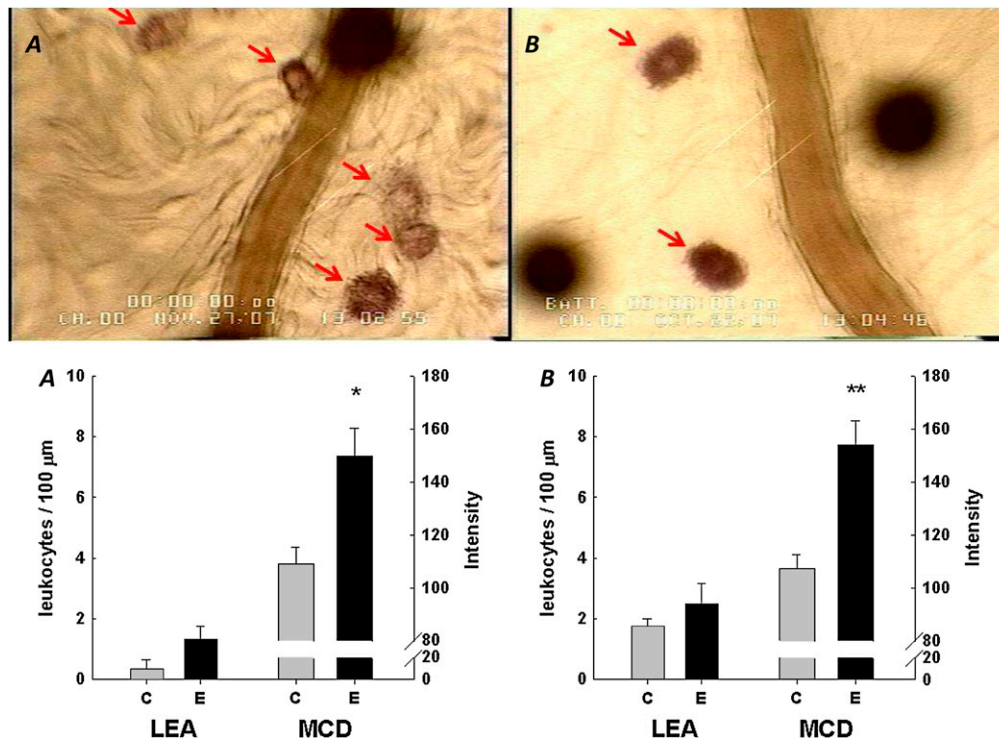


**Figure 2.** Role of the renin–angiotensin (Ang) system (RAS) on the response of the mesentery to hypoxic alveolar macrophage supernatant. *Top:* representative microphotographs of the mesenteric microcirculation after topical application of 10 nM AngII (A), hypoxic alveolar macrophage supernatant in rats pretreated with AngII receptor blocker (B), and hypoxic alveolar macrophage supernatant in rats treated with apocynin (C). The red arrows point to the mast cells; the blue arrows identify adherent leukocytes. *Bottom:* average values of LEA, leukocytes/100 μm, and intensity of MCD in arbitrary units. Presented are means (±SEM) of values at the end of the control (C) and the experimental (E) period, respectively; n = 5 in all cases. \*P < 0.05; \*\*\*P < 0.001.

gas mixture was 0% (actual PO<sub>2</sub>, ~5 mm Hg) than when equilibration was performed with 10% O<sub>2</sub> (actual PO<sub>2</sub>, ~65 mm Hg). The increase was transitory, reaching a peak at 15 minutes of equilibration, and gradually returning toward control. The increase in supernatant H<sub>2</sub>O<sub>2</sub> produced by equilibration with 10% O<sub>2</sub> was blocked by pretreatment with polyethylene

glycol catalase. Viability of alveolar macrophages, assessed by Trypan blue exclusion at the end of the experiments, was higher than 95%, and was not influenced by the PO<sub>2</sub>, even when it reached values as low as approximately 5 mm Hg.

Average peritoneal macrophage supernatant H<sub>2</sub>O<sub>2</sub> concentration under control conditions (10% O<sub>2</sub>) was 0.31 (±0.05)



**Figure 3.** Role of the RAS on the microvascular response to mast cell secretagogue C4880. *Top:* representative microphotographs of mesenteric microcirculation illustrating the effect of topical application of C4880 after pretreatment with AngII receptor blocker (A), or with apocynin (B). *Bottom:* average values of LEA, leukocytes/100 μm, and intensity of MCD in arbitrary units. Presented are means (±SEM) of values at the end of the control (C) and the experimental (E) period, respectively; n = 5 in all cases. \*P < 0.05; \*\*P < 0.01.

**TABLE 1. EFFECTS OF HYPOXIA ON H<sub>2</sub>O<sub>2</sub> RELEASE BY ALVEOLAR AND PERITONEAL MACROPHAGES**

Cell Type	% O <sub>2</sub>	Supernatant Δ [H <sub>2</sub> O <sub>2</sub> ]* (nM/10 <sup>6</sup> cells)				% Viability	No. of Cultures
		15 min	30 min	45 min	60 min		
Alveolar macrophages	21	-0.03 ± 0.02	-0.11 ± 0.03 <sup>†</sup>	-0.12 ± 0.03 <sup>†</sup>	-0.09 ± 0.04 <sup>‡</sup>	95.8 ± 0.4	5
	10	0.28 ± 0.07 <sup>†</sup>	0.09 ± 0.03 <sup>‡</sup>	-0.01 ± 0.03	-0.06 ± 0.04	96.9 ± 0.3	5
	0	1.05 ± 0.13 <sup>§</sup>	-0.03 ± -0.02	-0.02 ± 0.02	-0.02 ± 0.02	98.6 ± 0.6	5
	10 + CAT	0.02 ± 0.01	0.02 ± 0.02	0.00 ± 0.02	-0.01 ± 0.02	96.9 ± 0.5	5
Peritoneal macrophages	10	-0.02 ± 0.01	-0.02 ± 0.02	-0.01 ± 0.02	-0.02 ± 0.02	95.9 ± 0.9	5
	0	0.01 ± 0.01	0.01 ± 0.01	-0.01 ± 0.01	0.01 ± 0.02	95.2 ± 0.5	5

Definition of abbreviation: CAT, catalase.

\* Changes in the concentration of H<sub>2</sub>O<sub>2</sub> with respect to the control; each value (n = 5) is the average of the value at a given time minus the corresponding control.

<sup>†</sup> P < 0.01.

<sup>‡</sup> P < 0.05.

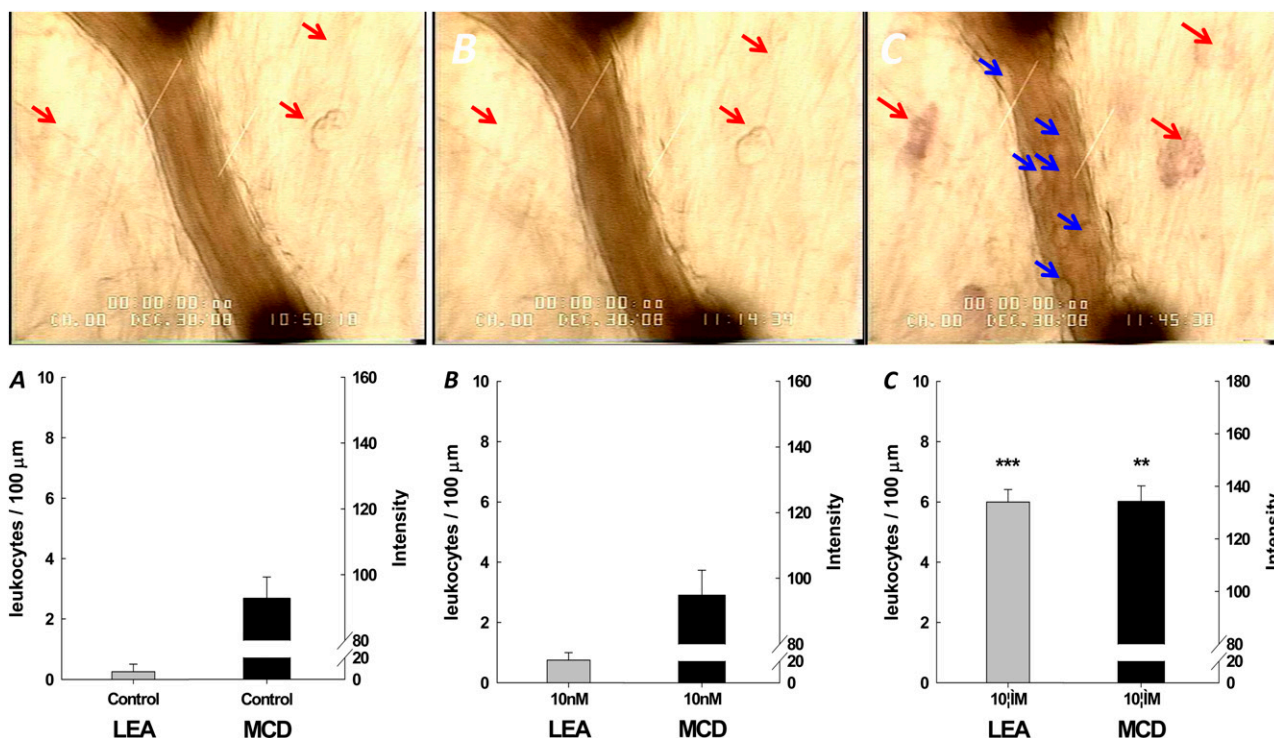
<sup>§</sup> P < 0.001.

nM/liter/10<sup>6</sup> cells, which was not significantly different from that observed under control conditions (21% O<sub>2</sub>) in alveolar macrophages. However, in contrast with alveolar macrophages, equilibration of peritoneal macrophages with 0% O<sub>2</sub> was not accompanied by H<sub>2</sub>O<sub>2</sub> release into the supernatant. Cell viability at the end of 1 hour of equilibration was also approximately 95%, and was not influenced by the supernatant PO<sub>2</sub>.

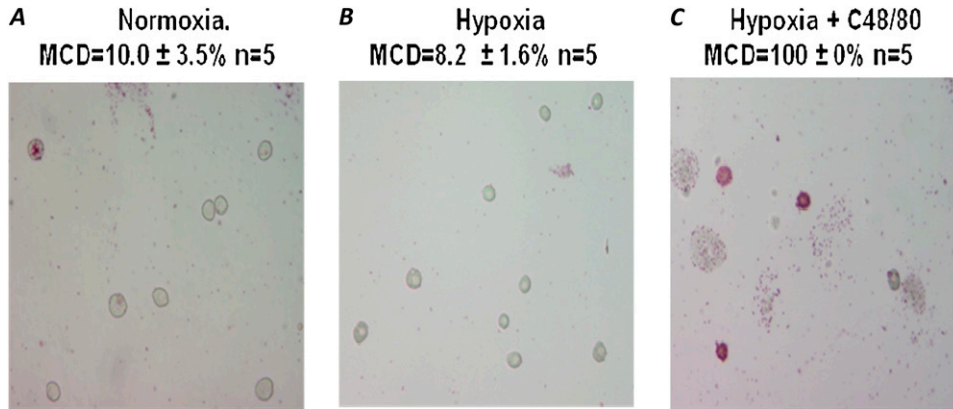
Because H<sub>2</sub>O<sub>2</sub> activates mast cells and induces inflammation, additional experiments in the mesenteric microcirculation were performed to determine whether the small amount of H<sub>2</sub>O<sub>2</sub> present in the alveolar macrophage supernatant after 30 minutes of hypoxia could be responsible for the inflammatory response illustrated in Figure 1A. In those experiments, supernatant of alveolar macrophages equilibrated with 10% O<sub>2</sub> was applied topically to the mesentery. Topical application of a solution of 10 nM H<sub>2</sub>O<sub>2</sub>, a concentration over fivefold higher

than that observed in the alveolar macrophage supernatant at 30 minutes of 10% O<sub>2</sub> equilibration, did not increase LEA or mast cell degranulation above that of the untreated control samples (compare Figures 4A and 4B). Only when a solution with a concentration of 10 μM was applied did LEA and mast cell degranulation become evident (Figure 4C, n = 5).

**Effect of hypoxia on peritoneal mast cell degranulation.** Fresh peritoneal mast cell cultures (Figure 5, n = 5 cell cultures studied) were successively exposed to gas mixtures with 10 and 0% O<sub>2</sub>, and 0% O<sub>2</sub> plus C4880. After 30 minutes of exposure to each condition, a small sample (5–10 μl, ~20,000 cells/μl) was removed and mixed with an equal volume of a solution of ruthenium red to assess mast cell degranulation. Exposure of peritoneal mast cells to 0% O<sub>2</sub> did not significantly increase uptake of ruthenium red from the levels observed during normoxic equilibration (compare Figures 5A and 5B). Addition



**Figure 4.** Effect of topical H<sub>2</sub>O<sub>2</sub> on the normoxic mesentery. *Top:* representative microphotographs of the mesenteric microcirculation illustrating the effect of topical application of H<sub>2</sub>O<sub>2</sub>. (A) Untreated, (B) after application of 10 nM H<sub>2</sub>O<sub>2</sub>, and (C) after application of 10 μM H<sub>2</sub>O<sub>2</sub>. Because H<sub>2</sub>O<sub>2</sub> bleaches ruthenium red, the photographs were taken after the H<sub>2</sub>O<sub>2</sub> was washed out from the surface, after which ruthenium red was applied. *Bottom:* average values of LEA, leukocytes /100 μm, and intensity of MCD in arbitrary units. n = 5 in all cases. \*\*P < 0.01; \*\*\*P < 0.001.



**Figure 5.** Effect of hypoxia on MCD. Representative photomicrographs of primary mast cell cultures. The cells represented in A–C were obtained from the same primary culture. The data on top of each photograph is the mean ( $\pm$ SEM) of the percent MCD in five separate experiments. (A) Normoxia (MCD =  $10.0 \pm 3.5\%$ ;  $n = 5$ ); (B) hypoxia (MCD =  $8.2 \pm 1.6\%$ ;  $n = 5$ ); (C) hypoxia plus C48/80 (MCD =  $100 \pm 0\%$ ;  $n = 5$ ).

of C4880 during hypoxia to cells obtained from the same cultures induced complete mast cell degranulation (Figure 5C), indicating that the hypoxic mast cells could respond normally to a general secretagogue.

**Interactions between isolated mast cells, alveolar macrophages, and peritoneal macrophages.** In these series, mast cells cultured in 10%  $O_2$  were centrifuged, and the pellet suspended in supernatant removed from fresh primary cell cultures of alveolar or peritoneal macrophages. The supernatants contained no macrophages. The mast cells resuspended in macrophage supernatant were equilibrated with 10%  $O_2$  in all cases.

Suspension of isolated mast cells in supernatant of alveolar macrophages that had been cultured in normoxia did not induce mast cell degranulation (Figure 6A,  $n = 5$  cell cultures studied); on the other hand, suspension in supernatant of alveolar macrophages cultured in hypoxia (10%  $O_2$ ) significantly increased mast cell degranulation (Figure 6B,  $n = 5$ ). In contrast, suspension of mast cells in supernatant of peritoneal macrophages that had been incubated with 0%  $O_2$  failed to produce mast cell degranulation (Figure 6C,  $n = 5$ ).

**Effects of hypoxia on selected cytokine levels.** Of all the agents screened in the supernatant of alveolar macrophages using a multianalyte ELISA array (IL-1 $\beta$ , -4, -6, -10, -12, and -17 $\alpha$ , IFN- $\gamma$ , MCP-1, TNF- $\alpha$ , and MIP-1 $\alpha$  and -1 $\beta$ ), only MCP-1 showed a marked increase with hypoxia. Subsequent individual ELISA assays using a specific antibody showed that MCP-1 increased almost fourfold in the supernatant of alveolar macrophages exposed to hypoxia compared with the normoxic control (Figure 7). In contrast, equilibration with nominal 0%  $O_2$  does not elicit release of MCP-1 from peritoneal macrophages or mast cells (Figure 7).

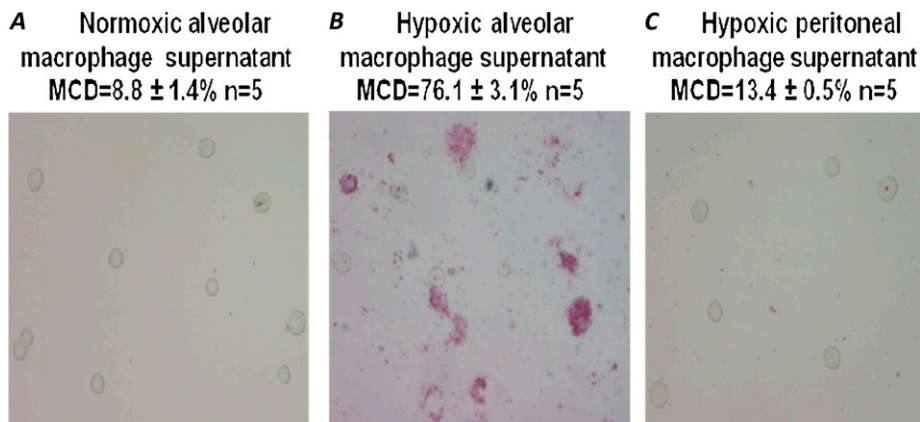
## DISCUSSION

The central findings of these experiments are the following: (1) supernatant of hypoxic alveolar macrophages elicited inflammation in the normoxic mesentery, whereas supernatant of peritoneal macrophages equilibrated with even lower  $PO_2$  values had no inflammatory effect; (2) hypoxia activated primary cultures of alveolar, but not of peritoneal macrophages; (3) mast cells in primary cultures did not degranulate when exposed to hypoxia, although degranulation occurred when mast cells were placed in contact with supernatant of hypoxic alveolar macrophages, but not of hypoxic peritoneal macrophages; (4) hypoxia induced release of MCP-1 from alveolar, but not peritoneal macrophages or mast cells.

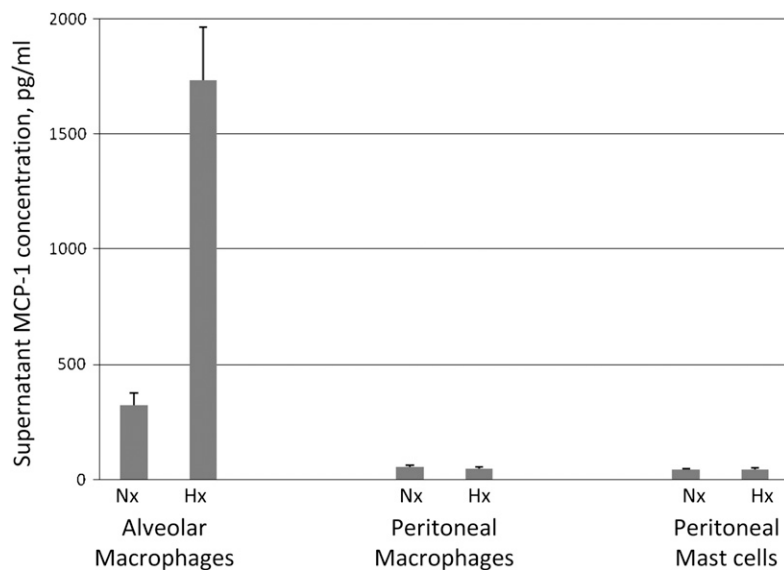
These results demonstrate a direct link between alveolar macrophage activation by hypoxia and mast cell degranulation, and rule out possible roles of low tissue  $PO_2$  and of resident tissue macrophages in the early phase of the inflammation of alveolar hypoxia. The results add a key piece of evidence that confirms our hypothesis that the inflammation produced by alveolar hypoxia is triggered by alveolar macrophage-borne mediator(s) carried by the circulation. The release by alveolar macrophages exposed to hypoxia of a mast cell secretagogue, MCP-1, provides evidence concerning the nature of a possible mediator of inflammation.

### Studies in the Mesenteric Microcirculation

There were two objectives of the *in vivo* studies in the mesenteric microcirculation. First, as the mast cells and resident tissue macrophages used in the present experiments were collected from the peritoneum, it was important to determine whether the responses of the primary cell cultures to the various



**Figure 6.** Effect of immersion of mast cells in supernatant of alveolar or peritoneal macrophages. Mast cells obtained for the same culture were immersed in supernatant of alveolar macrophages that had been equilibrated with 21%  $O_2$  (A), 10%  $O_2$  (B), or peritoneal macrophages equilibrated with 0%  $O_2$  (C). The supernatants contained no macrophages, and, after mast cell immersion, were equilibrated with 10%  $O_2$ . The data above each photograph is the mean ( $\pm$ SEM) of the percent of MCD in five separate experiments.



**Figure 7.** Effect of hypoxia on the release of monocyte chemoattractant protein (MCP)-1 by alveolar macrophages. Supernatant MCP-1 concentration of primary cultures of alveolar macrophages, peritoneal macrophages, and peritoneal mast cells exposed to 30 minutes of hypoxia (Hx) or normoxia (Nx) ( $n = 5$  in each case).

interventions were similar to those observed *in vivo*. Peritoneal mast cells and macrophages were selected because they can be harvested with minimal manipulation, and, therefore, their responses are least affected by the isolation procedures. The second objective of the *in vivo* studies was to demonstrate whether the response of the mesenteric microcirculation to hypoxic alveolar macrophage supernatant is similar to that observed previously in the cremaster. A difference in the responses of these two vascular beds would argue against our hypothesis that the widespread inflammation of alveolar hypoxia in the intact animal is triggered by a mediator released into the circulation by activated alveolar macrophages.

Topical application of supernatant of alveolar macrophages equilibrated with 10%  $O_2$  produced essentially the same response in the mesentery as that observed previously in the cremaster microcirculation (9): mast cell degranulation, increased LEA (Figure 1A), and activation of the local RAS (Figures 2 and 3). As observed in the cremaster, activation of the RAS in the mesentery is a result of mast cell degranulation (9, 10). The mechanism by which mast cell degranulation activates the RAS is not clear. Possible pathways of activation include mast cell AngI-converting chymase (15–17), or release of renin, which has been demonstrated in myocardial mast cells (18, 19).

In addition to demonstrating the similarity of responses in the cremaster and the mesentery, the present experiments expand our previous findings in the cremaster in two important respects. First, apocynin, a blocker of the assembly of NADPH oxidase, had essentially the same effects as the AngII receptor blocker in preventing the inflammation. This provides further insight into the mechanism of action of AngII by showing that its inflammatory effects are secondary to the assembly of NADPH oxidase. A role for NADPH oxidase-generated ROS has been documented in several conditions characterized by increased RAS activity, including diabetes (20), hypertension (21, 22), and ischemia-reperfusion (17). A second important new finding of these experiments was that supernatant of peritoneal macrophages exposed to a more severe level of hypoxia than alveolar macrophages did not initiate an inflammatory response in the mesentery (Figure 1C). This observation is consistent with the failure of hypoxia to induce a respiratory burst in cultures of peritoneal macrophages (Table 1). These findings, together with the absence of *in vivo* cremaster in-

flammation during selective cremaster hypoxia in the presence of normal alveolar  $PO_2$  (2, 3), indicate that resident tissue macrophages do not contribute to the initiation of the systemic inflammation of alveolar hypoxia.

The results show that the inflammatory response to supernatant of hypoxic alveolar macrophages has similar characteristics in the mesenteric and in the skeletal muscle microcirculations, and supports the hypothesis that the widespread inflammation of alveolar hypoxia is the result of mast cell activation by an agent released by alveolar macrophages.

Alveolar macrophages, but not peritoneal macrophages, responded with a transitory release of  $H_2O_2$  when exposed to hypoxia. The  $H_2O_2$  release is a manifestation of the respiratory burst that occurs during macrophage activation (23), and is characterized by superoxide generation, followed by dismutation to  $H_2O_2$ . In addition to an important role in inactivation of phagocytosed pathogens, ROS generated during the respiratory burst are thought to play a role in intracellular signal transduction (24, 25). In the present experiments, the amount of  $H_2O_2$  released by alveolar macrophages was largest at the lowest  $PO_2$ . The correlation between the magnitude of the respiratory burst and the severity of hypoxia in the presence of maintained cell viability indicates that this is a biological response of alveolar macrophages to reduced  $PO_2$ . On the other hand, peritoneal macrophages exposed to the same  $PO_2$  did not become activated. The dissimilar effects of hypoxia on activation of the two types of macrophages are paralleled by the different effects of their supernatants on mast cells: whereas supernatant of hypoxic alveolar macrophages elicited degranulation of mast cells both *in vivo* (Figure 1A) and *in vitro* (Figure 6B), supernatant of hypoxic peritoneal macrophages exposed to much lower  $PO_2$  had none of these effects (Figures 1C and 6C). Thus, evidence obtained both *in vivo* and *in vitro* points to a specific effect of reduced  $PO_2$  on alveolar macrophages.

The discrepancy between alveolar and peritoneal macrophages in the response to hypoxia is a manifestation of the different characteristics of these two cell types. Although both originate from a common precursor in the bone marrow, alveolar macrophages *in vivo* are normally exposed to a higher  $PO_2$  than peritoneal macrophages. These different environments may, in part, determine their different metabolic patterns (26), and perhaps explain the dissimilar effects of hypoxia.

The present experiments clearly demonstrate a link between activation of alveolar macrophages by hypoxia and mast cell degranulation: degranulation did not occur when mast cells were directly exposed to hypoxia, but was observed when the mast cells were immersed in supernatant of alveolar macrophages that had been equilibrated in hypoxia. This shows that hypoxic alveolar macrophages release an agent that produces mast cell degranulation. This agent is not released by normoxic alveolar macrophages or by hypoxic peritoneal macrophages, which show no evidence of activation. Our earlier *in vivo* finding, that selective cremaster hypoxia does not induce mast cell degranulation in rats with normal alveolar PO<sub>2</sub> (3), agrees with these *in vitro* observations, and demonstrates that neither reduced local PO<sub>2</sub> nor activation of resident tissue macrophages participate in the systemic inflammation of hypoxia.

A key issue in this phenomenon is the nature of the mediator released by alveolar macrophages in response to hypoxia. There is evidence that alveolar hypoxia leads to pulmonary inflammation in rats (27), and alveolar macrophages play an important role in this phenomenon. Reduction of alveolar PO<sub>2</sub> in rats leads to increased expression of mRNA for TNF- $\alpha$ , MIP-1 $\beta$ , and intercellular adhesion molecule-1 in the lung, all of which are reduced by depletion of alveolar macrophages (27); hypoxia also increases expression of TNF- $\alpha$  and MIP-1 $\beta$  in isolated alveolar macrophages (28). In addition, hypoxia acts as a synergist in the interaction of several pathogens with alveolar macrophages (29–31). However, although these responses may influence the development of pulmonary and systemic inflammation later on in the course of hypoxia, it is unlikely that the phenomenon described in the present experiments is a consequence of changes in gene expression. Given the rapid onset of the response to hypoxia, and the fact that the targets are the mast cells, the putative mediator of systemic inflammation is likely to be a mast cell secretagogue stored in alveolar macrophages. To provide additional information on this subject, several possible candidates were screened. MCP-1 was observed to increase significantly in the supernatant of alveolar macrophages exposed to hypoxia in the time frame used in these studies. Peritoneal macrophages or mast cells did not release MCP-1 when exposed to even lower PO<sub>2</sub>. MCP-1, a chemokine of the CC family, fits the criteria for a putative mediator of hypoxia-induced inflammation: MCP-1 induces chemotaxis of alveolar macrophages, mast cells, and human T lymphocytes (32). MCP-1 is released from alveolar macrophages *in vitro* in response to hypoxia and hypoxia/reoxygenation (33–35), influences distal organ damage in hemorrhagic shock (36), and activates mast cells to elicit microvascular inflammation (37, 38). Further studies are necessary to determine the mechanism underlying the release of MCP-1 by alveolar macrophages, the interaction of MCP-1 with mast cells, and whether other alveolar macrophage-borne agents participate in the activation of mast cells. Nevertheless, the demonstration of increased release of a mast cell secretagogue provides confirmation of our hypothesis that the systemic inflammation of hypoxia is initiated by an alveolar macrophage-borne mediator.

The present results highlight the role of alveolar macrophages in initiating an inflammatory response in the systemic microcirculation. Although most known functions of these cells take place within the lung, mounting evidence indicates that alveolar macrophage activation leads to systemic inflammation and microvascular function impairment. For example, after activation by phagocytosis of particulate matter, cytokines released by alveolar macrophages act on the bone marrow to mobilize platelets and leukocytes, which stimulate the release of acute-phase proteins, and lead to systemic inflammation (39, 40). The phenomenon described in the present study represents

an example of systemic effects initiated by activation of alveolar macrophages by another type of stimulus; in this case, reduction of alveolar PO<sub>2</sub>.

Several conditions featuring alveolar hypoxia are accompanied by systemic effects, and inflammation has been implicated as a causative or contributory factor in these systemic effects. These include the cachexia and muscle wasting (41, 42) and the cardiovascular abnormalities (43) of chronic obstructive pulmonary disease, the suboptimal erythropoiesis of pulmonary fibrosis (44), and the cardiovascular complications of sleep apnea (45, 46). Conditions in which hypoxia develops rapidly are also accompanied by a systemic inflammatory component. For instance, pneumonia is accompanied by systemic inflammation, and elevated circulating inflammatory markers are predictors of subsequent mortality (47, 48). Although these markers may, in part, reflect the response to bacterial infection, it is conceivable that the hypoxic environment in areas of lung consolidation may lead to activation of alveolar macrophages that contribute to the systemic inflammation. Acute altitude illnesses represent another example of systemic inflammatory response to alveolar hypoxia. Rapid ascent to high altitude is increasingly frequent, and may result in acute mountain sickness or high-altitude cerebral edema (49). Although the role of inflammation in these cases is still unclear, the presence of elevated levels of circulating and tissue inflammatory markers (50–52), and the effectiveness of dexamethasone, an anti-inflammatory steroid, in the treatment of acute mountain sickness (49, 53), suggest the contribution of an inflammatory component. Whether activation of alveolar macrophages plays a role in the pathogenesis of these and other conditions associated with alveolar hypoxia should be the subject of further research.

It is important to keep in mind that reduction of alveolar PO<sub>2</sub> in an intact organism is a complex stimulus, which sets in motion responses with different time courses; accordingly, a number of mechanisms may participate at different moments in the course of hypoxia. For example, increased leukocyte-endothelial adhesive interactions in response to hypoxia have been demonstrated in isolated human umbilical veins, in which the phenomenon described here clearly does not occur (54). However, the time course of the *in vitro* responses (hours vs. minutes) is quite different from that described in the present article, suggesting that the underlying mechanisms are different from those described here.

In summary, the present study provides key evidence in support of our hypothesis that the inflammation of alveolar hypoxia is initiated by the release of a mediator from alveolar macrophages. This evidence includes the identification of a possible mediator of this phenomenon, a mast cell secretagogue produced by alveolar macrophages. Further research is necessary to determine whether other agents participate, and to develop tools to antagonize the mediator's effects. This should help in understanding the possible contribution of this phenomenon to the pathogenesis of illnesses associated with reduced alveolar PO<sub>2</sub>, as well as its role in the overall strategies of adaptation of organisms to alveolar hypoxia.

**Conflict of Interest Statement:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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