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CLINICAL RESEARCH

The *ACE I/D* polymorphism is associated with nitric oxide metabolite and blood pressure levels in healthy Mexican men

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Abstract The *I/D* insertion/deletion polymorphism of the angiotensin-converting enzyme has been related to hypertension. This polymorphism also seems to have gender related implications. Angiotensin II contributes to the production and release of oxygen reactive species that react with nitric oxide, inactivating its effects.
Objective: To establish whether the *ACE I/D* polymorphism correlates with nitric oxide plasma metabolites in healthy men and women.
Methods: Among 896 subjects between 18 and 30 years of age range, 138 fulfilled inclusion criteria. The polymorphism was identified by polymerase chain reaction, and blood nitric oxide metabolites were analyzed following the method described by Bryan.
Results: Both systolic and diastolic arterial pressures were higher in men than in women (107/67 vs. 101/65 mmHg, $p < 0.001$). In terms of the *ACE* gene, there were differences in the concentration of nitric oxide metabolites in men with the *I/D* and *D/D* genotypes when compared to carriers of the *I/I* genotype (33.55 and 29.23 vs. 53.74 pmol/ml; $p = <0.05$), while there were

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no significant differences in women when compared by genotype. Men with the *D/D* genotype had higher systolic blood pressure than *I/D* carriers (111 vs. 104 mmHg, $p < 0.05$). We observed no arterial blood pressure differences in women when grouped by ACE genotype.
Conclusions: The *ACE D/D* genotype was associated with nitric oxide metabolite levels and systolic blood pressure in clinically healthy men while it had no effect in women.

PALABRAS CLAVE
Polimorfismo I/D ECA;
Oxido Nítrico;
Población sana

El polimorfismo I/D de la ECA se asocia con los niveles de metabolitos óxido nítrico y de presión arterial en hombres mexicanos sanos

Resumen El polimorfismo inserción/deleción del gen de la enzima convertidora de la angiotensina (polimorfismo I/D de la ECA), se relaciona con hipertensión y sus efectos podrían estar asociados al género. La angiotensina II contribuye a la producción y liberación de especies reactivas de oxígeno, que reaccionan con el óxido nítrico (ON), inactivándolo.
Objetivo: Conocer si existen diferencias en la concentración de metabolitos de ON en hombres y mujeres sanos que puedan estar influidas por el polimorfismo I/D de la ECA.
Métodos: De 896 sujetos de entre 18 y 30 años, 138 cumplieron los criterios de inclusión. El polimorfismo fue identificado usando reacción en cadena de la polimerasa y los metabolitos de ON fueron analizados en sangre usando el método de Bryan.
Resultados: Las presiones sistólica y diastólica fueron más elevadas en hombres que en mujeres (107/67 vs. 101/65 mmHg $p < 0.001$). En relación con el genotipo, existieron diferencias significativas en la concentración de metabolitos de ON en los hombres con genotipos I/D, D/D comparados con los portadores del genotipo I/I (33.55 y 29.23 vs. 53.74 pmol/ml, respectivamente; $p = <0.05$). No hubo diferencias significativas en las mujeres portadoras de los diferentes genotipos. Respecto a la presión arterial, los hombres con genotipo D/D presentaron mayor presión arterial sistólica que aquellos portadores de I/D (111 vs. 104 mmHg, $p < 0.05$). En las mujeres no se observaron diferencias significativas comparándolas por genotipo.
Conclusiones: El genotipo D/D de la ECA está asociado con el nivel de metabolitos de ON en plasma y la presión arterial sistólica en hombres clínicamente sanos; esta asociación no se observa en las mujeres.

Background

Angiotensin-converting enzyme (ACE) catalyzes the conversion of angiotensin I to angiotensin II (AII), a potent vasoactive peptide with several acute and chronic effects on the cardiovascular system. In humans, the *ACE* gene is located on chromosome 17q23.3 (dbSNP rs1799752) and exhibits an insertion/deletion (*I/D*) polymorphism consisting of the presence or absence of 287 base pairs in intron 16. This polymorphism has been suggested to modify ACE levels and activity in plasma.[1] Deletion of both alleles (*D/D* genotype) is associated with increased enzymatic activity when compared to the insertion/insertion (*I/I*) genotype. Consequently, heterozygote insertion/deletion (*I/D*) carriers exhibit intermediate ACE concentration and activity.[2]

AII has been reported to be involved in the atherosclerotic process, particularly in association with hypertension.[3–6] Several studies have also reported evidence of a significant relation between the *ACE I/D* polymorphism and hypertension in male but not in female subjects.[7–9]

However, this relation has not been consistently observed in males, suggesting that other factors such as age are involved in the impact of the *ACE I/D* polymorphism on blood pressure.[10]

Nitric oxide (NO), synthesized by the endothelium, is important for vascular tone regulation and blood pressure control in humans.[11] AII contributes to the production of reactive oxygen species (ROS) that react with NO forming peroxynitrites, thus inactivating the molecule's effects and facilitating the propagation of the oxidative process by free radicals.[12,13]

It is well known that total NO production in patients with essential hypertension is decreased,[14] but little is known on the possible influence of the *ACE I/D* polymorphism on NO production in healthy individuals.

Therefore, we conducted this study to establish whether the *ACE I/D* polymorphism contributes to NO plasma concentrations in healthy men and women. We also searched for differences in blood pressure that have been associated to these genotypes.

Methods

We selected a total of 896 clinically healthy individuals whose age ranged between 18 and 30, from the blood donor pool of the ''Ignacio Chávez'' National Cardiology Institute. After preliminary screening, we selected a subgroup that met the following criteria:

(a) Inclusion criteria: both genders, with no infectious disease manifestations[15] or drug intake within the previous 14 days, no chronic-degenerative diseases or other known disease and with no addictions.
(b) Exclusion criteria: patients with a body mass index (BMI) $\geq$25 kg/m^2, waist circumference (WC) $\geq$80 cm in women or $\geq$90 cm in men, systemic arterial blood pressure (systolic, SAP/diastolic, DAP) $\geq$130/80 mmHg, glucose $\geq$110 mg/dL, total cholesterol (TC) $\geq$200 mg/dL triglycerides (TG) $\geq$150 mg/dL, pregnant, breastfeeding or menstruating women, patients who refused to sign the informed consent form or had developed any disease within 14 days prior to the study.

The protocol was approved by the Ethics Committee of the National Institute of Cardiology. All participants provided a signed informed consent.

Laboratory assessment

All patients were instructed to avoid strenuous exercise and eat a light dinner the day before blood samples were drawn. These were obtained in EDTA tubes after a 12 h overnight fast, from an antecubital vein after subjects had been seated for 15 min. Samples were centrifuged at 3000 rpm/20 min at 4 °C; the plasma was separated and analyzed or frozen at −80 °C until analysis. Plasma glucose, total cholesterol (TC), and triglycerides (TG) were determined by commercially available enzymatic methods. The phosphotungstic acid-Mg^{2+} precipitation procedure was used to precipitate apoB-containing lipoproteins before quantifying HDL-cholesterol. Quality control of lipid measurements was assessed by standardization with the Center for Disease Control and Prevention (Atlanta, GA). Low-density lipoprotein cholesterol was estimated in samples with triglyceride values below 400 mg/dl. Serum levels of all lipids were determined within 48 h after drawing the blood samples.

Data collection

We applied a cardiovascular risk factor questionnaire to our subjects, on their personal and familial habits and that included questions on previous and current diseases. The physical examination included: height, weight, and waist circumference (measured according to the 2006 official Mexican standard procedure).[16] Arterial blood pressure was measured three times in both arms, with 3 min intervals and after a 15 min rest; the average reading was used for analysis.

Genotyping

DNA extraction was performed in lymphocytes obtained from the blood samples of each patient, following conventional techniques.[17] We used Marre's PCR protocol.[18] As primers, GIIS and FYM generate a specific product for the insertion sequence of 376 base pairs; it has been reported that in heterozygote samples there is preferential amplification of the D allele and although Marre's protocol includes gene verification in the same test, we conducted a second PCR to corroborate the *D/D* genotype in our samples.[19]

NO quantification

Nitric oxide (NO) metabolite quantification was performed with Bryan method.[20] Total antioxidant capacity (TAC) was quantified with the CUPRAC method described by Upak et al.[21]

Statistical analysis

Data were expressed as percentages and averages $\pm$ standard deviation, or medians according to the variable's distribution. Kolmogorov–Smirnov's test was performed to analyze the distribution of continuous variables. In the case of continuous variables with no normal distribution, comparison between groups was made with Kruskal–Wallis' test.

The Hardy–Weinberg equilibrium (HWE) and comparisons between categorical variables were obtained with the Chi^2 test; continuous variables were analyzed by ANOVA and Bonferroni's post hoc test. Tukey's test was used for multiple comparisons. The association of polymorphisms and NO was analyzed by logistic regression following five inheritance models: co-dominant, dominant, recessive, heterozygous and additive advantage. Results were considered statistically significant if $p < 0.05$.

Results

We recruited 896 apparently healthy subjects in the 18–30 age range. One hundred and seventy-two (172) fulfilled inclusion criteria but 34 were excluded due to abnormal laboratory tests, resulting in 138 healthy volunteers included in the study. The group's anthropometric parameters and biochemical results are shown in Table 1. The group was divided and analyzed by gender. Body mass indices were comparable between men and women but men had greater waist circumference values. Systolic and diastolic blood pressures, LDL-cholesterol and triglycerides were higher in men while HDL-cholesterol and total antioxidant power capacity were lower in men than in women. The general familial risk factors are shown in Table 2 but no gender differences were identified. The polymorphism was in Hardy–Weinberg equilibrium ($\chi^2 = 1.81$; $p = 0.22$). Allelic frequency in this sample of healthy individuals was 59% for the I allele and 41% for the D allele. Genotype frequencies were 37.6% ($n = 52$) for *I/I*, 42.7 for *I/D* ($n = 59$), and 19.5 ($n = 27$) for *D/D*. We found no differences between the groups in the analyzed parameters or upon analysis of the *I/D* polymorphism in five inheritance models (data not shown).

Table 1 Anthropometric and biochemical characteristics of the population divided by gender.

	All subjects ($n=138$)	Men ($n=81$)	Women ($n=57$)	p
Age (years)	23 ± 3	24 ± 3	23 ± 2	0.143
Weight (kg)	61.46 ± 7.72	65.33 ± 5.88	55.96 ± 6.64	<0.0001
Height (m)	1.68 ± 0.14	1.71 ± 0.11	1.67 ± 0.18	0.083
BMI (kg/m^2)	22.41 ± 1.85	22.6 7 ± 1.65	22.04 ± 2.05	0.057
WC (cm)	78.25 ± 7.07	80.25 ± 6.33	75.40 ± 7.13	<0.0001
SAP (mmHg)	105 ± 10	107 ± 9	101 ± 9	0.001
DAP (mmHg)	66 ± 7	67 ± 7	65 ± 6	0.033
HR (bpm)	65 ± 7	64 ± 7	66 ± 7	0.201
TC (mg/dL)	149.03 ± 25.46	150.11 ± 30.27	147.49 ± 16.51	0.515
C-HDL (mg/dL)	48.37 ± 15.68	44.61 ± 13.89	53.71 ± 16.63	0.001
C-LDL (mg/dL)	85.86 ± 25.11	89.30 ± 27.77	80.96 ± 20.00	0.042
TG (mg/dL)	88.28 ± 32.75	96.54 ± 34.02	76.53 ± 25.77	<0.0001
Glucose (mg/dL)	82.35 ± 8.91	83.53 ± 8.92	80.67 ± 8.71	0.062
PAO (mmol/L)	569.54 ± 293.36	512.20 ± 269.74	651.02 ± 308.36	0.007
NO (pmol/mL)	37.39 ± 28.70	39.76 ± 32.08	34.03 ± 22.91	0.223

Data are expressed as median ± standard deviation. P values were estimated using Student's *T*-test. BMI, body mass index m^2; WC, waist circumference; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; HR: heart rate; TC, total cholesterol; C-HDL, high density cholesterol; C-LDL, low density cholesterol; TG, triglycerides; PAO, antioxidant power; NO, nitric oxide.

Table 2 Familial risk factors in the study population.

Risk factor	All subjects ($n=138$)	Men ($n=81$)	Women ($n=57$)
Vascular risk			
HTA (%)	49.3	46.9	52.6
PTCA (%)	15.2	13.6	17.5
Stroke (%)	11.6	12.3	10.5
PVD (%)	31.9	34.6	28.1
Metabolic risk			
DLP (%)	37	33.3	42.1
DT2 (%)	59.4	54.3	66.7
OBE (%)	47.8	43.2	54.4

Data expressed in percentages (%). p values were estimated using Chi-square and Pearson's test. HTA, systemic arterial hypertension; PTCA, percutaneous transluminal coronary angioplasty; CVA, stroke; PVD, peripheral vascular disease; DLP, dyslipidemia; DT2, diabetes mellitus type 2; OBE, obesity.

Table 3 shows the relation between the ACE genotypes and the evaluated parameters when analyzed by gender. We showed significant differences in the systolic arterial pressure of men carrying the *I/D* genotype compared with men with the *D/D* genotype ($p=0.04$). NO levels were lower in men with the *I/D* and *D/D* genotype than in men carrying the *I/I* genotype ($p=0.03$ and $p=0.022$ respectively). Women carrying the *D/D* genotype had a significantly different BMI than *I/D* carriers ($p=0.026$). Logistic regression analysis (uni and multivariate) of risk factors and genotypes showed no statistically significant differences.

Discussion

ACE is a key enzyme in the renin–angiotensin–aldosterone and kallikrein–kinin systems, and plays an important role in blood pressure regulation and electrolyte balance.[22] ACE is encoded in chromosome 17q23.3 and the *ACE* gene exhibits different polymorphisms. The *ACE I/D* polymorphism is the most studied, and it may explain up to 47% of the total phenotypic variation in ACE serum levels and determine ACE enzymatic activity.[2] In this study, the participants' selection was very careful, excluding those subjects with physical, hemodynamic or biochemical parameters that were outside of the ''normal'' range, since the main objective was to assess the levels of NO metabolites in terms of the ACE genotype. Therefore, it was necessary to exclude patients with any entity known to be associated with endothelial dysfunction.

It is important to emphasize that NO has an extremely short life in biological systems (less than 1 s in circulating blood)[23] so consequently, its metabolites (nitrite and nitrate) in blood have been widely used as indicators of endothelial NO synthesis activity.[24,25] For this reason, the concentration of NO was estimated using its metabolites, but this approach is nevertheless unable to distinguish between peroxynitrates and NO synthesis.

The frequency of the I and D alleles of the *ACE* gene, in this sample of healthy individuals, was similar to that previously reported in the Mexican mestizo population.[26–28] Although our study only included participants with normal arterial blood pressure (≤130/80 mmHg), there were statistically significant differences between men and women whereby men's arterial pressure readings were higher than women's. In this regard, Fornage et al.[8] showed that in a group of young subjects with a mean age of 14.8 years, males had a significantly higher systolic pressure than females. Coelho et al.[29] reported that in teenage healthy individuals, males had greater ACE activity, regardless of the genotype; this may explain why the men in our study had higher baseline arterial blood pressures.

Pointing to the *ACE* gene as an attractive candidate in the study of human essential hypertension and in the context of

Table 3 Analysis of clinical parameters in the population divided by gender and genotype.

Parameter	Male population			Female population		
	I/I (*n* = 29)	*I/D* (*n* = 33)	*D/D* (*n* = 19)	*I/I* (*n* = 23)	*I/D* (*n* = 26)	*D/D* (*n* = 8)
BMI (kg/m^2)	22.62 ± 1.65	22.70 ± 1.86	22.68 ± 1.33	22.09 ± 1.67	21.50 ± 2.26	23.63 ± 1.59
WC (cm)	79.66 ± 6.34	80.88 ± 6.78	80.05 ± 5.73	75.61 ± 6.25	74.46 ± 8.02	77.88 ± 6.62
SAP (mmHg)	107.72 ± 8.58	104.85 ± 9.52	111.68 ± 11.02[a]	102.78 ± 10.25	101.04 ± 9.75	99.88 ± 8.20
DAP (mmHg)	68.38 ± 7.86	66.12 ± 6.95	70.63 ± 7.42	67.35 ± 6.83	63.96 ± 6.63	64.13 ± 5.93
HR (bpm)	63.10 ± 8.23	64.94 ± 8.13	65.05 ± 6.77	66.39 ± 7.69	65.19 ± 7.87	67.88 ± 8.96
TC (mg/dL)	153.66 ± 28.10	148.91 ± 28.29	146.79 ± 37.25	143.87 ± 18.29	147.85 ± 14.40	156.75 ± 15.78
C-HDL (mg/dL)	47.84 ± 12.22	43.33 ± 11.01	41.90 ± 19.58	50.86 ± 15.31	55.15 ± 19.88	57.25 ± 3.80
C-LDL (mg/dL)	90.44 ± 25.72	89.32 ± 25.35	87.53 ± 35.35	80.65 ± 21.73	79.88 ± 20.20	85.37 ± 15.24
TG (mg/dL)	91.17 ± 29.68	98.00 ± 33.38	102.21 ± 43.77	75.09 ± 32.95	74.73 ± 20.80	86.50 ± 14.90
Glucose (mg/dL)	81.59 ± 81.59	84.21 ± 10.28	85.32 ± 7.86	81.87 ± 7.60	80.31 ± 10.08	78.38 ± 7.17
PAO (mmol/L)	494.24 ± 287.57	498.05 ± 224.66	564.22 ± 318.25	689.26 ± 356.20	639.92 ± 299.65	577.19 ± 172.48
NO (pmol/mL)	53.74 ± 46.45	33.53 ± 15.87[*]	29.23 ± 16.34[*]	29.52 ± 15.95	36.61 ± 29.66	38.61 ± 11.95

BMI, body mass index m^2; WC, waist circumference; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; HR, heart rate; TC, total cholesterol; C-HDL, high density cholesterol; C-LDL, low density cholesterol; TG, triglycerides; PAO, antioxidant power; NO, nitric oxide.

Data are expressed as median ± standard deviation. *p* values were estimated using ANOVA and Tukey's test for multiple comparisons.

[*] Significant differences in *I/I* carrier $p < 0.05$.

[a] Significant differences in *I/D* carrier $p < 0.05$.

this disease, some studies have documented differences in arterial pressure between genders.[7–9] In the Suita study, the authors showed that men have higher arterial pressure levels than women in association with the D allele.[9]

In our study of healthy volunteers, we observed that men with the *I/I* genotype have the highest concentrations of NO metabolites when compared to women with the same genotype and even with men or women with the *I/D* and *D/D* genotype; if the concentration of NO metabolites in reality reflects an increase in NO production, it is possible that the *I/I* genotype could confer some protection to men carrying the allele and may delay hypertension development. This finding may explain, at least in part, why men with the DD genotype develop systemic arterial hypertension more frequently than men with the *I/I* genotype as shown in previous studies.[7–9]

With respect to the overall groups' metabolic behavior, men had higher C-LDL and TG levels than women and lower C-HDL and PAO. These findings could be related to gender solely.

Study limitations

The major pathway for NO metabolism is its stepwise oxidation to nitrite and nitrate. In plasma, NO is almost completely oxidized to nitrite and remains stable for several hours, allowing its measurement. Sample preparation is the most important step in NO metabolite quantification. In this study, extreme care was taken to preserve the samples as described by Bryan, but the possibility of artificially generating NO products or metabolites during sample preparation still exists. This is a limitation in all of the studies that measure NO metabolites, regardless of the method employed. The statistical power of our study was 0.24 because of the decrease in the sample size and this could be another study limitation.

Conclusions

The *ACE D/D* genotype was associated with NO metabolites' levels and systolic blood pressure in clinically healthy men. High levels of NO metabolites in men with the *I/I* allele suggest that perhaps, this genotype may delay the manifestation of arterial hypertension in males. On the other hand, these genotypes had no effect in women. As referred in some studies, we conclude that ACE polymorphism has gender implications.

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Conflict of interest

The authors declare no conflict of interest.

Privacy policy

The authors declare that they have followed the protocols of the workplace on the publication of patient data.

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