



HAL
open science

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

Gorka San José, Ana Fortuño, María Ujué Moreno, Pablo Alejandro Robador,
Julian Bidegain, Nerea Varo, Oscar Beloqui, Javier Díez, Guillermo Zalba

► To cite this version:

Gorka San José, Ana Fortuño, María Ujué Moreno, Pablo Alejandro Robador, Julian Bidegain, et al.. The angiotensin-converting enzyme I/D polymorphism is associated with phagocytic NADPH oxidase-dependent superoxide generation: potential implication in hypertension. *Clinical Science*, 2009, 116 (3), pp.233-240. 10.1042/CS20080057 . hal-00479427

HAL Id: hal-00479427

<https://hal.science/hal-00479427v1>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Authors and affiliations: Gorca San José,^a Ana Fortuño,^a María U. Moreno,^a Pablo A. Robador,^a Julián Bidegain,^a Nerea Varo,^a Oscar Beloqui,^b Javier Díez,^{a,c} Guillermo Zalba.^a

^aDivision of Cardiovascular Sciences, Centre for Applied Medical Research, University of Navarra, 31008, Pamplona, Spain.

^bDepartment of Internal Medicine, University Clinic, University of Navarra, Pamplona, Spain.

^cDepartment of Cardiology and Cardiovascular Surgery, University Clinic, University of Navarra, Pamplona, Spain.

Keywords: essential hypertension; NADPH oxidase; I/D polymorphism; MMP-9; superoxide.

Short title: ACE I/D polymorphism and NADPH oxidase

Author for correspondence: Dr. Guillermo Zalba, Área de Ciencias Cardiovasculares. Centro de Investigación Médica Aplicada. Avda. Pío XII 55, 31008 Pamplona (SPAIN). Phone: 34948194700, FAX: 34948194716. *E-mail:* gzalba@unav.es

ABSTRACT

The objective of the present study was to analyse the influence of the angiotensin-converting enzyme (ACE) Insertion/Deletion (I/D) polymorphism on NADPH oxidase-dependent superoxide ($\cdot\text{O}_2^-$) production, and to deep on the clinical implication of this association in hypertensive patients. A case-control study was performed in a random sample of the general population composed by 189 normotensive subjects and 223 hypertensive patients. ACE polymorphism was determined by PCR. NADPH oxidase-dependent $\cdot\text{O}_2^-$ production was quantified in phagocytic cells by chemiluminescence. Matrix metalloproteinase-9 (MMP-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. I/D polymorphism was not associated with hypertension. NADPH oxidase-dependent $\cdot\text{O}_2^-$ production was significantly higher in DD than in II and ID both in normotensives and hypertensives. Interestingly, plasma levels of angiotensin II were significantly higher in DD than in II and ID both in normotensives and hypertensives. Plasma levels of MMP-9 and systolic blood pressure values were significantly higher in DD than in II and ID in hypertensives, while no differences were found among genotypes in normotensives. Interestingly, NADPH oxidase-dependent $\cdot\text{O}_2^-$ production positively associated with plasma MMP-9 levels in hypertensives, which remained significant after adjustment for age and sex. In conclusion, we reported for the first time an association of the DD genotype of the ACE I/D polymorphism with phagocytic NADPH oxidase-mediated $\cdot\text{O}_2^-$ overproduction. Within the group of hypertensive patients, DD cases also associated with increased blood pressure values and with enhanced plasma levels of MMP-9.

INTRODUCTION

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

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

Since Ang II increases NADPH oxidase-dependent $\cdot\text{O}_2^-$ production [14,15,24-27], the main objective of the present study was to explore the influence of I/D polymorphism on NADPH oxidase activity. As a second aim we also analyzed the impact of this polymorphism on the cardiovascular profile of patients with essential hypertension.

METHODS

Subjects

The study population consisted of 223 hypertensive patients and 189 non-hypertensive healthy subjects consecutively referred to the University Clinic of Navarra for routine medical work-up after a 12-hour overnight fast. In the present population half of the individuals came from an apparently healthy cohort previously analysed [23].

According to institutional guidelines, subjects were aware of the research nature of the study and agreed to participate. The study was carried out in accordance with the Helsinki Declaration, and the Ethical Committee of the University Clinic approved the protocol. Participants were considered hypertensive if they had sitting systolic blood pressure (SBP) and/or diastolic blood pressure (DBP) of more than 139 and 89 mmHg, respectively, or if they were taking antihypertensive medication. Half of the hypertensive patients were under antihypertensive treatment. Blood pressure was measured on three occasions using a mercury sphygmomanometer and the mean of these three readings was recorded. All the patients had appropriate clinical, laboratory and radiological evaluations to exclude secondary hypertension.

Genotyping of ACE I/D polymorphism

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

NADPH oxidase mediated $\cdot\text{O}_2^-$ production

$\cdot\text{O}_2^-$ production was measured in peripheral mononuclear cells (monocytes and lymphocytes) as previously [15]. Briefly, mononuclear cells were isolated from blood samples with Lymphoprep TM (Axis-Shield) and $\cdot\text{O}_2^-$ production were measured in basal conditions and in response to stimulation with phorbol 12-myristate 13-acetate (PMA) 2

mg/L and using lucigenin 5 $\mu\text{mol/L}$ in a chemiluminescent method that correlated well with the ferricytochrome C assay. We have previously demonstrated that PMA-stimulated $\cdot\text{O}_2^-$ 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 $\cdot\text{O}_2^-$, completely abolished the chemiluminescence induced with PMA thus verifying the specificity of the lucigenin assay for $\cdot\text{O}_2^-$ generation. Collectively, these previous findings allow us to identify the NADPH oxidase complex as the main enzymatic source of $\cdot\text{O}_2^-$ in mononuclear cells exposed to PMA.

Determination of Ang II, MMP-9 and interleukin-6

Plasma and serum samples were obtained from venous blood between 9 and 10 a.m. after overnight fast, and stored at -80°C until analysis. Interleukin-6 (IL-6) concentrations were analyzed in serum samples by a high-sensitive immunoassays 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 metalloproteinase-1, was used to determine MMP-9 levels in plasma samples, as previously [23]. Ang II was measured by competitive radioimmunoassay after extraction of the plasma samples (Euro-Diagnostica).

Statistical analysis

Data are expressed as mean \pm SEM. χ^2 analysis was used to test for deviation of genotype distribution from Hardy-Weinberg equilibrium and to determine whether there were differences in genotype frequencies between cases and controls. Variations in the clinical data between normotensives and hypertensives were assessed by Student's *t* test for unpaired data or Mann-Whitney's *U* tests. Clinical characteristics, including $\cdot\text{O}_2^-$ production and plasma and serum measurements, were compared among the three genotype groups (DD, ID and II) using χ^2 tests for categorical variables or ANOVA for continuous variables. Pearson correlation test was used to assess correlations between $\cdot\text{O}_2^-$

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 normotensives and hypertensives are shown in Table 1. Hypertensives exhibited significantly higher values of body mass index, blood pressure and age than normotensives.

Subjects were genotyped for the I/D polymorphism by PCR. The distribution of genotypes was as expected for Hardy-Weinberg equilibrium. Distribution of genotypes and alleles in normotensives and hypertensives are present in Table 2. The I/D polymorphism was not associated with hypertension.

Analysis of the I/D polymorphism in normotensives

Clinical characteristics of normotensives according to genotypes of the I/D polymorphism are shown in Table 3. There were no differences in confounding factors such as age, sex and body mass index among the three groups. In addition, values of blood pressure, glucose, cholesterol and triglycerides were similar in the three groups of subjects.

No differences were found for basal NADPH oxidase-dependent $\cdot\text{O}_2^-$ production according to the genotypes of I/D polymorphism (II, 2.1 ± 0.5 counts/s; ID, 1.6 ± 0.3 ; DD, 3.1 ± 0.7). Nevertheless, PMA-induced NADPH oxidase-dependent $\cdot\text{O}_2^-$ production was higher ($P=0.003$) in DD normotensives (15.6 ± 1.4 counts/s) than in II (9.3 ± 1.5 counts/s) and ID (11.5 ± 1.2 counts/s) normotensives (Fig. 1a). No differences were found between II and ID normotensives in PMA-stimulated $\cdot\text{O}_2^-$ production. Interestingly, plasma levels of Ang II were higher ($P=0.035$) in DD (25 ± 2 pmol/L) normotensives than in ID (18 ± 2 pmol/L) and II (16 ± 3 pmol/L) normotensives.

There were no differences in plasma levels of MMP-9 among II (12.5 ± 1.4 ng/mL), ID (12.4 ± 1.1 ng/mL) and DD (13.8 ± 1.0 ng/mL) normotensive individuals (Fig. 2a). No differences in serum levels of IL-6 were found among II (1.6 ± 0.6 pg/mL), ID (1.7 ± 0.5 pg/mL) and DD (1.9 ± 0.6 pg/mL) normotensives.

Analysis of the I/D polymorphism in hypertensives

Clinical characteristics of hypertensives according to genotypes of the I/D polymorphism are shown in Table 4. There were no differences in confounding factors such as age, sex and body mass index among the three groups. DD hypertensives displayed significantly higher values of blood pressure than II and ID hypertensives. 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 $\cdot\text{O}_2^-$ production according to the genotypes of I/D polymorphism (II, 2.6 ± 0.8 counts/s; ID, 2.1 ± 0.4 ; DD, 3.5 ± 0.7). Nevertheless, PMA-induced NADPH oxidase-dependent $\cdot\text{O}_2^-$ production was increased ($P=0.001$) in DD hypertensives (20.9 ± 2.2 counts/s) compared with II (12.4 ± 2.2 counts/s) and ID (11.4 ± 0.9 counts/s) hypertensives (Fig. 1b). No differences in PMA-stimulated $\cdot\text{O}_2^-$ production were found between II and ID hypertensives. NADPH oxidase-dependent $\cdot\text{O}_2^-$ production was higher ($P=0.01$) in DD hypertensives than in DD normotensives. Phagocytic $\cdot\text{O}_2^-$ production in patients on antihypertensive treatment with Ang II type 1-receptor antagonists (ARA) or angiotensin-converting enzyme inhibitors (ACEI) (II: 12.2 ± 3.2 counts/s, ID: 10.4 ± 1.3 counts/s, DD: 20.2 ± 3.0 counts/s, $P < 0.05$) was similar as compared to patients without such treatment (II: 12.8 ± 2.8 counts/s, ID: 12.0 ± 1.1 counts/s, DD: 22.6 ± 1.9 counts/s $P < 0.05$). It is important to note that plasma levels of Ang II were higher ($P=0.031$) in DD (30 ± 3 pmol/L) hypertensives than in ID (23 ± 2 pmol/L) and II (18 ± 3 pmol/L) hypertensives. No significant differences in Ang II levels were found between ID and II hypertensives, and between DD normotensives and DD hypertensives.

Plasma levels of MMP-9 were increased ($P=0.009$) in DD hypertensives (17.1 ± 1.1 ng/mL) compared with II (11.2 ± 1.5 ng/mL) and ID (12.4 ± 1.1 ng/mL) hypertensives (Fig. 2b). No differences in MMP-9 levels were found between II and ID hypertensives. Finally, MMP-9 levels were higher ($P=0.04$) in DD hypertensives than in DD normotensives.

Serum levels of IL-6 were higher in DD (2.5 ± 0.7 pg/mL) hypertensives than in ID (2.1 ± 0.5 pg/mL) and II (1.8 ± 0.4 pg/mL) hypertensives, although the difference did not reach statistical significance.

Association analysis between $\cdot\text{O}_2^-$ production and plasma MMP-9

There was a significant positive bivariate correlation between phagocytic $\cdot\text{O}_2^-$ production and plasma levels of MMP-9 in the whole population, which remained highly significant after controlling for age and sex ($r=0.320$, $P<0.001$), thus confirming previous data of our group [23]. Interestingly, in the present study we found the association between $\cdot\text{O}_2^-$ production and MMP-9 levels, after controlling for age and sex, remained statistically significant in hypertensive patients ($r=0.376$, $P<0.001$) but failed in normotensive subjects ($r=0.210$, $P=0.102$) (Fig. 3). Within hypertensive patients, this association remained also significant in DD, ID and II genotype groups.

DISCUSSION

The main finding of the present study is that the ACE I/D polymorphism was associated with NADPH oxidase-dependent $\cdot\text{O}_2^-$ production in phagocytic cells. More precisely, DD carriers exhibit greater NADPH oxidase-dependent $\cdot\text{O}_2^-$ production than II and ID carriers both in normotensive subjects and hypertensive patients. Interestingly, the DD genotype was associated with increased blood pressure values and plasma levels of MMP9 just in hypertensive patients.

Ang II plays a key role in cardiovascular pathophysiology by promoting vascular inflammation and remodelling, and endothelial dysfunction [30,31]. Among other

mechanisms, vascular effects of Ang II are mediated by NADPH oxidases in vascular smooth muscle cells [14,25], endothelial cells [26], fibroblasts [27], platelets [24] and leukocytes [15]. In rats made hypertensive with Ang II infusion, vascular NADPH oxidase activity is increased and the administration of an AT1 receptor antagonist reduces $\cdot\text{O}_2^-$ production [30]. Our findings showing an increased NADPH oxidase-mediated $\cdot\text{O}_2^-$ production in DD subjects, suggest that this polymorphism may activate the NADPH oxidase by upregulating Ang II levels. In fact, there are strong and consistent published data which demonstrate that ACE levels and/or activity in carriers of the *ACE* DD genotype are significantly higher than those with the I allele in different cell types, including lymphocytes [6-9]. Indeed, DD genotype carriers convert more Ang I to Ang II thus presenting increased Ang II levels [10]. In agreement with this, we found a significant association of DD genotype with high levels of Ang II.

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

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 population than among low-risk/general population [35]. This

is of importance, as phagocytic NADPH oxidase-mediated $\cdot\text{O}_2^-$ production positively correlates with carotid IMT [22]. In addition, the NADPH oxidase-mediated $\cdot\text{O}_2^-$ 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, our present finding showing that DD hypertensives exhibit higher MMP-9 levels than II and ID hypertensives, suggest that the presence of the DD 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].

Concerning to the limitation of this study, we can not 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-angiotensi-aldosterone system, and with environmental stimuli [37]. On the other hand, ACE is able to inactive bradykinin, and the D allele has been associated with an increased degradation of bradykinin in humans [38]. Thus, we can not 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 medications [39], a potential confounder of the study would be the antihypertensive medication of treated patients. Nevertheless, this possibility is unlikely in the present study because no differences were found in antihypertensive medication among hypertensives according to genotypes of the I/D polymorphism. In this line, antihypertensive treatment with ACEI and ARA might exert a relevant effect on Ang II levels, thus modulating NADPH oxidase-dependent $\cdot\text{O}_2^-$ production. Nevertheless, our findings show similar $\cdot\text{O}_2^-$ production both in ACEI/ARA treated and in non-treated hypertensives. In this sense, most of treated hypertensive patients did not normalize blood pressure values (data not shown).

In summary, we reported for the first time an association of the DD genotype of the ACE I/D polymorphism with phagocytic NADPH oxidase-mediated $\cdot\text{O}_2^-$ 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 DD hypertensives [40].

ACKNOWLEDGMENTS

We gratefully acknowledge technical support by Raquel Ros, Raúl Calero and Ana Montoya. This project was funded through the agreement between the Foundation for Applied Medical Research (FIMA) and “UTE project CIMA”, by the red Temática de Investigación Cooperativa en Enfermedades Cardiovasculares (RECAVA) from the Instituto de Salud Carlos III, Ministry of Health, Spain (grant RD06/0014/0008), by the European Union (InGenious HyperCare, grant LSHM-CT-2006-037093), by the Department of Health of Government of Navarra (grant 25/2005), by the Ministry of Science and Education (grant SAF2004-07910), and by the Ministry of Science and Culture (grant SAF2007-62533), Spain.

REFERENCES

1. Sayed-Tabatabaei, F.A., Oostra, B.A., Isaacs, A., van Duijn, C.M. and Witteman, J.C. (2006) ACE polymorphisms. *Circ. Res.* **98**, 1123-1133
2. Dell'omo, G., Penno, G., Pucci, L., Lucchesi, D., Fotino, C., Del Prato, S. and Pedrinelli, R. (2006) ACE gene insertion/deletion polymorphism modulates capillary permeability in hypertension. *Clin. Sci.* **111**, 357-364
3. Takahashi, T., Ueno, H., Yasumoto, K., Kagitani, S., Tomoda, F., Inoue, H. and Takata, M. (2003) Angiotensin-converting enzyme-gene polymorphism is associated

- with collagen I synthesis and QT dispersion in essential hypertension. *J. Hypertens.* **21**, 985-991
4. Perticone, F., Maio, R., Di Paola, R., Sciacqua, A., Marucci, A., De Cosmo, S., Perticone, M., Sesti, G. and Trischitta, V. (2007) Role of PC-1 and ACE genes on insulin resistance and cardiac mass in never-treated hypertensive patients. Suggestive evidence for a digenic additive modulation. *Nutr. Metab. Cardiovasc. Dis.* **17**, 181-187
 5. Redón, J., Chaves, F.J., Liao, Y., Pascual, J.M., Rovira, E., Armengod, M.E. and Cooper, R.S. (2000) Influence of the I/D polymorphism of the angiotensin-converting enzyme gene on the outcome of microalbuminuria in essential hypertension. *Hypertension* **35**, 490-495
 6. Rigat, B., Hubert, C., Alhenc-Gelas, F., Cambien, F., Corvol, P. and Soubrier, F. (1990) An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J. Clin. Invest.* **86**, 1343-1346
 7. Costerousse, O., Allegrini, J., Lopez, M. and Alhenc-Gelas, F. (1993) Angiotensin I-converting enzyme in human circulating mononuclear cells: genetic polymorphism of expression in T-lymphocytes. *Biochem. J.* **290**, 33-40
 8. Danser, A.H., Schalekamp, M.A., Bax, W.A., van den Brink, A.M., Saxena, P.R., Riegger, G.A. and Schunkert, H. (1995) Angiotensin-converting enzyme in the human heart. Effect of the deletion/insertion polymorphism. *Circulation* **92**, 1387-1388
 9. Mizuiri, S., Hemmi, H., Kumanomidou, H., Iwamoto, M., Miyagi, M., Sakai, K., Aikawa, A., Ohara, T., Yamada, K., Shimatake, H. and Hasegawa, A. (2001) Angiotensin-converting enzyme (ACE) I/D genotype and renal ACE gene expression. *Kidney Int.* **60**, 1124-1130
 10. Ueda, S., Elliott, H.L., Morton, J.J. and Connell, J.M. (1995) Enhanced pressor response to angiotensin I in normotensive men with the deletion genotype (DD) for angiotensin-converting enzyme. *Hypertension* **25**, 1266-1269

11. Landmesser, U. and Harrison, D.G. (2001) Oxidative stress and vascular damage in hypertension. *Coron. Artery Dis.* **12**, 455-461
12. Alexander, R.W. (1995) Theodore Cooper Memorial Lecture. Hypertension and the pathogenesis of atherosclerosis. Oxidative stress and the mediation of arterial inflammatory response: a new perspective. *Hypertension* **25**, 155-161
13. Taniyama, Y. and Griendling, K.K. (2003) Reactive oxygen species in the vasculature: molecular and cellular mechanisms. *Hypertension* **42**, 1075-1081
14. Touyz, R.M. and Schiffrin, E.L. (2001) Increased generation of superoxide by angiotensin II in smooth muscle cells from resistance arteries of hypertensive patients: role of phospholipase D-dependent NAD(P)H oxidase-sensitive pathways. *J. Hypertens.* **19**, 1245-1254
15. Fortuño, A., Oliván, S., Beloqui, O., San José, G., Moreno, M.U., Díez, J. and Zalba, G. (2004) Association of increased phagocytic NADPH oxidase-dependent superoxide production with diminished nitric oxide generation in essential hypertension. *J. Hypertens.* **22**, 2169-2175
16. Pettit, A.I., Wong, R.K., Lee, V., Jennings, S., Quinn, P.A. and Ng, L.L. (2002) Increased free radical production in hypertension due to increased expression of the NADPH oxidase subunit p22(phox) in lymphoblast cell lines. *J. Hypertens.* **20**, 677-683
17. Guzik, T.J., West, N.E., Black, E., McDonald, D., Ratnatunga, C., Pillai, R. and Channon, K.M. (2000) Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. *Circ. Res.* **86**, E85-E90
18. Azumi, H., Inoue, N., Takeshita, S., Rikitake, Y., Kawashima, S., Hayashi, Y., Itoh, H. and Yokoyama, M. (1999) Expression of NADH/NADPH oxidase p22phox in human coronary arteries. *Circulation* **100**, 1494-1498

19. Kalinina, N., Agrotis, A., Tararak, E., Antropova, Y., Kanellakis, P., Ilyinskaya, O., Quinn, M.T., Smirnov, V. and Bobik, A. (2002) Cytochrome b558-dependent NAD(P)H oxidase-phox units in smooth muscle and macrophages of atherosclerotic lesions. *Arterioscler. Thromb. Vasc. Biol.* **22**, 2037-2043
20. Sorescu, D., Weiss, D., Lassegue, B., Clempus, R.E., Szocs, K., Sorescu, G.P., Valppu, L., Quinn, M.T., Lambeth, J.D., Vega, J.D., Taylor, W.R. and Griendling, K.K. (2002) Superoxide production and expression of nox family proteins in human atherosclerosis. *Circulation* **105**, 1429-1435
21. Azumi, H., Inoue, N., Ohashi, Y., Terashima, M., Mori, T., Fujita, H., Awano, K., Kobayashi, K., Maeda, K., Hata, K., Shinke, T., Kobayashi, S., Hirata, K., Kawashima, S., Itabe, H., Hayashi, Y., Imajoh-Ohmi, S. and Itoh, H. (2002) Yokoyama M. Superoxide generation in directional coronary atherectomy specimens of patients with angina pectoris: important role of NAD(P)H oxidase. *Arterioscler. Thromb. Vasc. Biol.* **22**, 1838-1844
22. Zalba, G., Beloqui, O., San José, G., Moreno, M.U., Fortuño, A. and Díez, J. (2005) NADPH oxidase-dependent superoxide production is associated with carotid intima-media thickness in subjects free of clinical atherosclerotic disease. *Arterioscler. Thromb. Vasc. Biol.* **25**, 1452-1457
23. Zalba, G., Fortuño, A., Orbe, J., San José, G., Moreno, M.U., Belzunce, M., Rodríguez, J.A., Beloqui, O., Páramo, J.A. and Díez, J. (2007) Phagocytic NADPH oxidase-dependent superoxide production stimulates matrix metalloproteinase-9: implications for human atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **27**, 587-593
24. Germano, G., Sanguigni, V., Pignatelli, P., Caccese, D., Lenti, L., Ragazzo, M., Lauro, R. and Violi, F. (2004) Enhanced platelet release of superoxide anion in systemic hypertension: role of AT1 receptors. *J. Hypertens.* **22**, 1151-1156
25. Griendling, K.K., Minieri, C.A., Ollerenshaw, J.D. and Alexander, R.W. (1994) Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ. Res.* **74**, 1141-1148

26. Zhang, H., Schmeisser, A., Garlachs, C.D., Plotze, K., Damme, U., Mugge, A. and Daniel, W.G. (1999) Angiotensin II-induced superoxide anion generation in human vascular endothelial cells: role of membrane-bound NADH-/NADPH-oxidases. *Cardiovasc. Res.* **44**, 215-222
27. Pagano, P.J., Chanock, S.J., Siwik, D.A., Colucci, W.S. and Clark, J.K. (1998) Angiotensin II induces p67phox mRNA expression and NADPH oxidase superoxide generation in rabbit aortic adventitial fibroblasts. *Hypertension* **32**, 331-337
28. Rigat, B., Hubert, C., Corvol, P. and Soubrier, F. (1992) PCR detection of the insertion/deletion polymorphism of the human angiotensin converting enzyme gene (DCP1) (dipeptidyl carboxypeptidase 1). *Nucleic. Acids Res.* **20**, 1433
29. Shanmugam, V., Sell, K.W. and Saha, B.K. (1993) Mistyping ACE heterozygotes. *PCR Methods Appl.* **3**, 120-121
30. Rajagopalan, S., Kurz, S., Munzel, T., Tarpey, M., Freeman, B.A., Griending, K.K. and Harrison, D.G. (1996) Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J. Clin. Invest.* **97**, 1916-1923
31. Touyz, R.M. (2005) Intracellular mechanisms involved in vascular remodelling of resistance arteries in hypertension: role of angiotensin II. *Exp. Physiol.* **90**, 449-455
32. Zalba, G., San José, G., Moreno, M.U., Fortuño, M.A., Fortuño, A., Beaumont, F.J. and Díez, J. (2001) Oxidative stress in arterial hypertension: role of NAD(P)H oxidase. *Hypertension* **38**, 1395-1399
33. Zalba, G., San José, G., Moreno, M.U., Fortuño, A. and Díez, J. (2005) NADPH Oxidase-Mediated Oxidative Stress: Genetic Studies of the p22(phox) Gene in Hypertension. *Antioxid. Redox Signal.* **7**, 1327-1336
34. Pedro-Botet, J., Covas, M.I., Martín, S. and Rubies-Prat, J. (2000) Decreased endogenous antioxidant enzymatic status in essential hypertension. *J. Hum. Hypertens.* **14**, 343-345

35. Sayed-Tabatabaei, F.A., Houwing-Duistermaat, J.J., van Duijn, C.M. and Witteman, J.C. (2003) Angiotensin-converting enzyme gene polymorphism and carotid artery wall thickness: a meta-analysis. *Stroke* **34**, 1634-1639
36. Ridker, P.M., Brown, N.J., Vaughan, D.E., Harrison, D.G. and Mehta, J.L. (2004) Established and emerging plasma biomarkers in the prediction of first atherothrombotic events. *Circulation* **109**, IV6-IV19
37. Li, Y., Zagato, L., Kuznetsova, T., Tripodi, G., Zerbini, G., Richart, T., Thijs, L., Manunta, P., Wang, J.G., Bianchi, G. and Staessen, J.A. (2007) Angiotensin-converting enzyme I/D and alpha-adducin Gly460Trp polymorphisms: from angiotensin-converting enzyme activity to cardiovascular outcome. *Hypertension* **49**, 1291-1297
38. Murphey, L.J., Gainer, J.V., Vaughan, D.E. and Brown, N.J. (2000) Angiotensin-converting enzyme insertion/deletion polymorphism modulates the human in vivo metabolism of bradykinin. *Circulation* **102**, 829-832
39. Arnett, D.K., Claas, S.A., Glasser, S.P. (2006) Pharmacogenetics of antihypertensive treatment. *Vasc. Pharmacol.* **44**, 107-118
40. Muthumala, A., Montgomery, H., Palmén, J., Cooper, J.A. and Humphries, S.E. (2007) Angiotensin-converting enzyme genotype interacts with systolic blood pressure to determine coronary heart disease risk in healthy middle-aged men. *Hypertension* **50**, 348-353

Table 1. Clinical characteristics in normotensives and hypertensives.

	Normotensives	Hypertensives	Significance
Sex, male/female	154/35	187/36	NS
Age, years	54.9±0.7	58.6±1.1	<i>P</i> <0.05
BMI, kg/m ²	28.3±0.5	29.5±0.4	<i>P</i> <0.05
SBP, mmHg	118.4±1.1	141.4±1.4	<i>P</i> <0.05
DBP, mmHg	77.1±0.7	86.2±0.8	<i>P</i> <0.05
PP, mmHg	41.3±0.4	55.2±0.5	<i>P</i> <0.05
MAP, mmHg	90.9±0.8	104.6±1.0	<i>P</i> <0.05
Glucose, mg/dL	100.2±1.7	101.6±1.4	NS
Total Cholesterol, mg/dL	221±4	221±4	NS
HDL Cholesterol, mg/dL	50±1	48±1	NS
LDL Cholesterol, mg/dL	150±4	149±4	NS
Triglycerides, mg/dL	118±5	125±5	NS
Antihypertensive medication			
ACE inhibitor, n (%)	---	49 (22)	
ARA, n (%)	---	46 (21)	
Other antihypertensives, n (%)	---	33 (15)	

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; ACE, angiotensin converting enzyme; ARA, angiotensin II type 1-receptor antagonist.

Table 2. Comparison of genotype and allele frequencies for the I/D polymorphism of the ACE gene in normotensive and hypertensive subjects.

I/D polymorphism	Normotensives	Hypertensives	Significance
II, n (%)	29 (15.4)	28 (12.5)	$\chi^2 = 14.65; P > 0.05$
ID, n (%)	92 (48.7)	108 (48.5)	
DD, n (%)	68 (35.9)	87 (39.0)	
I allele frequency	0.3968	0.3677	
D allele frequency	0.6032	0.6323	

Table 3. Clinical characteristics of normotensives classified according to genotypes of the I/D polymorphism of the ACE gene.

	II	ID	DD	Significance
Sex, male/female	25/4	77/15	52/16	NS
Age, years	55.6±3.3	54.5±1.1	55.1±1.4	NS
BMI, kg/m ²	26.9±0.8	28.1±0.5	29.1±0.7	NS
SBP, mmHg	119.1±2.3	117.8±1.3	119.0±1.9	NS
DBP, mmHg	78.1±1.4	76.4±0.8	77.7±0.9	NS
PP, mmHg	41.0±0.8	41.4±0.5	41.3±0.6	NS
MAP, mmHg	91.8±1.2	90.2±0.9	91.5±1.1	NS
Glucose, mg/dL	100.1±2.9	100.3±1.8	100.1±2.7	NS
Total Cholesterol, mg/dL	233±11	212±6	229±7	NS
HDL Cholesterol, mg/dL	53±3	49±1	49±2	NS
LDL Cholesterol, mg/dL	155±8	144±4	157±6	NS
Triglycerides, mg/dL	111±8	120±3	118±7	NS

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure.

Table 4. Clinical characteristics of hypertensives classified according to genotypes of the I/D polymorphism of the ACE gene.

	II	ID	DD	Significance
Sex, male/female	23/5	91/17	72/15	NS
Age, years	60.6±2.8	59.0±1.1	57.4±1.9	NS
BMI, kg/m ²	28.2±0.9	29.9±0.5	29.4±0.7	NS
SBP, mmHg	137.0±2.6	140.3±1.7	144.5±1.9	<i>P</i> <0.05
DBP, mmHg	85.3±0.8	85.8±0.6	87.1±1.2	NS
PP, mmHg	51.7±0.8	54.5±0.6	57.4±0.6	<i>P</i> <0.05
MAP, mmHg	102.5±1.7	104.0±1.1	106.2±1.4	NS
Glucose, mg/dL	102.3±2.1	100.4±1.5	102.7±1.9	NS
Total Cholesterol, mg/dL	227±8	222±4	217±5	NS
HDL Cholesterol, mg/dL	49±3	50±2	46±2	NS
LDL Cholesterol, mg/dL	149±6	149±3	148±5	NS
Triglycerides, mg/dL	136±14	119±6	129±8	NS
Antihypertensive medication				
ACE inhibitor, n (%)	7 (25)	24 (22)	18 (21)	NS
ARA, n (%)	5 (18)	22 (20)	19 (22)	NS
Other antihypertensives, n (%)	4 (14)	15 (14)	14 (16)	NS

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; ACE, angiotensin converting enzyme; ARA, angiotensin II type 1-receptor antagonist.

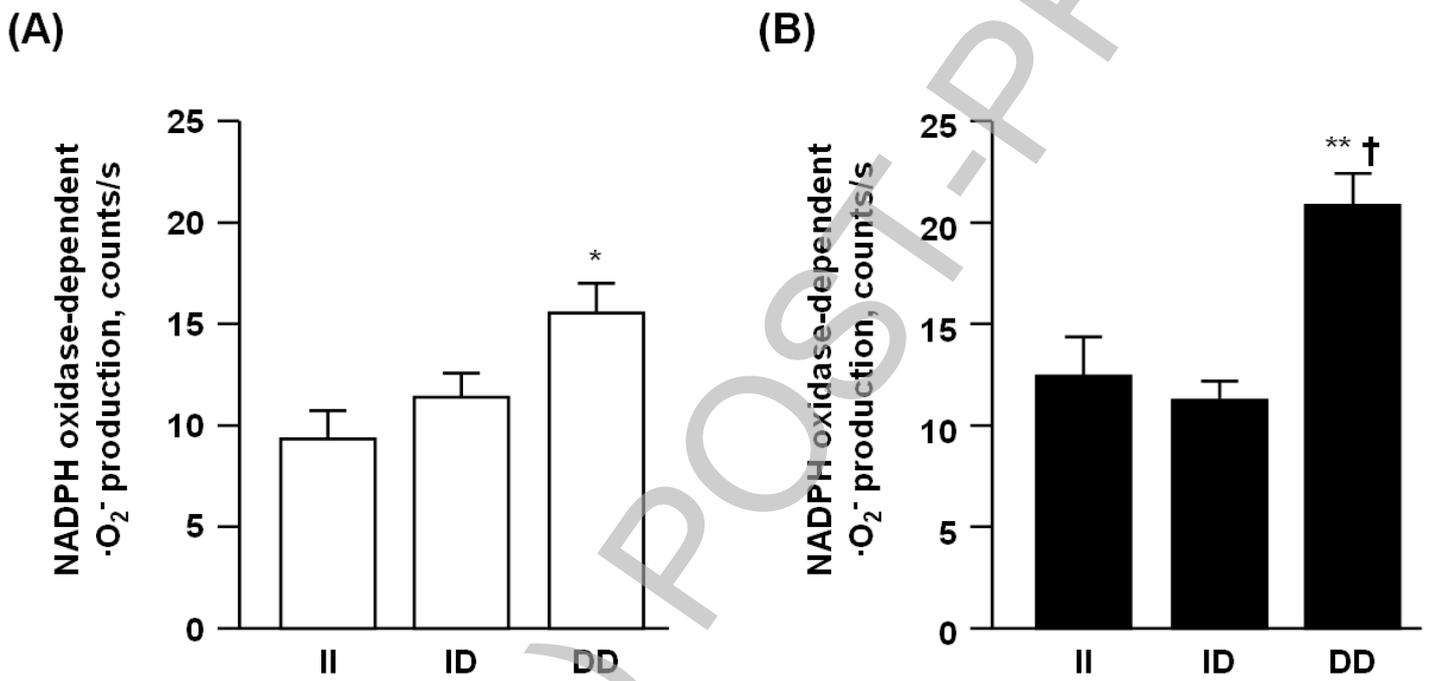
FIGURE LEGENDS

Figure 1. Effect of ACE I/D polymorphism genotype on NADPH oxidase-dependent $\cdot\text{O}_2^-$ production. NADPH oxidase-dependent $\cdot\text{O}_2^-$ production was determined by lucigenin chemiluminescence after phorbol 12-myristate 13-acetate (PMA) stimulation in peripheral mononuclear cells from 189 non-hypertensive healthy subjects (Panel A) and 223 hypertensive patients (Panel B). Bars show mean \pm SEM. * $P=0.003$ and ** $P=0.001$ compared with II and ID within the same group and † $P=0.01$ compared with DD normotensives.

Figure 2. Effect of ACE 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 (Panel A) and 223 hypertensive patients (Panel B). Bars show mean \pm SEM. * $P=0.009$ compared with II and ID within the same group, and † $P=0.04$ compared with DD normotensives.

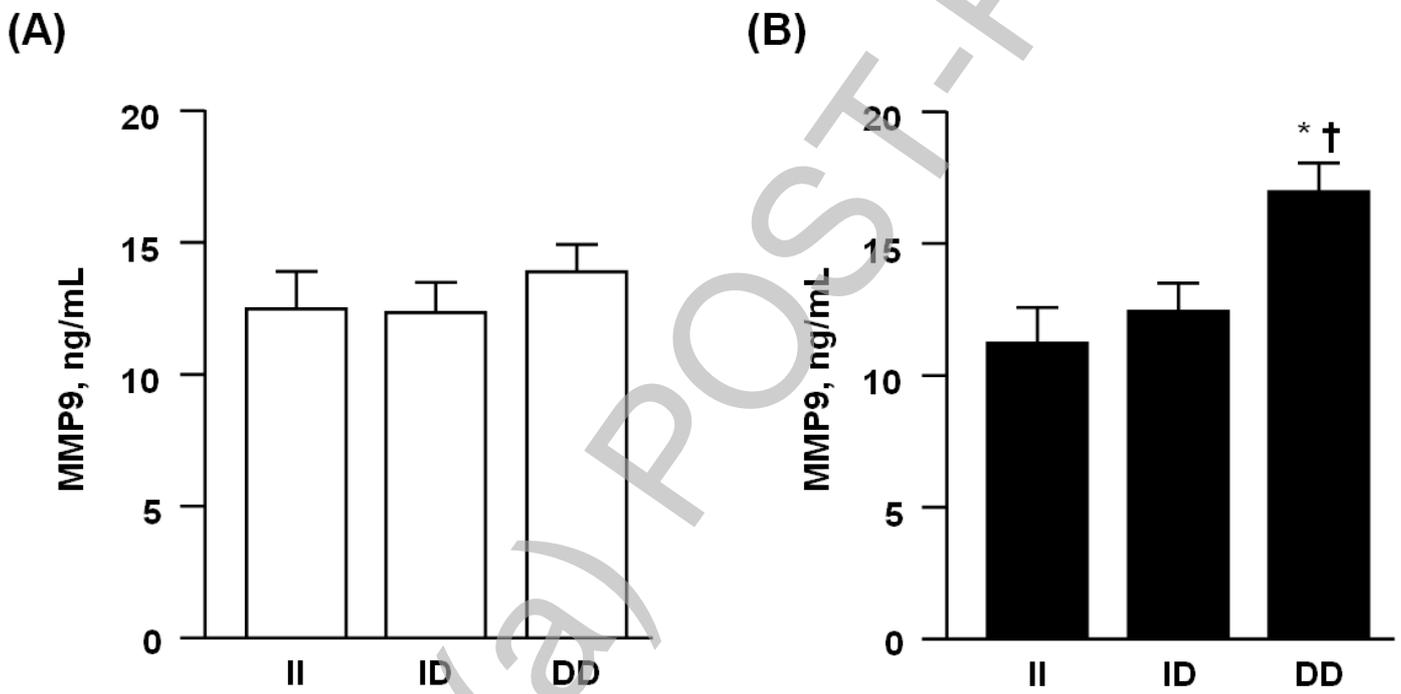
Figure 3. Association analysis between NADPH oxidase-dependent $\cdot\text{O}_2^-$ production and plasma levels of MMP-9 in normotensive subjects (Panel A) and in hypertensive patients (Panel B).

Figure 1



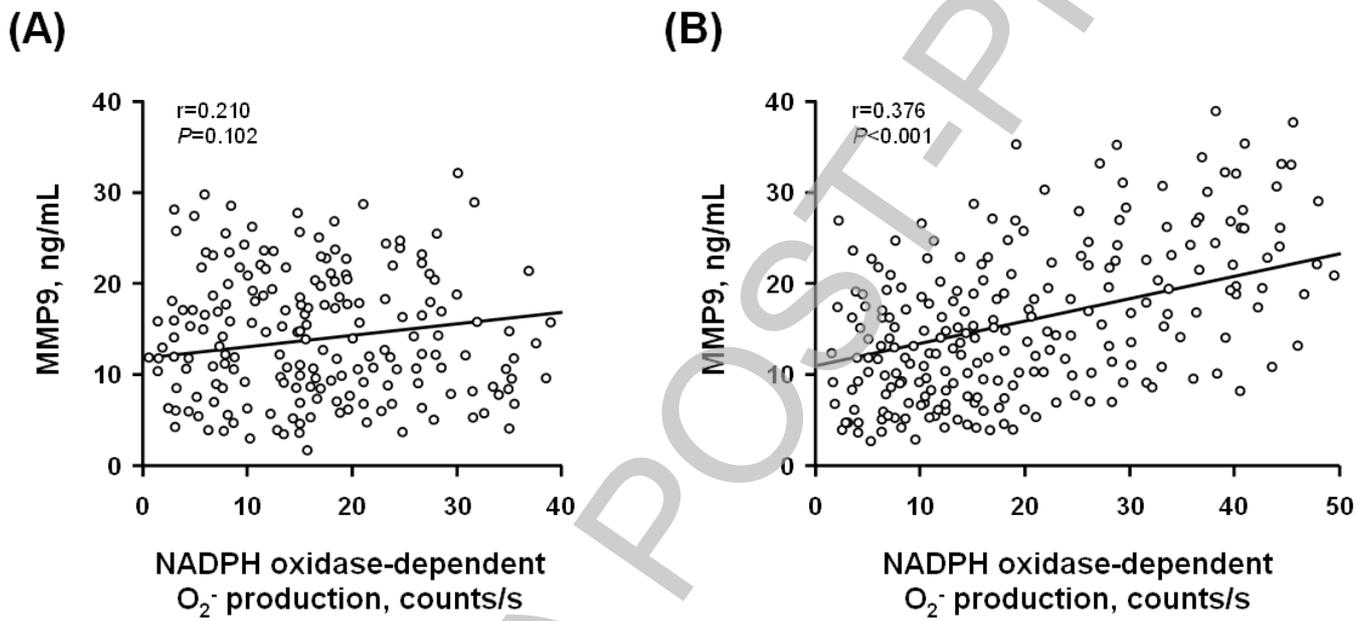
THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20080057

Figure 2



THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20080057

Figure 3



THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20080057