Neutral endopeptidase and angiotensin I converting enzyme insertion/deletion gene polymorphism in humans

JE Jalil[1], MP Ocaranza[1], C Oliveri[1,2], S Córdova[1], I Godoy[1], G Chamorro[1], S Braun[1], C Fardella[3], J-B Michel[4] and S Lavandero[2,5]

[1]*Department of Cardiovascular Diseases, Medical School, P. Catholic University of Chile, Marcoleta, Chile;* [2]*Department of Biochemistry and Molecular Biology, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago, Chile;* [3]*Department of Endocrinology, Medical School, P. Catholic University of Chile, Marcoleta, Chile;* [4]*INSERM, Unité 460 (Remodelage Cardiovasculaire), Paris, France;* [5]*Fondap Center for Cellular Molecular Studies*

Neutral endopeptidase (NEP) hydrolyses angiotensins (Ang) I and II and generates angiotensin-(1-7) [Ang-(1-7)]. In humans, the insertion/deletion (I/D) angiotensin-I converting enzyme (ACE) gene polymorphism determined plasma ACE levels by 40%. In rats, a similar polymorphism determines ACE levels which are inversely associated to NEP activity. The objective of this study is to evaluate the relationship between ACE expression and plasma NEP activity in normotensive subjects and in hypertensive patients. In total, 58 consecutive patients with hypertension, evaluated in our Hypertension Clinic, were compared according to their ACE I/D genotypes with 54 control subjects in terms of both plasma ACE activity and NEP activities. Plasma ACE activity was elevated 51 and 70% in both DD ACE groups (normotensives and hypertensives) compared with their respective ID and II ACE groups ($P<0.001$). A significant effect of the ACE polymorphism and of the hypertensive status on ACE activity was observed ($P<0.001$). In normotensive DD ACE subjects, NEP activity was 0.30 ± 0.02 U/ml, whereas in the normotensive II ACE and in the normotensive ID ACE subjects NEP activity was increased 65 and 48%, respectively ($P<0.001$). In the hypertensive DD ACE patients, NEP activity was 0.47 ± 0.03 U/mg. An effect of the I/D ACE genotypes on NEP activity ($P<0.04$) and an interaction effect between the I/D ACE genotype and the hypertensive status were also observed ($P<0.001$). These results are consistent with a normal and inverse relationship between the ACE polymorphism and NEP activity in normotensive humans (as is also observed in rats). This normal relationship is not observed in hypertensive patients.

Keywords: angiotensin converting enzyme; neutral endopeptidase; ACE polymorphism

Introduction

Neutral endopeptidase (NEP, enkephalinase, neprilysin, EC 3. 4.24.11), a zinc metalloendopeptidase, plays an important role in turning off peptide signalling events at the cell surface. NEP is an integral plasma membrane ectopeptidase of the M13 family of zinc peptidases,[1] distributed in endothelial cells, smooth muscle cells, cardiac myocytes, renal epithelial cells, and fibroblasts. NEP is found in the lung, gut, adrenal glands, brain, and in the heart[2,3] and cleaves several endogenous vasodilator and vasoconstrictor peptides and has a catalytic unit similar to that of the angiotensin I converting enzyme (ACE, kininase II, EC 3.4.15.1).[3]

NEP interacts within the renin angiotensin system (RAS) cleaving angiotensin I (Ang I) and angiotensin II (Ang II)[4,5] (Figure 1). NEP's action on Ang I generates angiotensin-(1-7) [Ang-(1-7)], an heptapeptide with vasodilator activity. The physiological effects of Ang-(1-7) are opposite to those of Ang II and would favour a blood-pressure-lowering effect under conditions of high Ang II activity.[4,5] On the other side, atrial natriuretic factor (ANF) is metabolized by NEP, an effect that could increase blood pressure. The final balance of the effects of NEP on the generation of Ang-(1-7) and metabolism of ANF contribute to determine the levels of blood pressure.

Correspondence: Dr JE Jalil, MD, Department of Cardiovascular Diseases, Medical School, P. Catholic University of Chile, Marcoleta 367, Santiago, Chile.
E-mail: jjalil@med.puc.cl

ACE plays a significant role in the regulation of the RAS by hydrolysing Ang I to Ang II and degradating bradykinin (BK) to BK-(1-7) and also Ang-(1-7) to Ang-(1-5), both inactive peptides (Figure 1). 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.[2] In hypertension, combined inhibition of ACE and NEP (vasopeptidase inhibition) reduces blood pressure.

In humans, the I/D ACE gene polymorphism is due to the presence (insertion, I) or absence (deletion, D) of a 287 bp sequence in the intron 16 on the chromosome 17q23[6] and determines plasma ACE levels by 40%. The D allele is associated with increased ACE activity.[7–9]

Recent evidence suggests that the DD ACE genotype is associated with higher risk of hypertension only in men.[10–12] However, this association is not seen in all populations.

It remains unknown whether NEP activity in humans—given its interaction with Ang I and Ang II and with BK—is modulated by ACE expression in a similar way as in the normotensive rat. Since higher ACE activity will decrease Ang I and BK levels and increase Ang II levels, we hypothesized here that an inverse relationship between ACE expression (genetically determined) and NEP activity might also exist. We also evaluated the effect of hypertension on this relationship. To test this hypothesis, NEP activity was determined in plasma from normotensive and hypertensive homozygous subjects with (a) the DD ACE genotype (genetically with higher plasma ACE activity) and (b) the ACE II genotype (genetically with lower ACE activity). These subjects were also characterized according to their ACE genotype and circulating ACE activity.

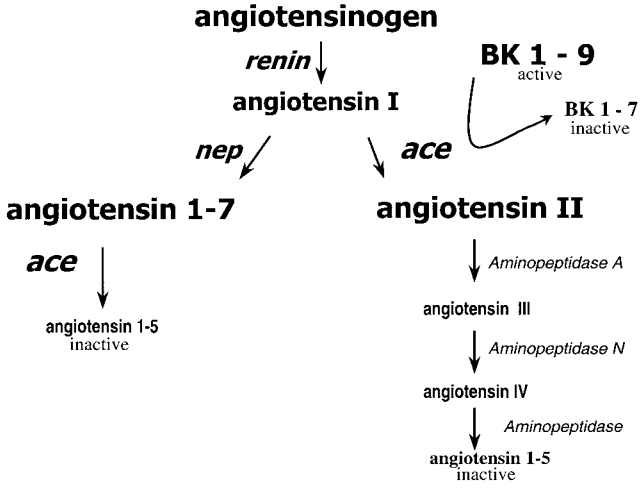

Figure 1 Interactions between NEP and ACE. Enzymes in the system are written in italics. Abbreviations: BK = bradykinin.

Methods

Study design

The study was approved by the Research Committee of the Medical School, P. Catholic University of Chile. Participants were consecutive patients with essential hypertension ($n = 58$) evaluated in our hypertension clinic (BP $\geqslant$ 140/90 mmHg measured twice in sitting position in 2 different days, not taking antihypertensive drugs), with normal renal function, nondiabetic, nonobese (body mass index $< 28\,kg/m^2$), without secondary hypertension. Their characteristics are depicted in Table 1. Controls ($n = 54$) were healthy normotensive subjects (BP < 140/90 mmHg measured twice in sitting position, not taking antihypertensive drugs), selected from a population-based study concerning prevalence of risk factors for chronic diseases, nonobese, and nondiabetics.[13] Blood pressure was determined twice in the sitting position and later at the time of echocardiographic examination. For the analysis, the last blood-pressure determinations were averaged. Their characteristics are described in Table 2.

Table 1 Demographics and laboratory results of the hypertensive patients (mean ± s.e.m.)

ACE I/D Genotype	*II*	*ID*	*DD*		
N	*18*	*22*	*18*	*F*	P
Age (years)	52.0 ± 1.4	50.9 ± 1.0	52.2 ± 0.9	0.4	NS
Males/females	7/11	12/10	9/9		NS*
Body weight (kg)	68.6 ± 2.3	72.0 ± 2.6	68.6 ± 2.0	0.7	NS
BMI (m/kg^2)	25.6** ± 0.5	27.2 ± 0.4	26.2 ± 0.3	3.6	0.034
Systolic BP (mmHg)	157.6 ± 5.3	161.5 ± 4.8	158.0 ± 3.8	0.2	NS
Diastolic BP (mmHg)	96.8 ± 1.7	100.4 ± 2.3	98.4 ± 2.0	0.7	NS
LV mass index (g/m^2)	80.4 ± 3.4	87.7 ± 7.5	89.3 ± 4.1	0.8	NS
Serum creatinine (mg/dl)	1.0 ± 0.1	0.9 ± 0.0	1.0 ± 0.0	0.8	NS
Haematocrit (%)	42.4 ± 1.1	41.5 ± 1.3	43.7 ± 0.8	1.2	NS

*χ^2.
**$P < 0.05$ *vs* the other genotypes.

Table 2 Demographics and laboratory results of the normotensive subjects (mean ± s.e.m.)

ACE I/D Genotype	*II*	*ID*	*DD*		
N	*17*	*18*	*19*	*F*	P
Age (years)	51.7 ± 0.9	51.9 ± 1.1	50.0 ± 0.9	1.5	NS
Males/females	9/8	8/11	8/10	0.5*	NS
Body weight (kg)	61.8 ± 1.9	65.8 ± 2.9	59.8 ± 2.4	1.5	NS
BMI (m/kg^2)	24.1 ± 0.5	24.5 ± 0.6	23.4 ± 0.7	0.8	NS
Systolic BP (mmHg)	123.2 ± 2.3	124.6 ± 2.3	125.7 ± 1.9	0.6	NS
Diastolic BP (mmHg)	73.8 ± 1.7	75.7 ± 1.6	76.2 ± 1.4	0.8	NS
LV mass index (g/m^2)	64.5 ± 3.3	65.7 ± 3.1	65.3 ± 4.0	0.0	NS
Serum creatinine (mg/dl)	0.84 ± 0.04	0.79 ± 0.03	0.79 ± 0.03	0.9	NS
Haematocrit (%)	44.6 ± 1.0	42.9 ± 0.8	43.0 ± 1.1	1.1	NS

*χ^2.
NS: not significant.

DNA extraction and ACE polymorphism determination

After subjects signed the informed consent, one blood sample was obtained and the ACE polymorphism was determined in DNA extracted from leucocytes and amplified by polymerase chain reaction (PCR) as previously described.[9] The sense oligonucleotide primer was 5′ CTG GAG ACC ACT CCC ATC CTT TCT 3′ (ACE1, Eurogentecs, France) and the antisense primer: 5′ GAT GTG GCC ATC ACA TCC GTC AGAT 3′ (ACE2, Eurogentecs, France).[9,14] As the D allele in heterozygous samples is preferentially amplified, each sample found to have the DD genotype was subjected to a second, independent PCR amplification with a primer pair that recognizes an insertion-specific sequence (ACE3, 5′TGG GAC CAC AGC GCC CGC CAC TAC 3′; ACE4, 5′ TCG CCA GCC CTC CCA TGC CCA TAA3′. The PCR procedure consisted of denaturation at 95°C for 30 s, annealing at 67°C for 45 s, and extension at 72°C for 2 min, repeated for 35 cycles, followed by a final extension at 72°C for 10 min.[15] This reaction yields a 335-bp amplicon only in the presence of an I allele, and no product in samples homozygous DD. We analysed all the samples with the DD allele using this procedure and none of them was misclassified. Additionaly, the Hardy–Weinberg equilibrium was confirmed.

Measurement of plasma ACE activity

Another blood sample was obtained in a chilled heparinized tube (after overnight fasting). The sample was then centrifuged within 3 h at 4°C, plasma was stored at −80°C and processed within 4 weeks. The method used was based on spectrofluorimetric determination of histidyl-L-leucine (HL) using Z-phenyl-histidyl-leucine (Bachem Bioscience Inc, USA) as an ACE substrate.[16–18] All determinations were made simultaneously in duplicate. Intraassay and interassay variation coefficients were both 1%.[18]

Plasma NEP activity determination

A blood sample was obtained in a chilled heparinized tube. The sample was centrifuged at 4°C and plasma was stored at −80°C. The NEP activity was measured fluorimetrically according to Florentin *et al.*[19] Briefly, plasma (15 μl) was incubated at 37°C during 40 min with 50 μmol/l Dansyl-D-Ala-Gly-Phe(pNO_2)-Gly (DAGPNG, Sigma, St Louis, MO, USA), 200 nmol/l enalapril (Laboratorio Saval, Santiago, Chile) and 50 mmol/l Tris-HCl buffer (pH 7.4) in the presence or absence of 20 nmol/l thiorphan (Sigma, St Louis, MO, USA). Enzymatic reactions were stopped by boiling at 95°C. The samples were then diluted with Tris-HCl buffer and centrifuged at 5000 g. The fluorescence in the supernatant was measured with a fluorescence spectrometer at 562 nm (λ_{ex} 342 nm). The calibration curve was prepared by adding increasing concentrations of Dansyl-D-Ala-Gly (DAG, Sigma, St Louis, MO, USA) (1–10 μmol/l) and decreasing concentrations of DAGPNG (50–40 μmol/l). Proteins were measured by the Bradford's method.[20] Plasma NEP activity was expressed in U/mg protein (1 U = 1 nmol DAG/min). All determinations were made in duplicate. Intraassay and interassay variation coefficients were 10 and 9.7%, respectively.

Echocardiographic measurements

They were obtained with a 3.5 MHz transducer at the same time of blood sampling with an Aloka SSD 875 equipment in order to evaluate LV mass. All measurements were performed according to the recommendations of the American Association of Echocardiography.[21] The following variables in the parasternal short axis were measured: interventricular septal thickness (IVSpTh) and posterior wall thickness (PWTh), end diastolic (EDD) and end systolic dimension (ESD). With these variables, LV mass and LV mass indexes were calculated according to the formula developed by Devereux and modified by the ASE.[22]

Statistical analysis

The results are presented as mean ± s.e.m. One way ANOVA followed by Student–Newmans–Keul test as well as ANOVA with two factors using I/D ACE genotypes and hypertension status (hypertensive or normotensive) as independent factors as well as linear regression and χ^2 tests were used (SPSS 10.1).

Results

Demographics, blood pressure and laboratory results (Tables 1 and 2)

In all, 112 subjects were consecutively evaluated (54 normotensives and 58 hypertensives). Genotype distribution between hypertensives and normotensives was the same. Both groups were similar in terms of age, sex distribution, renal function, and haematocrit. Blood pressure and LV mass index were higher ($P< 0.05$) in the hypertensive patients, without differences among the genotypes.

Plasma ACE activity

Figure 2 shows that plasma ACE activity was significantly elevated by 51 and 70% in both DD ACE homozygous groups (normotensives and hypertensives) compared with their respective ID and II ACE groups ($F=31$; $P<0.001$ and $F=24.5$; $P<0.001$, respectively). Plasma ACE activity increased by 19% ($P<0.01$) in the three hypertensive groups compared with the three normotensive groups.

By ANOVA with two factors (genotype and presence/absence of hypertension), a significant effect of the genotype and of the hypertensive status on plasma ACE activity was observed ($F=50.4$, $P<0.001$ and $F=12.4$, $P=0.001$, respectively) without interaction of these factors on plasma ACE activity.

Plasma NEP activity

In the normotensive homozygous DD ACE subjects, plasma NEP activity was 0.30 ± 0.02 U/ml, whereas in the normotensive homozygous II ACE and in the normotensive heterozygous ID ACE subjects plasma NEP activity was increased by 65 and 48%, respectively ($F=18.2$; $P<0.001$, Figure 3a).

In the hypertensive homozygous DD ACE patients, however, plasma NEP activity was 0.47 ± 0.03 U/mg, which was similar to that observed in hypertensive homozygous II ACE and in the hypertensive heterozygous ID ACE subjects ($F=2.5$; $P=0.093$, power 54%; Figure 3b).

A negative linear relationship was observed in the normotensive subjects between plasma ACE and plasma NEP activities ($r=-0.53$, $P<0.01$, Figure 4a).

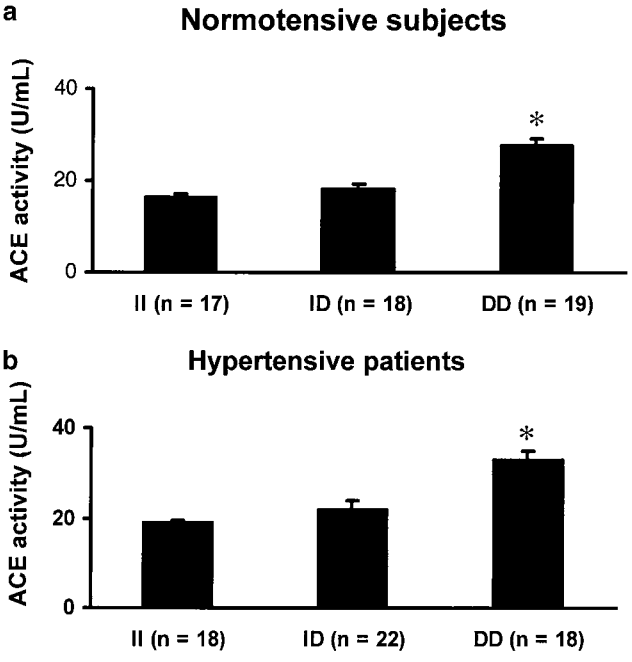

Figure 2 Plasma ACE activity in normotensive subjects ((a), $n=55$) and in hypertensive patients ((b), $n=58$). ACE activity was measured by the spectrofluorimetric determination of histidyl-L-leucine (HL) using Z-phenyl-histidyl-leucine as an ACE substrate. Symbol: *$P<$ 0.05 *vs* the ACE II and ACE ID groups after a significant ANOVA ($P<0.001$).

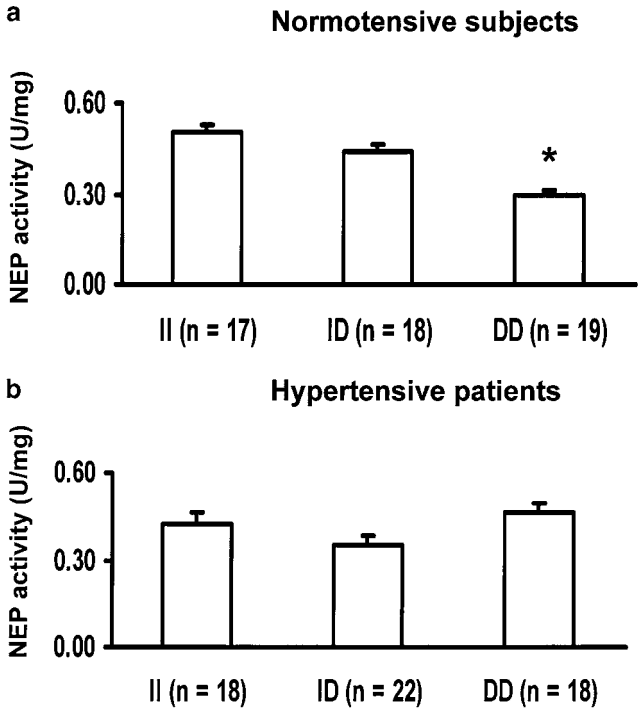

Figure 3 Plasma NEP activity in normotensive subjects (a) and in hypertensive patients (b). NEP activity was measured by fluorescence using Dansyl-D-Ala-Gly-Phe(pNO_2)-Gly (DAGPNG) as a substrate in the presence or absence of thiorphan. Symbol: *$P<0.05$ *vs* the ACE II and ACE ID groups after a significant ANOVA ($P<0.001$).

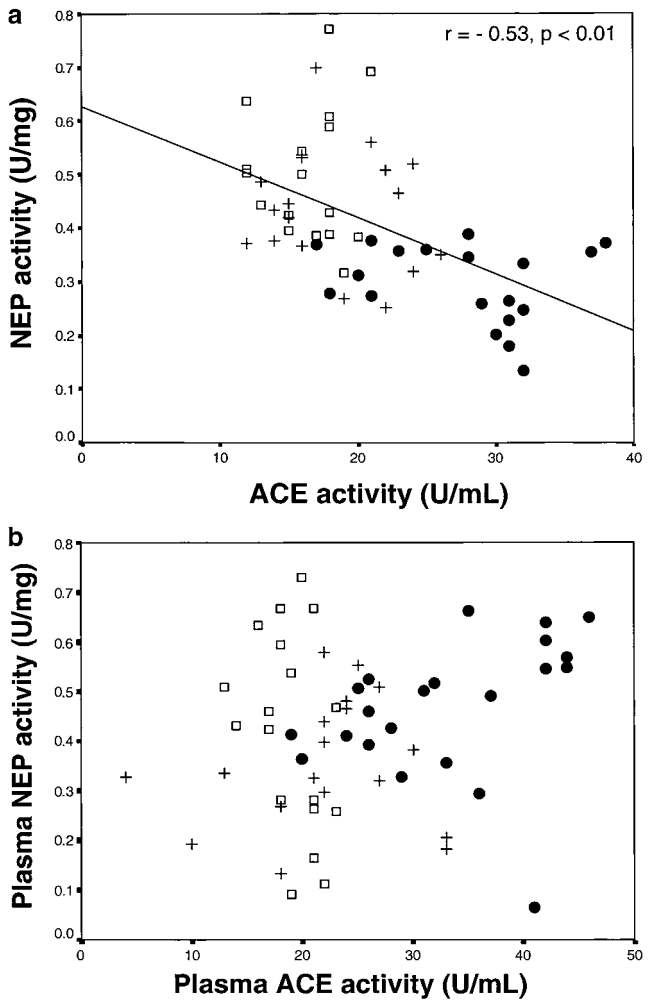

Figure 4 Relationship between plasma NEP and ACE activities. (a) Normotensive (control) subjects ($n = 54$). Symbols: normotensive ACE DD subjects (●); normotensive ACE ID subjects (+), normotensive ACE II subjects (○). (b) Hypertensive patients ($n = 58$). Symbols: hypertensive ACE DD subjects (●); hypertensive ACE ID subjects (+), hypertensive ACE II subjects (○).

In the hypertensive patients, no such linear relationship was observed (Figure 4b).

By ANOVA with two factors, a significant effect of the I/D ACE genotype on plasma NEP activity was observed ($F = 3.6$; $P < 0.04$) and an interaction effect between the I/D ACE genotype and the hypertensive status was observed ($F = 11.3$, $P < 0.001$; power 99%).

Discussion

The main findings of the present study were: (a) in normotensive homozygous DD ACE genotype subjects (with genetically determined higher plasma ACE activity), plasma NEP activity was significantly lower than in normotensive homozygous II ACE genotype and in normotensive heterozygous homozygous II ACE genotype subjects (with genetically determined lower plasma ACE activity) and (b) this difference in plasma NEP activity was not observed in the presence of hypertension.

NEP is constitutively expressed in endothelial cells from the kidney, lung, and vascular wall[23,24] and has a broad specificity for several substrates, such as BK, substance P, angiotensin, and adrenomedullin.[25–28] NEP also contributes to the degradation of extracellular BK (specially when ACE is inhibited).[29] In human cardiac tissue, NEP accounts for nearly 50% of the metabolism of BK.[30] NEP is a potent inactivator of vasoactive and inflammatory peptides, BK, atrial natriuretic peptide, Ang I, endothelins, and tachykinins.[24] NEP expression and regulation might influence local vasomotor and inflammatory responses in the macrovasculature as well as in the microvasculature.[24] Besides a constitutive expression of NEP, its induction in human endothelial cells is possible.[23]

Possible cellular origin of soluble NEP are neutrophils and alveolar epithelial cells.[31] NEP is known to be an integral membrane protease, and the mechanism for the release of a soluble form is unknown. One possible origin of soluble NEP is by the shedding process, which occurs in many eukaryotic cells with membrane-bound proteins being released with portions of plasma membrane or as proteolipid aggregates.[32] The soluble form of NEP found in plasma could, therefore, originate from the release of the entire membrane NEP by leakage in alveolar epithelial cells or activated neutrophils. A post-translational proteolytic cleavage, as observed in the case of ACE,[33] cannot be excluded.

In rats and in humans, there is a significant variation of plasma ACE activity due to a polymorphism of the ACE gene.[7–9] Recently, we demonstrated in normotensive rats the influence of an ACE gene polymorphism on different levels of circulating and tissue NEP activities, suggesting the existence of a modulatory effect of ACE expression on NEP activity in the rat.[34] NEP activity was significantly decreased in serum and tissues in rats with genetically high ACE activity, with an inverse relationship between NEP and ACE activities.[34] The inverse relationship between plasma ACE and plasma NEP activity observed here in normotensive human subjects is consistent with the previous experimental observation in rats. In humans, the D ACE allele has been associated with cardiovascular disease, which may be related to enhanced Ang-II production and/or to degradation of BK.[35] Brown *et al*[36] have observed a longer half-life of BK in serum from subjects with the II ACE genotype and low ACE activity. Another potential mechanism for explaining the association of the D ACE allele with cardiovascular disease—suggested by our current results—is the finding of low levels of NEP activity in normotensive subjects with the D ACE allele.

The increased circulating ACE activity observed in the present study in the hypertensive patients

compared to the normotensive subjects has been previously observed in a different population.[37]

The inverse relationship between plasma ACE (genetically determined) and plasma NEP activities in normotensive subjects was not observed here in the hypertensive patients with identical genotypes and plasma ACE levels distribution. It is difficult to explain the mechanism(s) of this different relationship between circulating ACE and NEP activities in the presence of hypertension with the current information.

In the present study neither angiotensins, kinins, nitric oxide, prostaglandins, or cytokine levels were measured, which could have helped to understand the mechanisms behind the relationships between ACE expression and NEP activity in normotensive and hypertensive subjects. This is one of the limitations of our study and a subject of further research. Possible cellular origins of reduced NEP activity in the presence of high ACE expression (or *vice versa*) in normotensive subjects could be by reduced Ang-(1-7) and BK levels or increased Ang-II levels through a receptor-mediated mechanism. A direct effect of ACE expression at the level of NEP expression through Ang-(1-7) or Ang II might also be possible. These cellular mechanisms in normotensive subjects with high ACE expression could be modified in the hypertensive patients resulting in a different relationship between ACE and NEP. Recently, a significant graded relationship between blood pressure and levels of sICAM-1 as well as IL-6 has been observed in men,[38] a relationship in which ACE (and angiotensins) probably has a role and which could be interacting with the NEP levels in the hypertensive patients with the DD ACE genotype.

The present findings could also help to understand the variability and sometimes unpredicted clinical efficacy of ACE inhibitors (and also of Ang-II receptor blockers as well as possibly of the new vasopeptidase inhibitors) in patients with hypertension or heart failure. The effects of these inhibitors could be dependent to some extent on the interaction of ACE expression with NEP activity and also with hypertension. Drummer *et al*[39] have suggested that chronic treatment with ACE inhibitors may differentially affect the NEP activity causing a substantial re-direction of the angiotensin metabolism. In the rat, separate inhibition of either ACE or NEP induces both enzymes.[40] Further studies using NEP and/or ACE inhibitors in humans with low and high ACE activity may clarify whether there is a differential pharmacological and biological response to these enzymatic inhibitors.

In conclusion, these results are consistent with a relationship between the ACE polymorphism and NEP activity in normotensive humans (as is also observed in rats). High ACE expression is associated with low circulating—and possibly tissue—NEP activity (and *vice versa*). This inverse relationship is not observed (or is lost) in hypertension and could explain some pathogenic mechanisms of hypertension, some of the effects associated with the presence of the D ACE allele or the observed variability of the clinical effects of some agents used in hypertension and heart failure.

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