

# Angiotensin I–Converting Enzyme Modulates Neutral Endopeptidase Activity in the Rat

Carla Oliveri, María Paz Ocaranza, Ximena Campos, Sergio Lavandero, Jorge E. Jalil

**Abstract**—Angiotensin I is a substrate for both ACE and for neutral endopeptidase 24.11 (NEP). We hypothesized that high ACE expression is related to low NEP activity. Accordingly, circulating and tissue NEP and ACE activities were measured by fluorometry in homozygous rats ( $F_0$  and  $F_2$ ) for the Lewis microsatellite allele (LL, low ACE) and for the Brown Norway microsatellite allele (BB, high ACE). Plasma, lung, and aortic ACE activities in  $F_0$  and  $F_2$  were higher in BB rats than in LL rats ( $P < 0.01$ ), whereas left ventricular ACE activity was similar in both genotypes. In contrast, NEP activity in the LL group was higher in the serum, aorta, and lungs in  $F_0$  and  $F_2$  homozygous ( $P < 0.05$ ). Plasma ACE activity was inversely correlated with serum ( $r = -0.6$  and  $-0.598$  in  $F_0$  and  $F_2$ , respectively;  $P < 0.03$ ) and lung NEP activities ( $r = -0.77$  in  $F_0$  and  $r = -0.59$  in  $F_2$ ,  $P < 0.01$ ). Aortic ACE and NEP activities were also correlated ( $r = -0.696$  and  $-0.584$  in  $F_0$  and  $F_2$ , respectively;  $P < 0.03$ ). In conclusion, genetically determined high ACE expression in rats is inversely related to tissue NEP activity, which could determine lower angiotensin-(1-7) tissue levels.

**Key Words:** angiotensin-converting enzyme ■ angiotensin ■ polymorphism ■ neutral endopeptidase

Angiotensin I–converting enzyme plays an important role in the regulation of the renin angiotensin system (RAS) by hydrolyzing angiotensin (Ang) I to Ang II and degrading bradykinin to bradykinin-(1-7) and Ang-(1-7) to Ang-(1-5), both inactive peptides. In this way, ACE activity influences circulating and tissue levels of Ang II, contributes to regulate the vasculature tone, and may have some effects on cardiac and vascular mass and structure.<sup>1</sup>

Neutral endopeptidase (NEP, enkephalinase, neprilysin, EC 3.4.24.11) is a transmembrane zinc metalloendopeptidase, present at the surface of several tissues.<sup>2</sup> NEP also participates in the RAS, cleaving Ang I and Ang II.<sup>3,4</sup> The action of NEP on Ang I generates Ang-(1-7), an heptapeptide with vasodilator activity. The physiological effects of Ang-(1-7) are opposite those of Ang II and would favor a blood pressure–lowering effect under conditions of high Ang II activity.<sup>3,4</sup>

In humans, the ACE gene insertion/deletion (I/D) polymorphism is due to the presence (insertion) or absence (deletion) of a 287-bp sequence in the intron 16 on the chromosome 17q23<sup>5</sup> and determines plasma ACE levels by 50%. The D allele is associated with increased ACE and possibly increased Ang II levels.<sup>6,7</sup> Recent evidence suggests that the DD genotype—with higher plasma ACE activity—is associated with higher risk of hypertension in men.<sup>8</sup> A similar polymorphism has been observed in normotensive rats,

whereas homozygous rats for the Lou and Lewis microsatellite alleles have lower ACE activity than that of homozygous rats for the Brown Norway microsatellite allele (BB).<sup>9</sup>

It remains unknown whether NEP activity—because of its interaction with Ang I and Ang II as well as with bradykinin—is modulated by ACE expression. Because higher ACE activity will decrease Ang I and bradykinin levels and increase Ang II levels, we hypothesized that an inverse relationship between ACE expression (genetically determined) and NEP activity might exist. To test this hypothesis, NEP activity was determined in serum and in different tissues from male Lewis homozygous (LL, genetically with low ACE activity) and BB rats (genetically with high ACE activity) in  $F_0$  and in homozygous  $F_2$ . These rats were also characterized by their ACE genotype and circulating and tissue ACE activity.

## Methods

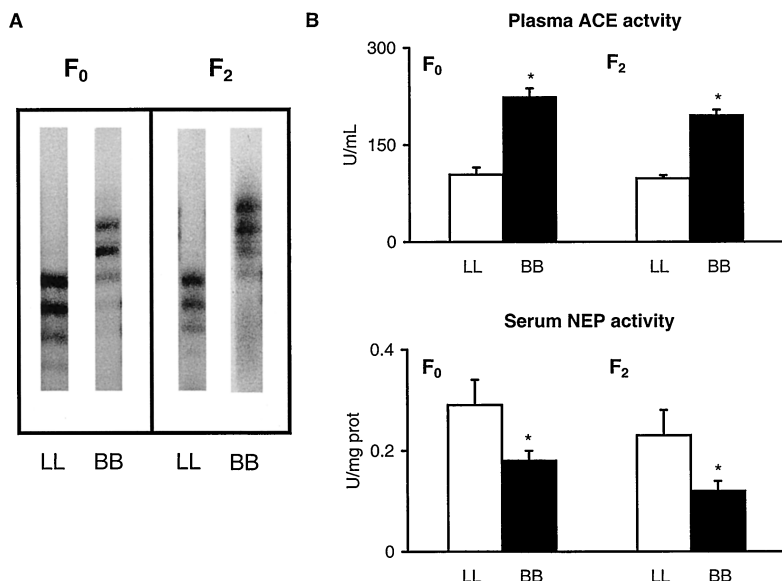
The experiments were performed following the recommendations of the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 93-23, revised 1985).

## Animals

Male normotensive rats were used. They were homozygous  $F_0$  and  $F_2$ . The  $F_2$  homozygous were obtained after mating male  $F_0$  Brown Norway with female Lewis (LL) inbred strains obtained from Charles Rivers (Wilmington, Mass). These  $F_0$  rats produced  $F_1$

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**Figure 1.** A, Genetic characterization of the ACE microsatellite marker in homozygous F<sub>0</sub> and F<sub>2</sub> rats. DNA was isolated from peripheral blood, and an ACE gene promoter region was amplified by polymerase chain reaction using specific sense and antisense oligonucleotides. The antisense oligonucleotide was previously radio-labeled with [ $\gamma$ -<sup>32</sup>P]ATP by polynucleotide kinase reaction. The radioactive-amplified fragments were separated by acrylamide-bisacrylamide gel. Finally, this gel was radioautographed for 16 hours at -80°C. B, Plasma ACE activity and serum NEP activity in homozygous F<sub>0</sub> and F<sub>2</sub> rats were measured fluorometrically by following the hydrolysis of Z-phenyl-L-histidyl-L-leucine and DAGPNG, respectively. Plasma ACE activity was expressed as U/mL; and serum NEP activity, as U/mg protein (n=6 to 9/group). 1 U=1 nmol/min. Values are mean±SEM. \*P<0.05.

hybrids that were mated to obtain the F<sub>2</sub> cohort. The animals were given a standard rat chow with salt and water ad libitum.

### ACE Polymorphism Determination

The polymerase chain reaction primers (sense oligonucleotide primer, 5'-ATT ACC ATA GAG GGC AGC AAG ATC-3'; anti-sense primer, 5'-CAG ACT TTT CAC CAA TTT TGA CAGC-3') and the procedure used to amplify the microsatellite located at the 5' end of the intron 13 inside the rat ACE gene and characterized by a (CA)<sub>n</sub> repeat were performed as described by Hilbert et al<sup>10</sup> and Challah et al<sup>9</sup>. DNA was extracted from circulating leukocytes using Chelex 100.<sup>11</sup>

### ACE Activity Determination

ACE activity was measured fluorometrically by following the hydrolysis of Z-phenyl-L-histidyl-L-leucine (Bachem Bioscience Inc), an analogous substrate for ACE.<sup>12,13</sup> The product L-histidyl-L-leucine was quantified by spectrofluorometry. Plasma ACE activity was expressed in U/mL (1 U=1 nmol/min).

For measurement of tissue ACE activity, 100 mg of tissue (lung, aorta, and left ventricle [LV]) were homogenized with TN buffer (50 mmol/L Tris-HCl at pH 8.0, 1% NaCl). The extracts were centrifuged at 1000g for 15 minutes at 4°C, and the supernatant was recentrifuged at 15 000g for 60 minutes at 4°C. The pellet was resuspended in 1 mL TN buffer and centrifuged at 15 000g for 5 minutes at 4°C. Finally, the pellet was resuspended in 1 mL cold TN buffer containing 8 mmol/L 3[(3-cholamidopropoyl)-dimethylammonio]-1-propane-sulfonate. Protein concentrations were measured by the method of Bradford,<sup>14</sup> using bovine globulin as standard. Tissue ACE activity was expressed in U/mg protein. All measurements were performed in duplicate.

### NEP Activity

The NEP activity was measured fluorometrically as described in detail by Florentin et al.<sup>15</sup> Briefly, 100  $\mu$ g protein was incubated for 30 minutes at 37°C with 50  $\mu$ mol/L dansyl-D-Ala-Gly-p-nitro-Phe-Gly (DAGPNG, Sigma), 200  $\mu$ mol/L enalapril (Laboratorio Saval), and 50 mmol/L Tris-HCl buffer (pH 7.4) in the presence or absence of 20 nmol/L thiorphan (Sigma). Enzymatic reactions were stopped by boiling at 95°C for 5 minutes. The samples were then diluted with Tris-HCl buffer and centrifuged at 5000g for 30 minutes. The fluorescence of the supernatant was measured with a fluorescence spectrometer at 562 nm ( $\lambda_{ex}$  342 nm). The calibration curve was prepared by adding increasing concentrations of dansyl-D-Ala-Gly (DAG, Sigma) (1 to 10  $\mu$ mol/L) and decreasing concentrations of

DAGPNG (50 to 40  $\mu$ mol/L). NEP activity was expressed in U/mg protein.

For measurement of tissue NEP activity, the tissues were quickly removed, washed extensively with cold saline solution to remove all blood, and homogenized in 10 volume cold 50 mmol/L Tris-HCl buffer (pH 7.4) using an Ultraturax homogenizer. The homogenate was centrifuged for 5 minutes at 1000g at 4°C. The pellet was discarded and the supernatant centrifuged at 60 000g for 60 minutes at 4°C. The resulting pellet was superficially washed 3 times with cold buffer and resuspended in 50 mmol/L Tris-HCl buffer (pH 7.4) and used as the enzymatic source. Protein was measured by the method of Bradford.<sup>14</sup>

### Statistical Analysis

Results are shown as mean±SEM. Comparisons between the 2 groups (LL and BB) were performed with Student's *t* test for independent measurements or with the Mann Whitney test. Linear correlation was also applied. A *P* value  $\leq$ 0.05 was considered statistically significant.

## Results

### ACE Genotypes and General Characteristics

Eight F<sub>0</sub> LL and 6 BB male rats as well as 9 F<sub>2</sub> LL and 6 F<sub>2</sub> BB male rats were killed. Genetic variability of ACE was determined by polymerase chain reaction from DNA extracted and purified from circulating leukocytes (Figure 1A). ACE microsatellite marker analysis in the F<sub>0</sub> and F<sub>2</sub> cohorts demonstrated that the difference between LL (rats with low ACE activity) and BB (with high ACE activity) rats was only in 4 bp, (168 and 171 bp, respectively). No differences were observed between the LL and BB genotypes in both cohorts in body weight, LV weight, right ventricle weight, or systolic blood pressure (Table 1).

### Circulating ACE and NEP Activities

In accordance with the genotypes, plasma ACE activity (Figure 1B) in the BB rats was 116% and 100% higher than that in the LL rats in the F<sub>0</sub> and F<sub>2</sub> groups, whereas serum NEP activity was significantly lower, 35% and 49%, in rats with the BB genotype in the F<sub>0</sub> and F<sub>2</sub> groups, respectively.

### Systolic Blood Pressure and Relative Cardiac Weight in Both Genotypes

	F <sub>0</sub>		F <sub>2</sub>	
	LL (n=8)	BB (n=6)	LL (n=9)	BB (n=6)
Systolic blood pressure, mm Hg	100±6	108±3	117±7	109±3
Relative LV weight (g LV×100/body weight)	0.25±0.01	0.22±0.01	0.27±0.01	0.26±0.01
Relative right ventricle weight (g RV×100/body weight)	0.10±0.01	0.07±0.01	0.06±0.04	0.07±0.02

Values are mean±SEM.

### Tissue ACE Activity

ACE activity showed important variations according to the nature of the sample and the ACE gene polymorphism. As shown in Figure 2A, lung and aortic ACE activities were significantly increased in the BB rats compared with LL rats in the F<sub>0</sub> and F<sub>2</sub> groups (69% and 240% in F<sub>0</sub> and 81% and 107% in F<sub>2</sub>, respectively;  $P<0.05$ ). No differences were observed in LV ACE activities between the 2 genotypes in both cohorts.

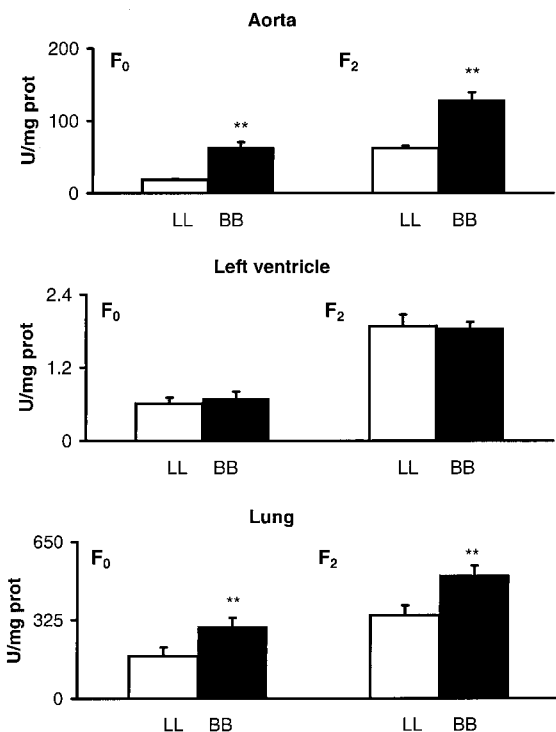
### Tissue NEP Activity

Tissue NEP activity was constantly higher in rats with the LL genotype in all the sampled tissues such as lung, LV, and

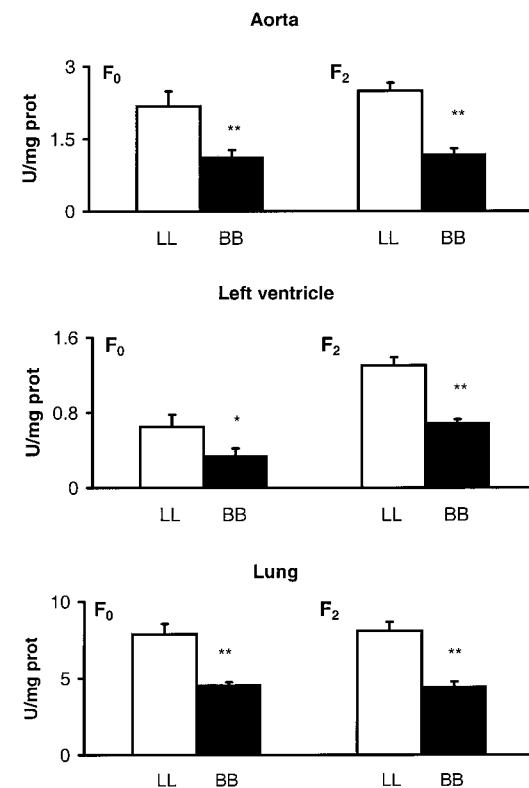
aorta (72%, 92%, and 94% in the F<sub>0</sub> generation and 81%, 93%, and 106%, in the F<sub>2</sub> generation, respectively;  $P<0.01$ ) (Figure 2b). NEP activity was also higher in rats with the LL genotype compared with those with the BB genotype in other tissues (measured in the F<sub>2</sub> generation) such as liver ( $0.79±0.08$  versus  $0.27±0.03$  U/mg protein,  $P<0.01$ ), right ventricle ( $0.88±0.16$  versus  $0.32±0.08$  U/mg protein,  $P<0.01$ ), and kidney ( $91.5±2.4$  versus  $71.3±6.2$  U/mg protein,  $P<0.03$ ).

A significant correlation was found between plasma ACE and serum NEP activities ( $r=-0.6$  and  $-0.598$  in F<sub>0</sub> and F<sub>2</sub>, respectively;  $P<0.03$ ) and also between aortic ACE and NEP activities ( $r=-0.696$  and  $-0.584$  in F<sub>0</sub> and F<sub>2</sub>, respectively;  $P<0.03$ ).

### A Angiotensin-converting enzyme activity



### B Neutral endopeptidase 24.11 activity



**Figure 2.** Aorta, LV, and lung ACE (A) and NEP (B) activities in rats with different ACE gene polymorphisms. LL and BB homozygous F<sub>0</sub> and F<sub>2</sub> male rats were genotyped by a polymerase chain reaction procedure and killed. Tissues were quickly removed, homogenized, and centrifuged. Protein was measured in the resulting pellet. ACE and NEP activities were measured fluorometrically by following the hydrolysis of Z-phenyl-L-histidyl-L-leucine or DAGPNG, respectively. Tissue activities (n=6 to 9/group) were expressed as U/mg protein, respectively. 1 U=1 nmol/min. Values are mean±SEM. \* $P<0.05$ , \*\* $P<0.01$ .

Plasma ACE activity was inversely correlated with lung NEP activity ( $r = -0.77$  in  $F_0$ , and  $r = -0.59$  in  $F_2$ ;  $P < 0.01$ ).

## Discussion

NEP is constitutively expressed in endothelial cells from kidney, lung, and vascular wall<sup>16</sup> and is inducible by thrombin via activation of the protein kinase C pathway.<sup>17</sup> This enzyme has specificity for a variety of substrates (specially for the peptide bond phenylalanine-leucine), such as bradykinin, substance P, and angiotensin,<sup>18–20</sup> and contributes to the degradation of extracellular bradykinin, especially when ACE is inhibited.<sup>21</sup> Because NEP is a potent inactivator of vasoactive and inflammatory peptides, such as the chemotactic peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine, bradykinin, atrial natriuretic peptide, Ang I, endothelins, and tachykinins, its expression and regulation might influence local vasomotor and inflammatory responses in the macrovasculature and microvasculature.<sup>17</sup> Besides a constitutive expression of NEP, induction of this peptidase is possible via phospholipase C and protein kinase C activation.<sup>17</sup> Possibly, multiple promoters are involved in regulating expression of the NEP gene. The level of expression of the NEP transcripts varies in different tissues.<sup>22</sup> The mRNAs derived from the NEP gene can be differentially expressed in a regional- and cell-specific manner.<sup>22</sup>

In normotensive rats and in humans, there is variation of the plasma ACE concentration, likely resulting from a polymorphism of the expression of the gene.<sup>9</sup> Our study in homozygous rats shows for first time the influence of an ACE gene polymorphism on different levels of circulating and tissue NEP activities, suggesting the existence of a modulating effect of ACE expression on NEP activity. NEP activity was significantly decreased in serum, lung, aorta, LV, kidney, and liver in rats with genetically high ACE activity. In serum and in all these tissues, NEP activity had an inverse relationship with ACE activity (genetically determined). In the LV, however, we did not observe different ACE activity between both genotypes, probably because of the young age of the animals.

We examined here 2 ACE genotypes: the BB and LL rats that exhibit contrasted levels of circulating and tissue ACE and NEP. The plasma ACE results confirm previous data obtained in an  $F_2$  intercross population between Brown Norway and Lou rats<sup>9</sup> and between the stroke-prone spontaneously hypertensive rats and the normotensive Wistar-Kyoto strain.<sup>23</sup> This degree of genetic determination of ACE expression in inbred rat strains offers an opportunity to study the interaction between genetic and environmental determinants of ACE<sup>9</sup> and NEP expression.

In humans, the ACE D allele is associated with cardiovascular disease, which may be related to enhanced Ang II production and to degradation of bradykinin.<sup>24</sup> In this respect, Brown et al<sup>25</sup> observed a significantly longer half-life of bradykinin in serum from subjects with the II genotype. Another new mechanism for explaining the association of the ACE D allele with cardiovascular disease—which is suggested by our results—is the possible finding of low levels of NEP activity in subjects with the ACE D allele.

In the present study, we did not measure angiotensins or kinins levels, which could have helped us to understand the mechanisms of ACE expression on modulation of NEP levels. Possible mechanisms of reduced NEP activity in the presence of high ACE expression (or vice versa) could be by reduced Ang-(1-7) and bradykinin levels or increased Ang II levels through a receptor-mediated mechanism. A direct effect of ACE expression or activity at the level of NEP expression by Ang-(1-7) or Ang II should be further investigated.

The findings of the present study could also help to understand the variability and sometimes unpredictable clinical efficacy of ACE inhibitors (and also of Ang II receptor blockers and possibly of the new vaso-peptidase inhibitors) in patients with hypertension or heart failure. The effects of these inhibitors could be dependent in some extent on the interaction of ACE expression with NEP activity. Drummer et al<sup>26</sup> have also suggested that chronic treatment with ACE inhibitor may differentially affect the NEP activity, causing a substantial re-direction of angiotensin metabolism. In the rat, separate inhibition of either ACE or NEP induces both enzymes, with varying induction in different tissues.<sup>27</sup> Further research using NEP or ACE inhibitors on the  $F_2$  generation of LL (low ACE) and BB (high ACE) rats may clarify whether there is a differential pharmacological and biological response to both inhibitors.

In conclusion, these results are consistent with a modulatory effect of the ACE polymorphism on NEP activity in the rat (and possibly in humans). High ACE expression is associated with low circulating and tissue NEP activity. This inverse relationship could explain some pathogenic effects due to the presence of the ACE D allele in humans and the observed variability of the clinical effects of the renin-angiotensin system blockers.

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