Characterization of angiotensin IV-degrading enzymes and receptors on rat mesangial cells

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Chansel, Dominique, Stanislas Czekalski, Sophie Vandermeersch, Emmanual Ruffet, Marie-Claude Fournie-Zaluski, and Raymond Ardaillou. Characterization of angiotensin IV-degrading enzymes and receptors on rat mesangial cells. Am. J. Physiol. 275 (Renal Physiol. 44): F535–F542, 1998.—Because mesangial cells (MC) are a target and a degradation site for angiotensin II (ANG II), we characterized the degrading enzymes and receptors of ANG IV, a metabolite of ANG II, on these cells. ANG IV was metabolized into its NH2-terminal deleted peptides, ANG II-(4–8), ANG II-(5–8), and ANG II-(6–8) by rat MC. Total protection of ANG IV was obtained only when PC-18, a specific aminopeptidase N (APN) inhibitor, and J FH-27A, a mixed inhibitor of dipeptidylaminopeptidase (DAP) and neutral endopeptidase (NEP), were simultaneously added. In contrast, thiorphan, an NEP inhibitor, was inactive. These results demonstrate the exclusive role of APN and DAP in ANG IV degradation.125I-labeled ANG IV binding was studied in the presence of PC-18 and J FH-27A to suppress ligand degradation. Under these conditions, ANG IV-specific receptors could be demonstrated with a KD of 1.8 nM and a density of 55 fmol/mg. In contrast with MC, no evidence for ANG IV receptors could be obtained in freshly isolated glomeruli. ANG IV stimulated cytosolic calcium concentration in MC, whereas its NH2-terminal deleted metabolites were inactive. Therefore, ANG IV must be protected from degradation by APN and DAP in studies on the nonimmediate biological effects of this peptide.

Methods

Materials. Reagents for these studies were obtained as follows: 125I-labeled ANG IV (74 TBq/mmol) was from the Radiochemical Centre (Amersham, UK); [3H]Leu-enkephalin (1,430 GBq/mmol) was from Isotopchim (Ganogobie-Peyruis, France); culture media, antibiotics, and cell culture supplies were from GIBCO (Paisley, UK); FCS was from Boehringer (Mannheim, Germany); ionomycin and the acetoxymethyl ester of fura 2 were from Calbiochem (San Diego, CA); ANG II-(3–8) (also referred to as ANG IV) and ANG II-(4–8) were from Peninsula (London, UK); and purified APN (25 U/mg), thiorphan, and captopril were from Sigma (St. Louis, MO). ANG II-(5–8), ANG II-(6–8), and ANG II-(3–7) were obtained in the laboratory by solid-phase peptide synthesis. Losartan, and its metabolite, EXP-3174, two nonpeptide AT1 antagonists, were donated by Merck, Sharp and Dohme Research

a) angiotensin IV-degrading enzymes and receptors on rat mesangial cells.

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c) ANG IV must be protected from degradation by APN and DAP in studies on the nonimmediate biological effects of this peptide.

d) Specific receptors for ANG IV were then characterized in the presence of inhibitors of these two enzymes, and the stimulatory effect of ANG IV, but not of its metabolites, on cytosolic calcium ([Ca2+]i) was demonstrated.
Laboratories (West Point, PA); CV-11974, another AT 1 antagonist, and PD-123177, an AT 2 antagonist, were donated by Takeda (Tokyo, Japan) and Parke-Davis (Ann Arbor, MI), respectively. PC-18, a specific inhibitor of APN, and JF-27A, an inhibitor of both DAP and neutral endopeptidase (NEP), were synthesized according to previously published techniques (7, 14).

Isolation of rat glomeruli and rat mesangial cell culture. Primary cultures of mesangial cells were obtained from collagenase-treated glomeruli as previously described (13). Kidneys were removed under pentobarbital anesthesia from 100- to 150-g male Sprague-Dawley rats, and glomeruli were isolated by sieving techniques and centrifugation. Collagenase-treated glomeruli were seeded in plastic flasks of 25 cm 2 in the presence of 5 ml of RPMI-1640 medium buffered with 20 mM Hepes (pH 7.4) and supplemented with 10% FCS, 50 U/ml penicillin G, 50 µg/ml streptomycin sulfate, and 2 mM glutamine. Culture medium was changed every 2 days. Mesangial cells began to grow from glomeruli after 7–8 days. These cells, stellate or fusiform in shape by phase-contrast microscopy, were subcultured at day 21. Confluent cells in the second subculture were studied in all experiments. They exhibited typical morphological and biochemical features of mesangial cells (13).

HPLC analysis. After a preincubation of 15 min with or without enzyme inhibitors, ANG IV was incubated during 20 min at 22°C under control conditions or in the presence of rat mesangial cells. Supernatants were collected, and samples (100 µl) were automatically injected (model SII-10A; Shimadzu, Kyoto, Japan) on a Vydac-C8 column (250 × 4.6 mm; particle size, 5 µm). The samples were analyzed using a linear gradient with two solvents: 0.05% trifluoroacetic acid (solvent A) and 0.038% trifluoroacetic acid-90% acetonitrile (solvent B). The program was as follows: 0–30 min, 15–30% solvent B at a flow rate of 0.8 ml/min. The cleavage products formed during the incubation were detected at 214 nm. The major metabolites were identified by comparison with synthetic markers. Their relative amounts were calculated by integration of peak areas and were expressed as percentages of the sum of their areas.

APN and DAP assay. APN activity was measured on intact cells as previously described (26, 27). Cells were suspended in 0.5 ml of Ca 2+ -free phosphate-buffered saline supplemented with 1 mM MgCl 2 . The enzymatic reaction was started by addition of 1 mM alanine p-nitroanilide as substrate. Incubation was carried out with gentle agitation for 5–20 min under zero-order kinetic conditions. The amount of substrate, mesangial cells were preincubated with 10 µM PC-18 and 0.1 µM thiopran in 200 µl of buffer to inhibit APN and NEP activity, respectively. The enzymatic reaction was initiated by addition of 2 pmol of [ 3 H]Leu-enkephalin. After 30 min of incubation, the reaction was terminated by heating 3 min at 80°C. The product of hydrolysis was separated by chromatography using a C 18 Sep-Pak column (Waters) initially washed successively with 10 ml ethanol and 10 ml water. [ 3 H]-labeled Tyr-Gly was eluted three times with 2 ml water, which was collected in a liquid scintillation vial. [ 3 H] radioactivity was measured in a liquid scintillator with a 60% retention times with those of synthetic markers (Fig. 1A). We verified the absence of degradation of ANG IV when incubated alone in the incubation medium (Fig.

RESULTS

ANG IV hydrolysis study. Incubation of 2 µM of ANG IV with rat mesangial cells for 20 min at 22°C resulted in the marked degradation of the peptide (Fig. 1C). Only 32 ± 5% (n = 6) of ANG IV persisted at the end of the incubation. The degradation products were only the NH 2 -terminal peptides. ANG II-(5–8) was the most important (45 ± 5%), suggesting the role of a DAP in its formation. ANG II-(4–8) was also detected (8 ± 3%), indicating the presence of APN. The third product identified was ANG II-(6–8) (15 ± 3%). Identification of these peaks was made possible by comparison of their retention times with those of synthetic markers (Fig. 1A). We verified the absence of degradation of ANG IV when incubated alone in the incubation medium (Fig.

Other assays. cAMP, cGMP, and inositol phosphate production by confluent rat mesangial cells were also measured according to techniques already described (9).

Binding studies. Isolated glomeruli in suspension in phosphate-buffered saline or confluent mesangial cells in 12-well plates that had been deprived of serum during 24 h were studied after having been rinsed three times with 0.15 M NaCl. Then, they were incubated at 22°C for 45 min with 125 I-ANG IV (0.25 nm) in 500 µl of buffer (335 mM NaCl, 20 mM Tris, 5 mM glucose, 10 mM KCl, 10 mM NaCH 3 COO, and 0.2% bovine serum albumin; pH 7.4) supplemented with 10 µM PC-18, an APN inhibitor, and 1 µM JF-27A, a DAP inhibitor. At the end of the incubation period, the medium was removed. The glomeruli were retained on a cellulosic filter after filtration under vacuum, and the cells were rinsed three times with 2 ml of ice-cold 0.15 M NaCl. The cells were then dissolved in 1 M NaOH and 125 I radioactivity was counted using an LKB gamma counter (Malmo, Sweden) with 60% counting efficiency. Kinetic studies, competitive binding experiments, and saturation binding experiments were performed. Nonspecific binding was measured in the presence of 1 µM unlabeled ligand, and specific binding was calculated as the difference between total and nonspecific binding. It was expressed as femtomoles of 125 I-ANG IV bound permilligram of protein. Cell proteins were measured using the Bradford technique (4).

The effect of ANG IV (1–100 nM) on 125 I-[Sar 1 ,Ala 8 ]ANG II binding to isolated glomeruli was also examined. Binding studies were performed according to a previously published technique (6).

Cytosolic free calcium determination. Cells were cultured on thin glass microscope coverslips precoated with 0.2% gelatin and were studied at subconfluence. Cells were loaded with 1.5 µM fura-2 AM at 37°C for 90 min. For measurements of fluorescence, each coverslip was placed on the stage of the inverted microscope, and one cell was selected. The superfusion was then superfused at a rate of 0.6 ml/min at 37°C with basal medium or with the solution to be tested. Fura 2 was alternatively excited at wavelengths of 340 and 380 nm using a 75-W xenon light source, filters, and a chopper (PTI Phototronics II System; Kontron). The fluorescence intensities (S at 340 nm and L at 380 nm) were calculated from the equation:

\[
\text{S} = K_{d} R_{min} / (R_{max} - R) L_{max} /	ext{L}_{min}
\]

where \( K_{d} \) = 244 nm, \( R_{min} \), \( R_{max} \), and \( R_{max} - R \) are the fluorescence values at 0 and saturating concentrations of calcium, respectively. \( L_{max} \), \( L_{min} \), \( R_{min} \), and \( R_{max} \) were determined by external calibration as previously described (29).

\[ d \]
Further experiments were designed to study the protective effect of two enzyme inhibitors, PC-18 and JFH-27A. PC-18 is a recently designed β-aminothiol that exhibits a high affinity (10 nM) for APN that is 100-fold better than for APA (7). Addition of 10 µM PC-18 (Fig. 1D) partially protected ANG IV from degradation. The peak of the peptide was 55 ± 8% at the end of incubation. Only one degradation product, ANG II-(5–8), was apparent and represented the remaining 45 ± 10%. This suggested the presence of a DAP activity that was still active after addition of PC-18, an inhibitor with a strict specificity for APN. JFH-27A is a hydroxamate dipeptide containing a retroamide bond with metal-chelating properties. It exhibits a high affinity for both NEP (K_i = 0.37 nM) and a membrane-bound zinc DAP (K_i = 0.8 nM) but not for APN (K_i = 510 nM) (14). Addition of 1 µM JFH-27A (Fig. 1E) only slightly inhibited ANG IV degradation with persistence of 62 ± 8% of the ANG IV peak. The peaks of the three degradation products were still apparent. Of note, ANG II-(5–8) was in smaller amount (18 ± 5%) than in control (45 ± 5%), thus confirming that NEP and converting enzyme were not implicated in ANG IV degradation. We also verified that ANG IV was degraded in the presence of a commercial source of APN. There was a two-thirds reduction of the ANG IV peak after 5-min incubation at 37°C with appearance of the NH_2-terminal deleted peptides, with the most abundant being ANG II-(4–8). Addition of PC-18 (10 µM) completely suppressed ANG IV hydrolysis (data not shown).

APN and DAP activities on rat mesangial cells and freshly isolated rat glomeruli. APN activity was present on mesangial cells with a mean value of 6.5 ± 0.9 nmol·min^{-1}·mg^{-1} (n = 10). It was inhibited by PC-18 in a concentration-dependent manner (Fig. 3). At the maximum concentration studied (10 µM), only 10% of basal APN activity persisted. Fifty percent inhibition was obtained with 20 nM PC-18 in agreement with the previously reported K_i of this agent for APN (14). DAP activity was also present at the surface of rat mesangial cells. Its mean value was 0.16 ± 0.011 pmol·min^{-1}·mg^{-1} (n = 5). Sixty percent of this activity was inhibited by 10 µM JFH-27A (Fig. 4). To verify whether APN and DAP activities also exist on glomeruli in vivo, we repeated the preceding assays with rat isolated glomeruli. APN activity was present on mesangial cells with a mean value of 83 ± 12 nmol·min^{-1}·mg^{-1} (n = 4). Ninety-four percent were inhibited in the presence of 10 µM PC-18. DAP activity amounted to 0.04 ± 0.01 pmol·min^{-1}·mg^{-1} (n = 4) with 75% inhibition by 10 µM JFH-27A.
Binding studies of $^{125}$I-ANG IV on intact rat mesangial cells and freshly isolated rat glomeruli in the presence of enzyme inhibitors. Saturation binding experiments were performed in the presence of 10 µM PC-18 and 1 µM JFH-27A to protect the ligand from degradation by the mesangial cell ectoenzymes. Under these conditions, the amount of $^{125}$I-ANG IV bound increased progressively as a function of $^{125}$I-ANG IV concentration and reached a plateau within 1–1.5 nM. Nonspecific binding did not exceed 20% of total binding at equilibrium. The Scatchard transformation of the data provided a straight line, suggesting a single class of receptors (Fig. 5). The $K_D$ and the $B_{max}$ derived from three such Scatchard analyses were $1.8 \pm 0.4$ nM and $55 \pm 6$ fmol/mg protein (30,000 sites/cell), respectively.

We verified that $^{125}$I-ANG IV bound was not displaced after addition of losartan, EXP-3174, or CV-11974, three AT₁ antagonists, or of PD-123177, an AT₂ antagonist. As expected, unlabeled ANG IV was a potent competitor. Fifty percent of $^{125}$I-ANG IV displacement was obtained at a concentration of 50 nM (Fig. 6). No specific binding of $^{125}$I-ANG IV could be detected on freshly isolated rat glomeruli within a large range of concentrations (50 pM to 2 nM), both with and without addition of PC-18 and J FH-27A.

We also examined the effect of ANG IV on $^{125}$I-[Sar¹,Ala⁸]ANG II binding. There was no competitive inhibition of binding within a large range of concentra-
ANG IV (1–100 nM). Fifty-four percent inhibition of binding was observed in the presence of 1 µM ANG IV (data not shown).

Biological effects of ANG IV on rat mesangial cells. ANG IV (100 nM) did not modify cAMP and cGMP production. It also did not modify inositol phosphate formation. The only biological effect observed was the ANG IV-stimulated \([Ca^{2+}]_i\) (Fig. 7). We confirmed that ANG II (100 nM) produced an immediate and sharp \([Ca^{2+}]_i\) peak. \([Ca^{2+}]_i\) reached 199 ± 13 nM (n = 10), corresponding to an increase above baseline of 72 ± 10 nM. Subsequent exposure of the cells to 100 nM ANG IV produced a peak of the same shape and magnitude, thus confirming that ANG II pretreatment did not result in desensitization of the ANG IV effect. Addition of 10 mM EDTA to the incubation medium suppressed the stimulatory effect of ANG IV. It was also verified that ANG II-(4–8) and ANG II-(3–8) had no stimulatory effect on \([Ca^{2+}]_i\) (data not shown).

DISCUSSION

Three types of observations have established that ANG IV is an active fragment of ANG II. First, specific binding sites for \(^{125}\)I-ANG IV distinct from AT1 and AT2 receptors have been found in different tissues and particularly in the kidney (9, 11, 12). Second, ANG IV has specific effects that differ from those of ANG II and ANG III. For example, ANG IV causes an increase in renal blood flow, whereas ANG II is vasoconstrictory (19). Third, some effects of ANG II such as the induction of type 1 inhibitor of plasminogen (PAI-1) are suppressed in the presence of an aminopeptidase inhibitor, suggesting that they depend on ANG II fragments (20). The mechanism of signal transduction of these ANG IV receptors is still unknown. Similarly, the degradation pathways of ANG IV have not yet been examined.
ANGiotensin IV and Mesangial cells

Despite the great interest to know which enzyme(s) must be inhibited to increase the availability of the peptide. The results observed in the present study demonstrate for the first time that APN and DAP are the two main enzymes degrading ANG IV in rat mesangial cells. With respect to APN, these conclusions are based on three lines of evidence: 1) the formation of ANG II-(4–8), the NH2-deleted degradation product of ANG IV, when ANG IV was incubated in the presence of rat mesangial cells; 2) the hydrolysis of ANG IV by purified APN; and 3) the fact that PC-18 suppressed the formation of ANG II-(4–8) in the presence of rat mesangial cells. Concerning DAP, there are also several reasons making it likely that this enzyme is involved: 1) the formation of ANG II-(5–8), the degradation product obtained by deletion of the two NH2-terminal amino acids when ANG IV was exposed to rat mesangial cells in the presence of PC-18 blocking APN activity; 2) the fact that JF-27A, a mixed inhibitor of NEP and DAP, inhibited the formation of ANG II-(5–8), when ANG IV was exposed to rat mesangial cells, whereas thiorphan, a specific NEP inhibitor, was inactive. A total protection of ANG IV was provided by the simultaneous addition of PC-18 and JF-27A, demonstrating the exclusive role of APN and DAP. Since APN is widely distributed and DAP is also present in the brain (8), it is highly likely that both enzymes are the main degrading enzymes of ANG IV in most preparations.

Confirmation of the presence of APN and DAP on rat mesangial cells was obtained from the assay of these enzyme activities using appropriate synthetic substrates and mesangial cells as the source of enzyme. Both activities could be detected with values (6.5 nmol·min⁻¹·mg⁻¹ and 0.16 pmol·min⁻¹·mg⁻¹, respectively) in the range of those found in other preparations. For example, APN activity has been reported equal to 2.5 ± 0.06 and 1.5 ± 0.01 nmol·min⁻¹·mg⁻¹ in human glomerular epithelial and mesangial cells, respectively (26, 27). DAP activity was 0.2 pmol·min⁻¹·mg⁻¹ in a homogenate of porcine brain (8). It was also possible to detect APN and DAP activities on isolated rat glomeruli, thus suggesting that their presence on mesangial cells did not result from a phenotypic change due to the culture (results not shown).

The two enzymes involved in ANG IV degradation are metallopeptases, which explains the high degree of protection of ANG IV by EDTA in the presence of rat mesangial cells. APN is a zinc metalloptase belonging to the thermolysin-like enzyme group. It has been cloned and corresponds to a glycoprotein of 110 kDa in the rat (23). APN exhibits a broad specificity for peptides with a NH2-terminal neutral or basic amino acid including alanine, arginine, and leucine. Its main physiological substrates are enkephalins and ANG III (1, 25). The selective ability of APN to metabolize ANG III has also been demonstrated by Kugler (21) using kidney homogenates. In this study, ANG III competitively inhibited the activity of APN with a K, of 3 µM, whereas ANG II was marginally effective as a noncompetitive inhibitor. ANG IV degradation has not been previously studied in detail. Bestatin, an aminopeptidase inhibitor, has been added to the incubation medium in 125I-ANG IV binding experiments in most of the published studies (9, 18, 28) to block the degradation of the labeled peptide. Formation of ANG II-(5–8), when ANG IV was incubated with rat mesangial cells in the presence of PC-18 suggested the implication of a DAP, in addition to APN, in ANG IV degradation. In various mammalian tissues, four types of DAP activities have been described according to the dipeptide moieties liberated from the NH2 terminus of various peptides (8). For example, DPP IV is a post-proline DAP, i.e., an enzyme whose substrate requirements are a free amino terminus and a penultimate proline residue. Although splitting off Val-Tyr instead of Tyr-Gly, the DAP present on rat mesangial cells exhibits properties that are close to those of the DAP previously purified from porcine brain, since activities of both enzymes are blocked by chelating agents. Moreover, JF-27A, a hydroxamate dipeptide containing a retro amide bond that has been characterized as a specific inhibitor of NEP and porcine brain DAP (14), also inhibits rat mesangial cell DAP. This DAP acts in the brain as an enkephalin-degrading enzyme (8). Our results suggest it could be also implicated in the metabolism of ANG IV, which exerts a variety of functions in the brain including control of learning, memory, and exploratory behavior (32) as well as vasodilation (19). The implications of enzymes other than aminopeptidases had been already suggested by Hall et al. (16), who reported that 48% of 125I-ANG IV was degraded in the presence of bovine vascular smooth muscle cells after a 120-min incubation period, despite the addition of bestatin.

To study 125I-ANG IV binding under appropriate conditions that would allow the nondegradation of the ligand in the incubation medium, we performed the binding studies in the presence of 10 µM PC-18 and 1 µM JF-27A. Specific receptors for ANG IV could be demonstrated with an affinity of 1.8 nM, very close to that which we previously observed in collecting duct cell membranes (9). Nonspecific binding did not exceed 20% of total binding, and ANG IV binding sites did not recognize AT1 (losartan, EXP-3174, and CV-11974) and AT2 (PD-123177) antagonists. These various characteristics of ANG IV receptors on rat mesangial cells resemble those already described in other preparations (9, 16, 28). The demonstration of an ANG IV-dependent increase of [Ca2+]i suggests that ANG IV receptors were implicated in this effect. ANG IV was as potent as ANG II in stimulating intracellular calcium. No cross-desensitization was observed, and intact ANG IV was needed to obtain [Ca2+]i stimulation. Moreover, the finding that ANG IV does not inhibit 125I-[Sar1,Ala8]ANG II binding to rat mesangial cells at concentrations stimulating [Ca2+]i, demonstrates that this effect of ANG IV was not mediated by the AT1 receptors. Such a stimulatory effect of ANG IV on [Ca2+]i has been already described by Dostal et al. (10) in vascular smooth muscle cells and by Dulin et al. (12) in opossum kidney cells. This observation suggests that ANG IV could produce cell contraction and promote mitogenesis.
via $\text{Ca}^{2+}$. The latter effect was previously demonstrated by Wang et al. (31) in cultured rabbit cardiofibroblasts.

$^{129}$-ANG IV did not specifically bind to freshly isolated rat glomeruli over a large range of concentrations, suggesting the absence of glomerular ANG IV receptors in vivo. This is in agreement with the recent report by Handa et al. (17), who were unable to detect any specific ANG IV binding sites on glomeruli with the autoradiographic method. Taken together, these results raise the question of the significance of the binding sites detected on cultured mesangial cells. Two hypotheses may be raised. 1) Specific ANG IV binding sites on mesangial cells represent a new phenotypic characteristic appearing under culture conditions and possibly in vivo under conditions of pathological induction that we did not examine in the present study. 2) These binding sites exist normally in vivo, but their small number and their localization deep within the glomerulus make them difficult to detect. Of note, the absence of ANG IV receptors on glomeruli was not associated with the absence of ANG IV-degrading enzymes, which were found both on isolated glomeruli and cultured mesangial cells.

In conclusion, this study demonstrates that mesangial cells in vitro are both the target and the site of degradation of ANG IV. Two enzymes, APN and DAP, are involved in the degradation process and can be inhibited by appropriate agents, PC-18 and J FH-27A, respectively. The receptor sites for ANG IV are only sensitive to this peptide but not to its fragments, and ANG IV receptor interaction results in $\text{Ca}^{2+}$ stimulation. Association of PC-18 and J FH-27A entirely protects ANG IV from degradation by cell ectoenzymes and is thus required to study ANG IV receptors in intact cells. Moreover, the effects of ANG IV, whenever they are not immediate, should be potentiated by adequate enzyme inhibition.

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