Pathways for angiotensin-(1–7) metabolism in pulmonary and renal tissues

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Allred, Alicia J., Debra I. Diz, Carlos M. Ferrario, and Mark C. Chappell. Pathways for angiotensin-(1–7) metabolism in pulmonary and renal tissues. Am J Physiol Renal Physiol 279: F841–F850, 2000.—Two of the primary sites of actions for angiotensin (ANG)-(1–7) are the vasculature and the kidney. Because little information exists concerning the metabolism of ANG-(1–7) in these tissues, we investigated the hydrolysis of the peptide in rat lung and renal brush-border membrane (BBM) preparations. Radiolabeled ANG-(1–7) was hydrolyzed primarily to ANG-(1–5) by pulmonary membranes. The ANG-converting enzyme (ACE) inhibitor lisinopril abolished the generation of ANG-(1–5), as well as that of smaller metabolites. Kinetic studies of the hydrolysis of ANG-(1–7) to ANG-(1–5) by somatic (pulmonary) and germinal (testes) forms of rat ACE yielded similar values, suggesting that the COOH-domain is responsible for the hydrolysis of ANG-(1–7). Pulmonary metabolism of ANG-(1–5) yielded ANG-(3–5) and was independent of ACE but may involve peptidyl or dipeptidyl aminopeptidases. In renal cortex BBM, ANG-(1–7) was rapidly hydrolyzed to mono- and dipeptide fragments and ANG-(1–4). Aminopeptidase (AP) inhibition attenuated the hydrolysis of ANG-(1–7) and increased ANG-(1–4) formation. Combined treatment with AP and nephrisin (Nep) inhibitors abolished ANG-(1–4) formation and preserved ANG-(1–7). ACE inhibition had no effect on the rate of hydrolysis or the metabolites formed in the BBM. In conclusion, ACE was the major enzymatic activity responsible for the metabolism of ANG-(1–7) in the lung, which is consistent with the ability of ACE inhibitors to increase the half-life of circulating ANG-(1–7) and raise endogenous levels of the peptide. An alternate pathway of metabolism was revealed in the renal cortex, where increased AP and Nep activities, relative to ACE activity, promote conversion of ANG-(1–7) to ANG-(1–4) and smaller fragments.

angiotensin-converting enzyme; nephrisin; lisinopril; SCH-39370; aminopeptidase; amastatin

ANGIOTENSIN (ANG)-(1–7) is one of several alternative products of the renin-angiotensin system that exhibits biological activity (7). The overall actions of ANG-(1–7) counterbalance the actions of ANG II (17). Recent studies demonstrate that ANG-(1–7) contributes to the blood-pressure-lowering actions either of ANG converting enzyme (ACE; EC 3.4.15.1) inhibition alone or when ACE inhibition is combined with AT1 receptor (an ANG receptor) blockade in spontaneously hypertensive rats (25–27). The laboratory of Campagnolo-Santos and colleagues (Britto et al., Ref. 4) showed that the ANG-(1–7) antagonist d-[Ala7]ANG-(1–7) reverses the resetting of the baroreflex response during ACE inhibition. Unlike the growth-promoting actions of ANG II, ANG-(1–7) exhibits an opposite effect in vascular smooth muscle cells that is not attenuated by treatment with AT1 or AT2 selective antagonists but is sensitive to the d-alanine antagonist (18). Strawn et al. (44) recently demonstrated that the peptide also exerts its antiproliferative actions in vivo, because a chronic infusion of ANG-(1–7) attenuated neointimal proliferation in injured rat carotid artery. In rabbit renal afferent arterioles, recent studies by Ren et al. (33) demonstrate that ANG-(1–7) is a vasodilator agent. Its actions were not blocked by AT1 or AT2 antagonists but were attenuated by d-[Ala7]ANG-(1–7), suggesting a non-AT1 and non-AT2 ANG-(1–7) receptor localized to the renal afferent arteriole (33).

At present, relatively little information is known regarding metabolism of circulating and tissue ANG-(1–7). The presence of ANG-(1–7) in plasma is dependent at least in part on nephrisin (5, 9, 26, 53). Studies in isolated cells of neural or vascular origin implicated prolyl endopeptidase (EC 3.4.24.26) and a thiol-sensitive endopeptidase, thimet oligopeptidase (EC 3.4.24.15) (11–12, 39, 51). Yamada et al. (52) reported that ANG-(1–7) has an exceptionally short half-life (t1/2 < 9 s) in the circulation of rats. Several studies now indicate that, similar to bradykinin, ACE may play an important role in regulating the circulating levels of ANG-(1–7) (10, 14). Chronic treatment with various ACE inhibitors substantially augmented (5- to 25-fold) the level of ANG-(1–7) in the circulation of several species, including humans. ACE inhibition also increased the t1/2 of ANG-(1–7) (>60 s) in both normotensive and hypertensive rats (52). Kinetic analysis of ANG-(1–7) metabolism by purified canine ACE revealed a high affinity for ACE [Michaelis-Menten constant (Km), 0.8 μM; catalytic efficiency (kcat/Km) of
2,200 mM⁻¹·s⁻¹) (10). Deddish et al. (14) reported similar kinetic values for ANG(1–7) with human ACE. Of particular interest, they found that the amino-terminal (NH₃) domain of ACE, the predominant form of ACE in intestinal fluid, was primarily responsible for hydrolysis of ANG(1–7), and that at high concentrations, the heptapeptide may function as an inhibitor at the carboxy (COOH) domain (14). Therefore, we examined the metabolism of ANG(1–7) by both somatic and germinal forms of ACE to determine which domain may contribute to the hydrolysis of the peptide in the rat. Furthermore, we determined the enzymatic routes for ANG(1–7) formation and degradation in the lung and kidney membranes of the rat.

MATERIALS AND METHODS

Animals. Studies were performed using tissues obtained from adult male 10- to 12-wk-old Sprague-Dawley rats (Hartmann, Indianapolis, IN). Animals were maintained on a normal diet, with free access to tap water in our Association for Assessment and Accreditation of the Laboratory Animal Care-approved facility with a 12:12-h light-dark cycle (lights on 7:00 AM to 7:00 PM). Animals were killed by decapitation; lungs and kidneys were removed immediately, and renal cortex was dissected out on ice. Pulmonary and renal cortical tissues were stored at −80°C until they were needed for each experiment.

Pulmonary membrane preparation. Lung tissue was minced and homogenized with the use of IKI Labortechnik Ultra-Turrax T25 at 30,000 g (Janke and Kunkel) in 25 mM HEPES, 125 mM NaCl, and 100 mM mannitol, pH 7.4 on ice. Membranes were washed twice by centrifugation at 30,000 g for 20 min, and the resulting pellet was reconstituted in the same buffer without mannitol; 10 μg of pulmonary membranes were used in the metabolism studies. Protein concentration was determined with a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA).

ACE purification. The two molecular forms of ACE were purified from a membrane fraction of the rat lung (somatic) or testis (germinal) by use of a lisinopril-coupled affinity column (10). Purified ACE was analyzed by SDS-PAGE (6% polyacrylamide, wt/vol) by use of the stacking method described by Laemmli (see Ref. 29). Proteins were visualized with a Pharmacia silver stain kit (Pharmacia Biotech, Piscataway, NJ). The fraction was purified by a second chromatographic separation and subjected to amino acid analysis (Protein Core Facility, Wake Forest Univ. School of Medicine).

RENAL BRUSH-BORDER MEMBRANE VESICLE PREPARATION. Brush-border membrane (BBM) vesicles were prepared according to the method of Kinne-Saffran and Kinne (28). In brief, frozen renal cortex was minced and then homogenized in 10 mM β-mannitol and 2 mM Tris-HCl, pH 7.1, on ice. Differential centrifugation and double precipitation with CaCl₂ produced a pellet of BBM. The pellet then was suspended in 100 mM β-mannitol and 20 mM Tris-HCl, pH 7.4, and centrifuged at 48,000 g for 20 min. This resulting pellet was reconstituted in 0.3 ml of 100 mM β-mannitol and 20 mM Tris-HCl, pH 7.4, and passed through a 1-ml 25-gauge tuberculin needle 10 times to promote formation of a right-side-out orientation for BBM vesicles (28). Enrichment of the brush border was indicated by a 5-fold increase in alkaline phosphatase activity, a 20-fold increase in leucine aminopeptidase activity, and a 12-fold decrease in Na⁺-K⁺-ATPase, a marker of the basolateral membranes.

Enzyme assays. Alkaline phosphatase activity of the prepared membrane vesicles was determined by the hydrolysis of p-nitrophenyl phosphate (pNPP, Sigma) in a glycine buffer with 1 mM MgCl₂, pH 6.5-8.0 (21). To establish contamination levels of the BBM by basolateral membranes, Na⁺-K⁺-ATPase activity in the BBM was determined with pNPP as substrate. Contribution of K⁺-activated phosphatases was determined by addition of potassium chloride (10 mM); potassium chloride-ouabain (10 mM KCl, 1 mM ouabain) was added to a second set of samples to determine the contribution of ouabain-sensitive phosphatases (34). To ensure linear reaction rates, enzyme activity was measured in 2–20 μg of protein. Aminopeptidase activity was measured with the fluorescent substrate 7-amido-4-methyl-coumarin (Leu-AMC) (41). ACE activity was determined in pulmonary membranes (1 μg) or BBM vesicle suspension (100 μg) with the synthetic substrate Hip-His-Leu (10). Neprilysin activity was determined in pulmonary membranes (2.5–5.0 μg) and BBM vesicle suspension (0.2–0.4 μg) with the use of the fluorescent substrate N-terminyl-Ala-Ala Phe-7-AMC (54).

Determination of ANG metabolism. The standard assay was conducted at 37°C in phosphate-buffered saline (14 mM NaH₂PO₄, 36 mM Na₂HPO₄, and 150 mM NaCl, pH 7.4) with brush-border or pulmonary membranes, the indicated concentrations of enzyme inhibitors, and 1.0–1.5 nM iodinated ANG peptides, in a final volume of 100 μl. Pulmonary membranes were used at a concentration of 10-μg/100-μl sample, and BBM vesicles were used at a concentration of 1-μg/100-μl sample. Membranes were allowed to preincubate for 5 min with enzyme inhibitors before addition of iodinated ANG peptides. At the specified times, the reaction was stopped by addition of ice-cold 0.4% phosphoric acid-acetonitrile (Phos/Acn), transferred immediately to dry ice, and stored at −80°C. For each experiment, zero time point controls for radiolabeled ANG(1–7), ANG(1–5), and ANG I were determined by addition of the labeled substrate to membranes (with or without inhibitors) containing the Phos/Acn solution. The HPLC analysis for all zero time point controls yielded essentially a single peak of radioactivity for each peptide in the presence or absence of peptide inhibitors.

Metabolism of unlabeled ANG peptides was carried out in a similar manner. Brush-border membrane vesicle suspensions (1–10 μg) were incubated at 37°C with ANG I alone (50 μM) or in the presence of 50–400 μM ANG II. Reactions were stopped as described above for the metabolism of iodinated ANG peptides.

To confirm the identity of the major product of unlabeled ANG(1–7) metabolism, 10 μM ANG(1–7) was incubated with BBM, and products were isolated by HPLC and then evaporated to dryness in a vacuum centrifuge (Savant, Farmingdale, NY). The fraction was purified by a second chromatographic separation and subjected to amino acid analysis (Protein Core Facility, Wake Forest Univ. School of Medicine).

HPLC analysis. Separation of the ¹²⁵I-labeled metabolic products was achieved by a modified reversed-phase HPLC method (10). Peptides were fractionated on a Perkin-Elmer HPLC with a Waters Nova-Pak C₁₈ column. Mobile-phase solvents were 0.1% phosphoric acid (vol/vol) in water (buffer A) and 80% acetonitrile in 0.1% phosphoric acid (buffer B). For separation of the metabolic products of ¹²⁵I-ANG(1–7), the column was eluted with a linear gradient of 10–30% of buffer B for 20 min at a flow rate of 0.35 ml/min. For separation of ¹²⁵I-ANG I metabolic products, the column was eluted with a linear gradient of 15–45% of buffer B for 25 min at a flow rate of 0.35 ml/min. HPLC fractions were collected at 1-min intervals and counted in a Cobra II Autogamma.
ANGIOTENSIN-(1–7) METABOLISM

(Packard, Meriden, CT) gamma counter. Products were identified by comparison with retention time of standard radioiodinated ANG peptides. ANG peptides were iodinated (125I-labeled sodium; NEN Life Sciences Products, Boston, MA) by chloramine T-sodium (10).

For the analysis of unlabeled ANG peptide metabolism, samples were fractionated on an Applied Biosystems HPLC (Foster City, CA) equipped with a 2.1-mm Nova-Pak C18 column and an Aquapore C8 guard column (10). Chromatographic separation was achieved with the solvent system described above, and the gradient consisted of an isocratic gradient of 15% of buffer B for 2 min, a linear gradient of 15–30% of buffer B for 15 min, and an isocratic gradient of 30% of buffer B for 10 min at a flow rate of 0.35 ml/min at ambient temperature.

Materials. ANG peptides were purchased from Bachem (Torrance, CA). Acetonitrile (Optima grade) was obtained from Fisher Scientific (Fair Lawn, NJ). Lisinopril, a converting enzyme (EC 3.4.15.1) inhibitor, was provided by Merck (West Point, PA). SCH-39370, a neprilysin (EC 3.4.24.11) inhibitor, was provided by Schering-Plough (Madison, NJ). Amastatin, bestatin, and all other reagents were obtained from Sigma.

Statistics. Differences in the generation of 125I-labeled peptides under various conditions were assessed by one-way ANOVA with Student-Newman-Keuls post hoc analysis. The statistical analysis was performed with GraphPad Prism and Stat Mate programs (GraphPad Software, San Diego, CA). The criterion for statistical significance was set at $P < 0.05$.

RESULTS

Pulmonary membranes. As shown in Fig. 1A, in rat pulmonary membranes, 125I-ANG-(1–7) was essentially hydrolyzed within 15 min. The primary metabolite of ANG-(1–7) eluted with a retention time corresponding to 125I-ANG-(3–5) and a minor peak identified as 125I-ANG-(3–5). Addition of the ACE inhibitor lisinopril (Lis) abolished the generation of 125I-ANG-(1–5) and substantially increased the peak of 125I-ANG-(1–7). At this time point, we observed little additional metabolism of 125I-ANG-(1–7) in the presence of the ACE inhibitor other than a small peak identified as 125I-ANG-(1–4).

In Fig. 1B, 125I-ANG-(1–5) was metabolized to 125I-ANG-(3–5) in pulmonary membranes under control conditions. Previous results using purified canine ACE demonstrated that ANG-(1–5) is not a substrate for this enzyme (10). Inhibition of neprilysin (SCH-39370) or ACE (Lis) did not alter the metabolism of ANG-(1–5) (data not shown). Addition of the aminopeptidase inhibitor amastatin reduced but did not completely prevent the degradation of 125I-ANG-(1–5) to 125I-ANG-(3–5). Preliminary characterization revealed that the chelating agents EDTA and EGTA (5 mM each) or the serine protease inhibitor phenylmethylsulfonyl fluoride (1 mM) did not attenuate production of 125I-ANG-(3–5), suggesting that the activity was not a serine or metallopeptidase.

To further characterize the hydrolysis of ANG-(1–7) by ACE, we purified both somatic and germinal forms of the enzyme and determined the kinetic constants. As shown in Fig. 2, somatic ACE isolated from pulmonary membranes exhibited a $K_m$ for ANG-(1–7) of 0.98 μM, a maximal velocity ($V_{max}$) of 0.37 μmol·min$^{-1}$·mg$^{-1}$, and a turnover constant ($K_{cat}$) of 1.10 s$^{-1}$. Germinal ACE, isolated from the testes, exhibited a $K_m$ of 1.20 μM for ANG-(1–7), a $V_{max}$ of 0.67 μmol·min$^{-1}$·mg$^{-1}$, and a $K_{cat}$ of 1.23 s$^{-1}$. Kinetic studies revealed similar kinetic constants for ANG-(1–7) with somatic and germinal ACE, suggesting involvement of the COOH-terminal domain in metabolism of ANG-(1–7). Shown in the SDS gel (Fig. 2, inset) are the purified preparations of both somatic ACE (170 kDa) and germinal ACE (110 kDa) lacking the NH$_2$-terminal active site domain.

In addition to ACE, a number of peptidase activities are localized to the pulmonary surface (37). To deter-
mine whether the pulmonary compartment contributes to generation of ANG-(1–7). 125I-ANG I was incubated in pulmonary membranes with and without Lis. As shown in Fig. 3, the major product of ANG I metabolism under control conditions was ANG II, with smaller peaks of ANG-(1–4) and ANG-(1–7). With the addition of Lis, ANG II production was greatly diminished, and the peak of ANG I is conserved. We observed no significant increase in production of ANG-(1–7) or ANG-(1–4). Furthermore, addition of the nephrilysin (Nep) inhibitor did not reduce the ANG-(1–4) or ANG-(1–7) peaks (data not shown). These results imply that the lung is not a site for ANG-(1–7) production, even under the conditions of ACE inhibition, but may be a site for metabolism of the peptide. Indeed, the assessment of enzyme activities in pulmonary membranes with synthetic substrates revealed that ACE is the most predominant peptidase compared with aminopeptidase or Nep activities (Fig. 3B).

Renal BBM. To assess the activities responsible for ANG-(1–7) metabolism in the kidney, we prepared a suspension of rat renal BBM from isolated renal cortex. Previous studies in porcine BBM have identified Nep as the primary enzyme contributing to the processing of ANG I to ANG-(1–7) (42). The radioligand 125I-ANG-(1–7) was incubated with BBM under control conditions and in the presence of enzyme inhibitors. Under control conditions, 125I-ANG-(1–7) was rapidly hydrolyzed to mono- and dipeptide fragments [tyrosine (Tyr) and valine-tyrosine (Val-Tyr)] that elute quite early in the chromatograph as a single peak (Fig. 4A). Because significant aminopeptidase activity is found along the brush border (45), amastatin was added to determine the contribution of aminopeptidase to the degradation of 125I-ANG-(1–7). Amastatin abolished formation of these fragments and revealed peaks corresponding to intact 125I-ANG-(1–7), 125I-ANG-(1–4), and 125I-ANG-(1–5). As shown in Fig. 4B, combined treatment with amastatin and SCH-39370 augmented the concentration of ANG-(1–7) while abolishing 125I-ANG-(1–4) formation. These conditions also revealed a small peak of ANG-(1–5) that appeared sensitive to the ACE inhibitor Lis (Fig. 4B). Although not shown, SCH-39370 alone did not significantly alter metabolism of 125I-ANG-(1–7) compared with control conditions. Additional studies examined the metabolism of unlabeled ANG-(1–7) in the BBM. In this case, amino acid analysis of the primary metabolite revealed its identity as ANG-(1–4); Nep inhibition abolished generation of ANG-(1–4) and attenuated ANG-(1–7) hydrolysis (data not shown).

Figure 5A illustrates the time course for the metabolism of 125I-ANG-(1–7) in BBM. Under control conditions, 125I-ANG-(1–7) is degraded with a t1/2 of 12.5 ± 0.75 min. Amastatin attenuated metabolism, prolonging the t1/2 of 125I-ANG-(1–7) to 24.7 ± 1.3 min (P < 0.01). However, the combination of amastatin and SCH-39370 essentially abolished the metabolism of 125I-ANG-(1–7). The addition of the ACE inhibitor Lis did not increase recovery of 125I-ANG-(1–7) compared with the combined inhibition of aminopeptidase and Nep. In contrast to the lung, ACE activity was substantially lower than that of leucine aminopeptidase or Nep in the brush border (Fig. 5B). These results confirm the lack of significant ACE activity in the BBM and the failure to observe a principal role for ACE in the metabolism of 125I-ANG-(1–7) in the tubular epithelium.

The metabolic products resulting from incubation of radiolabeled ANG I with BBM were also determined. In the absence of peptidase inhibitors, 125I-ANG I was metabolized to Tyr and Val-Tyr fragments as well as ANG-(1–4) and ANG-(1–7) (Fig. 6A). In the presence of amastatin, 125I-ANG I was metabolized primarily to 125I-ANG-(1–7) and 125I-ANG-(1–4) (Fig. 6B). Com-

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Fig. 2. Kinetic characterizations of ANG-(1–7) hydrolysis by rat somatic and germinal ACE. Varying concentrations of ANG-(1–7) (0.5–5 μM) were incubated with somatic (○) or germinal (●) ACE for 30 min at 37°C. The double reciprocal plot represents the mean of 2 separate experiments for each enzyme. Inset: silver-stained SDS-PAGE of affinity-purified rat pulmonary (somatic) and testicular (germinal) forms of ACE. Molecular mass is expressed in kDa. Km, Michaelis-Menten constant; Vmax, maximal velocity.

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Fig. 5B. Time course for the metabolism of 125I-ANG-(1–7) in rat renal BBM under control conditions (○), amastatin (●), and SCH-39370 (▲). The addition of amastatin and SCH-39370 prolonged the half-life of 125I-ANG-(1–7) to 24.7 ± 1.3 min (P < 0.01), with no significant recovery of 125I-ANG-(1–7) compared with control conditions. Additional studies examined the metabolism of unlabeled ANG-(1–7) in the BBM. In this case, amino acid analysis of the primary metabolite revealed its identity as ANG-(1–4); Nep inhibition abolished generation of ANG-(1–4) and attenuated ANG-(1–7) hydrolysis (data not shown).

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Fig. 6A. Identification of the primary metabolite of 125I-ANG-(1–7) in rat renal BBM. The addition of amastatin and SCH-39370 prolonged the half-life of 125I-ANG-(1–7) to 24.7 ± 1.3 min (P < 0.01), with no significant recovery of 125I-ANG-(1–7) compared with control conditions. Additional studies examined the metabolism of unlabeled ANG-(1–7) in the BBM. In this case, amino acid analysis of the primary metabolite revealed its identity as ANG-(1–4); Nep inhibition abolished generation of ANG-(1–4) and attenuated ANG-(1–7) hydrolysis (data not shown).

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Fig. 6B. Identification of the primary metabolite of 125I-ANG-(1–7) in rat renal BBM. The addition of amastatin and SCH-39370 prolonged the half-life of 125I-ANG-(1–7) to 24.7 ± 1.3 min (P < 0.01), with no significant recovery of 125I-ANG-(1–7) compared with control conditions. Additional studies examined the metabolism of unlabeled ANG-(1–7) in the BBM. In this case, amino acid analysis of the primary metabolite revealed its identity as ANG-(1–4); Nep inhibition abolished generation of ANG-(1–4) and attenuated ANG-(1–7) hydrolysis (data not shown).
combined treatment with amastatin and SCH-39370 increased the ANG I peak and diminished production of both ANG-(1-O7) and ANG-(1-O4). Although we also observed a minor peak corresponding to ANG II during combined peptidase inhibition, these data reveal the alternate Nep-dependent production of ANG-(1-O7) from ANG I.

Additional studies assessed the metabolism of unlabeled ANG I (50 μM) in the BBM preparation as determined by HPLC-ultraviolet (UV) analysis. As shown in Fig. 7A, the main metabolites of ANG I were identified as ANG-(1-O7) and ANG-(1-O4). Consistent with the findings for 125I-ANG I, the Nep inhibitor abolished production of both ANG-(1-O7) and ANG-(1-O4), effectively preserving 80–90% of ANG I (Fig. 7B). In this case, it is noteworthy that ANG II was not detected with the provision of high concentrations of ANG I either alone or with Nep inhibition. Because similar amounts of ANG I and ANG II have been found in the luminal fluid of rat proximal tubules (32), we further assessed the metabolism of ANG II in the presence of varying concentrations of ANG II in BBM (Fig. 8). Incubation of ANG II alone predominantly resulted in the formation of ANG-(1-O4) by Nep with no detectable levels of ANG-(1-O7) (data not shown). The coinubcation of ANG II and ANG I results in a greater quantity of ANG-(1-O4) compared with ANG I alone. Indeed, doubling the concentration of ANG II up to 400 μM yields an essentially linear increase in the production of ANG-(1-O4) (Fig. 8). However, even at the highest concentration of ANG II, the levels of ANG-(1-O7) were not diminished. These results suggest a high capacity for Nep to convert ANG I to ANG-(1-O7) in the brush border. Furthermore, ANG II may have a protective effect on ANG-(1-O7) by inhibiting further metabolism of the peptide to ANG-(1-O4).
DISCUSSION

ANG-(1–7) is generated directly from ANG I by at least three endopeptidases, including Nep, prolyl endopeptidase, and thimet oligopeptidase (7). The contribution of these enzymes to the formation of ANG-(1–7) is dependent on the tissue source or vascular compartment. For example, in the circulation, we and others have shown that ANG-(1–7) generation was primarily dependent on Nep (5, 26, 53), whereas prolyl endopeptidase activity formed ANG-(1–7) in isolated vascular endothelium (39, 50). Identification of alternate pathways for ANG-(1–7) metabolism is of relevance in explaining the antihypertensive effects of the peptide as well as the contribution of the peptide to the vasodilator effects of ACE inhibitors and the new class of vasopeptide inhibitors.

In keeping with previous findings, there was essentially complete conversion of ANG I to ANG II by pulmonary endothelial-bound ACE (38). Our results now show that the lung is a primary site for the metabolism of ANG-(1–7), because Lis inhibited ANG-(1–5) formation and attenuated the metabolism of ANG-(1–7). After conversion of ANG-(1–7) to ANG-(1–5) by ACE, the pentapeptide was further metabolized to ANG-(3–5) by amino- or dipeptidyl aminopeptidases. These data are consistent with previous studies demonstrating that ANG-(1–5) is not a substrate for ACE (10). Further evidence that the pulmonary bed contributes to the metabolism of ANG-(1–7) is the observation that even in the presence of ACE inhibition, the conversion of ANG I to ANG-(1–7) was a relatively minor pathway. In accordance with these results, measurement of ACE activity was overwhelmingly higher than Nep or leucine aminopeptidase in pulmonary membranes.

There are two isoforms of ACE: one form (somatic) is produced by the pulmonary vascular bed and other tissues, whereas the second form (germinal) resides exclusively in developing sperm cells of the adult testes (3, 40). The somatic form is a 170-kDa glycoprotein with two catalytically active sites (49). In comparison, the germinal form expresses only one active site corre-

Fig. 5. Time course for the metabolism of 125I-ANG-(1–7) in rat renal BBM. A: 125I-ANG-(1–7) was incubated with 1 μg of renal BBM at 37°C either alone (●) or with AM (○), AM and SCH-39370 (△), or the combination of AM, SCH-39370, and Lis (□). Data are means ± SE; n = 3. *P < 0.05 vs. control, **P < 0.01 vs. control, and #P < 0.01 vs. AM. B: comparison of AP, Nep, and ACE activity in BBM membranes (results are means ± SE; n = 5).

Fig. 6. Metabolism of 125I-ANG I by rat renal BBM. 125I-ANG I was incubated with 1 μg of BBM for 15 min at 37°C either alone (CON; ●), A) or with 1 μM AM (○) or with AM and 10 μM SCH-39370 (△) (B) and analyzed by HPLC. 125I-ANG I was rapidly hydrolyzed to ANG-(1–7), ANG-(1–4), and smaller metabolites. AM abolished the early eluting peak, and addition of SCH-39370 decreased production of both ANG-(1–7) and ANG-(1–4). The HPLC results are representative of 3 separate metabolism experiments.
We isolated both forms of the enzyme from rat to determine the extent to which somatic or germinal ACE hydrolyzed ANG-(1–7) to ANG-(1–5). The kinetic studies revealed essentially identical values for both somatic and germinal ACE: a $K_m$ for ANG-(1–7) of $<1 \mu M$ and a $K_{cat}/K_m$ of $1,100 \text{mM}^{-1}\cdot s^{-1}$. The similar kinetic values for germinal and somatic ACE suggest that the COOH-terminal active site primarily contributes to the hydrolysis of ANG-(1–7). In agreement with these findings, we showed previously that ANG-(1–7) hydrolysis by canine ACE was abolished by dinitrofluorobenzene. Moreover, the greater potency of Lis over captopril in inhibiting ANG-(1–7) metabolism implicated the COOH-domain as the active site involved in the degradation of the peptide (10). Andrade et al. (2), however, recently observed that an NH2-terminal fragment of somatic ACE from rat mesangial cells hydrolyzed ANG-(1–7) to a greater extent than the intact enzyme. In contrast to the present results, both recombinant forms of the NH2- and COOH-domains exhibited significantly higher activity than either the recombinant or mesangial somatic enzyme (2). Although human ACE also hydrolyzed ANG-(1–7) to ANG-(1–5) with kinetics comparable to both rat and canine, Deddish et al. (14) found that ANG-(1–7) was cleaved exclusively by the NH2-domain of human ACE. In their study, both the NH2-domain and somatic forms of human ACE exhibited identical kinetic values for ANG-(1–7) (14). Furthermore, because of the extremely slow turnover ($K_{cat} < 0.006 \text{ s}^{-1}$) of ANG-(1–7) at the COOH-domain, the peptide acted as a competitive inhibitor for COOH-domain hydrolysis (14). These data suggest that in addition to acetyl-Ser-Asp-Lys-Pro, ANG-(1–7) may be one of a few endogenous substrates exclusively hydrolyzed by the NH2-domain of human ACE. Corvol et al. (13) have noted that the NH2- and COOH-domains of ACE are not identical, exhibiting ~60% sequence similarity (13). Indeed, these investigators have recently identified a selective inhibitor for the NH2-terminal domain of human ACE (16). It is not known what functional significance is served by the selective hydrolysis of ANG-(1–7) by the NH2-domain of human ACE. However, two studies now show that ANG-(1–7) interacts with ACE to attenuate the desensitization of the bradykinin B2 receptor (14, 23). Roks et al. (35) demonstrated that ANG-(1–7) also reduces the ACE-dependent formation of ANG II in human mammary vessels albeit at high concentrations of the peptide (1–10 $\mu M$). These data may suggest a more complex relationship between ANG-(1–7) and ACE other than simply the hydrolysis of the peptide.

The kidney is another important target organ for the physiological actions of ANG-(1–7) (8, 15, 22, 47). ANG-(1–7) is found in significant concentrations in the renal tissue and in the urine from both rats and humans (8). In brush-border vesicles, preferential hydrolysis of ANG-(1–7) was mediated by aminopeptidases and the contribution of Nep after aminopeptidase blockade. The low levels of ACE activity in the

Fig. 7. Metabolism of ANG I by rat renal BBM. Unlabeled ANG I (50 $\mu M$) was incubated with 1 $\mu g$ of renal BBM for 30 min at 37°C under control conditions (A) or with the Nep inhibitor SCH-39370 (10 $\mu M$; B). The production of ANG-(1–7) and ANG-(1–4) was determined by HPLC-ultraviolet (UV) analysis at 220 nm, and the separation conditions are described in MATERIALS AND METHODS. Results are representative of 3 separate metabolism experiments.

Fig. 8. Metabolism of ANG I by rat renal BBM. Unlabeled ANG I (50 $\mu M$) was incubated with 1 $\mu g$ of renal BBM for 30 min at 37°C under control conditions or with ANG II at 50, 100, 200, or 400 $\mu M$. ANG-(1–7) and ANG-(1–4) were determined by HPLC-UV analysis. Data are means ± SE; n = 3.
brush border may account for our failure to observe any significant contribution of this enzyme to the metabolism of ANG-(1–7). Stephenson and Kenny (42) also noted the absence of ACE activity in contributing to the overall hydrolysis of ANG I or bradykinin in proximal tubules from pig. These investigators have described an additional metalloendopeptidase in the renal brush border of the rat, referred to as endopeptidase-2, or meprin (EC 3.4.24.18) (43). Meprin exhibits preference for peptide bonds flanked by hydrophobic residues and cleaves ANG I or ANG II to ANG-(1–4). However, the specific activity of meprin for ANG I and ANG II is 75- and 30-fold less than Nep, respectively; meprin is also resistant to Nep inhibitors such as phosphoramidon (43). Thus, to our knowledge, this is the first study to demonstrate that Nep has the capacity to hydrolyze the 4-tyrosine,5-isoleucine (Tyr⁴-Ile⁵) bond of ANG-(1–7) to ANG-(1–4) using either a radiolabeled or nonlabeled form of the heptapeptide. Our data may be of particular value in interpreting the metabolism of ANG I in BBM. We found that labeled or unlabeled ANG I was hydrolyzed primarily to ANG-(1–7) and ANG-(1–4); these results are identical to ANG I metabolism in brush-border vesicles from pig (42) as well as from purified preparations of renal Nep (20). Although the previous studies concluded that ANG-(1–4) was formed directly from ANG I, our present data suggest that ANG-(1–4) may arise, at least in part, from the continued hydrolysis of ANG-(1–7) by Nep. Indeed, coincubation of ANG II with ANG I did not significantly attenuate ANG-(1–7) levels in the brush border. In kinetic studies with human Nep, ANG I (Kₘ = 36 μM) exhibited a much higher Kₘ than ANG II (Kₘ = 280 μM) (20). If the Kₘ of ANG-(1–7) were comparable with that of ANG II, the addition of ANG II would compete for further hydrolysis of ANG-(1–7) to ANG-(1–4) by Nep. Moreover, the lower Kₘ of ANG I may allow for the formation of ANG-(1–7). Additional studies, however, are necessary to resolve this issue, particularly the kinetic analysis of ANG-(1–7) hydrolysis by Nep. Nevertheless, these studies revealed the existence of alternate pathways for ANG-(1–7) metabolism with activities depending on tissue, compartment, and concentration-dependent specificity.

Alternate pathways for ANG-(1–7) metabolism in the kidney may be of importance in view of the recent interest in Nep or “mixed” Nep/ACE inhibitors as therapies for hypertension and congestive heart failure (1, 36). Infusion of a Nep inhibitor causes diuresis and natriuresis and, in some hypertensive models, a significant reduction in mean arterial blood pressure. The renal actions of Nep agents are typically attributed to the preservation of either bradykinin (46) or the natriuretic peptides (36). However, protection of ANG-(1–7) from Nep hydrolysis could yield similar or additional actions, as ANG-(1–7) exhibits both natriuretic and diuretic properties when infused in the rat kidney (15, 22, 47) and has vasodilatory actions in the renal afferent arterioles (33). If ANG-(1–7) contributes to the actions of Nep inhibition, other endopeptidases or carboxypeptidases must be involved in the renal formation of the heptapeptide. Although the present studies and those of others (42) do not support a Nep-independent pathway in the brush border of proximal tubules, recent evidence suggests that the tubular fluid contains other enzymes capable of catalyzing the formation of ANG-(1–7) from ANG I or ANG II (6, 45). In view of the contribution of aminopeptidase activity to ANG-(1–7) hydrolysis in the BBM, the inhibition of both Nep and aminopeptidases may provide additional effects in the kidney. In this regard, functional studies with the ANG-(1–7) antagonist D-[Ala⁷]ANG-(1–7) are important for the assessment of whether ANG-(1–7) contributes to the renal actions of combined inhibitor treatments.

In conclusion, the present study elaborates on the differential pathways in the metabolism of ANG-(1–7). The intrarenal metabolism of ANG-(1–7) to ANG-(1–4) and smaller peptide fragments was catalyzed through aminopeptidase and Nep activities. ANG I was metabolized primarily to ANG-(1–7) and ANG-(1–4); Nep and aminopeptidase inhibition did not reveal formation of ANG II. These results contrast to that of ANG metabolism in the pulmonary membrane fraction, where ANG I was rapidly converted to ANG II, and, in the presence ACE inhibition, there was no appreciable formation of ANG-(1–7). However, ACE was the primary enzyme responsible for the hydrolysis of ANG-(1–7) to ANG-(1–5). These studies emphasize that the diversity in the formation of active ANG peptides is influenced by the differential enzyme activities that contribute to local renin-ANG systems.

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