*J. Enzyme Inhibition*, 1991, Vol. 4, pp. 347–358
Reprints available directly from the publisher
Photocopying permitted by license only





# CHARACTERISATION OF NEUTRAL ENDOPEPTIDASE 3.4.24.11 (NEP) IN THE KIDNEY: COMPARISON BETWEEN NORMOTENSIVE, GENETICALLY HYPERTENSIVE AND EXPERIMENTALLY HYPERTENSIVE RATS

RAINER SCHULZ[†], YUMI SAKANE, CAROL BERRY and RAJENDRA GHAI[‡]

*Research Department, Pharmaceutical Division, CIBA-GEIGY Corporation, Summit, New Jersey 07901*

*(Received 3 July 1990)*

Neutral endopeptidase 3.4.24.11 (NEP) has been identified as the major atrial natriuretic factor (ANF) degrading enzyme in rat kidney, therefore, suggesting a possible role for this enzyme in blood volume and pressure regulation. Various experimentally induced and genetically hypertensive rat models have been used to test NEP inhibitors. The presence of different isoforms of NEP in the various hypertensive rat models would have relevance when searching for novel NEP inhibitors. Therefore, we compared the properties of NEP in kidney cortex homogenates in order to test for possible differences in the following hypertensive rat models and their appropriate controls: spontaneously hypertensive rats (SHR), Wistar Kyoto strain (WKY), DOCA-salt hypertensive rats, and Sprague Dawley control rats (SD). No relevant differences were found when comparing the following parameters: (1) specific activity (mean: 204 U/mg protein), (2) Michaelis constant (mean: 280 $\mu$M), (3) $IC_{50}$ of thiorphan (mean: 6.5 nM) and phosphoramidon (mean: 54 nM), (4) pH profiles (optimum at pH 8.0), (5) heat inactivation profiles (half-life 20 min at 65°C), (6) immunotitration of kidney cortex homogenates, (7) molecular weight as determined by gel filtration (92,000 Dalton) and (8) affinity chromatography with concanavalin A. Without evidence for the presence of different NEP isoforms, it is unlikely that divergent findings in DOCA-salt rats and SHR using a given NEP inhibitor are due to isoforms of NEP.

KEY WORDS: Neutral endopeptidase 3.4.24.11, ANF, kidney, WKY, SHR, DOCA

## INTRODUCTION

Neutral endopeptidase 3.4.24.11 (NEP) is a metallopeptidase that inactivates numerous hormones by cleavage at the amino side of hydrophobic amino acids. It has been identified as the major atrial natriuretic factor (ANF) degrading enzyme in the kidney.[1] NEP cleaves the cysteine phenylalanine bond ($Cys^7$- $Phe^8$) and thereby inactivates ANF.[2] In rats the kidney is responsible for one sixth to one third of total ANF clearance.[3] In humans the importance of the kidney in ANF clearance is demonstrated by elevated plasma levels of ANF in renal failure.[4]

ANF is secreted from mammalian cardiac atria into the systemic circulation and is involved in the homeostasis of fluid balance and blood pressure.[5,6] Infusion of

[†]Present address: Human Pharmacology Institute, CIBA-GEIGY GmbH, Waldhoernlestr. 22, D-7400 Tuebingen, Federal Republic of Germany.
[‡]Correspondence.

pharmacological amounts of ANF into untreated patients with essential hypertension resulted in a sustained lowering of blood pressure.[7] Therefore, it is assumed that an increase in half-life of endogenous ANF could be beneficial in cardiovascular diseases, such as essential hypertension and heart failure.

Specific inhibitors of NEP, such as thiorphan, SQ 29,072, SCH 39,370, have been used successfully to inhibit ANF inactivation and to prolong blood pressure lowering and renal effects of ANF.[8–15] Various test systems have been employed in these studies, particularly normotensive, spontaneously hypertensive (SHR) and experimentally hypertensive (DOCA-salt) rats. It is generally assumed – but has not been shown – that the ANF degrading NEP activity in these different rat models is caused by the same enzyme protein. However, early studies found evidence for the existence of a phosphoramidon-sensitive (NEP-1) and a phosphoramidon-insensitive (NEP-2) enzyme form in rat and mouse kidney.[16,17] Also, differences have been observed in the depressor response of SHR and DOCA-salt rats to the NEP inhibitors thiorphan and SCH 39,370.[12,18] The properties of NEP in different rat models of hypertension have not been compared before. The existence of isoforms of NEP could have important implications for the drug discovery process of potential NEP inhibitors as the choice of a certain rat model for *in vivo* testing would become crucial and comparison of results from different models would be made more complex. Therefore, we studied the properties of NEP in SHR and DOCA-salt rats and compared them to their appropriate controls.

## MATERIALS

### *Chemicals*

Glutaryl-Ala-Ala-Phe-2-naphthylamide was purchased from Research Plus, Bayonne, NJ. Bisbenzimide trihydrochloride (Hoechst 33258), calf thymus DNA sodium salt, fast garnet, leucine aminopeptidase, Tris-HCl, maleate anhydride, deoxycorticosterone acetate (DOCA), EDTA, N-2-hydroxyethylpiperazine-N′2-ethanesulfonic acid (HEPES), methyl-α-D-mannopyranoside, sodium azide, phosphoramidon, thiorphan, molecular weight markers; β-amylase, bovine serum album, carbonic anhydrase and cytochrome C were purchased from Sigma Chemical Co.., St. Louis, MO. Sodium acetate, sodium bicarbonate, sodium carbonate, sodium chloride, sodium phosphate (dibasic), and Tween 20 were purchased from J.T. Baker Chem. Co., Phillipsburgh, NJ.

Methyoxyflurane (Metofane®was obtained from Pittman Moore, Inc., Washington Crossing, NJ. Concanavalin A, sepharose and Sephacryl S-200 was purchased from Pharmacia, AB Laboratory Separation Division, Uppsala, Sweden. Silastic implants were manufactured by Dow Corning Co., Midland, MI. Bovine serum albumin was purchased from Miles Laboratories Inc., Elkhart, Ind. Phenol reagent solution (Folin-Ciocalteau) was bought from Fisher Scientific Co., Fair Lawn, NJ. The rabbit anti-rat NEP (prepared using purified NEP from normotensive SD rats) polyclonal antiserum was obtained from Dr. Louis B. Hersh, Depts. of Pharmacology, Internal Medicine and Biochemistry, The University of Texas Health Sciences Center, Dallas, Texas 75235. Pansorbin®cells (*Staphylococcus aureus* cells) were procured from Calbiochem, LaJolla, Ca.

*Study Animals*

The rats used in this study were males between 12 and 15 weeks of age, purchased from Taconic Farms, Germantown, NY. The animals were divided into two groups namely, normotensive and hypertensive. Wistar-Kyoto rats Tac:N (WKY) fBR weighing 309 ± 20 g (mean ± S.D.) and Sprague Dawley rats Tac:N(SD) fBR weighing 387 ± 16 g (mean ± S.D.) were chosen as normotensive controls for spontaneously hypertensive rats Tac:N(SHR) fBR weighing 278 ± 10 g (mean ± S.D.) and DOCA-salt hypertensive rats, respectively.

*DOCA-salt hypertensive rat model* The DOCA salt hypertensive rats were prepared according to the method of Berecek *et al.*[19] using a slightly modified procedure. Briefly, the left kidney of SD rats was removed under methoxyflurane anesthesia. Each rat also received a subcutaneous silastic implant impregnated with deoxycorticosterone acetate (DOCA) at a dose of 75 mg/kg. Animals were returned to their home cages and given 1% NaCl and 0.2% KCl as drinking fluid. Rats received tap water in place of the 1% NaCl solution at the start of the fifth week following DOCA implantation. Animals were sacrificed during the fifth to sixth week after initiating the DOCA-salt hypertension, at a time when blood pressure had plateaued. At sacrifice, the rats weighted 389 ± 20g (mean ± S.D.).

*Sprague Dawley one-kidney rats (SD1Kd)* In order to separate the influence of treatment with DOCA from uinilateral nephrecotomy and salt treatment, the left kidney was removed from four male Sprague-Dawley rats as described above for the DOCA-salt rat model. However, no DOCA pellet was implanted. These animals were treated in the same way as the DOCA-salt rats, and weighed 345 ± 12 g (mean ± S.D.) when sacrificed.

## METHODS

*Preparation of Homogenate from Kidney Cortex*

The animals were weighed and sacrificed with carbon dioxide. The right kidney was removed, washed in ice-cold isotonic saline, weighed and frozen at −70°C until preparation of homogenate. Kidneys were dissected into halves, the cortex was separated from the medulla and washed in ice-cold isotonic saline. Kidney cortex of each rat was homogenized separately, six times for 15 s in five volumes (weight/volume) of 50 mM Tris-HCl buffer, pH 7.3 at 37°C, using a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY) at a setting of 7. The homogenate was centrifuged at 600 × g for 10 min at 4°C in a Sorvall RC-5B centrifuge. The supernatant was stored frozen in aliquots of 100 μl at −70°C until used.

*Determination of DNA*

Quantitative determination of DNA in homogenates was carried out according to the method of Labarca and Paigen.[20] The method is based on the enhancement of fluorescence measured when bisbenzimide (Hoechst 33258) binds to DNA. Calf thymus DNA was used as a standard. Briefly, samples and standards were diluted appropriately to a final concentration of 100 to 1000 ng DNA in 0.5 ml volume using

assay buffer (pH 7.4), which consisted of 0.05 M $NaH_2PO_4$, 2 M NaCl, 0.002 M EDTA and 0.02% Na Azide. A stock solution (200 $\mu$g/ml in $H_2O$) of bisbenzimide was diluted 1:1000 which assay buffer and 0.5 ml added to standards, samples and blanks (assay buffer alone). After mixing, another 2 ml of assay buffer were added to each sample for a final volume of 3 ml. Intensity of fluorescence was determined at 356 nm excitation and 458 nm emission. The amounts of DNA in the samples were calculated form the standard curve.

*Protein Determination*

Protein assays were carried out in duplicates according to the method of Lowry *et al.*[21] using bovine serum albumin as a standard.

*Enzyme Assay*

NEP activity was determined in duplicates by the hydrolysis of the substrate glutaryl-Ala-Ala-Phe-2-naphthylamide using a modified procedure of Orlowski and Wilk.[22] The incubation mixture (total volume of 250 $\mu$l) contained 0.5 M Tris buffer, pH 7.4, 500 $\mu$M substrate (final concentration), and leucine aminopeptidase M (5 $\mu$g). The mixture was incubated for 10 min at 37°C and 1 ml of fast garnet (50 $\mu$g fast garnet/ml of 10% Tween 20 in 1 M sodium acetate, pH 4.2) was added to stop the reaction. Enzyme activity was measured spectrophotometrically at 530 nm. One unit (U) of NEP activity is defined as 1 nmol of 2-naphthylamine release per min at 37°C at pH 7.4.

*Kinetic Studies*

$K_m$ determinations were performed in 0.05 M Tris-HCl buffer, pH 7.4. Various concentrations (0.125 to 2 mM) of the substrate glutaryl-Ala-Ala-Phe-2-naphthylamide were used and the assay carried out in triplicates. $K_m$ values were determined by least-square analysis of linear transformations calculated according to the method of Lineweaver-Burk.[23]

*pH Profile*

The enzymatic assay was performed at different pH values using the following buffers: 0.05 M Tris-maleate buffer for pH 6 and pH 6.5; 0.05 M Tris-HCl buffer for pH 7, 7.4, and 8; 0.05 M carbonate-bicarbonate buffer for pH 8.5 and 9.5.

*Heat inactivation*

Homogenates from all the rat models were incubated at 65°C. Initial activity was adjusted to the same level. Protein concentration was adjusted to the same value (1 mg/ml final concentration) by addition of bovine serum albumin. Aliquots of the heated material were withdrawn at given time intervals and assayed for the remaining activity. Results are expressed as a percentage of the initial enzyme activity.

*Immunotitration*

Kidney cortex homogenates from WKY, SHR, SD, SD1Kd, and DOCA salt rats containing 15 units NEP activity were incubated with increasing amounts of NEP antiserum. The mixture was made up to a volume of 0.1 ml with Tris buffer, pH 7.4. After 60 min at room temperature, Pansorbin® (30 ul) was added to the mixture and the incubation continued for an additional 10 min. The reaction mixture was centrifuged at 15,000 × g for 5 min, and the remaining activity in the supernatant was determined in duplicates. Control tubes contained normal rabbit serum. Results are expressed as a percentage of these control values.

*Concanavalin A Column Chromatography*

Kidney cortex homogenates, containing 700 units of NEP activity in a total volume of 250 $\mu$l, were loaded onto a 3 ml Con A affinity column, previously equilibrated with 5 mM HEPES buffer, pH 7.4. The column was washed 3 times with 4.2 ml of 5 mM HEPES buffer, pH 7.4 to remove non-bound (i.e. non-glycosylated) NEP. The amount of adsorbed (i.e. glycosylated) protein was calculated as the difference between the loading activity minus the non-bound activity.

*Determination of Molecular Weight*

The molecular weight of NEP from all rat models was determined by chromatography on Sephacryl S-200. The column was equilibrated in 50 mM Tris-HCl buffer pH 7.4 containing 0.15 M NaCl and calibrated using $\beta$-amylase ($M_V$ 200,000), bovine serum albumin (68,000), carbonic anhydrase (29,000) and cytochrome C (12,400). Kidney cortex homogenates from each model containing 2000 units of NEP were separated by column chromatography. The elution profile of the collected fractions was followed by protein absorbance of 230 nm and by enzymatic activity. The molecular size of NEP was calculated according to the method of Andrews.[24]

*Inhibitiion of NEP Activity*

The specific inhibitors phosphoramidon and thiorphan were tested for their ability to inhibit NEP activity. The homogenates were preincubated for 10 min at 37°C with increasing concentrations of inhibitor. The $IC_{50}$ values were derived from inhibition curves with the non-linear regression curve-fitting program of Research System/1 (RS/; Bolt, Berenik, and Newman, Inc.) software package on a VAX 1/750 computer equipped with VMS operating system (Digital Equipment Corporation).

*Statistical Analysis*

Means with standard error (SE) or standard deviation (S.D.) are given. Differences were tested for overall significance by analysis of variance (ANOVA, factorial design). Multiple comparisons were performed using Fisher's protected least significant difference method (Fisher's PLSD, Snedecor and Cochran).[25] P-values less than 0.05 were considered significant.

## RESULTS

Kidney weights from each group are shown in Figure 1. As expected, kidney weight increased significantly ($p < 0.001$) in unilaterally nephrectomized SD rats as compared to control SD rats. Kidneys from SHR were slightly smaller ($p < 0.05$) than WKY kidneys (1.34 $\pm$ 0.03 g vs. 1.10 $\pm$ 0.02 g; mean $\pm$ SE). No other significant differences were found between the groups. The kidney weight normalized by body weight (KW/BW) increased significantly ($\pi < 0.001$) by 98% (DOCA) and 97% (SD1Kd) compared to SD (Table I).

The protein content of the kidney cortex homogenate from DOCA and SD1Kd was significantly lower than either SD control or SHR (Table II). In addition, SD1Kd rats showed a significantly lower protein than that of WKY. However, there was no difference beween WKY, SHR and SD groups. In contrast, the DNA content of kidney cortex was not significantly different except in the Sd1Kd group which was 26% higher than that of the SD control group (Table II). The DNA/protein ratio was significantly higher in the DOCA-salt and SD1Kd group compared to the SD control group indicating that hyperplasia is part of the compensatory renal growth after unilateral nephrectomy (Table II). This ratio in the SD was lower than WKY and

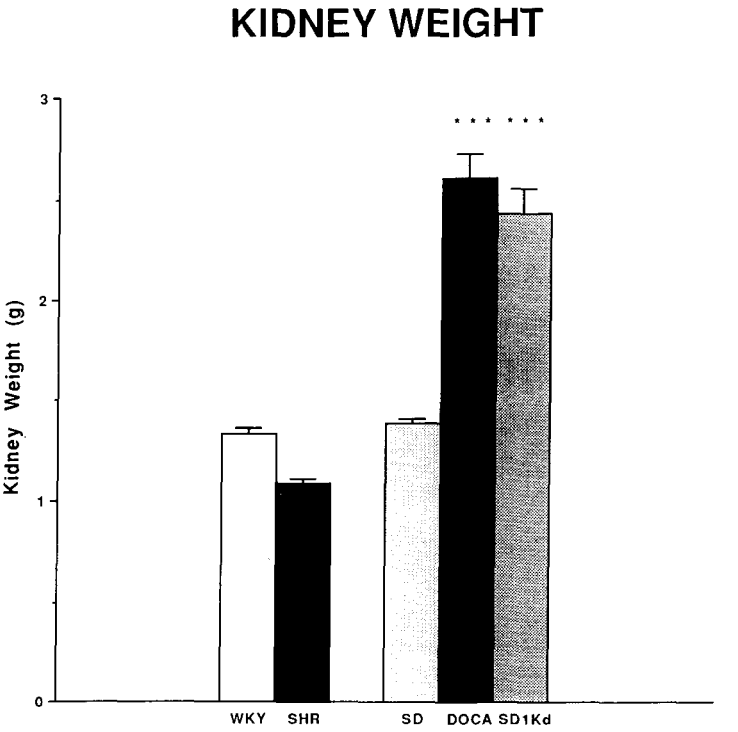


FIGURE 1 Right kidney wet weight. Values are mean $\pm$ SE. * Inidcates a lower kidney weight compared to WKY ($p < 0.05$); *** indicates a higher kidney weight compared to SD ($p < 0.001$).

TABLE I
Kidney weight/body weight ratio in hypertensive rat models.

| Group (n) | KW/BW ratio (mg/g) | comparison to | |
|---|---|---|---|
| | | WKY | SD |
| WKY (8) | 4.34 ± 0.08 | | *** |
| SHR (8) | 3.95 ± 0.05 | * | * |
| SD (8) | 3.60 ± 0.04 | *** | |
| DOCA (4) | 7.13 ± 0.25 | *** | *** |
| SD1Kd (4) | 7.08 ± 0.28 | *** | *** |

Mean values ± SE are given. n indicates number of animals. * = $p < 0.05$; *** = $p < 0.001$. There are no significant difference between DOCA and SD1Kd group. The ratio for SHR was significantly lower ($p < 0.001$) than for DOCA and SD1Kd.

TABLE II
Protein and DNA content of kidney cortex of rat models.

| Group (n) | Protein μg/mg wet weight | DNA | DNA/protein ratio ($\times 10^{-3}$) |
|---|---|---|---|
| WKY(8) | 198 ± 3.7 | 3.13 ± 0.11 | 16 ± 1 |
| SHR(8) | 207 ± 6.3 | 3.17 ± 0.10 | 15 ± 2 |
| SD(8) | 213 ± 4.0 | 2.75 ± 0.12 | 13 ± 1 |
| DOCA(8) | 174 ± 43.9** | 3.18 ± 0.25 | 19 ± $1^{+++}$ |
| SD1Kd(4) | 162 ± 1.5** | 3.48 ± $0.34^{+}$ | 21 ± $5^{+++}$ |

Mean values ± SE are given. n indicates number of animals. **indicates a significantly lower ($p < 0.01$) protein content in DOCA and SD1Kd rats compared to SD. $^{+}$indicates a significant higher DNA content in SD1Kd rats versus SD rats ($p < 0.05$). $^{++}$indicates a significantly higher ($p < 0.001$) ratio in DOCA and SD1Kd rats compared to SD. There was no significant difference between the DOCA and the SD1Kd group.

SHR ($p < 0.05$). No differences were found between WKY versus SHR and DOCA versus SD1Kd group.

Table III shows that the specific activity of NEP in units/mg protein and units/μg DNA in kidney cortex homogenates. The results show that the specific activities are similar in the SHR, WKY and SD rats. No significant differences are seen in the DOCA and SD1Kd rats when compared to the SD control rats. However, the specific

TABLE III
Specific activity of NEP in rat kidney cortex

| group (n) | (Units/mg protein) | Units/μg DNA) |
|---|---|---|
| WKY (8) | 217.9 ± 7.5 | 13.8 ± 0.5 |
| SHR (8) | 224.2 ± 6.5 | 14.7 ± 0.5 |
| SD (8) | 208.2 ± 6.9 | 16.4 ± 0.7 |
| DOCA (8) | 184.1 ± $15.5^{+}$ | 10.2 ± 1.2*** |
| SD1Kd (4) | 186.0 ± $10.6^{+}$ | 9.0 ± 1.2*** |

Mean values ± SE are given. n indicates number of animals. $^{+}$indicates a lower ($p < 0.05$) specific activity of DOCA when compared to WKY and SHR and of SD1Kd when compared to SHR. *** indicates a lower ($p < 0.001$) specific activity in DOCA and SD1Kd rat compared to SD. DOCA and SD1Kd were also lower when compared to SHR ($p < 0.001$) and WKY ($p < 0.01$).

activity of DOCA-salt rats was lower when compared to the WKY and SHR. Also, the specific activity of SD1Kd rats was significantly lower than that for the SHR rats. The specific activity based on the DNA content of the homogenate is an estimate of the average cellular NEP activity in euploid renal cells in kidney cortex and eliminates differences in protein content (Table III). Again, there were no differences between WKY and SHR. However, specific activities in DOCA and SD1Kd rats were found to be significantly lower ($p < 0.001$) when compared to the SD control rats. They were also lower when compared to the SHR ($p < 0.001$) and WKY ($p < 0.001$). NEP activity of WKY rats was lower in comparison with SD rats ($p < 0.05$).

The $K_m$ values obtained in all five groups (average $K_m$ 280 $\mu$M) (Table IV) were not significantly different ($p < 0.05$). NEP molecular weight obtained from Sephacryl S-200 chromatography showed a mean molecular weight of 92,000 daltons. Concanavlin A chromatography indicated that 90% of NEP molecules are glycosylated in all five groups. Table V lists the $IC_{50}$ values of thiorphan and phosphoramidon for each animal model. The $IC_{50}$ for thiorphan was 6.5 $\pm$ 0.3 nM and 54.0 $\pm$ nM for phosphoramidon. No differences in affinity toward these two inhibitors of NEP were found between the groups. Figure 2 shows the pH profiles of NEP activity; no relevant differences were observed. The pH optimimum was 8.0 in all five groups. At pH 7.4 (the assay condition employed) the enzyme shows 81% of the activity obtained at pH 8.0. All five models yield identical heat inactivation curves (Figure 3). In the plot of logarithm of residual activity against time the curves exhibit a linear decrease of activity. Approximately 50% of the initial activity was lost after 20 min at 65°C. In immunotitration experiments a non-linear decrease of enzyme activity was obtained in all five groups indicating decreased enzyme inhibition by adding constant amounts of antiserum (Figure 4). Five microlitres of the antiserum at a dilution of 1:50 reduced about 50% of enzyme activity in SD, DOCA, and SD1Kd rats. However, twice the amount of antiserum was required to precipitate the same number of enzyme units in WKY and SHR.

TABLE IV
Michaelis constant of NEP

| group (n) | $K_m$ ($\mu$M) |
|---|---|
| WKY (4) | 267 $\pm$ 12 |
| SHR (4) | 280 $\pm$ 6 |
| SD (4) | 267 $\pm$ 2 |
| DOCA (4) | 278 $\pm$ 8 |
| SD1Kd(4) | 306 $\pm$ 21 |

Mean values $\pm$ SE are given. n indicates number of animals.

TABLE V
$IC_{50}$ values for inhibition of NEP activity ($IC_{50}$)

| group (n) | thirophan (nM) | phosphoramidon (nM) |
|---|---|---|
| WKY (4) | 6.9 $\pm$ 1.2 | 56.5 $\pm$ 3.3 |
| SHR (4) | 6.6 $\pm$ 0.8 | 55.6 $\pm$ 3.2 |
| SD (4) | 6.5 $\pm$ 1.0 | 52.9 $\pm$ 4.9 |
| DOCA (4) | 6.4 $\pm$ 0.6 | 51.7 $\pm$ 4.0 |
| SD1Kd(4) | 6.0 $\pm$ 0.6 | 53.1 $\pm$ 2.7 |

Mean values $\pm$ SE are given. n indicates number of animals

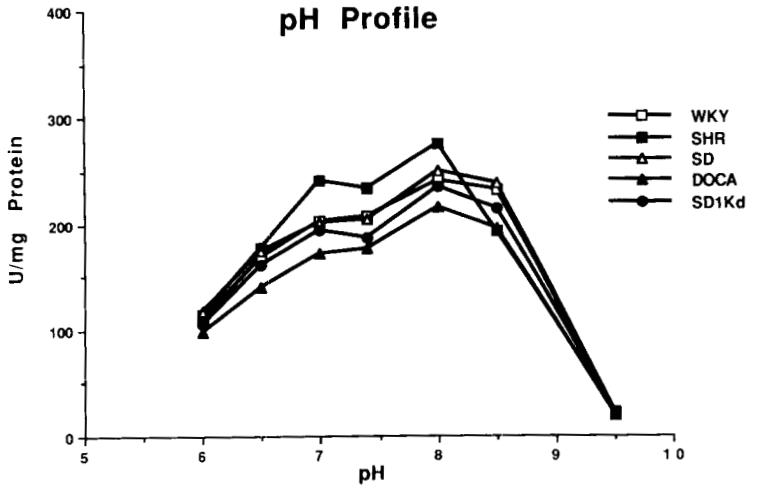


FIGURE 2 pH-activity profiles of NEP activity in kidney cortex homogenates. Each data point represents the mean value of separate experiments with homogenates from four different animals.

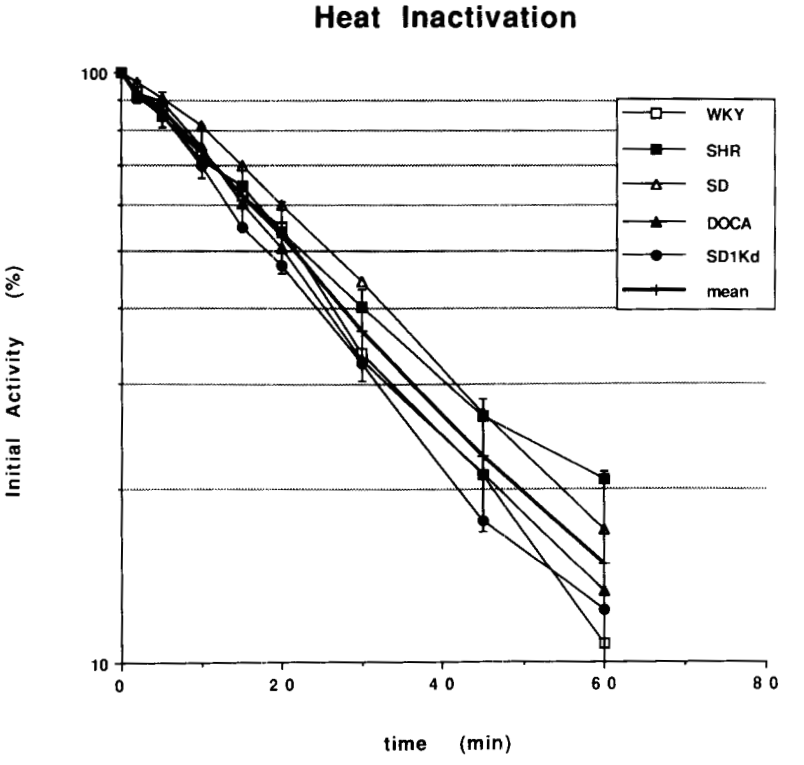


FIGURE 3 Heat inactivation of NEP activity in kidney cortex homogenates at 65°C. Each data point represents the mean value of separate experiments with homogenates from four different animals. As there were no significant differences between the heat-inactivation curves of the five different groups at any time point, mean values ± S.D. for all experiments performed across the different animals models are also given (bold line).

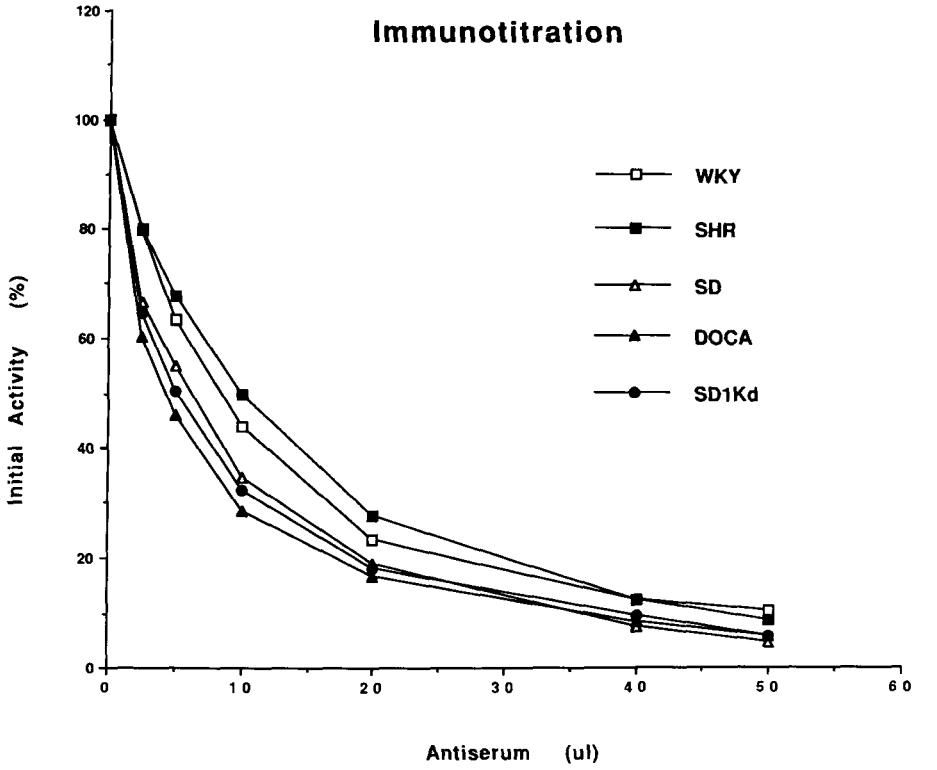


FIGURE 4 Immunotitration of NEP activity in kidney cortex homogenate using polyclonal rabbit anti-rat NEP antiserum. Each data point represents the mean value of separate experiments with homogenates from four different animals.

## DISCUSSION

The hypertensive rat models selected for this study were of two types; genetic (SHR) or experimentally induced (DOCA-salt). In the DOCA-salt model unilateral nephrectomy induces compensatory renal growth i.e., hypertrophy and hyperplasia.[26] In the present study, DNA content increased significantly by 26% following unilateral nephrectomy in SD rats. This is comparable to what has been reported for total DNA content of the whole kidney.[27] A non-significant increase of 16% was found in the DOCA group. The well known effects of corticosteroids to inhibit the synthesis of DNA is apparently responsible for this discrepancy. Nevertheless, the DNA/protein ratio, indicative of hyperplasia, increased significantly both in the DOCA group (46%) and in the SD1Kd group (62%). This is due to a decrease in protein content (Table II), which can be explained by water retention in these two groups with unilateral nephrectomy and 1% NaCl in the drinking fluid. DOCA treatment can be excluded as a causal factor, as the protein content between the DOCA group and the SD1Kd group is not different.

We compared the properties of NEP in the kidney cortex homogenates from WKY,

SHR, SD rats, and DOCA-salt rats. Contrary to previous findings[16] our results do not indicate the presence of any isoforms of NEP in these homogenates. A direct comparison between our findings and the results of Kenney *et al.*[16] is not possible because, (a) they do not specify their rat strain and (b) they used the beta chain of insulin as a substrate for their enzyme assay. Similar to our findings, rabbit, pig and man do not show any isoforms of NEP in the kidney (Kenny *et al.*)[16]

The specific activity of NEP based on protein content was not significantly different in DOCA-salt rat and SD1Kd rats when compared to the SD control rats. However, NEP activity based on DNA activity based on DNA content decreased significantly by 38% (DOCA) and 45% (SD1Kd). This parallels the increase of the DNA/protein ratio suggesting that the changes in NEP activity are due to changes in protein and/or DNA content and not to the presence of different NEP isoenzymes with lower specific activity.

Further evidence that there are no different NEP isoenzymes with lower specific activities is provided by the observation of similar $K_m$ values in all groups and the lack of any differences in affinity of NEP towards the two specific inhibitors thiorphan and phosphoramidon. Also, the heat inactivation experiments showed no differences between the different rat models. The average half-life of NEP at 65°C was 20 min. A pH optimum of 8.0 was found in all groups. It should be noted that the pH profile using Glutaryl-Ala-Ala-Phe-2-naphthylamide as substrate resembles the one where ANF was used as a substrate.[28] However, the optimum pH using ANF was found to be 9.0. Using Concanavalin A affinity column chromatography it appears that about 90% of the NEP activity is glycosylated in all five groups. Sonnenberg *et al.*[1] reported about 80% of the NEP activity to be glycosylated.

Immunotitration experiments revealed no differences between SHR and the normotensive WKY group and between DOCA-salt rats and SD rats. The differences found between WKY/SHR and SD rats (including DOCA-salt and SD1Kd rats) may be explained as follows; since 90–95% of NEP activity can be immunoprecpitated, the enzyme from all five models shows identical antigenic sites.[29] Therefore, the differences seen may be due to the presence of different amounts of enzymatically inactive or partially inactive but cross-reacting proteins.

In summary, our results do not indicate the presence of isoforms of NEP 3.4.24.11 in the rat models studied. This finding helps to validate the concept of using various hypertensive rat models in preclinical studies for the testing of novel NEP inhibitors. Furthermore, it can now be ruled out that divergent results of a given NEP inhibitor in DOCA-salt rats and SHR are due to isoforms of NEP.

## *References*


1. 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.
2. Schiller, P.W., Bellini, F., Dionne, G., Maziak, L.A., Garcia, R., de Lean, A. and Cantin, M. (1986) *Biochem. Biophys. Res. Commun.*, **138,** 880.
3. Krieter, P.A. and Trapani, A.J. (1989) *Drug. Met. Disp.*, **17,** 14.
4. Hasegawa, K., Matsushita, Y., Inoue, T., Morii, H., Ishibashi, M. and Yamaji, T. (1986) *J. Clin. Endocr. Metab.*, **63,** 819.
5. Inagami, T. (1989) *J. Biol. Chem.*, **264,** 3043.
6. Raine, A.E.G., Firth, J.G. and Ledingham, J.G.G. (1989) *Clin. Sci.*, **76,** 1.
7. Singer, D.R.J., Markandu, N.D., Buckley, M.G., Weir, L.M., Sugden, A.L., Jeeves, M., Sagnella, G.A. and MacGregor G.A. (1988) *Am. J. Hypert.*, **1**(3), 93A.
8. Fennell, S.A., Swerdel, J.N., Delaney, N.G. and Seymour, A.A. (1988) *FASEB. J.*, **2,**A936.

9. McMartin, C., Hagen, M.E., Sytwu, I., Webb, R.L., Wennogle, L.P. and Zimmerman, M.B. (1988) *FASEB. J.*, **2,** A936.
10. Olins, G.M., Krieter, P.A., Trapani, A.J. and Spear, K.L. (1988) *FASEB. J.*, **2,** A936.
11. Seymour, A.A., Delaney, N.G., Swerdel, J.N., Fennell, S.A., Neubeck, R., Druckman, S.P., Cushman, D.W. and DeForrest, J.M. (1988) *FASEB. J.*, **2,** A936.
12. Sybertz, E.J., Chiu, P.J.S., Vemulapalli, S., Pitts, B., Foster, C.J., Watkins, R.W., Barnett, A. and Haslanger, M.F. (1989) *J. Pharmacol. Exp. Ther.*, **250,** 624.
13. Trapani, A.J., Smits, G.J., McGraw, D.E., Spear, K.L., Koepke, J.P., Olins, G.M. and Blaine, E.H. (1988) *FASEB. J.*, **2,** A936.
14. Vemulapalli, S., Chiu, P.J.S., Foster, C. and Sybertz, E.J. (1988) *Am. J. Hypert.*, **2**(3), 106A.
15. Zimmerman, M.B., McMartin, C., Yassay, G.D., Wennogle, L.P. and Webb, R.L. (1988) *FASEB. J.*, **2,** A937.
16. Kenny, A.J., Fulcher, I.S., Ridgwell, K. and Ingram, J. (1981) *Acta Biol. Med. Germ.*, **40,** 1465.
17. Mulligan, M.T., Bond, J.S. and Beynon, R.J. (1982) *Biochem. Int.*, **5,** 337.
18. Baum, T., Becker, F.T., Sybertz, E.J., Sabin, C. and Desiderio, D.M. (1983) *Eur. J. Pharmacol.*, **94,** 85.
19. Berecek, K.H., Barron, K.W., Webb, R.L. and Brody, J. (1982) *Hypertension*, **4**(3) II131.
20. Labarca, C. and Paigen, K. (1980) *Analyt. Biochem.*, **102,** 344.
21. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.*, **193,** 265.
22. Orlowski, M. and Wilk, S. (1981) *Biochemistry*, **20,** 4924.
23. Segel, I.H. (1975) *Biochemical Calculations*, 2nd edition. New York: John Wiley and Sons, Inc.
24. Andrews, P. (1964) *Biochem. J.*, **91,** 222.
25. Snedecor, G.W. and Cochran, W.G. (1980) *Statistical methods*. 7th edition. Ames, Iowa: The Iowa State University Press.
26. van Urk H., Malamud, D., Soler-Montesinos, L. and Malt, R. (1978) *Lab. Invest.*, **38,** 674.
27. Preuss, H.G. and Goldin, H. (1983) *Kidney Inst.*, **23,** 635.
28. Koehn, J.A., Norman, J.A., Jones, B.N., LeSuer, L., Sakane, Y. and Ghai, R.D. (1987) *J. Biol. Chem.*, **262,** 11623.
29. Reznick, A.Z. and Gershon, D. (1977) *Mech. Ageing Dev.*, **6,** 345.