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# Renin released from mast cells activated by circulating MCP-1 initiates the microvascular phase of the systemic inflammation of alveolar hypoxia

Jie Chao,<sup>1</sup> Gustavo Blanco,<sup>1</sup> John G. Wood,<sup>1,2</sup> and Norberto C. Gonzalez<sup>1</sup>

Departments of <sup>1</sup>Molecular and Integrative Physiology and <sup>2</sup>Surgery, University of Kansas Medical Center, Kansas City, Kansas

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Chao J, Blanco G, Wood JG, Gonzalez NC. Renin released from mast cells activated by circulating MCP-1 initiates the microvascular phase of the systemic inflammation of alveolar hypoxia. Am J Physiol Heart Circ Physiol 301: H2264-H2270, 2011. First published September 30, 2011; doi:10.1152/ajpheart.00461.2011.-Reduced alveolar Po2 in rats produces a rapid systemic inflammation characterized by reactive O<sub>2</sub> species generation, mast cell (MC) degranulation, leukocyte-endothelial interactions, and increased vascular permeability. The inflammation is not initiated by the low systemic Po2 but rather by the release of monocyte chemoattractant protein-1 (MCP-1) from alveolar macrophages (AMO) activated by alveolar hypoxia. Circulating AMO-borne MCP-1 induces MC degranulation, which activates the local renin-angiotensin system (RAS) and mediates the microvascular inflammation. This study was directed to determine the mechanism of RAS activation by MCP-1-induced MC degranulation. Experiments in isolated rat peritoneal MCs showed the following: 1) Western blots and immunocytochemistry demonstrated the presence of renin and angiotensin-converting enzyme (ACE) in MCs and their release upon degranulation; 2) MCP-1-induced degranulation of MCs incubated in plasma produced an increase in angiotensin II (ANG II) concentration; and 3) this increase was inhibited completely by the following agents: the MCP-1 receptor antagonist RS-102895, the specific rat renin inhibitor WFML, or the ACE inhibitor captopril administered separately. Captopril also inhibited ANG II generation by MCs incubated in culture medium plus ANG I. The results show that peritoneal MCs contain active renin, which activates the RAS upon degranulation, and that peritoneal MCs are a source of ACE and suggest that conversion of ANG I to ANG II is mediated predominantly by ACE. This study provides novel evidence of the presence of active renin in rat peritoneal MCs and helps explain the mechanism of activation of the RAS during alveolar hypoxia.

monocyte chemoattractant protein-1; angiotensin-converting enzyme

REDUCTION OF INSPIRED Po<sub>2</sub> in rats produces a rapid and widespread systemic inflammation characterized by microvascular generation of reactive O<sub>2</sub> species (30), mast cell (MC) degranulation (22), increased leukocyte-endothelial interactions (31), and extravasation of albumin (32). This response, which has been observed directly in the mesentery (4, 5, 22, 30–32) and skeletal muscle (6, 10, 21) and pial microcirculations (13), is not initiated by the low tissue Po<sub>2</sub> but rather by the rapid release into the circulation of a chemokine, monocyte chemoattractant protein-1 (MCP-1), also known as CCL2, from alveolar macrophages (AMO) activated by the low alveolar Po<sub>2</sub> (4). Circulating MCP-1 induces degranulation of perivascular MCs (4), which, in turn, leads to activation of the reninangiotensin system (RAS) and microvascular inflammation (10).

The possibility that the systemic inflammation is initiated by a mediator released from a distant site and transported by blood had been suggested by two critical findings: the first is a dissociation observed between tissue microvascular Po2 and inflammation: MC degranulation and leukocyte recruitment occur only when alveolar Po<sub>2</sub> is reduced, independent of the systemic  $Po_2$  (6, 22). The second finding is that plasma from hypoxic rats evokes an inflammatory response in tissues of normoxic rats (16). The evidence supporting an inflammatory cascade initiated by AMO activation by low alveolar Po<sub>2</sub> is the following: 1) Reduction of Po<sub>2</sub> in primary cultures of AMO results in rapid (<15 min) release of  $H_2O_2$  and MCP-1. In contrast, neither primary cultures of peritoneal macrophages or peritoneal MCs are activated by exposure to low Po<sub>2</sub> for 30 min (5). The lack of response of systemic tissue macrophages and MCs is in agreement with the in vivo observation that selective reduction of tissue Po2 does not elicit inflammation if alveolar Po<sub>2</sub> is normal (21). 2) The supernatant of hypoxic AMO, but not of hypoxic systemic tissue macrophages, induces MC degranulation in vivo and in vitro. (4, 5). 3) Plasma MCP-1 concentration increases rapidly in rats breathing 10% O<sub>2</sub>; this increase does not occur in AMO-depleted rats (4). This is consistent with previous observations that AMO depletion prevents the systemic inflammation of alveolar hypoxia and that plasma from intact hypoxic rats, but not of AMO-depleted rats, elicits inflammation in normoxic tissues (5, 9). 4) MCs undergo degranulation when immersed in plasma from intact hypoxic rats but not in plasma from AMO-depleted hypoxic rats (5). 5) MCP-1 induces concentration-dependent MC degranulation (4). 6) MCP-1 administration to normoxic rats replicates the inflammation of hypoxia; conversely, MCP-1 receptor antagonist blocks the systemic inflammation of hypoxia in intact rats (4).

Degranulation of MCs by circulating AMO-borne MCP-1 is followed by activation of the local RAS. The evidence that MC degranulation causes activation of the local RAS and generation of ANG II is convincing. First, alveolar hypoxia does not induce an increase in circulating plasma renin activity or ANG II concentration in conscious intact rats (10), suggesting that activation of the RAS in this case occurs at the local level, rather than as a result of renin release by the renal juxtaglomerular cells. Second, it is clear that RAS activation does mediate the microvascular response to alveolar hypoxia: blockade of the RAS with ANG II receptor antagonists or with angiotensin-converting enzyme (ACE) inhibitors prevents the systemic inflammation of alveolar hypoxia, as well as that elicited by related stimuli like topical application of plasma from hypoxic rats, supernatant of hypoxic AMO, and MCP-1

Address for reprint requests and other correspondence: N. C. Gonzalez, Dept. of Molecular and Integrative Physiology, Mail Stop 3043, Univ. of Kansas Medical Center, 3901 Rainbow Blvd, Kansas City, KS 66160 (e-mail: ngonzale@kumc.edu).

(4, 5, 10); however, RAS blockade does not prevent the MC degranulation initiated by these stimuli. Third, the inflammatory response to ANG II does not involve MC degranulation (5, 17, 26); and finally, the inflammation elicited by MC degranulation with compound 4880 (C4880), a MC secretagogue, is prevented by blockade of the RAS (5, 10).

While the mechanisms underlying the inflammatory cascade initiated by activation of AMO are understood, a question remaining is the mechanism by which MC degranulation leads to activation of the local RAS. One possibility is the presence of renin in MCs, which would be released after MC degranulation to activate the local RAS. Renin was demonstrated in myocardial MCs (14, 20) and more recently in MCs of the lungs (1, 25), although its pathophysiological role at this site is debated (11). The role of activation of the RAS in the systemic inflammation of alveolar hypoxia was demonstrated in the mesentery and skeletal muscle microcirculations (4, 10); however, it is not known if MCs in those sites contain renin. A second unanswered question relates to the transformation of ANG I to ANG II. In addition to endothelial and circulating ACE, ANG II may be generated from ANG I by chymase, which is contained in MCs (19).

The objective of these experiments was to answer these remaining questions. Specifically, we inquired whether renin is contained in peritoneal MCs and whether release of renin after MC degranulation could explain the activation of the RAS during alveolar hypoxia. Peritoneal MCs were selected because their role in the systemic inflammation of alveolar hypoxia has been studied extensively in vivo and in vitro (4, 5, 22). The results show that active renin is contained in peritoneal MCs and that its release after degranulation of MCs with MCP-1 leads to the generation of ANG II. The data further show that MC are a source of ACE and suggest that ANG II is formed largely, if not uniquely, by conversion of ANG I by ACE.

#### METHODS

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, approved all procedures.

### Isolation of Peritoneal MCs

Sprague-Dawley rats (250-300 g) were anesthetized with 45 mg/kg ketamine and 0.4 mg/kg atropine im, and a catheter was placed in the jugular vein. Twenty milliliters of DMEM with 10% serum containing penicillin (100 U/ml), streptomycin (100 µg /ml), and heparin (5 U/ml) were injected into the peritoneal cavity, followed by gentle massage and recovery of fluid into cooled polypropylene tubes. Usually, cells obtained from two rats were pooled and used for each experiment. Before the lavage was started, 3 ml of blood were obtained from each rat and plasma was separated by centrifugation and used for MC incubation. After the lavage was completed, the rats were euthanized with an overdose of sodium pentobarbital (150 mg/kg iv). The cells were centrifuged at 400 g for 15 min at room temperature and washed twice with serum-free DMEM. Cell pellets were resuspended in 0.75 ml serum-free DMEM, and macrophages were separated from MCs by differential centrifugation using a Percoll solution as described before (18). MCs isolated by this procedure exceed 95% in purity (4, 5, 18) as determined by light microscopy. Approximately  $1 \times 10^6$  MCs are usually harvested from one rat. The separated MCs were resuspended in 2 ml of DMEM with 10% serum containing penicillin (100 U/ml) and streptomycin (100

 $\mu$ g/ml) 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 min. The MCs were collected by centrifugation at 3,000 rpm for 2 min. The supernatant was discarded with a pipette, and the MCs were resuspended in 2 ml of serum-free DMEM. MCs did not adhere to the culture plate; rather, they were suspended in plasma, and the cell suspension was equilibrated with humidified gas mixtures (10% O<sub>2</sub>-5% CO<sub>2</sub>-85% N<sub>2</sub>) via an 18-gauge needle placed on the flask's cap. Forty five minutes later, the suspension was divided into aliquots of 0.4 ml containing 0.4 × 10<sup>6</sup> MCs each. The different pharmacological agents described in *Experimental Protocol* were administered at this step. The cells were then incubated for an additional 20 min after which the aliquots were centrifuged at 3,000 rpm for 2 min. The supernatants were collected for ANG II concentration measurement

#### ELISA Analysis for ANG II Measurement

A single-analyte ELISA was performed using the sandwich-based ELISA technique to measure ANG II concentration in plasma or supernatant samples. A 50- $\mu$ l aliquot of each sample was added to the well for measurement according to the instructions of the ANG II ELISA kit (Cayman Chemical). Each sample was tested in triplicate.

#### Immunocytochemistry

MCs were suspended in DMEM and plated on coverslips coated with poly-D-lysine at a cell density of 10<sup>4</sup> cells/ml. After 30 min, the coverslips were rinsed twice with PBS. The cells were then fixed with paraformaldehide, 4% in PBS, and were permeabilized for 30 min at room temperature with a solution containing 0.3% Triton X-100 dissolved in PBS. After three washes with 0.3% Triton X-100 in PBS, the cells were blocked with 10% normal goat serum, 0.3% Triton X-100 in PBS at room temperature for 2 h. After three washes with 0.3% Triton X-100 in PBS, the cells were blocked with 10% normal goat serum, 0.3%Triton X-100 in PBS at room temperature for 2 h. Next, the permeabilized cells were exposed to goat anti-renin (sc-27318; 1:100; Santa Cruz) and rabbit anti-ACE (sc-20791; 1:100; Santa Cruz) antibodies in 10% normal goat serum, 0.3% Triton X-100 in PBS at 4°C overnight. Both these antibodies recognize the rat forms of each polypeptide. Following three washes with PBS, the MCs were exposed to secondary fluorescence antibody (donkey anti-goat Alexa 594-conjugated antibody and donkey-anti-rabbit Alexa 647-conjugated antibody; 1:500; Invitrogen) for 2 h. As a negative control, MCs were processed as described above but without the primary antibody. After being washed with PBS three times, the coverslips were mounted with Prolong Gold containing DAPI to stain nuclei from Molecular Probe (Eugene, OR) at room temperature. The slides were examined with a Leica TSC STED confocal microscope.

#### Western Blotting Analysis

Each suspension of MCs was divided into two aliquots each; one of them was treated with the MC secretagogue C4880 (10 mg/l) for 20 min, and the other remained untreated. The cell suspensions were centrifuged at 3,000 rpm for 5 min at room temperature; the supernatants were collected and the cell pellets were lysed using the mammalian cell lysis kit (Sigma, St. Louis, MO). Supernatants and lysates containing equal amounts of proteins were electrophoresed in a SDS-PAGE under reducing conditions and were transferred to PVDF membranes. The supernatant from untreated MCs contained negligible amounts of proteins; in this case, a volume equal to that of the supernatant from C4880-treated MCs was loaded for comparison. The blots were blocked with 5% nonfat dry milk in PBS and were probed with antibodies recognizing renin (sc-27318; 1:500; Santa Cruz) or ACE (sc-20791; 1:500; Santa Cruz) of rat origin. The secondary antibodies were alkaline phosphatase conjugated to donkey anti-goat or rabbit IgG (1:5,000). Signals were detected by chemiluminescence (Pierce Thermo). After signal measurement, Western blot

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membranes were striped with stripping buffer (Thermo) to remove primary and secondary renin antibodies and were reprobed with ACE; the process was repeated and the membranes were reprobed with GAPDH antibodies using the same protocol. Western blots from three individual MC cultures were prepared using this protocol.

#### **Experimental Protocol**

The mechanism by which MC degranulation leads to activation of the RAS was studied by determining the effect of different pharmacological agents on the generation of ANG II by MCs. MCP-1 was used since this is the agent that activates MCs during alveolar hypoxia. With the exception of the last series of experiments (see below), MCs were suspended in plasma since it provides all the substrates necessary to generate ANG II. ANG II generation, determined by the difference between untreated and MCP-1-treated plasma ANG II concentration, was used as a marker for activation of the RAS by MC degranulation. Aliquots of 400 µl, containing an average of  $0.4 \times 10^6$  cells each, were used in the experiments described below. In all cases, MCs were incubated for 20 min in the presence of the specific agent after which plasma was removed and frozen for measurement of ANG II with ELISA. The following experiments were carried out.

Effect of MCP-1 on ANG II generation by MCs. MC aliquots were incubated in plasma for 20 min with the following concentrations of MCP-1 = 0, 15, and 30 ng/ml. A fourth aliquot was incubated with MCP-1 (30 ng/ml) plus the CCR2 receptor blocker RS-102895 (10  $\mu$ M).

*Effect of renin inhibition on ANG II generation by MCs.* In a separate series of experiments using the same protocol, aliquots of MCs were incubated in plasma with MCP-1 (0, 15, and 30 ng/ml) in

the presence and in absence of the rat renin inhibitor acetyl-His-Pro-Phe-Val-Statine-Leu-Phe (WFML; ANASPEC, San Jose, CA; 3  $\mu$ M).

Effect of ACE inhibition on the generation of ANG II by MCs. Aliquots of MCs were incubated in plasma with 0 and 30 ng/ml MCP-1 in the presence and in the absence of the ACE inhibitor captopril (1 mM).

Effect of ACE inhibition on ANG II generation by MCs suspended in medium containing ANG I. In these experiments, MCs were suspended in serum-free culture medium containing ANG I (100 nM). In this case, MC degranulation was elicited with the generic MC secretagogue C4880 (10 mg/l), rather than MCP-1

#### **Statistics**

Data are presented as means  $\pm$  SD. In each set of experiments, the data of three separate MC suspensions subjected to each of the different treatments were averaged. Comparisons between treated and control groups were carried out with a one-way ANOVA followed by Bonferroni tests for multiple comparisons. A *P* value <0.05 was considered to indicate a significant difference.

#### RESULTS

Figure 1 shows a series of confocal immunocytochemistry images demonstrating the presence of renin and ACE in peritoneal MCs, and their release upon degranulation induced by the general MC secretagogue C4880. The presence of renin and ACE in MCs is confirmed by the immunoblots shown in Fig. 2. Both renin and ACE protein are detected in the MC lysates but not in the supernatant of the untreated MC suspen-



Fig. 1. Representative immunofluorescence images showing renin and angiotensin-converting enzyme (ACE) in peritoneal mast cells (MCs). DAPI staining (blue fluorescence) identifies DNA that was used as a nucleus marker. Bright field (BF) pictures show MC morphology, both before and after degranulation with C4880. A: images in the absence of primary antibodies (negative controls). B: images showing presence of renin (red fluorescence) and ACE (pink fluorescence) in intact, isolated peritoneal MCs. C: images showing renin and ACE in the granules released from MCs after degranulation with MC secretagogue C4880.



Fig. 2. *A*: Western blots of MC lysates and supernatant. Blots from 2 individual MC suspensions show the presence of renin and ACE protein in untreated MC lysates and in MC lysates and supernatant samples obtained after degranulation of MC with C4880. Neither renin nor ACE proteins are detected in the supernatant from MC cultures not treated with C4880 in which MCs did not degranulate. Minute amounts of GAPDH protein in the supernatant of unreated MCs is likely due to the small amount MCs ( $\sim$ 5–10% of the total number) that undergo degranulation during the isolation procedure. *B*: average densitometry ratio with respect to GAPDH. Given absence of renin and ACE in the untreated supernatant, densitometry ratios could not be calculated. Each bar represents the means ± SE of data obtained in 3 separate MC suspensions.

sions. After degranulation of MCs induced by C4880, both proteins are detected in the lysate as well as the supernatant. The demonstration of renin and ACE in the supernatant in this case shows that renin and ACE contained in the MCs are released into the supernatant after degranulation with C4880. The barely detectable signal corresponding to GAPDH protein in the supernatant of the untreated MCs is likely due to the small number of MCs ( $\sim$ 5–10% of the total MC number) that undergo degranulation during the separation procedures (4, 5).

Incubation with MCP-1 produced a concentration-dependent increase in plasma ANG II concentration. This increase was not observed when MCs were incubated with the MCP-1 receptor antagonist RS 102895, 10  $\mu$ M (Fig. 3). The depen-



Fig. 4. Effect of the specific rat renin inhibitor WFML on the generation of ANG II mediated by MCP-1. Data are means  $\pm$  SD. Each bar represents the average of 3 separate experiments. \*P < 0.05 vs. corresponding untreated group.

dence of ANG II generation by MCs on the MCP-1 concentration parallels the relationship between MCP-1 concentration and MC degranulation observed before (4). The inhibitory effect of the MCP-1 receptor antagonist indicates that the generation of ANG II is a specific effect of MC activation by MCP-1.

Figure 4 depicts results of a separate series of experiments showing that the increase in plasma ANG II that follows MC activation with MCP-1 was inhibited by pretreatment with the specific rat renin inhibitor WFML (Fig. 4). These results show that MC renin, released from MC upon degranulation with MCP-1, initiates the reaction that culminates in the generation of ANG II.

Captopril, an ACE inhibitor, blocks the generation of ANG II mediated by MCP-1 (Fig. 5). The concentration of ANG II in the captopril-treated cultures was not significantly different from that seen in the untreated cultures in which MCP-1 was not added. This suggests that the generation of ANG II from ANG I was mediated largely, if not exclusively, by ACE.

The experiments depicted in Fig. 6 were carried out to determine if MC degranulation would lead to conversion of ANG I into ANG II in a system in which ACE is not present in the supernatant. C4880 was used instead of MCP-1 because a cofactor, normally expressed in plasma, is necessary for MCP-1 to induce MC degranulation at biologically relevant concentrations (4). When MCs were incubated in culture medium in the presence of 100 nM ANG I, the levels of ANG II



800 - Untreated 600 - Captopril 400 - 200 - 0 MCP-1: 0 30 ng/ml

Fig. 3. Effect of monocyte chemoattractant protein-1 (MCP-1) on ANG II generation by MC suspended in plasma. RS 102895 is an MCP-1 receptor antagonist. Data are means  $\pm$  SD. Each bar represents the average of 3 separate experiments . \**P* < 0.05 vs. MCP-1 = 0. §*P* < 0.05 vs. MCP-1 = 15 ng/ml.

Fig. 5. Effect of captopril on the generation of ANG II induced by 30 ng/ml MCP-1 Data are means  $\pm$  SD. Each bar represents the average of 3 separate experiments. \**P* < 0.05 vs. corresponding captopril-treated culture.



Fig. 6. Effect of captopril on the generation of ANG II by MCs suspended in culture medium in the presence of ANG I. MCs were suspended in serum-free DMEM culture medium containing 100 nM ANG I. Effect of C4880 in cultures of MCs incubated in plasma is represented for comparison in the last bar. Data are means  $\pm$  SD. Each bar represents the average of 3 separate experiments. \**P* < 0.05 vs. corresponding captopril.

were negligible. Incubation with C4880 resulted in an increase in ANG II concentration to levels comparable to those seen in the cultures in which 30 ng/ml of MCP-1 were used. The increase in ANG II produced by C4880 was blocked by captopril, indicating that the conversion of ANG I to ANG II was mediated by ACE. The increase in ANG II induced by C4880 in MCs immersed in medium containing 100 nM ANG I was similar to that induced by the same concentration of C4880 in MCs incubated in plasma. This indicates that incubation of MCs in medium plus 100 nM ANG I provided conditions similar to those of plasma for the generation of ANG II.

# DISCUSSION

The results of these experiments show that renin is contained in peritoneal MCs and that degranulation of MCs with MCP-1 leads to the release of renin and the resulting generation of ANG II. The data also show that peritoneal MCs are a source of ACE and suggest that the conversion of ANG I to ANG II is largely mediated by ACE. The present results provide an answer to an outstanding question, namely the mechanism by which MC degranulation activates the RAS during the systemic inflammation elicited by alveolar hypoxia (5, 10).

Since the discovery of renin in cardiac MCs, evidence has been accumulating regarding the consequences of activation of the myocardial RAS by MC renin in response to phenomena such as ischemia/reperfusion (14, 20) The results presented here help explain the mechanisms of activation of the RAS initiated by a different stimulus, namely MCP-1 released by AMO activated by alveolar hypoxia. The presence of active renin in peritoneal MCs expands the previous findings of renin in cardiac and pulmonary MCs and suggests a widespread role for MC renin in the activation of the local RAS. While the present experiments were carried out in peritoneal MCs, the demonstration of similar in vivo interactions between MCs and the RAS in mesentery and skeletal muscle demonstrate a generalized role of the RAS in the systemic inflammation of hypoxia and suggest a possible role for MC renin in various organ systems (5, 10)

Renin in peritoneal MCs was identified by immunohistochemistry coupled with demonstration of renin protein using

Western blot (Fig. 1). These findings were complemented by experiments that determined the functional role of MC renin in the activation of the RAS during alveolar hypoxia, using a relatively simple experimental system consisting of peritoneal MCs immersed in fresh plasma. Peritoneal MCs were used because activation of the RAS by MCs has already been demonstrated in the mesentery (5), as well as in skeletal muscle (10), and the role of peritoneal MCs in the systemic inflammation of alveolar hypoxia has been characterized extensively in vivo and in vitro in our previous work (4, 5, 22). In addition, peritoneal MCs can be harvested with minimal manipulation so their responses are least affected by the isolation procedures. MCP-1 was selected to induce MC degranulation because this is the AMO-borne agent that activates MCs in vivo to initiate the systemic inflammation of alveolar hypoxia (4). The highest concentration of MCP-1 used in these experiments, 30 ng/ml, is close to the plasma concentration detected in conscious rats breathing 10% O<sub>2</sub> (4). Accordingly, the conditions of these in vitro experiments approximate those prevailing in vivo during alveolar hypoxia. The cells were immersed in fresh plasma to provide all the necessary substrates for the generation of ANG II. Generation of ANG II was assessed by the change in plasma ANG II concentration as measured by ELISA, a method that provides a reliable estimate of the concentration of ANG II in biological fluids. Special care was taken to use the same number of MCs in the different experiments; under these conditions, differences in concentration of ANG II reflect differences in the quantities of renin released by MC degranulation.

MCP-1 produced a concentration-dependent increase in the generation of ANG II (Fig. 3). Incubation of MCs with plasma MCP-1 concentrations of 15 and 30 ng/ml produce degranulation of  $\sim$ 40 and 80% of immersed MCs, respectively (4). The increase in plasma ANG II shown in Figs. 3 and 4 in response to MCP-1 roughly parallels the concentration dependence of MC degranulation induced by MCP-1 (4). Thus, as greater numbers of MCs undergo degranulation, more renin is released into plasma with the resulting increase in the generation of ANG II. The effect of WFML, a specific rat renin inhibitor (15), provides independent evidence that the generation of ANG II after MC degranulation is mediated by active MC renin.

Captopril blocked the increase in plasma ANG II concentration produced by MCP-1, showing a participation of ACE in the conversion of ANG I to ANG II. The fact that plasma ANG II concentration in the MC suspensions treated with captopril was not different from that seen in the untreated controls (Fig. 5) suggests that the generation of ANG II was largely, if not exclusively, mediated by ACE. The present findings are consistent with earlier data in skeletal muscle: in this case, equally effective inhibition of the MC-induced activation of the RAS was obtained using either [Sar<sup>1</sup>,Thr<sup>8</sup>] ANG II, a combined ANG II type 1 and 2 receptor antagonist, or teprotide, an ACE inhibitor (9). Mounting evidence indicates that in addition to ACE, MC chymase plays an important role in the conversion of ANG I to ANG II in humans (3, 7, 23). However, it appears that the relative contribution of ACE and chymase in the generation of ANG II is highly species dependent and that the role of MC chymase in rodents is much lower or nonexistent, in contrast with other species like humans, rabbits, and dogs, (2, 12). Rat connective tissue MCs, such as peritoneal MCs,

express RMCP-5, an  $\alpha$ -chymase that does not convert ANG I, and RMCP-1, a  $\beta$ -chymase that generates ANG II with a rather slow kinetics and that degrades ANG II (19, 28). Thus the general consensus is that ANG II generation via MC chymase in the rat is minimal (8). It is possible, however, that under some conditions, chymase-dependent ANG II generation in rodent MC may become more significant. For instance, evidence of chymase activity was obtained in mice myocardial MCs (27). In this case, however, it would appear that expression of myocardial MC chymase is secondary to the elevated bradykinin levels that accompany prolonged ACE inhibition. While an increased chymase expression in peritoneal MCs during prolonged ACE inhibition cannot be ruled out, the present data indicate that the generation of ANG II induced by MCP-1 was largely, if not exclusively, mediated by ACE.

The experiments depicted in Fig. 6 show that MC degranulation results in ANG II generation in a system in which ACE is not present in the supernatant. In these experiments, compound C4880 was used to induce MC degranulation, because MCP-1 is ineffective in activating MCs immersed in culture medium or other artificial solutions. In previous studies, we demonstrated that MC degranulation with physiologically relevant MCP-1 concentrations like those used in this study requires the presence of an as yet unidentified cofactor present in plasma (4). The available evidence indicates that this cofactor is constitutively expressed in normoxic alveolar macrophages and is secreted into plasma (4). Since investigating MCs as a source of ACE required incubation in an ACE-free medium, C4880 was used to induce MC degranulation. The concentration of C4880 used here results in MC degranulation of an extent similar to that produced by 30 ng/ml of MCP-1 (4). This is consistent with the observation that both agents resulted in similar increases in ANG II concentration (compare Fig. 6 with Figs. 3-4). It is also evident that the concentration of ANG I present in the medium was sufficient to support the generation of ANG II elicited by MC degranulation of this magnitude. The increase in ANG II produced by C4880 was blocked by captopril, indicating that the conversion of ANG I to ANG II was mediated by ACE. Since no ACE was contained in the medium, it is reasonable to conclude that the generation of ANG II was the result of conversion of ANG I by MC ACE released into the medium upon MC degranulation.

The results support the idea that the generation of ANG II after MC degranulation by MCP-1 is largely, if not uniquely, mediated by ACE and, furthermore, that MC ACE contributes to this phenomenon. While the results of Fig. 6 clearly show that ACE released upon MC degranulation can effectively generate ANG II when adequate amounts of ANG I are present in the medium, it is unclear what the contribution of MC ACE would be in the context of the intact animal. In addition to pulmonary and renal endothelial cells, ACE is present in the circulation and interstitial fluid of most tissues. Accordingly, it is possible that its presence in MCs may be another example of biological redundancy.

In summary, this study demonstrates the presence of active renin and ACE in peritoneal MCs and helps explain the mechanism of the activation of the RAS that follows degranulation of perivascular MCs during alveolar hypoxia. In a wider sense, the results suggest that the role of MC renin may be more widespread than what is currently known and provide another example of the role of the MC-initiated activation of the local RAS in cardiovascular inflammation.

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# DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

#### AUTHOR CONTRIBUTIONS

J.C. performed experiments; J.C. and N.C.G. analyzed data; J.C., G.B., J.G.W., and N.C.G. interpreted results of experiments; J.C. prepared figures; J.C. drafted manuscript; J.C., G.B., J.G.W., and N.C.G. edited and revised manuscript; J.C., G.B., J.G.W., and N.C.G. approved final version of manuscript; N.C.G. conception and design of research.

## REFERENCES

- Andersson CK, Mori M, Bjermer L, Löfdahl CG, Erjefält JS. Novel site-specific mast cell subpopulations in the human lung. *Thorax* 64: 297–305, 2009.
- Balcells E, Meng QC, Johnson WH Jr, Oparil S, Dell'Italia LJ. Angiotensin II formation from ACE and chymase in human and animal hearts: methods and species considerations. *Am J Physiol Heart Circ Physiol* 273: H1769–H1774, 1997.
- Caughey GH, Raymond WW, Wolters PJ. Angiotensin II generation by mast cell alpha- and beta-chymases. *Biochim Biophys Acta* 1480: 245–257 2000.
- Chao J, Donham P, van Rooijen N, Wood JG, Gonzalez NC. MCP-1/ CCL2 released from alveolar macrophages mediates the systemic inflammation of acute alveolar hypoxia. *Am J Respir Cell Mol Biol* 45: 53–61, 2011.
- Chao J, Wood JG, Blanco VG, Gonzalez NC. The systemic inflammation of alveolar hypoxia is initiated by alveolar macrophage-borne mediator(s). Am J Respir Cell Mol Biol 41: 573–582, 2009.
- Dix R, Orth T, Allen J, Wood JG, Gonzalez NC. Activation of mast cells by systemic hypoxia, but not by local hypoxia, mediates increased leukocyte-endothelial adherence in cremaster venules. *J Appl Physiol* 95: 2495–2502, 2003.
- Doggrell SA, Wanstall JC. Cardiac chymase: pathophysiological role and therapeutic potential of chymase inhibitors. *Can J Physiol Pharmacol* 83: 123–130, 2005.
- Gallwitz M, Enoksson M, Hellman L. Expression profile of novel members of the rat mast cell protease (rMCP)-2 and (RMCP)-8 families, and functional analyses of mouse mast cell protease (mMCP)-8. *Immunogenetics* 59: 391–405, 2007.
- Gonzalez NC, Allen J, Blanco VG, Schmidt EJ, van Rooijen N, Wood JG. Alveolar macrophages are necessary for the systemic inflammation of acute alveolar hypoxia. J Appl Physiol 103: 1386–1394 2007.
- Gonzalez NC, Allen J, Schmidt EJ, Casillan AJ, Orth T, Wood JG. Role of the renin-angiotensin system in the systemic microvascular inflammation of alveolar hypoxia. *Am J Physiol Heart Circ Physiol* 292: H2285–H2294, 2007.
- Krop M, Özünal ZG, Chai W, de Vries R, Fekkes D, Bouhuizen AM, Garrelds IM, Danser AHJ. Mast cell degranulation mediates bronchoconstriction via serotonin and not via renin release. *Eur J Pharmacol* 640: 185–189, 2010.
- Kunori Y, Muroga Y, Iidaka M, Mitsuhashi H, Kamimura T, Fukamizu A. Species differences in angiotensin II generation and degradation by mast cell chymases. J Recept Signal Transduct Res 25: 35–44, 2005.
- Mc Donald J, Gonzalez NC, Wood JG. Mast cell degranulation promotes the cerebral microvascular inflammatory response to hypoxia (Abstract). *FASEB J* 17: A1282, 2003.
- Mackins CJ, Kano S, Seyedi N, Schafer U, Reid AC, Machida T, Silver RB, Levi R. Cardiac mast cell-derived renin promotes loa angiotensin formation, norepinephrine release and arrhythmias in ischemiareperfusion. J Clin Invest 116: 1063–1070, 2006.
- 15. Nakamura S, Averill DB, Chappell MC, Diz DI, Brosnihan KB, Ferrario CM. Angiotensin receptors contribute to blood pressure homeo-

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stasis in salt-depleted SHR. Am J Physiol Regul Integr Comp Physiol 284: R164-R173, 2003.

- Orth T, Allen JA, Wood JG, Gonzalez NC. Plasma from conscious hypoxic rats stimulates leukocyte-endothelial interactions in normoxic cremaster venules. J Appl Physiol 99: 290–297, 2005.
- Piqueras L, Kubes P, Alvarez A, O'Connor E, Issekutz AC, Esplugues JV, Sanz MJ. Angiotensin II induces leukocyte-endothelial cell interactions in vivo via AT1 and AT2 receptor mediated P-selectin upregulation. *Circulation* 102: 2118–2123, 2003.
- Reilly CF, Tewksbury DA, Schechter Poole TJ, Zetter BR. Stimulation of peritoneal mast cell migration by tumor-derived peptides. *Cancer Res* 43: 5857–5861, 1983.
- NM, Travis J. Rapid conversion of angiotensin I to angiotensin II by neutrophils and mast cell proteinases. J Biol Chem 257: 15, 8619–8622 1982.
- Silver RB, Reid AC, Mackins CJ, Askwith T, Schaefer U, Herzlinger D, Levi R. Mast cells: a unique source of renin. *Proc Natl Acad Sci USA* 101: 13607–13612, 2004.
- Shah S, Allen J, Wood JG, Gonzalez NC. Dissociation between skeletal muscle microvascular Po<sub>2</sub> and hypoxia-induced microvascular inflammation. *J Appl Physiol* 94: 2323–2329, 2003.
- Steiner DR, Gonzalez NC, Wood JG. Mast cells mediate the microvascular inflammatory response to systemic hypoxia. *J Appl Physiol* 94: 325–334, 2003.
- Takai S, Jin D, Muramatsu M, Okamoto Y, Miyazaki M. Therapeutic applications of chymase inhibitors in cardiovascular diseases and fibrosis. *Eur J Pharmacol* 501: 1–8, 2004.
- Urata H, Nishimura H, Ganten D. Chymase-dependent angiotensin II forming systems in humans. Am J Hypertens 9: 277–284, 1996.

- 25. Veerappan A, Reid AC, Estephan R, O'Connor N, Thadani-Mulero M, Salazar-Rodirguez A, Levi R, Silver RB. Mast cell renin and a local angiotensin system in the airway: role in bronchoconstriction. *Proc Natl Acad Sci USA* 105: 1315–1320, 2008.
- Wan MX, Wang Y, Liu Q, Schramm R, Thorlacius H. CC chemokines induce P-selectin-dependent neutrophil rolling and recruitment in vivo: intermediary role of mast cells. *Br J Pharmacol* 138: 698–706, 2003.
- Wei CC, Hase N, Inoue Y, Bradley EW, Yahiro E, Li M, Navqi N, Powel PC, Shi K, Takahashi Y, Saku K, Urata H, Dell'Italia LJ, Husain A. Mast cell chymase limits the cardiac efficacy of Ang-I converting enzyme inhibitor therapy in rodents. *J Clin Invest* 120: 1229– 1239, 2010.
- Wintroub BU, Schechter NB, Lazarus GS, Kaempfer CE, Schwartz Angiotensin I LB. conversion by human, and rat chymotryptic proteinases. J Invest Dermatol 83: 336–339, 1984.
- Wolny A, Clozel JP, Rein J, Mory P, Vogt P, Turino M, Kiowski W, Fischli W. Functional and biochemical analysis of angiotensin II-forming pathways in the human heart. *Circ Res* 80: 219- 227, 1997.
- Wood JG, Johnson JS, Mattioli LF, Gonzalez NC. Systemic hypoxia promotes leukocyte-endothelial adherence via reactive oxidant generation. *J Appl Physiol* 87: 1734–1740, 1999.
- Wood JG, Mattioli LF, Gonzalez NC. Hypoxia causes leukocyte adherence to mesenteric venules in nonacclimatized, but not in acclimatized rats. *J Appl Physiol* 87: 873–881, 1999.
- Wood JG, Johnson JS, Mattioli LF, Gonzalez NC. Systemic hypoxia increases leukocyte emigration and vascular permeability in conscious rats. *J Appl Physiol* 89: 1561–1568, 2000.

