Role of the renin-angiotensin system in the systemic microvascular inflammation of alveolar hypoxia

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Hypoxia, induced by a reduction of inspired \( P_{O_2} \), results in rapid microvascular inflammation in rats (31). This response, observed in several systemic microcirculatory beds (12), is characterized by increased levels of reactive \( O_2 \) species (29), elevated leukocyte-endothelial adhesive interactions (31), and increased vascular permeability and leukocyte emigration (30). Mast cell activation is a key early phenomenon (27), and lipid mediators released by mast cells, such as platelet-activating factor and leukotriene \( B_4 \), play a role in the inflammatory response (5). The inflammation is attenuated by administration of nitric oxide donors (26, 31), antioxidants (26, 29), and cromolyn, a mast cell stabilizer (8, 26, 27).

Recent studies in cremaster muscle suggest that the inflammation is not triggered by the reduction of local microvascular \( P_{O_2} \), but rather by a mediator released from a distant site and transported by the circulation (8, 24). Selective reduction of cremaster microvascular \( P_{O_2} \) does not elicit leukocyte-endothelial adherence or mast cell degranulation as long as arterial blood \( P_{O_2} \) is not reduced; conversely, inhalation of 10% \( O_2 \) and reduction of arterial \( P_{O_2} \) produces leukocyte-endothelial adherence and mast cell stimulation in the cremaster microcirculation, even when cremaster microvascular \( P_{O_2} \) is maintained at normoxic levels (8, 24). More direct evidence of the participation of a circulating mediator was provided by the observation that topical application of plasma obtained from conscious rats exposed to hypoxia for 5 min produced an inflammatory response in the normoxic cremaster (20). No inflammation was observed when plasma from normoxic control rats was applied, ruling out a nonspecific effect of plasma. The inflammation was still observed when mast cell activation and leukocyte-endothelial adherence induced by alveolar hypoxia in the donor rat were prevented by administration of cromolyn, a mast cell stabilizer. This suggests that the response of the recipient normoxic cremaster was not due to inflammatory products released by adherent leukocytes or activated mast cells into the plasma of the hypoxic donor. Finally, plasma separated after in vitro equilibration of blood with hypoxic gas mixtures did not elicit an inflammatory response, suggesting that the mediator substance does not originate in blood cells (20).

Arteriolar vasoconstriction accompanies the increase in venular leukocyte-endothelial interactions and the mast cell activation produced in the normoxic cremaster by plasma from hypoxic rats (20). This suggests either that the substance triggering the inflammation has vasoactive properties or that it elicits the formation/release of a vasoconstrictor. Angiotensin II (ANG II) is a potent vasoconstrictor implicated in inflammatory processes in the cardiovascular system (4, 6, 10). In addition to the circulating renin-angiotensin system (RAS), local tissue RAS plays an important role in several pathophysiological processes, including cardiovascular inflammation (22). ANG II affects all steps of the inflammatory response: increased leukocyte-endothelial interactions and leukocyte recruitment, increased vascular permeability, and eventually tissue remodeling (9, 10). Similar to systemic hypoxia, ANG II stimulates leukocyte-endothelial interactions in postcapillary venules via generation of reactive \( O_2 \) species (28); these effects can be blocked by antioxidant administration (29). ANG II is generated from angiotensin I (ANG I) by angiotensin-converting enzyme (ACE). This reaction occurs in a variety of tissues, including myocardium, vascular smooth muscle, endothelium, and skeletal muscle (22). Depending on the tissue and the animal species, mast cell chymase may participate in the formation of ANG II from ANG I (3, 18). The recent discovery of renin in cardiac mast cells adds a new dimension to the interaction between mast cells and the RAS at the tissue level (25).
The objective of the present experiments was to study the possible participation of the RAS in the microvascular inflammation of alveolar hypoxia. Specifically, we tested the hypothesis that the response of normoxic skeletal muscle to plasma from hypoxic rats is the result of activation of the RAS in the cremaster microcirculation. This observation would suggest a role of the RAS in the inflammation induced by alveolar hypoxia and provide important information as to the mechanism underlying this response. The results indicate that the RAS does mediate the inflammatory effect of plasma from hypoxic rats and support the idea that the putative mediator released from a distant site during alveolar hypoxia induces microvascular inflammation via mast cell-mediated stimulation of the local RAS.

METHODS

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

Intravitral Microscopy

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. Arterial blood pressure was continuously measured with a digital blood pressure monitor connected to the carotid artery catheter. A tracheotomy was performed, and a PE-240 catheter was connected to a rodent nonbreathing two-way valve. The animals breathed spontaneously throughout the experiment.

The right cremaster muscle was prepared for intravitral 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. 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 of one another) within the selected area of the vessel. The average of the five values was used as the venule diameter. Care was taken to select the same points in all measurements in a given muscle. An optical Doppler velocimeter was used to measure venular center line red blood cell (RBC) velocity. Average RBC velocity was calculated as center line velocity/1.6 (7). Venule shear rate (s⁻¹) was calculated as 8 × [average RBC velocity (mm/s)/venular diameter (mm)] (14). Shear rate is the velocity gradient between layers of a fluid circulating with laminar flow. Since postcapillary venules are devoid of vascular smooth muscle, venular diameter remains relatively unchanged; therefore, the calculated shear rate is proportional to the average blood velocity and, ultimately, blood flow. Accordingly, venular shear rate, like blood flow, is a function of perfusion pressure, vessel diameter, and blood viscosity. Shear rate, as well as blood velocity in venules with essentially constant diameter, provide an estimate of the intensity of the forces that oppose the adherence of leukocytes to the endothelium of postcapillary veins. Adherent leukocytes were defined as those leukocytes that remained stationary for 30 s or longer. Leukocyte adherence was expressed as the number of adherent leukocytes per 100 μm of vessel length.

Second- or third-order arterioles (15- to 30-μm diameter), not adjacent to the venules chosen for observation, were also observed in some of the experiments. In these cases, arterioles and veins were observed alternatively every 5 min. Arteriolar RBC velocity was measured with an optical Doppler velocimeter. Arteriolar diameter was measured with the analySIS Software Image System, using the same approach described for venule diameter. Changes in arteriolar diameter were expressed as the ratio experimental/control for individual experiments, where “control” was the average diameter of all the measurements obtained in the 10-min control period.

Measurement of Extravasation of Fluorescent Albumin

Arterial and venous catheters were placed under anesthesia, and the cremaster was dissected as described above. 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 a 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. Care was taken to analyze the same section of the microcirculation in all the images of a given experiment. The magnitude of albumin extravasation was estimated by the ratio of extravascular 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 breathing 10% O₂ in the donor rat (20, 27). Thirty minutes after cromolyn administration, the rats were placed into a Lucite chamber where 10% O₂–90% N₂ was applied topically onto the cremaster of a normoxic rat prepared for intravitral microscopy as described above. At 5 min of exposure to hypoxia, a 3-ml blood sample was obtained in a 5-ml syringe coated with heparin (1,000 USP units/ml) and the blood was centrifuged for 10 min. Plasma was separated and applied topically onto the cremaster of a normoxic rat prepared for intravitral microscopy as described above. Approximately 12–15 min elapsed between blood withdrawal from the donor rat and the first topical application of plasma onto the cremaster. During this time no attempts were made to avoid equilibration of plasma gas tensions with ambient air and the resulting increase in donor plasma Po₂. In preliminary experiments we observed that the response to plasma was not affected by gas equilibration with room air.

Plasma renin activity (PRA) and ANG II concentration of the donor plasma were measured in four rats exposed to hypoxia for 5 min and four rats maintained in normoxia. In these cases, mean arterial blood pressure (MABP) was recorded immediately before and after the
with withdrawal of 3 ml of blood. Plasma was separated by centrifugation, frozen in liquid N2, and stored at −70°C until assayed for PRA and ANG II concentration by radioimmunoassay with a commercially available kit.

Experimental Protocol

The animals were assigned randomly to the various experimental groups described below.

Series 1: Effect of blockade of RAS on response of normoxic cremaster to application of plasma from hypoxic rats. After a 30-min postsurgery stabilization period, the cremaster microcirculation was observed for 10 min. The Saran Wrap covering the muscle was then removed, and a ~0.75 ml of donor rat plasma was distributed evenly over the entire surface of the cremaster. The muscle was covered again with Saran Wrap, and the microcirculation was observed for 10 min. A second plasma aliquot was applied, and the microcirculation was observed for another 10 min. With this general format, separate experiments were carried out, using bright-field microscopy to investigate leukocyte-endothelial interactions and vasomotor responses on one hand and fluorescence microscopy to assess albumin extravasation on the other. The following experiments were carried out.

EXPERIMENT 1A: PLASMA FROM HYPOXIC RATS APPLIED TO NORMOXIC CREMASTERS. After a 10-min control period, plasma from a hypoxic donor rat was applied topically onto the normoxic cremaster as described above.

EXPERIMENT 1B: PLASMA FROM HYPOXIC RATS APPLIED TO NORMOXIC CREMASTERS PRETREATED WITH ANG II RECEPTOR BLOCKER. After a control period of 10 min, the type 1 and type 2 ANG II receptor antagonist [Sar1,Thr3]ANG II (30 μg·kg−1·min−1) was infused intravenously throughout the experiment. Plasma from hypoxic rats was topically applied 10 and 20 min after initiation of the infusion. Ten minutes after the second plasma application, a solution of 1 μM ANG II was applied topically onto the cremaster to assess the adequacy of the receptor blockade.

EXPERIMENT 1C: PLASMA FROM HYPOXIC RATS APPLIED TO NORMOXIC CREMASTERS PRETREATED WITH ACE INHIBITOR. After a 10-min control period, the ACE inhibitor teprotide (100 μg/ml) was applied topically onto the cremaster. Plasma from hypoxic rats was applied as in experiments 1a and 1b. Teprotide application was repeated ~2 min before the first application of plasma. Ten minutes after the last plasma application, a solution of 1 μM ANG I was applied topically onto the cremaster to assess the adequacy of ACE inhibition.

EXPERIMENT 1D: PLASMA FROM HYPOXIC RATS APPLIED TO NORMOXIC CREMASTERS PRETREATED WITH ACE INHIBITOR AND BRA-DYKININ B2 RECEPTOR BLOCKER. Since ACE inhibition may not only reduce ANG II generation but also increase bradykinin (BK) levels, a fourth series of experiments was included in which pretreatment with teprotide as described above was accompanied by administration of the BK B2 receptor blocker (BK RB) HOE-140 (50 μg/kg iv). The format of this experiment was the same as that in experiment 1c. Ten minutes after the last plasma application, 5 μg/kg BK was infused as a bolus to assess the adequacy of BK receptor blockade.

EXPERIMENT 1E: PLASMA FROM HYPOXIC RATS APPLIED TO NORMOXIC CREMASTERS PRETREATED WITH ACE INHIBITOR PLUS ANG II INFUSION. These experiments were designed to determine whether the expected reduction in microvascular ANG II levels secondary to ACE inhibition could play a role in the response of the normoxic cremaster to plasma from hypoxic rats. After a control period of 10 min, teprotide was applied topically as described above. Ten minutes later, an intravenous infusion of ANG II (5 μM, 1 ml/h) was initiated and maintained until the end of the experiment. Preliminary experiments showed that this rate of infusion produced a return of arteriolar diameter to values close to those observed before ACE inhibition. In some experiments, minor adjustments (~20%) in the rate of infusion were necessary to maintain arteriolar diameter at the desired value. Ten minutes after the ANG II infusion was started, plasma from a conscious hypoxic rat was applied onto the normoxic cremaster. This was followed by a second plasma application 10 min later. The microcirculation was observed to document changes in arteriolar diameter and in leukocyte adherence to the venular endothelium as described above.

Series 2: Comparison of effects of mast cell stabilization on response to plasma from hypoxic rats and to topical ANG II. The effects of stabilization of mast cells on the response of the normoxic cremaster to plasma obtained from hypoxic rats and to topical application of ANG II were investigated in normoxic cremasters pretreated with cromolyn. As in series 1, separate experiments investigated the leukocyte-endothelial interactions in one group and the extravasation of albumin in the other.

EXPERIMENT 2A: TOPICAL APPLICATION OF PLASMA FROM HYPOXIC RATS. After a 10-min control period, cromolyn (0.11 mg/ml) was applied topically onto the cremaster. Ten minutes later, plasma from hypoxic rats was applied topically as described for series 1.

EXPERIMENT 2B: TOPICAL APPLICATION OF ANG II. After a 10-min control period, cromolyn was applied topically onto the cremaster. Ten minutes later 1 mM ANG II was applied onto the cremaster, followed by a second application of 10 mM ANG II 20 min later. The cremaster was observed for 20 min after the second ANG II application.

Series 3: Effect of blockade of RAS on leukocyte-endothelial interactions induced by mast cell stimulation. Cremaster mast cells were stimulated by topical application of compound 48/80 (C48/80, 15 μg/ml), and the microcirculation was observed in bright field for 30 min. In additional groups of experiments, the animals were pretreated with ANG II receptor blocker (ANG RB) or with topical ACE inhibitor before mast cell stimulation.

EXPERIMENT 2C: Effect of blockade of RAS on leukocyte-endothelial interactions of systemic hypoxia. The animals were prepared for bright-field intravital microscopy of the cremaster microcirculation as described above. After a control period breathing air, the animals were breathed 10% O2 spontaneously from a two-way nonbreathing valve connected to the tracheal catheter. Hypoxic gas breathing was maintained for 10 min, after which the animal returned to room air breathing. The following experiments were performed.

EXPERIMENT 2A: UNTREATED. No pharmacological agents were administered.

EXPERIMENT 2B: ANG II RECEPTOR BLOCKADE. The ANG II RB was infused continuously as described above, starting 10 min before initiation of hypoxia.

EXPERIMENT 2C: ACE INHIBITION. Teprotide was applied topically 10 min before the initiation of hypoxia.

Statistics

Each animal served as its own control; the effects of treatment were assessed by calculating the difference experimental – average control, where “experimental” represents the data obtained during the last 3 min of a given treatment period and “average control” is the mean of all the measurements during the 10-min control period. The differences were analyzed by a two-way repeated-measures ANOVA with time and treatment effect as independent factors. Significance was established by using t-tests with the Bonferroni correction for multiple comparisons. A P value <0.05 was considered to indicate a significant difference. All statistical analyses were performed with SPSS (version 14). Data in Figures 1–5 and Tables 1 and 2 are presented as means ± SE.
Table 1. Mean arterial blood pressure, plasma renin activity, and plasma angiotensin II concentration in conscious donor rats in normoxia and after hypoxia

<table>
<thead>
<tr>
<th>MABP, mmHg</th>
<th>PRA, ng ANG I *-ml⁻¹-h⁻¹</th>
<th>ANG II, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>Hypoxia</td>
<td>Normoxia</td>
</tr>
<tr>
<td>I: 105±3</td>
<td>94±2*</td>
<td>5.6±2.4</td>
</tr>
<tr>
<td>II: 101±2</td>
<td>91±1*</td>
<td>5.6±2.4</td>
</tr>
</tbody>
</table>

Data are means ± SE. MABP, mean arterial blood pressure; PRA, plasma renin activity; ANG II, plasma angiotensin II concentration; ANG I, angiotensin I. MABP values measured immediately before blood withdrawal; II, MABP values recorded immediately after the removal of ~3 ml of blood. *P < 0.05 vs. corresponding normoxia.

RESULTS

Effects of 5-min Hypoxia on Blood Pressure, PRA, and Plasma ANG II Concentration in Conscious Rats

In agreement with previous data (20), Table 1 shows that 5 min of alveolar hypoxia resulted in a modest reduction of MABP in conscious rats. No significant differences between normoxic and hypoxic animals were observed in either PRA or plasma ANG II concentration.

Effects of Plasma From Hypoxic Rats

Figure 1 shows the effects of application of plasma from hypoxic rats on normoxic untreated cremasters (n = 6) and on cremasters pretreated with ANG II RB (n = 6), ACE inhibition (n = 6), and ACE inhibition plus BK RB (n = 6).

As expected from previous studies (20), topical application of plasma from a hypoxic donor rat produced a rapid and significant increase in leukocyte-endothelial adherence (Fig. 1A), a decrease in venule shear rate (Fig. 1B), and arteriolar vasoconstriction (Fig. 1C) in the normoxic cremaster. In addition, experiments in additional groups of animals measuring FITC fluorescence intensity (each group consisted of 6 animals; Fig. 1D) resulted in the novel observation that hypoxic rat plasma also results in increased extravasation of albumin, suggesting elevation of vascular permeability in the normoxic cremaster. It is likely that the increase in blood viscosity resulting from the elevated vascular permeability, combined with the arteriolar vasoconstriction, led to the decrease in venule blood flow reflected in the reduction in venular shear rate that followed plasma application.

ANG II RB completely abolished the increase in leukocyte-endothelial interaction produced by hypoxic rat plasma (Fig. 1A). The lack of effect of topical ANG II at the end of the experiment indicates adequate receptor inhibition. ANG II RB elicited an increase in venule shear rate (Fig. 1B) and in arteriolar diameter (Fig. 1C) before the application of plasma; this probably reflects suppression of basal ANG II vasoconstrictor tone, with a resulting increase in arteriole and venule flow and increased venule shear rate. Blockade of ANG II receptors prevented the decrease in venule shear rate (Fig. 1B) and in arteriolar diameter (Fig. 1C) elicited by hypoxic rat plasma, suggesting that the vasoconstrictor response to plasma is mediated by ANG II. Figure 1D shows that ANG II RB also prevented the increased extravasation of albumin elicited by plasma from hypoxic rats.
ACE inhibition had essentially the same effects as ANG II RB on the response of leukocyte-endothelial adherence to plasma from hypoxic rats (Fig. 1A). The microhemodynamic effects of ACE inhibition, however, were different from those of ANG II RB. ACE inhibition also increased arteriolar diameter before application of plasma and prevented the arteriolar vasoconstriction that follows plasma application (Fig. 1C); however, venule shear rate did not increase after teprotide and decreased progressively after plasma application (Fig. 1B). A decrease in venule blood flow, indicated by the lower venular blood velocity, in the presence of arteriolar dilation is probably the result of fluid extravasation due to increased vascular permeability. Increased capillary pressure due to arteriolar vasodilation in the presence of constant perfusion pressure may have contributed to edema. This interpretation of the data is supported by the steady increase in albumin extravasation induced by ACE inhibition (Fig. 1D), which started after application of teprotide and before administration of plasma. The elevated vascular permeability, in turn, is most likely due to elevated levels of BK, which is a potent mediator of edema and increases after ACE inhibition (15). The results of the experiments in experiment 1d indicate that this is indeed the case: BK RB combined with ACE inhibition abolished the increase in albumin extravasation (Fig. 1D) and the decrease in venular shear rate produced by ACE inhibition. The results of the combined ACE inhibition plus BK RB also indicate that the effects of teprotide on leukocyte adherence and vasoconstriction produced by hypoxic rat plasma are not due to increased BK levels, but rather to reduced ANG II generation, since simultaneous administration of BK RB did not modify the effect of teprotide in these variables.

In summary, the data presented so far indicate that an intact RAS is necessary for the inflammatory response elicited in the normoxic cremaster by plasma obtained from hypoxic rats.

Effects of Plasma From Hypoxic Rats During ANG II Infusion After ACE Inhibition

Figure 2 shows that inhibition of ACE was followed by an increase in arteriolar diameter (Fig. 2, bottom) similar to that seen in the same conditions in the experiments shown in Fig. 1. Infusion of ANG II restored arteriolar diameter to control values and resulted in a moderate increase in MABP (Fig. 2, middle). Application of plasma from hypoxic rats under these conditions did not increase leukocyte-endothelial adherence or elicit vasoconstriction (Fig. 2, top). These results show that even under conditions in which ANG II in the cremaster microcirculation is maintained at levels that restore arteriolar diameter to control values, ACE inhibition still prevents leukocyte adherence and vasoconstriction after the application of plasma.

Effect of Cromolyn on Cremaster Responses to Plasma From Hypoxic Rats and to ANG II

Pretreatment of the normoxic cremaster with cromolyn completely abolished the increase in leukocyte-endothelial adherence (Fig. 3A), the vasoconstriction (Fig. 3B), the decrease in venular shear rate (Fig. 3C), and the increase in albumin extravasation (Fig. 3D) that follow the administration of plasma from hypoxic rats. These results support previous evidence indicating that the inflammation produced by plasma from hypoxic rats is secondary to activation of mast cells in the normoxic cremaster (20). ANG II resulted in small reductions in arteriolar diameter (Fig. 3F) that did not reach statistical significance. On the other hand, leukocyte-endothelial adherence (Fig. 3E) and albumin extravasation (Fig. 3H) increased with both ANG II concentrations, and these responses were not modified by pretreatment with cromolyn. The reduction in
venule shear rate produced by ANG II (Fig. 3G) most likely reflects reduction in venule blood flow and increased blood viscosity resulting from the fluid leak that follows the increase in venule permeability (Fig. 3H). The effect of ANG II on venule shear rate was not altered by pretreatment with cromolyn (Fig. 3H). The lack of effect of cromolyn indicates that the inflammation elicited by ANG II in the normoxic cremaster is not mediated by mast cell activation.
Effect of RAS Blockade on Leukocyte-Endothelial Adherence Produced by Mast Cell Activation

Figure 4 shows that mast cell activation with C48/80 increased leukocyte-endothelial adherence; this was attenuated by pretreatment with ANG II RB and with ACE inhibitor, both of which had similar effects. None of the treatments significantly modified venule shear rate. The attenuation by ANG II receptor blockade and ACE inhibition of C48/80-induced increased leukocyte-endothelial adherence suggests that the RAS participates in the inflammatory response initiated by mast cell activation.

Effect of RAS Blockade on Inflammatory Response to Alveolar Hypoxia

As previously demonstrated (8, 12, 20, 24), hypoxia induced by breathing 10% O2 is followed by a rapid increase in leukocyte-endothelial adherence and a decrease in venule shear rate in the cremaster microcirculation (Fig. 5). The latter is in large measure the result of the reduction in arterial blood pressure that follows systemic hypoxia in anesthetized rats (Table 2). In contrast, alveolar hypoxia in conscious rats induces only a modest reduction in MABP (Table 1; Refs. 19, 20). A transitory arteriolar vasoconstriction (20) may also contribute to the reduction in venular shear rate observed in the anesthetized rats breathing 10% O2 (Fig. 5). Pretreatment with ANG II RB or with ACE inhibitor prevented the increases in leukocyte-endothelial adherence produced by alveolar hypoxia, without modifying the decrease in venule shear rate. These results imply a participation of RAS activation in the systemic inflammation of alveolar hypoxia.

DISCUSSION

The central finding of this study is that blockade of the RAS attenuates the inflammation that follows the application of plasma obtained from hypoxic rat donors. These results point to an involvement of the RAS in this phenomenon and imply that the RAS plays a role in the initiation of the systemic inflammation produced by a reduction of inspired PO2.

Experimental Approach

The impetus for the study of a role for the RAS in this phenomenon originated from our previous studies suggesting that the inflammation induced by alveolar hypoxia is triggered by a mediator released from a distant site and carried by the circulation (8, 24). The effects of application of plasma from hypoxic rats on the normoxic cremaster suggested the presence of an agent that elicited vasoconstriction as well as inflammation (20); accordingly, exploring a possible role for the RAS, which mediates both cardiovascular inflammation and vasoconstriction, appeared to be a reasonable line of inquiry.

The RAS was blocked at two different sites: the conversion of ANG I to ANG II and the interaction between ANG II and its receptors. The ANG II RB used blocks both type 1 and type 2 ANG II receptors. While most effects of ANG II are mediated by type 1 receptor stimulation (4), there is evidence that activation of both receptor types is involved in the increased leukocyte-endothelial interactions promoted by exogenous ANG II (23). This, plus the fact that the aim of these studies was to make an initial exploration of the possible involvement of the RAS in the phenomena studied, led us to select an ANG II RB that would inhibit both receptor types. Teprotide was selected as an ACE inhibitor because it can be used topically in relatively high concentrations, thus restricting inhibition of ANG I to ANG II conversion to the cremaster microcirculation.

In general, an attempt was made to introduce treatments that would selectively modify the cremaster microcirculation without affecting the systemic circulation; thus ACE inhibition, mast cell stabilization and activation, and administration of ANG II were all done via topical application of pharmacological agents on the cremaster. However, this was not always

Fig. 4. Effect of blockade of the RAS on leukocyte-endothelial adherence (top) and venule shear rate (bottom) after mast cell stimulation with compound 48/80 (C48/80). The mast cell secretagogue C48/80 (15 μg/ml) was applied topically in all groups after a 10-min control period (vertical bar). ANG II RB infusion (30 μg-kg⁻¹-min⁻¹ iv throughout experiment; light gray bars) or teprotide (100 μg/ml topically; dark gray bars) was administered before the control. Data are means of 6 experiments/group. Error bars indicate SE. E/C, experimental/control.
possible: the ANG II RB selected has a short half-life, and continuous intravenous infusion is needed. Also, it was necessary to infuse ANG II intravenously in experiment 1 to attain physiological concentrations of ANG II within the cremaster microcirculation.

**Effects of RAS Blockade on Response to Plasma From Hypoxic Rats**

ANG II RB inhibited all of the inflammatory responses to plasma from hypoxic rats investigated in this study (Fig. 1). With the exception of a direct effect on vascular permeability and the subsequent decrease in venule shear rate (Fig. 1, B and D), the ACE inhibitor had effects similar to those of ANG II RB: it completely blocked the increase in leukocyte-endothelial adherence and the vasoconstriction elicited by plasma from hypoxic rats. The increase in albumin extravasation following ACE inhibition before plasma application is most likely the result of increased vascular permeability due to elevated BK levels, as evidenced by the observation that simultaneous ACE inhibition and BK receptor blockade eliminated the increased albumin extravasation. Addition of BK RB to ACE inhibition did not modify the effects of teprotide on the leukocyte-endothelial adherence and the vasoconstriction elicited by hypoxic rat plasma. This indicates that attenuation of these responses by ACE inhibition was due to reduction of ANG II rather than elevation of BK levels in the microcirculation.

The data in Fig. 1 indicate that an intact RAS system is necessary for the inflammatory response to plasma from hypoxic rats to occur and suggests that ANG II is the mediator of the inflammation. An alternative explanation would be that parallel mechanisms intervene in this response and that, rather than being the specific mediator, ANG II acts as a permissive factor. If this were the case, conditions in which ANG II levels are reduced, or its receptors blocked, would also attenuate the effect of plasma from hypoxic rats since the specific agonist would be ineffective in the absence of the permissive factor. This possibility was tested in the experiments summarized in Fig. 2, which shows that ACE inhibition blocked the plasma-induced inflammation even during infusion of ANG II. Since ACE inhibition was limited to the cremaster microcirculation, it is likely that the circulating levels of ANG II in the systemic circulation were elevated by exogenous ANG II administration, as suggested by the modest elevation of MABP that accompanied the infusion (Fig. 2). On the other hand, arteriolar diameter in the cremaster increased during ACE administration and returned to pre-teprotide values during the infusion of ANG II, suggesting that the rate of infusion selected was adequate to maintain physiological levels of ANG II within the cremaster microcirculation. Certainly, it is unlikely that ANG II levels in the cremaster microcirculation were reduced during the infusion. The fact that, under these conditions, plasma from hypoxic rats failed to elicit an inflammatory response supports the idea that ANG II is the specific mediator of this response, rather than a permissive factor.

**Interaction Between Mast Cells and RAS**

The inhibition by cromolyn of the inflammatory response to plasma from hypoxic rats (Fig. 3) is consistent with previous observations that demonstrate a key role of mast cell activation in the early stages of the inflammation of alveolar hypoxia (8, 20, 27) and suggests that mast cell activation is necessary for the inflammation originated by hypoxic rat plasma. Mast cells degranulate early after the onset of alveolar hypoxia in both the
mesentery (27) and the cremaster microcirculation (8, 20). Cromolyn prevents mast cell degranulation and attenuates the inflammation that follows alveolar hypoxia (8, 20, 27). Activation of mast cells of normoxic rats with C48/80 increases inflammatory markers in a manner analogous to that of alveolar hypoxia (8, 27). However, if the reduction of PO2 is restricted to the cremaster microcirculation, no inflammation is evident and mast cells do not degranulate; conversely, alveolar hypoxia produces mast cell degranulation and inflammation even if cremaster microvascular PO2 is maintained elevated (8, 24). These observations suggest that alveolar hypoxia is needed to induce mast cell activation and the subsequent inflammation, and that mast cell activation is independent of the local PO2. Together with these facts, the findings that plasma obtained from hypoxic rats induces inflammation (Fig. 1) and mast cell activation in the normoxic cremaster (20) support the notion that the inflammation is triggered by a mediator released from a distant site, such as the lungs, and transported by the circulation. Mast cell stabilization inhibits the effects of hypoxic rat plasma on the normoxic cremaster (Fig. 2), suggesting that the putative mediator initiates the inflammatory cascade by stimulating mast cells.

To this picture, the present study adds the observation that blockade of the RAS prevents the inflammation produced by plasma from hypoxic rats. It is unlikely that the inflammatory response is the result of elevated levels of renin or ANG II in the donor plasma since PRA and plasma ANG II concentrations of rats exposed to hypoxia for 5 min were not different from the values observed in normoxic controls (Table 1). Additional evidence against an effect of elevated RAS components in the donor plasma is the observation that cromolyn blocks the inflammatory response to plasma but does not have an effect on the inflammation elicited by topical ANG II. These observations, together with the fact that both ACE inhibition and ANG II RB block the inflammation produced by hypoxic rat plasma, suggest that ANG II is generated in the cremaster microcirculation and that mast cell activation is necessary for ANG II formation.

The mechanism by which mast cells interact with the RAS to elicit microvascular inflammation after application of plasma is far from clear. Components of the RAS, including mRNA and protein for renin and angiotensinogen, have been demonstrated in the microvessel wall of the rat cremaster (1). Also, vessel wall ANG II concentration >10-fold higher than circulating ANG II levels (1) suggests local formation of ANG II. Thus the skeletal muscle appears to contain all the elements necessary for the local generation of ANG II. Mast cells from some species, including humans, contain chymase, which acts as a converting enzyme. However, most of the available evidence indicates that rodent mast cells have relatively low levels of chymase compared with humans, dogs, and rabbits (3, 18). The essentially identical magnitude of the responses to teprotide and ANG II RB suggests that ANG I-to-ANG II conversion is mediated exclusively by ACE, and seems to rule out the participation of other converting enzymes such as mast cell chymase. Renin has been recently demonstrated in human, mice, and guinea pig myocardial mast cells (17, 25). The presence of preformed ANG II in cardiac mast cells has also been reported (13). At the moment we are not aware of a similar finding in skeletal muscle mast cells. Whether the stimulation of the RAS observed in this study is the result of release of mast cell renin or of another RAS component should be the subject of further study. While the present data support the notion that plasma from hypoxic rats leads to activation of the local RAS, and there is evidence of elements of the RAS in the cremaster microcirculation (1), some interaction with circulating components of the RAS could also occur.

If the general scenario described above is correct, it would be expected that RAS blockade would attenuate the inflammatory effects of a mast cell secretagogue. The results shown in Fig. 4 indicate that this is the case and support the idea of mast cell-mediated RAS stimulation by the donor plasma: both ANG II RB and ACE inhibition prevented the increase in leukocyte-endothelial adherence that follows mast cell stimulation with C48/80 (Fig. 4). In contrast with secretagogues that activate mast cells via surface receptor activation by IgE-bound antigens (16), C48/80 is a basic mast cell secretagogue that produces exocytosis by direct, receptor-bypassing activation of G proteins (11). Since multiple signaling pathways are activated by C48/80 (21), it is possible that the effects of mast cell stimulation with C48/80 may not be identical to those produced by the substance(s) contained in plasma from hypoxic rats, which may stimulate mast cells by activation of specific receptors. Nevertheless, these results clearly indicate that one of the effects of mast cell activation is stimulation of the local RAS, with the resulting increase in leukocyte-endothelial adherence. These results are consistent with previous observations that stimulation of cardiac mast cells with C48/80 produced release of renin and activation of the local RAS (17).

Role of RAS in Inflammatory Response to Alveolar Hypoxia

If activation of the RAS is involved in the initiation of the inflammation induced by alveolar hypoxia, it would be expected that RAS blockade would attenuate the inflammatory response elicited by reduction of inspired PO2. This appears to be the case: both ANG II RB and ACE inhibition attenuated one of the earliest indexes of inflammation, the increase in leukocyte-endothelial adherence that follows reduction of inspired PO2 (Fig. 5). Alveolar hypoxia in an intact animal is a complex stimulus, and it is possible that several factors may participate in the response of the cremaster microcirculation under these circumstances. Nevertheless, the results of these experiments clearly point to an involvement of the RAS in the microvascular inflammation of alveolar hypoxia.

In summary, the results of the present experiments, together with our previous observations discussed here, suggest that stimulation by alveolar hypoxia of cells located in a distant site results in the release of an intermediary substance that is transported via the circulation. The site of origin of the intermediary is unknown. The inflammation occurs rapidly and is widespread, and the mediator appears to be transported by the circulation. These features suggest an organ in which the reduction to inspired PO2 is rapidly sensed and which has a high rate of blood flow, such as the lungs. The putative intermediary activates the mast cells, which, in turn, lead to stimulation of the local RAS and generation of ANG II that initiates the inflammatory cascade characterized by increased leukocyte-endothelial interactions, vasoconstriction, and elevated vascular permeability. This suggests that the mediator is a mast cell secretagogue; the nature of the response also suggests a substance that is either preformed or rapidly syn-
thesized. Although several aspects of this process need further study, the widespread nature of the inflammation (12), the dissociation of inflammation from local PO2 values (8, 24), the effects of plasma obtained from hypoxic rats (20), and the role of mast cell activation in the inflammatory response (8, 20, 27), together with the results shown in the present study, are all consistent with this general scenario.

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