Several studies have postulated the existence of alternative pathways to angiotensin (ANG)-converting enzyme (ACE) for ANG II generation in tissues of different species based on evidence derived from experiments carried out with a combination of protease inhibitors and ANG II receptor antagonists. In a pioneering study (9) in this area, it was demonstrated that the vasoconstrictor response induced by ANG I in blood vessels of the hamster cheek pouch was blocked only partially by ACE inhibitors but completely abolished by ANG II receptor antagonists, leading to the conclusion that this vascular bed converts significant amounts of ANG I to ANG II by a route that does not involve ACE. In other investigations, the partial or total blockade of ANG II formation by different combinations of ACE inhibitors with chymostatin or other protease inhibitors has provided clues as to the nature of the enzymes involved in ANG I conversion in a particular tissue or pharmacological preparation (23, 45). Since the advent of [Pro11,D-Ala12]-ANG I, a biologically inactive precursor that selectively yields ANG II on incubation with chymases but not with ACE or carboxypeptidases (22), several reports (18, 22, 38, 41, 43, 61, 63) have described the relative contribution of chymases in ANG II formation in isolated preparations derived from various species.

The vascular endothelium actively participates in the control of vascular tone through the synthesis and metabolism of several vasoactive substances (1). In particular, the vascular endothelium has been shown to be a major site of conversion of circulating ANG I to ANG II by ACE located on its luminal surface (2). Also, cultured endothelial cells have been shown to contain renin and angiotensinogen and to be capable of synthesizing and secreting angiotensins (14, 25, 28, 36, 57, 62). Thus endothelial cells have the potential to play an important role in the vascular formation of ANG II. The finding that captopril does not completely block the synthesis of ANG II in endothelial cells (25, 57) suggests the existence of non-ACE pathways for ANG II formation and raises the question as to whether ACE is the sole enzyme responsible for ANG II generation in endothelial cells. Indeed, the existence of alternative ANG II-forming pathways in the vascular wall has been reported (17, 25, 26, 31, 32, 40, 42, 44, 58, 60, 65).

We (47, 52) have recently described the biochemical, enzymatic, and inhibitory properties of a chymostatin-sensitive ANG II-forming elastase-2 found in the perfusate of the isolated rat mesenteric arterial bed (MAB). One of the most interesting findings was that purified elastase-2 from the rat MAB perfusate was...
also capable of efficiently forming ANG II from [Pro11,D-Ala12]-ANG I, suggesting that in vivo formation of ANG II ascribed to chymases may have been overestimated in previous investigations of ANG II-forming pathways. It was also demonstrated that N-acetyl-Ala-Ala-Pro-Leu-chloromethylketone (Ac-AAPL-CK), an effective active site-directed inhibitor of human pancreatic elastase-2 (29), efficiently blocked the ANG II-generating activity of the rat elastase-2 (52). The cloned and sequenced cDNA for this ANG II-generating elastase-2 was found to be identical to that for rat pancreatic elastase-2 (37), whose corresponding mRNA was shown to be expressed in the rat lung but not in the aorta (51). A functional role for this elastase-2 has been proposed by studies carried out on the isolated rat MAB, indicating its participation in an ACE-independent pathway responsible for the pharmacological effects of both ANG I and the renin substrate tetradecapeptide (TDP) in this preparation (17, 32). The fact that elastase-2 is released into the rat MAB perfusate suggests that mesenteric endothelial cells (MECs) are a potential source of this enzyme. Additionally, the finding that mRNA for the elastase-2 is also found in the pancreas and lung (51) raises questions as to the extension of expression of this enzyme considering the functional diversity of rat tissues. Thus the main objectives of this work were 1) to study the contribution of rat elastase-2 for ANG II generation in the isolated rat MAB with the aid of different ANG II precursors and protease inhibitors; 2) to investigate whether MECs express rat elastase-2; and 3) to assess the distribution profile of mRNA expression for this enzyme in different rat tissues.

**MATERIALS AND METHODS**

**MAB isolation and removal.** All animal protocols were approved by the Medical College of Wisconsin and School of Medicine of Ribeirão Preto Institutional Animal Care and Use Committees. The surgical procedures for isolation and removal of the MAB were performed as previously described (39, 50). Briefly, male Sprague-Dawley or Wistar rats (200–250 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). A polyethylene (PE) cannula (PE-50, Clay Adams; Parsippany, NJ) was inserted into the superior mesenteric artery at its origin from the abdominal aorta, and blood was removed from the vascular bed by perfusing 10 ml of Krebs solution (in mM: 120 NaCl, 4.7 KCl, 3.0 CaCl2, 1.43 MgCl2, 25 NaHCO3, 1.17 KH2PO4, and 0.03 EDTA) containing heparin (25 IU/ml) through the cannula. The mesentry was then cut away from the intestine near the intestinal border; the mesentry was removed from the animal and placed ready for perfusion in an organ bath at 37°C.

**MAB perfusion pressure.** The isolated Wistar rat MAB was perfused with Krebs solution equilibrated with 95% O2–5% CO2, pH 7.4, at a constant rate of 4 ml/min, using a peristaltic pump (LKB-2115 multiperplex pump; Bromma). Perfusion pressure was recorded via a side arm of the mesenteric artery perfusion cannula with a HP-1280C pressure transducer and a HP-7754B recorder (Hewlett-Packard; Palo Alto, CA). After a 30-min stabilization period, phenylephrine was added to the perfusion solution (3–6 μM) until a stable perfusion pressure (100–120 mmHg) was achieved to avoid ANG II tachyphylaxis (27). Dose-response curves for ANG II (2–100 pmol), ANG I (20–400 pmol), TDP (150–2,400 pmol), and [Pro11,D-Ala12]-ANG I (140–2,400 pmol) were obtained by a bolus injection of 40 μl of the peptide solution (diluted in Krebs solution) into the perfusion stream before the pump. Injection of 40 μl of Krebs solution did not affect basal perfusion pressure. Single responses to ANG II (10 pmol), ANG I (100 pmol), TDP (1,000 pmol), and [Pro11,D-Ala12]-ANG I (1,000 pmol) were determined in the same preparation before and after the addition of 1) the ACE inhibitor captopril (36 μM), 2) the chymase inhibitor chymostatin (100 μM), 3) the combination of captopril (36 μM) and chymostatin (100 μM), 4) the elastase-2 inhibitor Ac-AAPL-CK (50 μM) (29, 51), and 5) the combination of captopril (36 μM) and Ac-AAPL-CK (50 μM). The interval between the injections was at least 15 min to avoid tachyphylactic responses. In another set of experiments, the effect of the ANG II receptor antagonist [Sar4,Ile8,Ala9]-ANG II (saralasin; 50 nM) on the responses elicited by ANG II (10 pmol) and [Pro11,D-Ala12]-ANG I (1,000 pmol) was also investigated. Saralasin and the inhibitors were added to the perfusion solution and allowed to act for 30 min before the pressor response induced by the agonists was tested.

**Harvesting of MECs.** MEC harvesting was performed by a modification of the method described previously (20). Initially, the isolated Sprague-Dawley rat MAB was perfused with Krebs solution at a flow rate of 1.0 ml/min for 15 min to ensure thorough removal of blood substances, followed by perfusion with Krebs solution containing 0.2% type I collagenase (Worthington Biochemical; Lakewood, NJ) at a flow rate of 2 ml/min for 90 min. The perfusate collected during the first 30 min was discarded; the perfusate was then collected every 10 min for 60 min and centrifuged at 1,000 g for 10 min. The cells were resuspended in Cellgro RPMI media containing 20% fetal bovine serum (Sigma; St. Louis, MO), 1% of 100× antibiotic/antimycotic solution (Sigma), and gentamycin (2 μg/ml, Life Technologies; Frederick, MD), plated onto a six-well plate, and incubated at 37°C in 5% CO2 in room air. After the cells reached confluence (5–7 days), they were passaged with trypsin-EDTA (Sigma) and plated on 100-mm-diameter cell culture dishes (1.8 × 106 cells/dish).

**Immunocytochemistry for CD31.** Confluent MECs were passaged, counted with a hemocytometer, and plated at 6 × 104 cells/ml on a chamber slide (Fisher; Pittsburgh, PA). Cells were allowed to grow 1–2 days in a CO2 incubator at 37°C to reach subconfluence. Slides were rinsed with Dulbecco’s phosphate-buffered saline (DPBS; Sigma), fixed in cold methanol at −20°C for 5 min, blocked in 3% goat serum for 1 h, and then incubated at room temperature for 2 h in mouse anti-rat CD31 antibody (BD Pharmingen; San Diego, CA) diluted in 0.1% goat serum (1:100). Cells were rinsed with DPBS and incubated for 1 h in anti-mouse IgG (Alexa 488, Molecular Probes; Eugene, OR) diluted in 0.5% BSA (1:100). Cells were rinsed in DPBS and mounted with Anti-Fade (Oncor; Gaithersburg, MD). Primary antibody was omitted in negative control slides.

**Isolation of RNA.** Total RNA from cells was isolated following the manufacturer’s instruction (RNeasy Mini Kit, Qiagen; Valencia, CA). RNA from different tissues (pancreas, MAB, lung, heart, kidney, liver, spleen, and aorta) was isolated as previously described (51). RNA was treated with DNase (Amersham Pharmacia; Piscataway, NJ) for 15 min at room temperature to remove any potential genomic DNA contamination. RNA concentration was measured spectrophotometrically at 260 nm.

**Detection of rat elastase-2 and von Willebrand factor mRNAs using RT-PCR.** First-strand cDNA synthesis was performed in a 33-μl reaction volume using 10 μg total
DNase-treated RNA, 0.2 µg random hexadeoxynucleotides, RT buffer [45 mM Tris-HCl (pH 8.3), 68 mM KCl, and 9 mM MgCl₂], 0.08 mg/ml BSA, 15 mM DTT, 1.8 mM dNTPs, and 150 units murine leukemia virus reverse transcriptase, as provided in the first-strand cDNA synthesis kit from Amersham Pharmacia. cDNA was synthesized during a 60-min incubation at 37°C, and the reaction was terminated by heating at 80°C for 5 min. RT products (3 µl) served as the template for PCR amplification, using the following primers synthesized by Research Genetics (Huntsville, AL): (1) sense 5’-ACA GAC TAC CAC GGA CAC AC-3’ and antisense 5’-GTG CGT TCC CAA GGT GAC-3’ (rat elastase-2; final PCR product 875 bp) (51) and (2) sense 5’-CCC TGC CTC AAT GAG GTG GT-3’ and antisense 5’-GCC TCC TCA CAT GTG TCA CAG CA-3’ (von Willebrand factor; final PCR product 587 bp) (24). All PCR reactions were performed in a total volume of 50 µl, which was composed of 20 pmol (0.4 µM) of each primer, PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 1.0 mM MgCl₂], 0.2 mM dNTPs, and 2.5 units recombinant Taq DNA polymerase (Life Technologies). Temperature cycling consisted of an initial denaturation step for 2 min at 94°C, followed by 40 cycles of amplification. Each round consisted of denaturation for 45 s at 94°C, annealing for 30 s at 58°C, and extension for 90 s at 72°C. Samples were incubated for additional 10 min at 72°C (terminal elongation) after the completion of the final cycle. For each set of primers, RT-PCR was performed on sterile water to check for contamination. A 9-round PCR amplification was performed on RNA to check for genomic DNA contamination. A 9-sample was electrophoretically size fractionated on a 1.5% agarose gel containing ethidium bromide (0.64 µg/ml). DNA was visualized under ultraviolet light to detect the presence of PCR amplification products at the anticipated sizes. The size of the PCR products was determined by comparison with the 100-bp ladder (Amersham Pharmacia).

**Enzymatic assays and inhibition.** All assays were carried out at 37°C by incubating the specified substrate with the enzyme samples in Tris-buffered saline (TBS; 0.03 M Tris·HCl containing 0.15 M NaCl; pH 8.1). Affinity-purified rat MAB elastase-2 was prepared as previously described (47), and human skin chymase was purchased from Calbiochem (San Diego, CA). To measure the enzymatic activity of elastase-2, the chromogenic substrates N-succinyl-Ala-Ala-Pro-Phe-pNA; 560 M), N-succinyl-Ala-Ala-Pro-Leu-p-nitroanilide (N-suc-AAPL-pNA; 560 M) or N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (N-suc-AAPF-pNA; 730 M), 100 µl of each sample were incubated for a period ranging from 10 min to 20 h with the substrate in a clear 96-well plate in a final volume of 300 µl. The absorbance of each reaction mixture resulting from the release of p-nitroaniline was read in a universal microplate reader (ELX 800, Bio-Tek Instruments; Winooski, VT) at 405 nm, and the amount of product formed was determined by comparison with a standard curve of p-nitroaniline ranging from 1 to 64 nmol in 300 µl of the assay buffer. The enzymatic generation of ANG II from ANG I was measured by radioimmunoassay (RIA). Properly diluted enzyme samples (10 µl) were individually incubated with ANG I (36 µM) for 30 min in a final volume of 100 µl TBS, and the reactions were terminated by heating at 100°C for 3 min. The ANG II formed after HPLC separation from other components of the reaction mixture was measured by RIA, as previously described (49). A standard curve for ANG II, ranging from 0.32 to 82.5 fmol, allowed quantification of the conversion reactions. The inhibitory effects of distinct substances were determined by incubating the samples with the inhibitor for 15 min at 37°C before the activity using N-suc-AAPL-pNA, N-suc-AAPF-pNA, or ANG I as the substrate was assayed, as described above.

**Collection of MEC-conditioned media.** After 1 (n = 4) and 48 h (n = 4) of exposure of cultured MEC to RPMI media 1640 (without phenol red), conditioned media samples were collected (3 ml/dish). Enzymatic activity in the conditioned media toward the substrate N-suc-AAPL-pNA was measured as described in Enzymatic assays and inhibition. Cellular viability was assessed by trypan blue exclusion. Conditioned media collected after 48 h of cell exposure (n = 12) were used for rat elastase-2 purification.

**Rat elastase-2 purification from MEC-conditioned media.** Conditioned media samples of cultured MEC were pooled and concentrated 24-fold by ultrafiltration (Centriplus YM-10, Millipore; Bedford, MA). Rat elastase-2 was chromatographically purified using a two-step procedure (47) by monitoring the ANG II-forming activity of the fractions using ANG I as the substrate, as described above. Briefly, 1 ml of the concentrated conditioned media was chromatographed over a Sepharyl S-300 column (10 × 920 mm) equilibrated with TBS and developed at room temperature at a flow rate of 6.0 ml/h. Fractions of 1.0 ml were collected, and enzyme activity was assayed. Rat elastase-2 was further purified by affinity chromatography on an ovoinhibitor-agarose column by percolating the pooled fractions from the gel filtration purification step through the affinity column (10 × 20 mm) at room temperature at a flow rate of 10 ml/h. Loosely bound proteins were removed by washing the column with 20 ml TBS. The enzyme was then eluted with 20 ml of 0.02 M HCl, with care being taken to neutralize any acid excess in the fractions immediately after collection. The fractions containing enzymatic activity toward ANG I were pooled and stored at 4°C.

**RESULTS**

**Functional experiments performed on the isolated rat MAB in the absence and presence of different protease inhibitors.** ANG II and its precursors (ANG I, TDP, and [Pro¹¹,D-Ala¹²]-ANG I) produced a dose-dependent increase in perfusion pressure of the isolated rat MAB, with a similar maximal pressor response. ANG II showed greater potency compared with all the precursors. [Pro¹¹,D-Ala¹²]-ANG I showed a very similar profile of vasconstrictor response compared with that of TDP (Fig. 1A). This is the first demonstration of the effectiveness of [Pro¹¹,D-Ala¹²]-ANG I in this preparation and confirms ACE-independent production of ANG II. The ANG II receptor antagonist saralasin abolished the vasconstrictor effect induced by [Pro¹¹,D-Ala¹²]-ANG I (1,000 pmol) as well as that by ANG II (10 pmol) (Fig. 1B).

As expected, captopril did not influence the vasconstrictor response elicited by [Pro¹¹,D-Ala¹²]-ANG I but partially reduced the pressor responses elicited by ANG I and TDP by 58% and 38%, respectively (Fig. 2A). Chymostatin almost completely abolished the vasconstrictor effect induced by [Pro¹¹,D-Ala¹²]-ANG I and partially inhibited that elicited by TDP, whereas it did not affect the pressor response to ANG I (Fig. 2B). The combination of captopril and chymostatin, however, reduced the vasconstrictor responses elicited by ANG I and TDP to 15–20% of their response in the absence of the inhibitors. It is noteworthy that this combination of inhibitors did not result in any further
decrease on the vasoconstrictor response elicited by [Pro$^{11},$D-Ala$^{12}$]-ANG I compared with that attained by chymostatin alone (Fig. 2C). Furthermore, in the presence of captopril and/or chymostatin, the responses induced by ANG II were not affected.

The vasoconstrictor effect induced by [Pro$^{11},$D-Ala$^{12}$]-ANG I was almost completely abolished by the inhibitor Ac-AAPL-CK, whereas the pressor response to TDP was only partially reduced by this inhibitor. The pressor response to ANG I was not affected by Ac-AAPL-CK (Fig. 3A). The vasoconstrictor responses elicited by ANG I, TDP, and [Pro$^{11},$D-Ala$^{12}$]-ANG I were greatly reduced by the combination of captopril and Ac-AAPL-CK. This combination of inhibitors, however, did not result in any additional decrease on the vasoconstrictor response elicited by [Pro$^{11},$D-Ala$^{12}$]-ANG I compared with the inhibitory effect of Ac-AAPL-CK alone (Fig. 3B).

Characterization and Validation of MECs. The MAB perfusion method described in this study proved to be an effective technique for the culture of rat vascular endothelial cells from the MAB. MECs were validated...
and characterized by the typical cobblestone appearance at confluence and immunocytochemical detection of the endothelial marker CD31 (Fig. 4) and by the presence of von Willebrand factor mRNA in the cell extracts (Fig. 5).

Detection of rat elastase-2 mRNA in MECs. RT-PCR, performed on total RNA from MECs with primers specific for rat elastase-2, yielded a clear single band with the predicted size of 875 bp (Fig. 5). This result prompted us to investigate the presence of the enzyme itself in cultured MECs.

Detection of rat elastase-2 activity in MEC-conditioned media. MEC-conditioned media showed enzymatic activity toward the chromogenic substrate N-suc-AAPL-pNA, a reaction strongly inhibited by 50 μM Ac-AAPL-CK (data not shown). Rat MAB elastase-2 hydrolyzes N-suc-AAPL-pNA with a catalytic efficiency of 10.6 min⁻¹·μM⁻¹ and is inhibited by Ac-AAPL-CK at low micromolar concentrations (52). On the other hand, the substrate N-suc-AAPL-pNA is refractory to the action of both rat peritoneal mast cell chymase-like proteases (52) and human skin chymase (data not shown); this latter enzyme was shown to be inhibited by 50 μM Ac-AAPL-CK when assayed with a convenient substrate, N-suc-AAPF-pNA. Altogether, these data indicate that a functional elastase-2 is secreted by cultured MECs, but the lack of selectivity of the inhib-
ACE-resistant substrate [Pro11,D-Ala12]-ANG I on the constrictor effect of ANG II, ANG I, TDP, and the
ments carried out to demonstrate the potential vaso-
tion of ANG II in the isolated rat MAB. The experi-
porting a functional role for elastase-2 in the genera-
from MEC-conditioned media
Inhibitor profile of rat elastase-2 purified from MEC-conditioned
media and inhibitory effect of different substances on enzyme activity. Rat elastase-2 was purified from MEC-conditioned media by the method previously described (47). Table 1 shows that the ANG II generation from ANG I by the enzyme purified from MEC-conditioned media was not affected by captopril (1 mM) but was inhibited in a concentration-dependent fashion by chymostatin and Ac-AAPL-CK (1–100 μM).

Tissue distribution of rat elastase-2 mRNA. Rat elastase-2 mRNA could be detected in the pancreas, MAB, lung, heart, kidney, liver, and spleen by RT-PCR (Fig. 6). The same strategy failed to reveal the presence of elastase-2 mRNA in the aorta, confirming our previous data (51).

**DISCUSSION**

This study provides different lines of evidence supporting a functional role for elastase-2 in the generation of ANG II in the isolated rat MAB. The experiments carried out to demonstrate the potential vasoconstrictor effect of ANG II, ANG I, TDP, and the ACE-resistant substrate [Pro11,D-Ala12]-ANG I on the isolated MAB clearly showed the existence of an ACE-independent pathway for ANG II generation, which is native to the rat MAB. However, as established in literature, rat chymase is mainly an angiotensinase (6, 30, 62), which argues in favor of elastase-2 being responsible for the ACE-independent pathway for ANG II generation in the rat MAB because it does not degrade ANG II (47, 52). To date, the enzymes known to be capable of forming ANG II from [Pro11,D-Ala12]-ANG I are either homologous to human skin chymase toward the substrates ANG I and N-suc-AAPF-pNA (data not shown). Therefore, our attempt to introduce an experimental approach for discriminating between chymases and elastases based on their sensitivity to Ac-AAPL-CK failed for lack of selectivity of the inhibitor. Similarly, chymostatin inhibits both chymases (56, 59) and elastase-2 (47), so the use of Ac-AAPL-CK and chymostatin cannot unequivocally indicate the relative contribution of different serine proteases in the generation of ANG II in the isolated rat MAB or elsewhere. However, as established in literature, rat chymase is mainly an angiotensinase (6, 30, 62), which argues in favor of elastase-2 being responsible for the ACE-independent pathway for ANG II generation in the rat MAB because it does not degrade ANG II (47, 52). To date, the enzymes known to be capable of forming ANG II from [Pro11,D-Ala12]-ANG I are either homologous to human heart chymase (22, 38, 41, 43, 63) or rat elastase-2 (52), so this latter enzyme is the only known rat protease fitting the experimental evidence described for the ACE-independent pathway for ANG II generation in the rat MAB.

The vasoconstrictor response elicited by bolus injections of [Pro11,D-Ala12]-ANG I in the isolated MAB was abolished by the ANG II receptor antagonist saralasin (50 nM) in the perfusion solution (Fig. 1). These data indicate that the biologically inactive peptide [Pro11,D-Ala12]-ANG I has to be converted into ANG II to produce vasoconstrictor responses in the isolated rat MAB, as previously shown for ANG I and TDP (32). The effects of protease inhibitors on the pressor responses to bolus injection of these three ANG II precursors were studied in the rat MAB in an attempt to determine the relative importance of ACE and non-

![Fig. 5. Ethidium bromide-stained agarose gel of RT-PCR products from MECs. cDNAs were amplified by PCR with gene-specific primers for rat elastase-2 (E-2; 875 bp) as well as von Willebrand factor (vWF; 587 bp). M, 100-bp markers; no RT, PCR of RNA.](http://ajpheart.physiology.org/)

![Fig. 6. Ethidium bromide-stained agarose gel of RT-PCR products from different tissues. cDNAs were amplified by PCR with gene-specific primers for rat E-2 (875 bp) as well as β-actin (351 bp).](http://ajpheart.physiology.org/)
ACE pathways for ANG II generation (Figs. 2 and 3). Previous reports have indicated two shortcomings inherent in this approach to reveal the predominance of ACE and non-ACE pathways in different experimental models: first, the outcome is highly dependent on the concentrations of the substrate used, with higher concentrations favoring the apparent contribution of non-ACE pathways (26); and second, the overcapacity among the ANG II generating proteases tends to diminish the relative importance of selectively inhibited enzymes (61). Notwithstanding, the synergistic inhibitory effects of captopril and chymostatin (Fig. 2) and captopril and Ac-AAPL-CK (Fig. 3) on the pressor responses to ANG I injection show a marked participation of a non-ACE pathway for ANG II production in the isolated rat MAB, even considering the overcapacity of ACE revealed by the little effects of chymostatin alone or Ac-AAPL-CK alone. The results shown in Figs. 2 and 3 also indicate that ACE and non-ACE pathways are equally important for converting TDP to ANG II in the isolated MAB; this substrate is converted to ANG II by the successive removal of three dipeptides by the action of ACE (13) or endoproteolytically by rat MAB elastase-2 (47). Additionally, the substrate [Pro₁₁,D-Ala₁₂]-ANG I is converted to ANG II predominantly by non-ACE pathways in the isolated and perfused MAB; whereas chymostatin alone or Ac-AAPL-CK alone almost completely blocked the pressor response elicited by [Pro₁₁,D-Ala₁₂]-ANG I injection, captopril had no effect. Although this ANG II precursor was designed that [Pro₁₁,D-Ala₁₂]-ANG I is a suitable substrate for ANG II generation in the isolated rat MAB and suggest that [Pro₁₁,D-Ala₁₂]-ANG I is a suitable substrate for revealing the role of non-ACE pathways in ANG II-mediated responses in this pharmacological preparation.

This study also provides evidence that cultured MECs synthesize rat elastase-2. First, RT-PCR performed on total RNA from MECs yielded a single band with the predicted size for rat elastase-2 (51); second, enzymatic activity toward the elastase-2 substrate N-suc-AAPL-pNA (12) was found in MEC-conditioned media. The release of rat elastase-2 into the MAB perfusate (46, 47) and MEC-conditioned media was predictable because molecular cloning and sequencing of cDNA for this enzyme revealed a message encoding a serine protease with a secretory protein leader peptide (51).

A remarkable feature of rat elastase-2 as an ANG I-converting protease is that it does not destroy the product ANG II (46, 47, 52) despite its broad proteolytic specificity toward somatostatin, mellitin, and oxidized insulin B-chain (47, 54). This property is shared with some chymases such as human (59), baboon (22), and dog (4), but not rodent chymases, which split ANG I at its two potential chymotryptic cleavage sites, Tyr⁴-Ile⁵ and Phe⁸-His⁹ (6, 30, 62). Only recently, a rat chymase that forms and does not cleave ANG II was described in vascular smooth muscle cells (21). One feature that differentiates this latter enzyme from rat elastase-2 is that it is not secreted, whereas rat elastase-2 is found free in both the MAB perfusate (46, 47) and MEC-conditioned media, as described here.

Rat elastase-2 was purified from MEC-conditioned media by the procedure originally described for the purification of the enzyme from the rat MAB perfusate (47). The inhibitory effect of various substances on the ANG II-forming activity of this affinity-purified enzyme from MECs (Table 1) showed it to be indistinguishable from its MAB counterpart (51). Current evidence indicate that chymases and rat elastase-2 are equally sensitive to most serine protease inhibitors (21, 47, 59), thus rendering the use of these inhibitors unsuitable for unambiguous identification of functionally relevant ANG II-forming enzymes in the rat vasculature. The use of orally active trifluoromethylketone elastase inhibitors has been recently documented in a model of pulmonary hypertension in rats and has resulted in reversal of the pathology (10). Because rat elastase-2 is an efficient ANG II-forming enzyme whose mRNA was also found in the rat lung (51), among other tissues (Fig. 6), it would be interesting to investigate the inhibitory effects of these new trifluoromethylketone inhibitors on purified rat elastase-2 in an attempt to explain, at least in part, the beneficial effects observed in therapy with these orally active inhibitors. Among these inhibitors, the compound ZD-0892, which is active toward neutrophil elastase (16) as well as other elastases (11), seems a promising inhibitor of rat elastase-2, an enzyme that has a large binding pocket for substrates and inhibitors with specificity directed to a special motif in the peptide ligand, particularly those displaying a proline residue at the penultimate position (12, 29, 54).

The finding that mRNA for rat elastase-2 was detected in various rat tissues (Fig. 6) may indicate that this enzyme has a role in ANG II formation in other tissues besides the rat MAB. In this regard, recent work from our laboratory (53) demonstrated that elastase-2 is expressed in the rat heart because the protein itself could be purified from the perfusate of the Langendorff preparation. The existence of locally formed renin-ANG system (RAS) components in multiple tissues has led to the assumption that ANG II may act as a local hormone. It may either potentiate systemic functions or have entirely separate activities that meet tissue needs (15, 48). An accumulating body of evidence strongly indicates the participation of a local RAS in the pancreas of various species that, in some cases, are markedly activated by experimental chronic hypoxia and acute pancreatitis (5, 34). The notion of a local RAS and its potential role in the pancreas have been previously reported in the dog (7, 8), rat (19, 35), mouse (33), and human (55). The data suggested that the local RAS may play an autocrine/paracrine role in the control of the endocrine/exocrine functions of the pancreas, regulating islet blood flow and thus the endocrine function of insulin secretion in the rat (3). As shown in Fig. 6,
the pancreas is one of the tissues with the highest expression of rat elastase-2 mRNA, raising the possibility that this enzyme participates in the pancreatic RAS by supporting a local ACE-independent pathway for ANG II generation.

It is worth mentioning that chymostatin and [Pro^{14},p-Ala^{12}]-ANG I, substances considered as selective for chymases, show a remarkable interaction with rat elastase-2 purified from the MAB perfusate (Refs. 47 and 52 and present data). Therefore, conclusions derived from experiments performed in rats with these substances should acknowledge that elastase-2 could also be the enzyme responsible for the alternative pathway to ACE for ANG II generation under investigation.

In conclusion, in the present study, we have provided pharmacological evidence that rat elastase-2 has a functional role for ANG II generation in the isolated rat MAB. We also demonstrated that cultured MECs synthesize elastase-2 and that the mRNA for this enzyme could be detected in different rat tissues. The intravascular localization of MAB elastase-2 and its ability to generate ANG II and not destroy it indicate that this enzyme might play a role in the rat cardiovascular system as an ANG II-forming agent.

The authors are grateful to Lisa Henderson, Luanne Kelly, Glenn Slocum, and Osmar Vetere for excellent technical assistance.

DISCLOSURES

This work was supported by National Heart, Lung, and Blood Institute Grant HL-29587 (to A. S. Greene) and the Fundação de Amparo a Pesquisa do Estado de São Paulo. C. F. Santos was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico Fellowship 200588/00-1.

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