Characterization of neutral endopeptidase 24.11 in dog glomeruli

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Neutral endopeptidase (NEP; also known as neprilysin and enkephalinase; EC 3.4.24.11) is a cell-surface metallopeptidase that is present in many mammalian tissues. It is particularly abundant on the brush-border membranes of the kidney proximal tubule. In this paper, the presence of NEP in purified glomeruli from dog kidney was assessed by measuring phosphoramidon- and thiorphan-sensitive [D-Ala²,Leu⁵]enkephalin-degrading activity. Using this assay, the K_m and k_{cat} of the glomerular enzyme were found to be identical to those of the tubular enzyme. By Western blotting the apparent M_r of the glomerular enzyme was found to be 104 000, compared with 94 000 for the tubular enzyme. This might be due to a different glycosylation pattern, since endoglycosidase F treatment of NEP obtained from both

tissues yielded deglycosylated enzymes with similar electrophoretic mobilities. The glomerular enzyme also appears to be membrane-bound, since it was retained in the detergent-rich phase after phase separation with Triton X-114. Autoradiography experiments performed with RB104, a new highly selective and potent NEP inhibitor, showed that NEP was expressed in both glomeruli and proximal tubules. The presence in glomeruli of NEP and some other brush-border peptidases (dipeptidyl-dipeptidase IV, aminopeptidase N and angiotensin I-converting enzyme) suggests that cell-surface peptidases might play an important role as regulators of plasma-derived peptides in this part of the nephron.

INTRODUCTION

Neutral endopeptidase (NEP; also known as neprilysin and enkephalinase; EC 3.4.24.11) is a cell-surface endopeptidase with an extracellular active site. The enzyme was first isolated from the brush-border membranes of the kidney (Kerr and Kenny, 1974) and was subsequently found in a wide range of tissues, including the central nervous system (Malfroy et al., 1978; Matsas et al., 1983; Erdős and Skidgel, 1989). More recently, the common acute lymphoblastic leukaemia antigen (CALLA, or CD10) has been found to be identical with human NEP (Letarte et al., 1988; Shipp et al., 1989). As an endopeptidase, NEP plays an important role in initiating the degradation of several active peptides which might be further degraded by exopeptidases such as aminopeptidases or carboxypeptidases. In the mammalian brain the enzyme is believed to play a key role in the catabolism of Met- and Leu-enkephalins (Malfroy et al., 1978; Matsas et al., 1983) as well as of substance P (Almenoff et al., 1981). Accordingly, several potent inhibitors of this peptidase have been examined in clinical trials as possible analgaesic agents (Roques and Beaumont, 1990).

The role of NEP at other locations is, however, less well understood. In the kidney, NEP appears to be involved in the degradation of small active peptides such as atrial natriuretic factor (ANF) (Sonnenberg et al., 1988; Margulies et al., 1990; Chiu et al., 1991) and bradykinin (Erdős and Skidgel, 1989). It had been clearly demonstrated that substantial renal degradation of ANF can take place rapidly (Condra et al., 1988) and that the ANF Cys⁷–Phe⁸ bond is the major cleavage site of NEP in that

organ (Olins et al., 1987; Stephenson and Kenny, 1987). This cleavage inactivates ANF, since the intact ring structure (Cys⁷–Cys²³) is essential for its biological activity (Currie et al., 1984; Garcia et al., 1985).

Enzymic degradation is not the only mechanism involved in ANF inactivation. Several studies indicate that another mechanism, involving a receptor-mediated clearance process, operates in the renal inactivation of ANF (Hirata et al., 1985; Maack et al., 1987; Almeida et al., 1989). High densities of clearance receptors and functional natriuretic-peptide receptors coupled to guanylate cyclase have been identified in the glomeruli (De Léan et al., 1985; Huang et al., 1986; Wilcox et al., 1991). Lower concentrations of ANF receptors are also present in basolateral tubular membranes (Hori et al., 1985), but no sites of ANF action have been clearly identified along the intraluminal membrane of the nephron. By contrast, NEP has been described so far as a typical brush-border enzyme. The presence of NEP in the apical brush-border membrane might thus be irrelevant to the termination of the effects of ANF in the kidney.

The aim of the present study was to characterize NEP activity in isolated dog glomeruli and to attempt the first biochemical characterization of the glomerular enzyme. We report that NEP is present in dog kidney glomeruli, and that the brush-border and glomerular enzymes have similar enzymic properties. Since glomeruli also contain high levels of ANF receptors, the presence of NEP in this part of the nephron might be more relevant to the rapid inactivation of the endogenous ANF *in vivo* and could account for the pharmacological effects of NEP inhibitors on diuresis and natriuresis.

Abbreviations used: NEP, neutral endopeptidase (neprilysin, endopeptidase 24.11; EC 3.4.24.11); ANF, atrial natriuretic factor; CALLA, common acute lymphoblastic leukaemia antigen; PEPCK, phosphoenolpyruvate carboxykinase; TBS, Tris-buffered saline; TFA, trifluoroacetic acid; RP-h.p.l.c., reverse-phase h.p.l.c.; mAb, monoclonal antibody; HACBO-Gly, (3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl)glycine; DPPIV, dipeptidyl-dipeptidase IV; APN, aminopeptidase N; ACE, angiotensin I-converting enzyme.

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MATERIALS AND METHODS

Isolation of glomeruli and proximal tubules

Glomeruli and proximal tubules were isolated from normal dogs as previously described (Tejedor et al., 1988). Phosphoenolpyruvate carboxykinase (PEPCK) activity, which is found exclusively in proximal tubules, was used as a marker to detect tubular contamination in glomeruli preparations. PEPCK activity was measured as described by Seubert and Huth (1965) and modified by Pogson and Smith (1975). Contamination was consistently less than 3%.

Protein preparation

Glomerular and proximal tubular fractions were solubilized with 1% (w/v) *n*-octyl β -D-glucoside in 50 mM Tris-buffered saline (TBS) (1 mg of protein/ml) for 1 h at 4 °C (Aubry et al., 1987), and then centrifuged at 10000 *g* for 30 min at 4 °C and stored at -20 °C until used.

Plasma membrane preparation

Glomerular and proximal tubular fractions were homogenized at 4 °C with a Polytron homogenizer (Kinematica, Lucern, Switzerland) in 5 ml of 5 mM Tris/HCl, pH 7.5. Homogenates were diluted 5-fold and layered over 10 ml of a 41% (w/v) solution of sucrose diluted in the homogenization buffer, and then centrifuged at 95000 *g* for 1 h at 4 °C in a Beckman SW 27 rotor (Bawab et al., 1992). The cytosolic fractions and the white interfacial band of plasma membranes were collected separately. The plasma membranes were pelleted by centrifugation at 95000 *g* for 30 min at 4 °C. Membranes were solubilized as described above.

Enzymic assays

The enzymic activity of NEP was assayed by measuring degradation of the tritiated substrate [*tyrosyl*-3,5-³H][*p*-Ala²,Leu⁵]-enkephalin (50 Ci/mmol; Commissariat à l'Energie Atomique, Gif-Sur-Yvette, France). Enzymic assays were carried out as previously described (Lemay et al., 1990). The *K_m* values were determined by the isotopic dilution method with unlabelled substrate (Peninsula Laboratories). In order to calculate the *k_{cat}* values, the amount of enzyme was quantified by Western blot analysis using purified rabbit kidney NEP as a standard (Le Moual et al., 1991). The effects of thiorphan [*N*-(DL-2-benzyl-3-mercaptopropionyl)glycine] (Roques et al., 1980) and phosphoramidon [*N*-(α -rhamnopyranosyloxy-hydroxyphosphinyl)-L-leucyl-L-tryptophan] (Sigma Chemical Co.) were determined by serial dilution of the inhibitor in enzymic assays at a substrate concentration of 50 nM. Under these conditions the *K_i* values corresponded to the IC₅₀ values, since the concentration of substrate was well below its *K_m*. IC₅₀ values were calculated using the Enzfitter Program (Elsevier-Biosoft, Cambridge, U.K.).

Enzymic determinations were performed also for the following brush-border hydrolases: dipeptidyl-dipeptidase IV (DPPIV), aminopeptidase N (APN) and angiotensin I-converting enzyme (ACE). The enzymic assays for DPPIV were as previously described (Jumarié and Malo, 1991). APN activity was determined using the same conditions as described for NEP, except that we used a non-protected substrate, [*tyrosyl*-3,5-³H][Leu⁵]-enkephalin (New England Nuclear, Boston, MA, U.S.A.). APN activity was then evaluated in the presence of phosphoramidon (1 μ M) and captopril (1 μ M). Finally, the enzymic activity of

ACE was assayed using the same conditions as described for NEP; in this case, phosphoramidon (1 μ M) and bestatin (10 μ M) were used as inhibitors of NEP and aminopeptidase respectively.

Endoglycosidase digestions and Western blotting

Endoglycosidase digestions were performed by adding endo- β -*N*-acetylglucosaminidase F or endo- β -*N*-acetylglucosaminidase H directly to aliquots of glomerular or proximal tubule samples, and the treated proteins were analysed by immunoblotting as described before (Lemay et al., 1990). For immunological detection of NEP, filters were preincubated for 1 h in 100 mM TBS containing 3% (w/v) BSA and 1% (w/v) dried milk and then incubated with a monoclonal antibody (mAb 18B5) raised against purified rabbit kidney NEP. The blots were then incubated with a biotinylated anti-(mouse immunoglobulin). The positions of the bands were visualized using the Vectastain ABC-Immunoperoxidase kit (Vector Laboratories, Burlingame, CA, U.S.A.).

Treatment of plasma membrane proteins with Triton X-114

The plasma membrane proteins (2 mg/ml) were resuspended in 3 ml of 10 mM Tris/HCl, pH 7.4, and 150 mM NaCl. They were then treated for 1 h at 4 °C with 1.5 ml of 5% (w/v) Triton X-114 (Sigma Chemical Co.) (Gee and Kenny, 1985). Detergent-insoluble material was pelleted at 31 500 *g* for 2 h at 4 °C before allowing phase separation of the supernatant at 30 °C (Gee and Kenny, 1985). The pretreatment of the detergent and the phase separation were as described previously (Bordier, 1981). Aliquots of the separated phases were analysed by immunoblotting. The enzymic activity of NEP was also measured in the separated phases of both glomerular and tubular fractions.

RB104 iodination and purification

The potent inhibitor of NEP, 2-[(3-iodohydroxy)phenylmethyl]-4-*N*-[3-hydroxyamino-3-oxo-1-phenylmethylpropyl]-amino-4-oxobutanoic acid (RB104) was synthesized as described (Fournié-Zaluski et al., 1992), and iodinated. Briefly, RB104 (5 μ g) was dissolved in 20 μ l of 5 mM phosphate buffer, pH 6.0, and iodinated in the presence of 0.5 mCi of Na¹²⁵I and 5 μ l of chloramine T (2 mg/ml). After an incubation of 10 s at room temperature, the reaction was stopped with 5 μ l of sodium metabisulphite (4 mg/ml). NaOH (10 M) was then added until the pH reached 10. The solution was left for 5 min at room temperature and the pH was re-adjusted to 1.5 with HCl (6 M). The iodinated mixture was diluted with 1 ml of 0.5% trifluoroacetic acid (TFA) in aqueous solution and applied to a Sep-Pak C₁₈ cartridge to eliminate unreacted substrates. The iodinated RB104 was then eluted with a 50% solution of acetonitrile in 0.5% TFA. [¹²⁵I]RB104 was further purified by reverse-phase h.p.l.c. (RP-h.p.l.c.) using a C₁₈ μ Bondapak column (Waters Associates, Milford, MA, U.S.A.).

Binding of [¹²⁵I]RB104 to blots of kidney fractions

The solubilized proteins were separated by SDS/PAGE (7.5% gels) in the absence of reducing agent (Laemmli, 1970) and then transferred to a nitrocellulose membrane. Binding of [¹²⁵I]RB104 was performed directly on the nitrocellulose membrane. Briefly, the membrane was incubated in a TBS solution containing 0.2% Tween 20 (TTBS) for 30 min at room temperature. It was then incubated for 1 h in a TTBS solution containing 100 pM [¹²⁵I]RB104 in the absence and presence of (3-hydroxy-

aminocarbonyl-2-benzyl-1-oxopropylglycine (HACBO-Gly) (10 μ M) or phosphoramidon (1 μ M). Samples were subsequently washed three times in TTBS (5 min each), dried and exposed to a Kodak XAR-5 film for 2 days at -70°C .

Autoradiography of [^{125}I]RB104 binding in dog kidney tissues

Dog kidney was perfused with 60 ml of Krebs solution at 4°C and then immersed in liquid nitrogen. Frozen dog kidney sections (8 μm) were fixed with a solution of 0.5% glutaraldehyde in PBS at room temperature for 10 min. Sections were then washed four times (10 min each) in PBS and once in Tris/HCl, pH 7.4. [^{125}I]RB104 (140 pM in Tris/HCl) was applied to each section and incubated at room temperature for 2 h. Control sections were incubated in the presence of HACBO-Gly (10 μ M) or phosphoramidon (1 μ M). Slides were then washed four times in Tris/HCl (30 s each) and dipped in distilled water (5 s). After drying, slides were dipped in photographic emulsion (NTB-2; Kodak) and exposed for 4 days. Slides were then stained with Harris haematoxylin and eosin and counterstained with lithium carbonate. Microphotography was performed with a Zeiss Axiophot photomicroscope.

RESULTS

Characterization of NEP activity

NEP activity was assayed in purified homogenates of either dog glomeruli or dog proximal tubules by measuring the rate of [^{125}I]-[tyrosyl-3,5- ^3H][D-Ala²,Leu⁵]enkephalin hydrolysis. All reactions were carried out in the presence of 10 μ M bestatin and 1 μ M captopril, which inhibit APN and ACE, respectively. As shown in Table 1, the specific activity of the glomerular NEP was in the same range as that of the proximal tubule enzyme. The K_m values found for glomerular and proximal tubule fractions were also very similar (Table 1). These values are in good agreement with the K_m of 59 μ M obtained using the same conditions for NEP purified from rabbit proximal tubules (Lemay et al., 1990). The catalytic rate constant (k_{cat}) of the glomerular enzyme was also found to be very close to that of the tubular enzyme (Table 1).

The sensitivity of the dog glomerular and tubular enzymes to thiorphan and phosphoramidon, two potent inhibitors of NEP, was assayed next. The concentrations of thiorphan necessary to achieve 50% inhibition (IC_{50}) were found to be very close for the glomerular and tubular enzymes (Table 1). Similarly, the values estimated with phosphoramidon were also equivalent in both cases (Table 1). These values agree closely with those determined for the inhibition of NEP activity in rat kidney membranes, where the IC_{50} values were 1.9 nM $\pm$ 0.2 thiorphan and 3.8 nM $\pm$ 1.7 for phosphoramidon (Malfroy and Schwartz, 1982).

In order to compare the relative levels of some other known brush-border peptidases, the enzymic activities of three other hydrolases were measured in both glomerular and tubular fractions (Table 2). Our results indicate that APN, DPPIV and ACE were all enzymically detectable in dog glomeruli as well as in tubules. Aminopeptidase activity measured by the hydrolysis of [^{125}I]-[tyrosyl-3,5- ^3H][Leu⁵]enkephalin should correspond to APN activity, since aminopeptidase A only attacks peptides with an acidic N-terminal amino acid (Stefanovic et al., 1992a). However, our assay does not discriminate between the membrane-bound APN and a possible contaminating cytosolic enzyme.

Immunoblotting of NEP

To characterize further the NEP present in dog glomeruli, the proteins solubilized from glomerular and tubular fractions were resolved by SDS/PAGE and revealed by immunoblotting using

mAb 18B5 (Figure 1). The apparent M_r of the dog glomerular protein was slightly higher (104000) than that of the dog kidney brush-border enzyme (94000) (Figure 1, compare lanes 2 and 3). However, the enzymes from dog and rabbit brush borders had the same M_r (Figure 1, compare lanes 1 and 3). Removal of N-linked sugar moieties from the dog glomerular and brush-border enzymes and from the rabbit brush-border NEP by treatment with endo- β -N-acetylglucosaminidase F produced polypeptides that migrated with similar mobilities on SDS/PAGE (Figure 1, lanes 4–6), suggesting that the sugar moieties are responsible for the major differences in apparent M_r . In all cases, NEP was found to be resistant to endo- β -N-acetylglucosaminidase H (Figure 1, lanes 7–9), indicating that complex sugars had been added in the Golgi apparatus. Different glycosylation patterns were also observed in the various organs expressing NEP in

Table 1 Kinetic parameters of NEP from glomeruli and proximal tubules in dog kidney

The enzymic assays were as described in the Materials and methods section. Initial velocities were measured at approx. 10% substrate hydrolysis using [^{125}I]-[tyrosyl-3,5- ^3H][D-Ala²,Leu⁵]enkephalin (50 nM) as substrate. The values are the means $\pm$ S.D. of four determinations performed on two independent preparations.

	Glomeruli	Tubules
Specific activity (fmol/min per μg)	2.3 $\pm$ 0.4	7.6 $\pm$ 1.3
K_m (μM)	76 $\pm$ 16	70 $\pm$ 16
k_{cat} (min^{-1})	1243 $\pm$ 85	1141 $\pm$ 81
IC_{50} (thiorphan) (nM)	0.8 $\pm$ 0.2	1.8 $\pm$ 0.2
IC_{50} (phosphoramidon) (nM)	2.9 $\pm$ 0.2	4.3 $\pm$ 0.1

Table 2 Relative levels of brush-border peptidases in dog glomerular and tubular fractions

All enzymic assays are described in the Materials and methods section. In this experiment, the Percoll gradient was omitted during the preparation of renal tubules. The values are the means $\pm$ S.D. of at least three independent determinations.

Enzyme	Substrate	Specific activity (fmol/min per μg of protein)	
		Glomeruli	Tubules
NEP	[D-Ala ² ,Leu ⁵]enkephalin	2.6 $\pm$ 0.5	7 $\pm$ 2
ACE	[D-Ala ² ,Leu ⁵]enkephalin	0.053 $\pm$ 0.008	0.160 $\pm$ 0.044
DPPIV	Gly-Pro- β -nitroanilide	(386 $\pm$ 37) $\times 10^3$	(366 $\pm$ 81) $\times 10^3$
APN	[Leu ⁵]enkephalin	0.559 $\pm$ 0.018	14.42 $\pm$ 3.89

Figure 1 Western blot of NEP from dog glomeruli and proximal tubules

The enzyme was revealed with mAb 18B5 to rabbit kidney NEP, both before and after endoglycosidase treatment. Lanes 1, 4 and 7, 60 ng of rabbit kidney NEP; lanes 2, 5 and 8, 70 μg of total dog glomerular protein; lanes 3, 6 and 9, 70 μg of dog membrane proximal tubule protein. Samples were left untreated (lanes 1–3) or were digested with endo- β -N-acetylglucosaminidase F (lanes 4–6) or endo- β -N-acetylglucosaminidase H (lanes 7–9). Positions of M_r markers are indicated on the right.

Figure 2 Triton X-114 extraction of glomerular and tubular NEP

Membranes from glomeruli (Glom) and proximal tubules (Tub) were extracted with Triton X-114 and both detergent and aqueous phases were separated on SDS/PAGE, transferred to nitrocellulose, reacted with mAb 18B5 and revealed by immunoperoxidase. A, Detergent phase; B, aqueous phase; C, purified rabbit NEP (94 000 M_r).

Table 3 Distribution of NEP activities following phase separation of glomerular and tubular proteins with Triton X-114

Membrane preparations were treated with Triton X-114 and subjected to phase separation and enzymic determination as described in the Materials and methods section. The results are expressed as enzyme activity recovered in the detergent and aqueous phases as a percentage of the total. The results are the means for two experiments.

Membrane fraction	Distribution (%)	
	Detergent phase	Aqueous phase
Proximal tubule	94	6
Glomerulus	93	7

Figure 3 Autoradiography with RB104 of glomerular and proximal tubule proteins

The solubilized proteins were separated by SDS/PAGE (7.5% gels) in the absence of reducing agent (Laemmli, 1970) and then electrotransferred on to a nitrocellulose membrane. The membrane was treated as described in the Materials and methods section. Lanes 1 and 2 were incubated with $[^{125}\text{I}]\text{RB104}$; lanes 3 and 4 (controls) were incubated with $[^{125}\text{I}]\text{RB104}$ in the presence of phosphoramidon (1 μM). Lanes 1 and 3 contained 45 μg of total glomerular proteins; lanes 2 and 4 contained 35 μg of proximal tubule proteins.

different species (Relton et al., 1983). The significance of these differential glycosylation patterns is unknown.

Glomerular NEP is a membrane glycoprotein

In order to determine whether the NEP activity found in glomeruli was associated with a soluble or a membrane protein,

Figure 4 Autoradiography of $[^{125}\text{I}]\text{RB104}$ binding to dog kidney sections

Frozen kidney sections were incubated with $[^{125}\text{I}]\text{RB104}$ (140 pM in Tris/HCl) as described in the Materials and methods section. Slides were dipped in photographic emulsion (NTB-2; Kodak) and exposed for 4 days. Slides were then stained with Harris haematoxylin and eosin and counterstained with lithium carbonate. (a) Silver grains over glomeruli (GLOM) and proximal tubule (PT). (b) Control section incubated in presence of phosphoramidon (1 μM). Microphotography was performed with a Zeiss Axiophot photomicroscope. Magnification, $\times 240$.

we centrifuged the glomerular homogenates on to sucrose cushions, and both the membrane-associated fraction and the supernatant were tested for NEP activity. NEP activity was associated with the membrane fraction, while no activity was found in the supernatant, suggesting that the glomerular NEP was membrane-bound.

The properties of Triton X-114, a non-ionic detergent, were used to confirm the membrane-bound nature of the glomerular enzyme. Total membranes of the proximal tubular and the glomerular fractions were solubilized with Triton X-114 and submitted to phase separation. The detergent and aqueous phases of each fraction were analysed by immunoblotting. As shown in Figure 2, both enzymes were recovered exclusively within the detergent phase, suggesting that both proteins are integral membrane proteins. Table 3 demonstrates that NEP was extracted mostly into the detergent-rich phase after phase separation of either glomerular or tubular membrane proteins.

Histological localization of NEP with [¹²⁵I]RB104

RB104, a new potent inhibitor of NEP, can be radioiodinated and used to detect nanogram quantities of the enzyme in a crude tissue extract. It is highly specific and was shown to recognize NEP in different tissues and species (Fournié-Zaluski et al., 1992). Blotting experiments were performed on dog glomerular and proximal tubular proteins separated by SDS/PAGE. RB104 bound to a single molecular species with an apparent M_r of 104000 in the glomerulus and 94000 in the tubule (Figure 3, lanes 1 and 2). As controls, the addition of phosphoramidon (10 μ M) (Figure 3, lanes 3 and 4) or HACBO-Gly (10 μ M) (results not shown) completely inhibited the binding of RB104 to the glomerular and the proximal tubular enzymes. [¹²⁵I]RB104 was next used to localize NEP on dog kidney sections. Figure 4(a) shows that the silver grains were localized over the proximal tubules as well as over the glomeruli. The parietal epithelium and the visceral core of the Bowman's capsule appeared to be labelled. As a control, sections were incubated in presence of HACBO-Gly (10 μ M) or phosphoramidon (1 μ M) (Figure 4b). These inhibitors completely abolished the binding of [¹²⁵I]RB104 to both the glomerular and the proximal tubular NEPs.

DISCUSSION

Immunochemical studies in the kidney (Metzgar et al., 1981; Erdős et al., 1985; Tauc et al., 1988) have led to contradictory data about the presence of NEP in the glomeruli when investigated in different species. However, even in same species (human), Metzgar et al. (1981), using an anti-CALLA monoclonal antibody, have reported intense glomerular staining in kidney sections, whereas a more recent report has disputed this finding (Erdős et al., 1985).

Our results indicate that NEP is present in both canine glomeruli and proximal tubules. This statement is supported by the following results: (a) fractions of purified glomeruli and proximal tubules hydrolysed the substrate [*tyrosyl*-3,5-³H][D-Ala²,Leu⁵]enkephalin with similar kinetic parameters (K_m and k_{cat}); (b) phosphoramidon and thiorphan inhibited the hydrolysis of [*tyrosyl*-3,5-³H][D-Ala²,Leu⁵]enkephalin by glomerular fractions, with IC_{50} values similar to those found in the proximal tubule fraction; (c) a specific mAb (18B5) raised against rabbit kidney NEP recognized the glomerular enzyme in the dog; (d) after enzymic deglycosylation, both enzymes migrated with an M_r of 85000 during SDS/PAGE; and (e) RB104, which is a highly specific NEP inhibitor, bound to both glomerular and tubular enzymes.

We suggest here that the two forms of NEP in dog kidney are not isoenzymes, but result from differential *N*-glycosylation of the same polypeptide, since endo- β -*N*-acetylglucosaminidase-F-digested enzymes from both tubular and glomerular fractions migrate with similar electrophoretic mobilities. Differences in the size of NEP from various tissues have been observed previously and attributed to differences in glycosylation (Relton et al., 1983).

It is very unlikely that the glomerular fractions are contaminated with proximal tubular enzymes, for the following reasons. First, the glomerular enzyme appeared to have a different M_r than the one from the tubule. Second, the activity of PEPCK, a typical tubular enzyme, was very low in the glomerular fraction, suggesting a contamination of glomerular fractions by tubules of less than 3 %, whereas the specific activities of NEP were of the same magnitude in both preparations.

Anion-exchange chromatography was shown to resolve charge variants of NEP in detergent-solubilized membranes from human

(Gafford et al., 1983), pig (Fulcher and Kenny, 1983) and rat (Vida and Hersh, 1992) kidneys. As those studies were performed on total or cortical homogenates, these variants could not be associated with any particular segments of the nephron. Further experiments will be required to determine whether, in these species, glycosylation variants correspond to the glomerular and proximal tubular enzymes, as suggested by our results in the dog.

Although NEP from virtually all sources is an intrinsic membrane-bound protein, a soluble form originating from an unknown source is normally present in human urine (Skidgel et al., 1987) and is detected in the blood of patients with respiratory distress syndrome (Johnson et al., 1985). Here we have shown that the glomerular enzyme appeared to be membrane-bound, since it was retained within the detergent phase of Triton X-114. Thus the glomerular NEP is probably not the source of the soluble enzyme.

Although most of our antibodies reacted with both the glomerular and the brush-border enzymes in immunoblotting experiments, they failed to recognize the glomerular enzyme in tissue sections. This might be explained by the fact that the tertiary structure of NEP depends on the composition of lipid or proteins of the membrane into which it is inserted. To circumvent this difficulty we used RB104, a new potent inhibitor of NEP which recognizes the native NEP from diverse tissues or species (Fournié-Zaluski et al., 1992). With dog kidney frozen sections, RB104 binding sites appeared to be localized over both the glomeruli and the proximal tubules. In the glomerulus, the parietal epithelium of the Bowman's capsule and the core of the glomerulus appeared to be labelled. The core of the renal glomerulus contains three cell types: (1) endothelial cells, which line the glomerular capillaries, (2) epithelial cells, which display multiple and interdigitated foot processes (podocytes), and (3) mesangial cells, which are embedded in the intercapillary matrix (Latta, 1973). Unfortunately, it was not possible to identify which one of these cell types expresses the glomerular enzyme. Another explanation might be that binding of RB104 occurred on neutrophils, which are known to express CALLA (Cossman et al., 1983). This, however, appears very unlikely since the kidneys were perfused before processing for autoradiography and were therefore largely devoid of blood cells, as observed under the light microscope.

Although the presence in glomeruli of cysteine and aspartic proteinases is well documented (Yokota et al., 1986; Baricos et al., 1990), only limited information is available concerning peptidases present in glomeruli. Strategically, the glomerulus might be an important site of regulation of plasma-derived peptides, since blood filtration occurs primarily at this site. In particular, membrane-bound glomerular peptidases may play a critical role as regulators of peptide hormone action or in glomerular haemodynamics. As shown in Table 2, APN, DPPIV and ACE, three cell-surface peptidases expressed in proximal tubules, were enzymically detectable in dog glomeruli. These results are mostly in agreement with other reports concerning the expression of these hydrolases in this part of the nephron. Indeed, DPPIV activity has been found in the glomeruli of several animal species (Stiller et al., 1991). In rat glomeruli, DPPIV antigens are expressed on the apical domains of both the endothelium and podocytes (Dekan et al., 1990). Although DPPIV was not detected in the glomeruli of the normal human kidney, Stiller et al. (1991) reported that under pathological conditions DPPIV is expressed in human glomeruli, and this seems to be correlated with some clinical findings, e.g. hypertension. More recently, aminopeptidase A and APN, two cell-surface exopeptidases, have been described in human glomerular epithelial cells (Stefanovic et al., 1992a). Interestingly, the renal

APN, which is essentially localized in the kidney brush border, was unexpectedly found to be expressed on podocytes and cultured mesangial cells (Stefanovic et al., 1992b). Here, in agreement with these previous results, we observed that APN was also expressed in dog glomeruli, albeit at much lower levels than those found in tubules. However, the relative contributions of the soluble cytosolic and membrane-bound aminopeptidase activities could not be assessed in our study. The presence of ACE in rabbit glomeruli was not detected by immunological methods (Tauc et al., 1988). Using a different approach, our results showed that ACE was enzymically detectable in dog glomeruli, although the relative level was three times lower than the one measured in tubular fractions. In any case, further investigations will be required to define the exact role of these hydrolases in the glomerulus. In addition to a possible role in some renal pathology, cell-surface peptidases might act as modulators of growth and differentiation, as suggested in some previous reports (Kenny et al., 1989; Stefanovic et al., 1992b).

In the kidney, NEP is believed to be involved in the degradation of several peptides, including ANF (Sonnenberg et al., 1988; Margulies et al., 1990; Chiu et al., 1991). The proposed mechanisms for clearance of ANF from the circulation include receptor-mediated internalization (Hirata et al., 1985; Maack et al., 1987; Almeida et al., 1989) as well as an enzymic process involving NEP (Olins et al., 1987; Stephenson and Kenny, 1987; Sonnenberg et al., 1988). However, the relative importance of these pathways has not been determined conclusively. In particular, no intraluminal sites for ANF action have been clearly identified along the nephron. It should be recalled that tubular NEP is localized on the apical side of the epithelial cells in the proximal tubule, and therefore might be irrelevant for the rapid metabolic inactivation of circulating ANF. Because of the presence of functional natriuretic-peptide receptor/guanylate cyclase in either the mesangial or epithelial cells of the glomerulus (Wilcox et al., 1991), the presence of active NEP in glomeruli is of greater physiological relevance.

Our results confirm the presence of NEP in the glomerulus and provide the first biochemical and enzymic characterization of this enzyme in this part of the nephron. They also provide a biochemical explanation for the potentiation of the glomerular actions of ANF by NEP inhibitors as reported previously (Shima et al., 1988). Whether NEP is also present in the glomeruli of other species remains to be determined by a more systematic approach based on the separation of nephron fractions as performed in our study.

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REFERENCES

- Almeida, F. A., Suzuki, M., Scarborough, R. M., Lewicki, J. A. and Maack, T. (1989) *Am. J. Physiol.* **256**, R469–R475.
- Almenoff, J., Wilk, S. and Orlowski, M. (1981) *Biochem. Biophys. Res. Commun.* **102**, 206–214.
- Aubry, M., Berteloot, A., Beaumont, A., Roques, B. P. and Crine, P. (1987) *Biochem. Cell Biol.* **65**, 398–404.
- Baricos, W. H., Cortez, S. L., Le, Q. C., Zhou, Y., Dicarlo, R. M., O'Connor, S. E. and Shah, S. V. (1990) *Kidney Int.* **38**, 395–401.
- Bawab, W., Querido, E., Crine, P. and DesGroseillers, L. (1992) *Biochem. J.* **286**, 967–975.
- Bordier, C. (1981) *J. Biol. Chem.* **256**, 1604–1607.
- Chiu, P. J. S., Tetzloff, G., Romano, M. T., Foster, C. J. and Sybertz, E. J. (1991) *Am. J. Physiol.* **260**, R208–R216.
- Condra, C. L., Leidy, E. A., Bunting, P., Colton, C. D., Nutt, R. F., Rosenblatt, M. and Jacobs, J. W. (1988) *J. Clin. Invest.* **81**, 1348–1354.
- Cossmann, J., Neckers, L. M., Leonard, W. J. and Greene, W. C. (1983) *J. Exp. Med.* **157**, 1064–1069.
- Currie, M. G., Geller, D. M., Cole, B. R., Siegel, N. R., Fok, K. F., Adams, S. P., Eubanks, S. R., Galluppi, G. R. and Needleman, P. (1984) *Science* **223**, 67–69.
- Dekan, G., Miettinen, A., Schnabel, E. and Farquhar, M. G. (1990) *Am. J. Pathol.* **137**, 913–927.
- De Léan, A., Vinay, P. and Cantin, M. (1985) *FEBS Lett.* **193**, 239–242.
- Erdős, E. G. and Skidgel, R. A. (1989) *FASEB J.* **3**, 145–151.
- Erdős, E. G., Schulz, W. W., Gafford, J. T. and Defendini, R. (1985) *Lab. Invest.* **52**, 437–447.
- Fournié-Zaluski, M. C., Soleilhac, J. M., Turcaud, S., Lai-Kuen, R., Crine, P., Beaumont, A. and Roques, B. P. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6388–6392.
- Fulcher, I. S. and Kenny, A. J. (1983) *Biochem. J.* **211**, 743–753.
- Gafford, J. T., Skidgel, R. A., Erdős, E. G. and Hersh, L. B. (1983) *Biochemistry* **22**, 3265–3271.
- Garcia, R., Thibault, G., Seidah, N. G., Lazure, C., Cantin, M., Genest, J. and Chretien, M. (1985) *Biochem. Biophys. Res. Commun.* **126**, 178–184.
- Gee, N. S. and Kenny, J. (1985) *Biochem. J.* **230**, 753–764.
- Hirata, Y., Takata, S., Tomita, M. and Takaichi, S. (1985) *Biochem. Biophys. Res. Commun.* **132**, 976–984.
- Hori, R., Inui, K. I., Saito, H., Matsukawa, Y., Okumura, K., Nakao, K., Morii, N. and Imura, H. (1985) *Biochem. Biophys. Res. Commun.* **129**, 773–779.
- Huang, C. L., Ives, H. E. and Cogan, M. G. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8015–8018.
- Johnson, A. R., Coalson, J. J., Ashton, J., Larumbide, M. and Erdős, E. G. (1985) *Am. Rev. Respir. Dis.* **132**, 1262–1267.
- Jumarie, C. and Malo, C. (1991) *J. Cell. Physiol.* **149**, 24–33.
- Kenny, A. J., Gusterson, B. A. and O'Hare, M. J. (1989) *Lancet* **II**, 785–787.
- Kerr, M. A. and Kenny, A. J. (1974) *Biochem. J.* **137**, 477–488.
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
- Latta, H. (1973) *Handb. Physiol. Sect. 8: Renal Physiol.* pp. 1–29.
- Lemay, G., Zollinger, M., Waksman, G., Roques, B. P., Crine, P. and Boileau, G. (1990) *Biochem. J.* **267**, 447–452.
- Le Moual, H., Devault, A., Roques, B. P., Crine, P. and Boileau, G. (1991) *J. Biol. Chem.* **266**, 15670–15674.
- Letarte, M., Vera, S., Tran, R., Addis, J. B., Onizuka, R. J., Quackenbush, E. J., Jongeneel, C. V. and McInnes, R. R. (1988) *J. Exp. Med.* **168**, 1247–1253.
- Maack, T., Suzuki, M., Almeida, F. A., Nussenzweig, D., Scarborough, R. M., McEnroe, G. A. and Lewicki, J. A. (1987) *Science* **236**, 675–678.
- Malfroy, B. and Schwartz, J. C. (1982) *Biochem. Biophys. Res. Commun.* **106**, 276–285.
- Malfroy, B., Swerts, J. P., Guyon, A., Roques, B. P. and Schwartz, J. C. (1978) *Nature (London)* **276**, 523–526.
- Margulies, K. B., Caverio, P. G., Seymour, A. A., Delaney, N. G. and Burnett, J. C., Jr. (1990) *Kidney Int.* **38**, 67–72.
- Matsas, R., Fulcher, I. S., Kenny, A. J. and Turner, A. J. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3111–3115.
- Metzgar, R. S., Borowitz, M. J., Jones, N. H. and Dowell, B. L. (1981) *J. Exp. Med.* **154**, 1249–1254.
- Olins, G. M., Spear, K. L., Siegel, N. R., Reinhard, E. J. and Zurcher-Neely, H. A. (1987) *Eur. J. Biochem.* **170**, 431–434.
- Pogson, C. I. and Smith, S. A. (1975) *Biochem. J.* **152**, 401–408.
- Relton, J. M., Gee, N. S., Matsas, R., Turner, A. J. and Kenny, A. J. (1983) *Biochem. J.* **215**, 519–523.
- Roques, B. P. and Beaumont, A. (1990) *Trends Pharmacol. Sci.* **11**, 245–249.
- Rocques, B. P., Fournié-Zaluski, M. C., Soroca, E., Lecomte, J. M., Malfroy, B., Llorens, C. and Schwartz, J. C. (1980) *Nature (London)* **288**, 286–288.
- Seubert, W. and Huth, W. (1965) *Biochem. Z.* **343**, 176–191.
- Shima, M., Seino, Y., Torikai, S. and Imai, M. (1988) *Life Sci.* **43**, 357–363.
- Shipp, M. A., Vijayaraghavan, J., Schmidt, E. V., Masteller, E. L., D'Adamo, L., Hersh, L. B. and Reinherz, E. L. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 297–301.
- Skidgel, R. A., Schulz, W. W., Tam, L. T. and Erdős, E. G. (1987) *Kidney Int. Suppl.* **20**, S45–S48.
- Sonnenberg, J. L., Sakane, Y., Jeng, A. Y., Koehn, J. A., Ansell, J. A., Wennogle, L. P. and Ghai, R. D. (1988) *Peptides* **9**, 173–180.
- Stefanovic, V., Vlahovic, P., Ardaillou, N., Ronco, P. and Ardaillou, R. (1992a) *Kidney Int.* **41**, 1571–1580.
- Stefanovic, V., Vlahovic, P., Ardaillou, N., Ronco, P., Nivez, M. P. and Ardaillou, R. (1992b) *Cell. Physiol. Biochem.* **2**, 60–72.
- Stephenson, S. L. and Kenny, A. J. (1987) *Biochem. J.* **243**, 183–187.
- Stiller, D., Bahn, H. and August, C. (1991) *Acta Histochem.* **91**, 105–109.

-
- Tauc, M., Chatelet, F., Verroust, P., Vandewalle, A., Poujeol, P. and Ronco, P. (1988) *J. Histochem. Cytochem.* **36**, 523–532
- Tejedor, A., Noël, J., Vinay, P., Boulanger, Y. and Gougoux, A. (1988) *Can. J. Physiol. Pharmacol.* **66**, 997–1009
- Vida, T. A. and Hersh, L. B. (1992) *Neuropeptides* **21**, 245–255
- Wilcox, J. N., Augustine, A., Goeddel, D. V. and Lowe, D. G. (1991) *Mol. Cell. Biol.* **11**, 3454–3462
- Yokata, S., Tsuji, H. and Kato, K. (1986) *J. Histochem. Cytochem.* **34**, 899–907

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