The angiotensin-converting enzyme insertion/deletion polymorphism is associated with phagocytic NADPH oxidase-dependent superoxide generation: potential implication in hypertension

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ABSTRACT

The objective of the present study was to analyse the influence of the ACE (angiotensin-converting enzyme) gene I/D (insertion/deletion) polymorphism on NADPH oxidase-dependent O2•− (superoxide radical) production, and to investigate the clinical implication of this association in hypertensive subjects. A case-control study was performed in a random sample of the general population composed of 189 normotensive subjects and 223 hypertensive subjects. The ACE polymorphism was determined by PCR. NADPH oxidase-dependent O2•− production was quantified in phagocytic cells by chemiluminescence. MMP-9 (matrix metalloproteinase-9), a marker of atherosclerosis previously reported to be associated with NADPH oxidase overactivity, was quantified by ELISA in plasma samples. The distribution of genotypes was in Hardy–Weinberg equilibrium. The I/D polymorphism was not associated with hypertension. NADPH oxidase-dependent O2•− production was significantly higher in D/D (deletion/deletion) than in I/I (insertion/insertion) and I/D, both in normotensive and hypertensive subjects. Interestingly, plasma levels of angiotensin II were significantly higher in D/D than in I/I and I/D, both in normotensive and hypertensive subjects. Plasma levels of MMP-9 and systolic blood pressure values were significantly higher in D/D than in I/I and I/D hypertensive subjects, whereas no differences were found among genotypes in normotensive subjects. Interestingly, NADPH oxidase-dependent O2•− production positively associated with plasma MMP-9 levels in hypertensive subjects, which remained significant after adjustment for age and gender. In conclusion, in the present study we have reported for the first time an association of the D/D genotype of the ACE I/D polymorphism with phagocytic NADPH oxidase-mediated O2•− overproduction. Within the group of hypertensive patients, D/D cases also associated with increased blood pressure values and with enhanced plasma levels of MMP-9.

Key words: angiotensin-converting enzyme (ACE), essential hypertension, I/D polymorphism, matrix metalloproteinase-9 (MMP-9), NADPH oxidase, superoxide.

Abbreviations: ACE, angiotensin-converting enzyme; ACEi, ACE inhibitor(s); AngII, angiotensin II; AngI, angiotensin I; ARA, AngII type 1-receptor antagonist; BMI, body mass index; BP, blood pressure; DBP, diastolic BP; D/D, deletion/deletion; I/D, insertion/deletion; I/I, insertion/insertion; IMT, intima-media thickness; IL-6, interleukin-6; MMP-9, matrix metalloproteinase-9; O2•−, superoxide radical; SBP, systolic BP.

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INTRODUCTION

ACE (angiotensin-converting enzyme) plays a key role in the development of hypertension and in the establishment of end-organ damage, including atherosclerosis. The D allele of the I/D (insertion/deletion) polymorphism of the ACE gene has been largely studied in cardiovascular disease [1]. In cardiovascular disease, association of the I/D polymorphism with target organ damage has been described in hypertensive subjects [2–5]. Interestingly, subjects with the D/D (deletion/deletion) genotype have higher concentrations of ACE in plasma, lymphocytes, heart and kidney [6–9]. Thus it is expected that subjects with the D/D genotype may be exposed to higher AngII (angiotensin II) levels than those with the I/I (insertion/insertion) or I/D genotype. Indeed, D/D genotype carriers convert more AngI (angiotensin I) into AngII, thus presenting increased AngII levels [10].

Oxidative stress induced by O$_2^{•–}$ (superoxide radical) is increased in arterial hypertension [11], and constitutes a possible mechanism which predisposes to the development of atherosclerosis in hypertension [12]. Previously, NADPH oxidases have been associated with cardiovascular diseases [13]. Thus NADPH oxidases are activated in human hypertension [14–16], and also play a key role in human atherosclerosis [17–21]. In atherosclerosis, enhanced phagocytic NADPH oxidase-dependent O$_2^{•–}$ production correlates positively with carotid IMT (intima-media thickness) [22], a surrogate marker of atherosclerosis, and with plasma levels of MMP-9 (matrix metalloproteinase-9) [23], a biomarker of vascular remodelling and an independent risk factor for atherothrombotic events.

Since AngII increases NADPH oxidase-dependent O$_2^{•–}$ production [14,15,24–27], the main objective of the present study was to explore the influence of the I/D polymorphism on NADPH oxidase activity. As a second aim we also analysed the impact of this polymorphism on NADPH oxidase activity. As a second objective we also analysed the impact of this polymorphism on NADPH oxidase activity. As a second objective we also analysed the impact of this polymorphism on NADPH oxidase activity.

Genotyping of the ACE gene I/D polymorphism

Genotyping of the I/D polymorphism was performed by PCR [28]. In order to identify mistyped I/D heterozygotes, an additional PCR was performed [29].

NADPH oxidase-mediated O$_2^{•–}$ production

O$_2^{•–}$ production was measured in peripheral mononuclear cells (monocytes and lymphocytes) as previously described [15]. Briefly, mononuclear cells were isolated from blood samples with Lymphoprep™ (Axis-Shield), and O$_2^{•–}$ production was measured in basal conditions and in response to stimulation with 2 mg/l PMA and using 5 μmol/l luciferin in a chemiluminescent method that correlated well with the ferricytochrome C assay. We have previously demonstrated that PMA-stimulated O$_2^{•–}$ production is inhibited by diphenylene iodonium, a flavoprotein inhibitor, and by apocynin, a specific intracellular inhibitor of NADPH oxidase assembly [15]. In addition, superoxide dismutase, a scavenger of O$_2^{•–}$, completely abolished the chemiluminescence induced with PMA, thus verifying the specificity of the luciferin assay for O$_2^{•–}$ generation. Collectively, these previous findings allow us to identify the NADPH oxidase complex as the main enzymatic source of O$_2^{•–}$ in mononuclear cells exposed to PMA.

Determination of AngII, MMP-9 and IL-6 (interleukin-6)

Plasma and serum samples were obtained from venous blood between 09:00 hours and 10:00 hours after an overnight fast, and were stored at −80°C until analysis. IL-6 concentrations were analysed in serum samples using a high-sensitive immunoassay system (Quantikine-HS IL-6; R&D systems). A sandwich ELISA (Amersham Biosciences), an assay that quantifies both the precursor form and the active form complexed with the tissue inhibitor of metalloproteinases-1, was used to determine MMP-9 levels in plasma samples, as previously described [23]. AngII was measured by competitive RIA after extraction of the plasma samples (Euro-Diagnostica).
Table 1  Clinical characteristics of the normotensive subjects and hypertensive subjects
Values are means ± S.E.M. PP, pulse pressure; MAP, mean arterial pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NS, not significant.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normotensivesubjects</th>
<th>Hypertensive subjects</th>
<th>Significance (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female (n)</td>
<td>154/35</td>
<td>187/36</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years)</td>
<td>54.9 ± 0.7</td>
<td>58.6 ± 1.1</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.3 ± 0.5</td>
<td>29.5 ± 0.4</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>118.4 ± 1.1</td>
<td>141.4 ± 1.4</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>77.1 ± 0.7</td>
<td>86.2 ± 0.8</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>PP (mmHg)</td>
<td>41.3 ± 0.4</td>
<td>55.2 ± 0.5</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>100.2 ± 0.8</td>
<td>106.4 ± 1.0</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>141.6 ± 2.3</td>
<td>151.6 ± 2.4</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>221 ± 4</td>
<td>221 ± 4</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>50 ± 1</td>
<td>48 ± 1</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)</td>
<td>150 ± 4</td>
<td>149 ± 4</td>
<td>NS</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>118 ± 5</td>
<td>125 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>Antihypertensive medication (n)</td>
<td>–</td>
<td>49 (22 %)</td>
<td>–</td>
</tr>
<tr>
<td>ACEI</td>
<td>–</td>
<td>46 (21 %)</td>
<td>–</td>
</tr>
<tr>
<td>ARA</td>
<td>–</td>
<td>33 (15 %)</td>
<td>–</td>
</tr>
</tbody>
</table>

Statistical analysis
Values are expressed as means ± S.E.M. χ² analysis was used to test for deviation of the genotype distribution from the Hardy–Weinberg equilibrium and to determine whether there were differences in genotype frequencies between cases and controls. Variations in the clinical data between normotensive and hypertensive subjects were assessed by Student’s t tests for unpaired data or Mann–Whitney’s U tests. Clinical characteristics, including O₂²⁻ production, and plasma and serum measurements, were compared among the three genotype groups (D/D, I/D and I/I) using χ² tests for categorical variables or ANOVA for continuous variables. A Pearson correlation test was used to assess correlations between O₂²⁻ production and plasma MMP-9 levels. Statistical analysis was performed with SPSS for Windows 13.0, and statistical significance was established as P < 0.05.

RESULTS

General characteristics of the study populations
Clinical characteristics of normotensive and hypertensive subjects are shown in Table 1. Hypertensive subjects had significantly higher values of BMI (body mass index), BP and age than normotensive subjects.

Subjects were genotyped for the I/D polymorphism by PCR. The distribution of genotypes was as expected for the Hardy–Weinberg equilibrium. The distribution of genotypes and alleles in normotensive and hypertensive subjects are shown in Table 2. The I/D polymorphism was not associated with hypertension.

Analysis of the I/D polymorphism in normotensive subjects
Clinical characteristics of normotensive subjects according to genotypes of the I/D polymorphism are shown in Table 3. There were no differences in confounding factors such as age, gender and BMI among the three groups. In addition, values of BP, glucose, cholesterol and triacylglycerols (triglycerides) were similar in the three groups of subjects.

No differences were found for basal NADPH oxidase-dependent O₂²⁻ production according to the genotypes of the I/D polymorphism (I/I, 2.1 ± 0.5 counts/s; I/D, 1.6 ± 0.3 counts/s; D/D, 3.1 ± 0.7 counts/s). Nevertheless, PMA-induced NADPH oxidase-dependent O₂²⁻ production was higher (P = 0.003) in D/D normotensive
Figure 1  Effect of the ACE gene I/D polymorphism genotype on NADPH oxidase-dependent O$_2^•$− production
NADPH oxidase-dependent O$_2^•$− production was determined by lucigenin chemiluminescence after PMA stimulation in peripheral mononuclear cells from 189 non-hypertensive healthy subjects (A) and 223 hypertensive subjects (B). Values are means ± S.E.M. *P = 0.003 and **P = 0.001 compared with I/I and I/D within the same group, and †P = 0.01 compared with D/D normotensive subjects.

Figure 2  Effect of the ACE gene I/D polymorphism genotype on MMP-9 plasma levels
Plasma MMP-9 levels were quantified using a sandwich ELISA in 189 non-hypertensive healthy subjects (A) and 223 hypertensive subjects (B). Values are means ± S.E.M. *P = 0.009 compared with I/I and I/D within the same group, and †P = 0.04 compared with D/D normotensive subjects.

subjects (15.6 ± 1.4 counts/s) than in I/I (9.3 ± 1.5 counts/s) and I/D (11.5 ± 1.2 counts/s) normotensive subjects (Figure 1A). No differences were found between I/I and I/D normotensive subjects in PMA-stimulated O$_2^•$− production. Interestingly, plasma levels of AngII were higher (P = 0.035) in D/D (25 ± 2 pmol/l) normotensive subjects than in I/D (18 ± 2 pmol/l) and I/I (16 ± 3 pmol/l) normotensive subjects.

There were no differences in plasma levels of MMP-9 among I/I (12.5 ± 1.4 ng/ml), I/D (12.4 ± 1.1 ng/ml) and D/D (13.8 ± 1.0 ng/ml) normotensive individuals (Figure 2A). No differences in serum levels of IL-6 were found among I/I (1.6 ± 0.6 pg/ml), I/D (1.7 ± 0.5 pg/ml) and D/D (1.9 ± 0.6 pg/ml) normotensive subjects.

Analysis of the I/D polymorphism in hypertensive subjects
Clinical characteristics of hypertensive subjects according to genotypes of the I/D polymorphism are shown in Table 4. There were no differences in confounding factors such as age, gender and BMI among the three groups. D/D hypertensive subjects displayed significantly higher values of BP than I/I and I/D hypertensive subjects. There were no differences in other clinical and biochemical parameters or in the antihypertensive treatment among the three groups.

No differences were found for basal NADPH oxidase-dependent O$_2^•$− production according to the genotypes of I/D polymorphism (I/I, 2.6 ± 0.8 counts/s; I/D, 2.1 ± 0.4 counts/s; D/D, 3.5 ± 0.7 counts/s). Nevertheless, PMA-induced NADPH oxidase-dependent O$_2^•$− production was increased (P = 0.001) in D/D hypertensive subjects (20.9 ± 2.2 counts/s) compared with I/I (12.4 ± 2.2 counts/s) and I/D (11.4 ± 0.9 counts/s) hypertensive subjects (Figure 1B). No differences in PMA-stimulated O$_2^•$− production were found between I/I and I/D hypertensive subjects. NADPH oxidase-dependent O$_2^•$− production was higher (P = 0.01) in D/D hypertensive subjects than in D/D normotensive subjects. Phagocytic O$_2^•$− production in subjects on antihypertensive treatment with ARAs (AngII type 1-receptor antagonists) or ACEi (ACE inhibitors) (I/I, 12.2 ± 3.2 counts/s; I/D, 10.4 ± 1.3 counts/s; D/D, 20.2 ± 3.0 counts/s; P < 0.05, D/D compared with I/I and I/D) was similar when compared with subjects without such treatment (I/I, 12.8 ± 2.8 counts/s; I/D, 12.0 ± 1.1 counts/s;
D/D, 22.6 ± 1.9 counts/s; *P* < 0.05, D/D compared with I/I and I/D). It is important to note that plasma levels of AngII were higher (*P* = 0.031) in D/D (30 ± 3 pmol/l) hypertensive subjects compared with I/D (23 ± 2 pmol/l) and I/I (18 ± 3 pmol/l) hypertensive subjects. No significant differences in AngII levels were found between I/D and I/I hypertensive subjects, and between D/D normotensive and D/D hypertensive subjects.

Plasma levels of MMP-9 were increased (*P* = 0.009) in D/D hypertensive subjects (17.1 ± 1.1 ng/ml) compared with I/I (11.2 ± 1.5 ng/ml) and I/D (12.4 ± 1.1 ng/ml) hypertensive subjects (Figure 2B). No differences in MMP-9 levels were found between I/I and I/D hypertensive subjects. Finally, MMP-9 levels were higher (*P* = 0.04) in D/D hypertensive subjects than in D/D normotensive subjects.

Serum levels of IL-6 were higher in D/D (2.5 ± 0.7 pg/ml) hypertensive subjects than in I/D (2.1 ± 0.5 pg/ml) and I/I (1.8 ± 0.4 pg/ml) hypertensive subjects, although the difference did not reach statistical significance.

**DISCUSSION**

The main finding of the present study is that the ACE I/D polymorphism was associated with NADPH oxidase-dependent O$_2^-$ production in phagocytic cells. More precisely, D/D carriers exhibit greater NADPH oxidase-dependent O$_2^-$ production than I/I and I/D carriers both in normotensive and hypertensive subjects. Interestingly, the D/D genotype was associated with increased BP values and plasma levels of MMP-9 just in hypertensive patients.

AngII plays a key role in cardiovascular pathophysiology by promoting vascular inflammation and remodelling, and endothelial dysfunction [30,31]. Among other mechanisms, vascular effects of AngII are mediated by NADPH oxidases in vascular smooth muscle cells [14,25], endothelial cells [26], fibroblasts [27], platelets [24] and leucocytes [15]. In rats made hypertensive with AngII infusion, vascular NADPH oxidase activity is increased and the administration of an ARA reduces O$_2^-$ production [30]. Our findings showing an increased NADPH oxidase-mediated O$_2^-$ production in D/D subjects suggest that this polymorphism may activate the NADPH oxidase by up-regulating AngII levels. In fact,
there are strong and consistent published data which demonstrate that ACE levels and/or activity in carriers of the ACE D/D genotype are significantly higher than those with the I allele in different cell types, including lymphocytes [6–9]. Indeed, D/D genotype carriers convert more AngI into AngII, thus having increased AngII levels [10]. In agreement with this, we found a significant association of the D/D genotype with high levels of AngII.

Among the mechanisms involved in the pathophysiology of hypertension, oxidative stress plays a major role. Experimental and human studies have demonstrated a relationship between NADPH oxidase-mediated oxidative stress and hypertension [32,33]. In this way, we found an association between NADPH oxidase-mediated O$_2^{•−}$ production and the I/D polymorphism in hypertension. In fact, although both D/D normotensive and D/D hypertensive subjects had increased NADPH oxidase-mediated O$_2^{•−}$ production compared with I/I and I/D genotype groups, the increase was higher in hypertensive than in normotensive subjects. It could be speculated that, in normotension, increased NADPH oxidase-mediated O$_2^{•−}$ production in D/D carriers might be compensated by an effective antioxidant defence, which may prevent increases in BP values. In contrast, in hypertension, antioxidant defences are attenuated [34] and, thus, an increased O$_2^{•−}$ production in D/D carriers might be actively involved in BP elevation.

A modest association between the I/D polymorphism and atherosclerosis has been reported [1], although this association is reinforced among high-risk populations such as hypertension. The D allele positively correlates with carotid IMT, although this association is stronger among high-risk populations than among low-risk/general populations [35]. This is of importance as phagocytic NADPH oxidase-mediated O$_2^{•−}$ production positively correlates with carotid IMT [22]. In addition, the NADPH oxidase-mediated O$_2^{•−}$ production positively correlates with plasma levels of MMP-9 in asymptomatic subjects [23]. Available evidence substantiates that MMP-9 levels represent an independent risk factor for atherothrombotic events that can provide a useful emerging plasma biomarker [36]. Thus the findings of the present study showing that D/D hypertensive subjects exhibit higher MMP-9 levels than I/I and I/D hypertensive subjects suggest that the presence of the D/D genotype may be associated with an increased risk of atherothrombotic events in hypertension. The clinical relevance of this finding is underlined by a recent study demonstrating that NADPH oxidase activation increases MMP-9 expression and activation in human monocytes, and that this activation is involved in human atherosclerosis [23].

With regards to the limitation of the present study, we cannot exclude the influence of interactions of the I/D polymorphism with other gene polymorphisms in this gene and in other genes, especially those implicated in the renin-angiotensin–aldosterone system, and with environmental stimuli [37]. On the other hand, ACE is able to inactivate bradykinin, and the D allele has been associated with an increased degradation of bradykinin in humans [38]. Thus we cannot rule out some effects mediated by this vasodilator. Because some studies suggest that subjects with essential hypertension with different I/D genotype may respond differentially to treatment with different antihypertensive medication [39], a potential confounder of the present study would be the antihypertensive medication of the treated subjects. Nevertheless, this possibility is unlikely in the present study because no differences were found in antihypertensive medication among hypertensive subjects according to genotypes of the I/D polymorphism. Antihypertensive treatment with ACEi and ARAs might exert a relevant effect on AngII levels, thus modulating NADPH oxidase-dependent O$_2^{•−}$ production. Nevertheless, our findings show similar O$_2^{•−}$ production both in ACEi/ARAtreated and in non-treated hypertensive
subjects. In this respect, most of the treated hypertensive subjects did not normalize BP values (results not shown).

In summary, in the present study we have reported for the first time an association of the D/D genotype of the ACE I/D polymorphism with phagocytic NADPH oxidase-mediated $O_2^{-*}$ overproduction. In addition, our findings suggest that NADPH oxidase-mediated oxidative stress can be a potential mechanism involved in the development of atherosclerotic complications previously reported in D/D hypertensive subjects [40].

ACKNOWLEDGEMENTS

We gratefully acknowledge technical support by Raquel Ros, Raül Calero and Ana Montoya.

FUNDING

This work was supported by the agreement between the Foundation for Applied Medical Research and ‘UTE project CIMA’, by the red Temática de Investigación Cooperativa en Enfermedades Cardiovasculares from the Instituto de Salud Carlos III, Ministry of Health, Spain [grant number RD06/0014/0008]; the European Union [grant number LSHM-CT-2006-037093]; the Department of Health of Government of Navarra [grant number 25/2005]; the Ministry of Science and Education [grant number SAF2004-07910]; and by the Ministry of Science and Culture Spain [grant number SAF2007-62533].

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Received 20 February 2008/27 June 2008; accepted 22 July 2008
Published as Immediate Publication 22 July 2008, doi:10.1042/CS20080057