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Title: The angiotensin-converting enzyme I/D 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 angiotensin-converting enzyme (ACE) Insertion/Deletion (I/D) polymorphism on NADPH oxidase-dependent superoxide ($\cdot O_2^-$) 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 $\cdot O_2^-$ 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 $\cdot O_2^-$ 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 $\cdot O_2^-$ 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 $\cdot O_2^-$ overproduction. Within the group of hypertensive patients, DD cases also associated with increased blood pressure values and with enhanced plasma levels of MMP-9.

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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 ($\cdot O_2^-$) 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 $\cdot O_2^-$ 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 $\cdot O_2^-$ 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.

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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 $\cdot O_2^-$ production

$\cdot O_2^-$ 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 $\cdot O_2^-$ production were measured in basal conditions and in response to stimulation with phorbol 12-myristate 13-acetate (PMA) 2

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mg/L and using lucigenin 5 µmol/L in a chemiluminescent method that correlated well with the ferricytochrome C assay. We have previously demonstrated that PMA-stimulated $\cdot O_2^-$ 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 $\cdot O_2^-$, completely abolished the chemiluminescence induced with PMA thus verifying the specificity of the lucigenin assay for $\cdot O_2^-$ generation. Collectively, these previous findings allow us to identify the NADPH oxidase complex as the main enzymatic source of $\cdot O_2^-$ 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±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 $\cdot O_2^-$ 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 $\cdot O_2^-$

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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 $\cdot O_2^-$ production according to the genotypes of I/D polymorphism (II, 2.1±0.5 counts/; ID, 1.6±0.3; DD, 3.1±0.7). Nevertheless, PMA-induced NADPH oxidase-dependent $\cdot O_2^-$ 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 $\cdot O_2^-$ 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.

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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 $\cdot O_2^-$ production according to the genotypes of I/D polymorphism (II, 2.6±0.8 counts/; ID, 2.1±0.4; DD, 3.5±0.7). Nevertheless, PMA-induced NADPH oxidase-dependent $\cdot O_2^-$ 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 $\cdot O_2^-$ production were found between II and ID hypertensives. NADPH oxidase-dependent $\cdot O_2^-$ production was higher (P=0.01) in DD hypertensives than in DD normotensives. Phagocytic $\cdot O_2^-$ 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.

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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 $\cdot O_2^-$ production and plasma MMP-9

There was a significant positive bivariate correlation between phagocytic $\cdot O_2^-$ 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 $\cdot O_2^-$ 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 $\cdot O_2^-$ production in phagocytic cells. More precisely, DD carriers exhibit greater NADPH oxidase-dependent $\cdot O_2^-$ 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

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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 $\cdot O_2^-$ production [30]. Our findings showing an increased NADPH oxidase-mediated $\cdot O_2^-$ 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 $\cdot O_2^-$ production and I/D polymorphism in hypertension. In fact, although both DD normotensives and DD hypertensives exhibited increased NADPH oxidase-mediated $\cdot O_2^-$ 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 $\cdot O_2^-$ 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 $\cdot O_2^-$ 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

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is of importance, as phagocytic NADPH oxidase-mediated $\cdot O_2^-$ production positively correlates with carotid IMT [22]. In addition, the NADPH oxidase-mediated $\cdot O_2^-$ 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 $\cdot O_2^-$ production. Nevertheless, our findings show similar $\cdot O_2^-$ 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).

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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 $\cdot O_2^-$ 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].

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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	$P<0.05$
BMI, kg/m^2	28.3±0.5	29.5±0.4	$P<0.05$
SBP, mmHg	118.4±1.1	141.4±1.4	$P<0.05$
DBP, mmHg	77.1±0.7	86.2±0.8	$P<0.05$
PP, mmHg	41.3±0.4	55.2±0.5	$P<0.05$
MAP, mmHg	90.9±0.8	104.6±1.0	$P<0.05$
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.

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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	

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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.

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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	$P<0.05$
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	$P<0.05$
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.

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FIGURE LEGENDS

Figure 1. Effect of ACE I/D polymorphism genotype on NADPH oxidase-dependent $\cdot O_2^-$ production. NADPH oxidase-dependent $\cdot O_2^-$ 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 ± 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 ± 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 $\cdot O_2^-$ production and plasma levels of MMP-9 in normotensive subjects (Panel A) and in hypertensive patients (Panel B).

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Figure 1

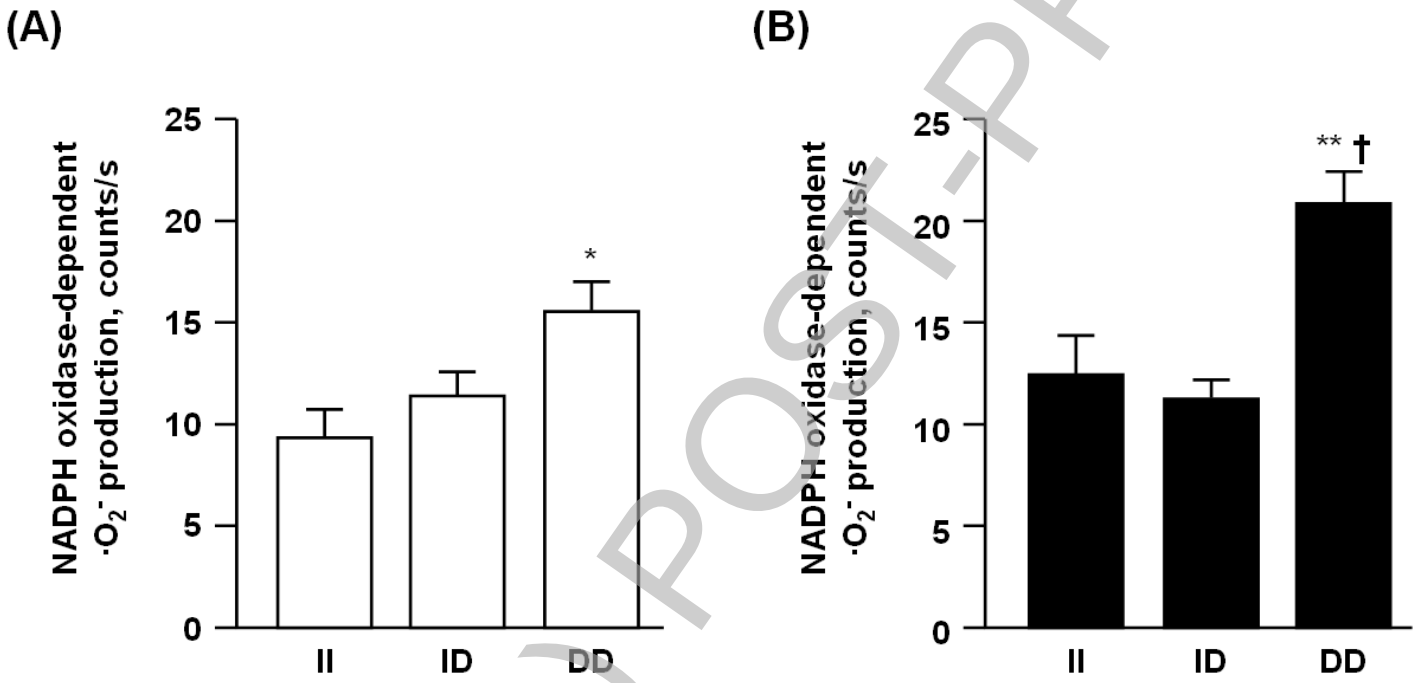

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Figure 2

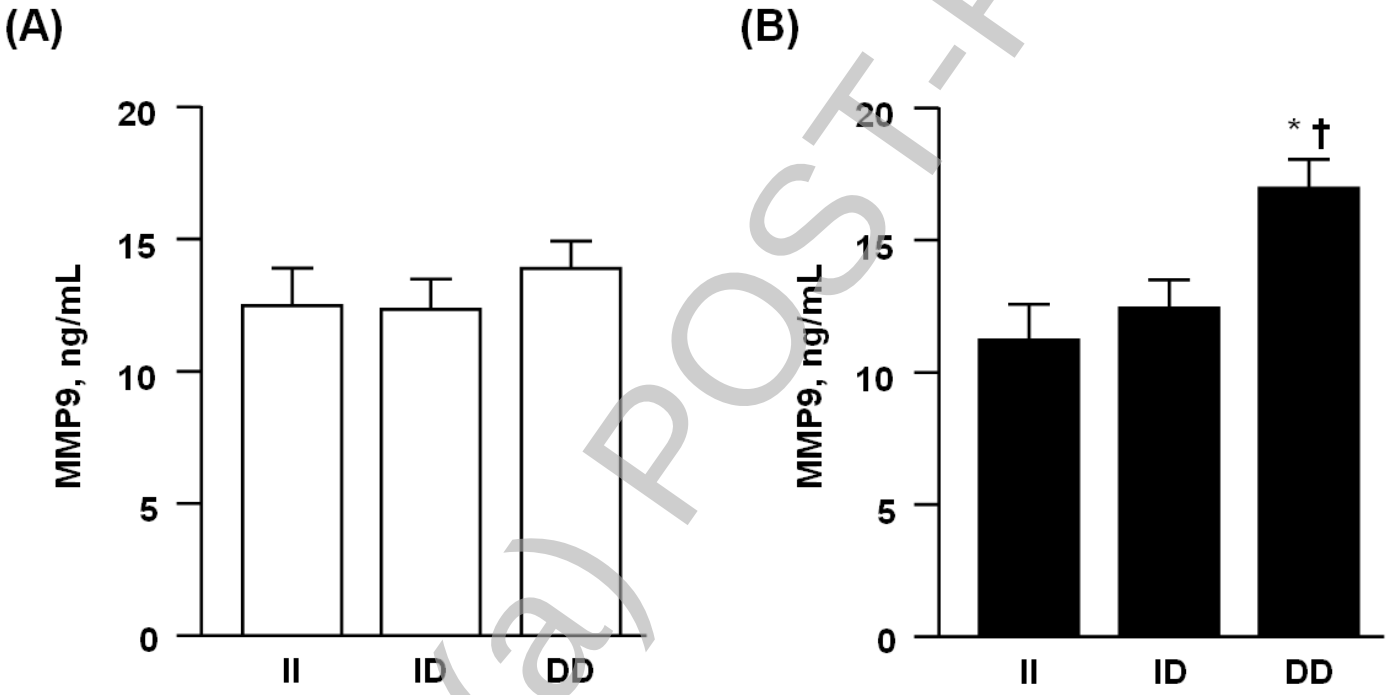

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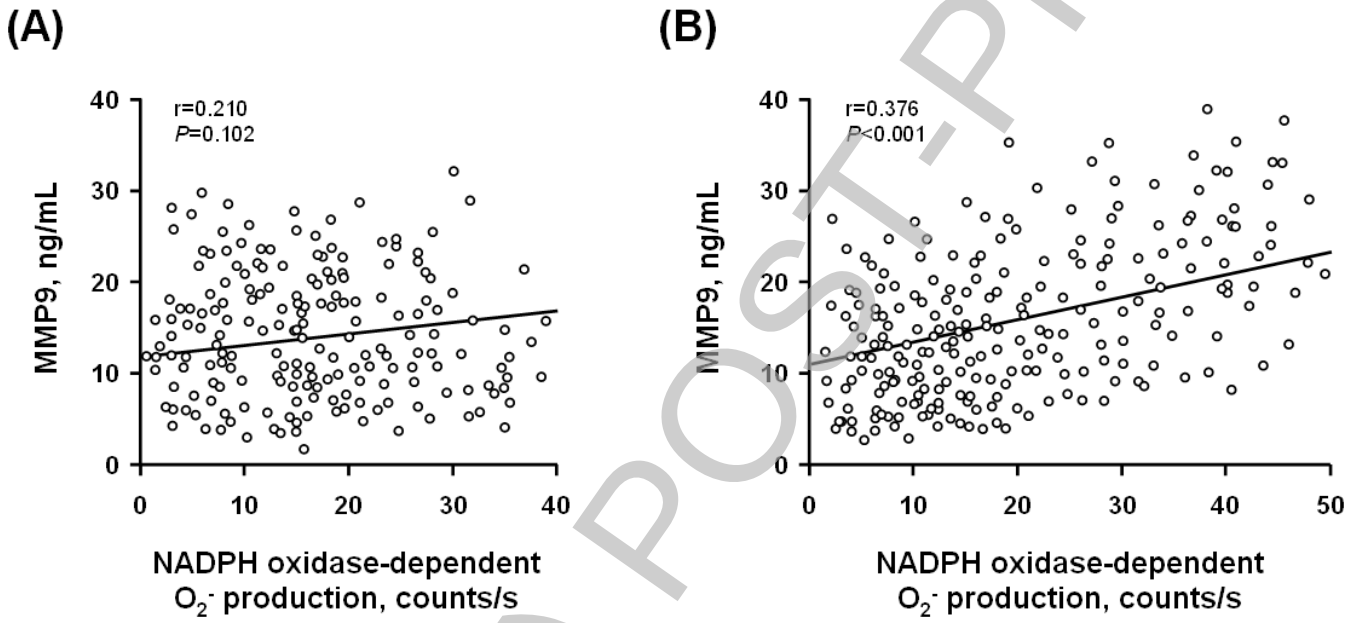
Figure 3



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