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Endothelial constitutive nitric oxide synthase, angiotensin converting enzyme, angiotensin II type 1 receptor gene polymorphisms and endothelial functions in healthy individuals

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Abstract. – INTRODUCTION: Endothelial dysfunction is recognized as an early and initiating event in the pathogenesis of coronary artery disease. Gene polymorphisms of endothelial constitutive nitric oxide synthase (ecNOS), angiotensin converting enzyme (ACE) and angiotensin II type 1 receptor (AT1R) have been found to be associated with atherosclerosis. We aimed to investigate the possible effects of ecNOS, ACE and AT1R gene polymorphisms on endothelial functions in healthy population.

MATERIALS AND METHODS: In 255 healthy subjects (male/female: 119/136 mean age 35.1±2.3 years) ecNOS, ACE and AT1R gene polymorphisms were assessed by polymerase chain reaction (PCR). Endothelium dependent (EDD, flow-mediated) and endothelium independent vasodilation (EID) were measured by high resolution brachial artery ultrasound and 0.5mg sublingual nitroglycerine respectively.

RESULTS: ecNOS and ACE genes had no significant effect on EDD and EID. However, subjects with AT1RAC+CC genotypes had lower EDD compared to subjects with AT1RAA genotype in females (19.4 ± 6.6% vs 21.5 ± 7.8%, p = 0.041). EDD and EID were significantly negatively associated with age, body mass index, serum creatinine, glucose, uric acid and hemoglobin levels. When the data on age, uric acid, BMI, glucose, creatinine, and hemoglobin were split into 3 as low-1/3, mid-1/3 and high 1/3, there was significant graded decrease in EDD and EID with these parameters. In multiple regression analysis, age and presence of AT1RAC+CC genotype retained as significant independent factors predicting endothelial functions.

CONCLUSIONS: Gene polymorphisms of endothelial constitutive nitric oxide synthase and angiotensin converting enzyme had no effect on endothelial functions. However, the presence of angiotensin II type 1 receptor polymorhism (AT1RAC+CC genotype) seemed to adversely affect the endothelial functions as reflected by impaired endothelium dependent and independent vasodilatation in healthy individuals.

Key Words:
Angiotensin converting enzyme polymorphism, Angiotensin II type 1 receptor polymorphisms, Endothelial constitutive nitric oxide synthase polymorphism, Endothelial dysfunction, Endothelium dependent vasodilatation, Endothelium independent vasodilatation.

Introduction

Endothelial dysfunction is recognized as an early and initiating event in the pathogenesis of coronary artery disease (CAD)[1-3]. Endothelial function also has a long term prognostic importance in terms of progression of cardiovascular diseases[4,5]. Gene polymorphisms of endothelial constitutive nitric oxide synthase (ecNOS)[6,7], angiotensin converting enzyme (ACE)[8,9] and angiotensin II type 1 receptor (AT1R)[9] have been found to be associated with atherosclerosis and CAD. Herein, we aimed to investigate the possible effects of ecNOS, ACE and AT1R gene polymorphisms on endothelial functions measured as both endothelium dependent and independent vasodilatation in healthy population.

Subjects and Methods

Study Population

Two hundred and fifty-five healthy subjects (male/female: 119/136, mean age 35.1 ± 12.3 years) were enrolled into the study. Each subject

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was evaluated by history, physical examination, and screening laboratory tests. Exclusion criteria included evidence or history of diabetes mellitus, hypertension, cardiovascular disease and chronic medication use including hormone replacement therapy. The study was approved by the Ethics Committee of the Istanbul Medical Faculty, University of Istanbul (No: 2009/925). Informed consent was obtained from each subject and the Declaration of Helsinki on Biomedical Research on Humans was followed for the study.

Laboratory Analysis

Major cardiovascular risk factors such as body mass index (BMI), smoking, family history (myocardial infarction or sudden death in first degree consanguinity in men before 55, in women before 65 years of age), glucose, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglyceride and uric acid were measured and recorded. Biochemical analyses were performed by standard methods in the clinical biochemical laboratory of Istanbul Faculty of Medicine.

Analysis of the Variable Number of Tandem repeats (VNTRs) Polymorphism of the Endothelial Constitutive Nitric Oxide Synthase (ecNOS) Gene

ecNOS genotypes were determined by polymerase chain reaction (PCR) using oligonucleotide primers (sense: 5'-AGGCCCTATGGTAGTGCCTTT-3'; antisense: 5'-TCTCTTAGTGCTGTGG TCAT-3') that flank the region of the 27 base pair (bp) direct repeat in intron 4 as described previously with minor modifications[10]. Reactions were performed in a total volume of 50 (l containing 500 ng genomic DNA, 10 pmol of each primer, 0.2 mM dNTP, 0.5 U Taq DNA polymerase (MBI Fermentas Inc., New York, NY, USA), 5(l PCR buffer (500 mmol/l KCl, 100 mmol trihidroxymethylaminomethane hydrochloride and 0.8% Nonidet P40; MBI Fermentas Inc.). The thermocycling procedure consisted of initial denaturation at 940°C for 5 minutes, 35 cycles of denaturation for 1 minute, annealing at 550°C for 1 minute, extension at 720°C for 1 minute. The PCR products were analyzed using 3% agarose gel electrophoresis and visualized by ethidium bromide staining.

Analysis of the Insertion/Deletion Polymorphism of the Angiotensin Converting enzyme (ACE) Gene

Genomic DNA was isolated from leukocytes obtained from peripheral venous blood samples using the method described by Miller et al[11]. Sixteenth intron of the ACE gene was amplified with PCR as described by Rigat et al[12]. In the first PCR, a 490-bp fragment representing the I allele and a 190-bp fragment representing the D allele were obtained (sense: 5'-CTGGAGACCA CTCCCATCCTTTCT-3'; antisense: 5'-GATGTGGCCATCACATTC GTCAGAT-3'). Second PCR reaction is necessary for the confirmation of the ACE genotype by an insertion specific PCR amplification with the primer pair of Lindpaintner et al[13] obtained (sense: 5'-TGGGACCACA GCGCCCGCCACTAC-3'; antisense: 5'-TCGCCAGCC CTCCCATGCCCATAA-3'). The first PCR product was electrophoresed on a 3% agarose gel. A 1.5% agarose gel was used for visualizing the second PCR product. The gels were stained with ethidium bromide and photographed under UV light.

Analysis of the A1166C Polymorphism of the Angiotensin II Type 1 Receptor (AT1R) Gene

Genomic DNA was extracted from white blood cells. The region of the AT1R located between nucleotides 423 and 1278 of the cDNA (14) was amplified by using oligonucleotides 5'-GGCTT TGCTTTGTCTTGTTG and 5'-AATGCTTGTAGCC AAAGTCACCT as sense and

Table I. Genotype and allele frequencies (n = 255).

ecNOS Gene Polymorphism		AT1R gene polymorphism		ACE gene polymorphism	
aa genotype	3	AA genotype	184	II genotype	45
ab genotype	42	AC genotype	68	ID genotype	111
bb genotype	210	CC genotype	3	DD genotype	99
a allele	0.09	A allele	0.85	I allele	0.39
b allele	0.91	C allele	0.15	D allele	0.61

Table II. The comparison of cardiovascular risk factors according to sex (*Myocardial infarction or sudden death in first degree consanguity in men before 55, in women before 65 years of age).

	Male (n=119) Mean ± SD	Female (n=136) Mean ± SD	*p* value
Age (years)	37 ± 12	33 ± 13	0.009
Smoking	36.1%	20.1%	0.002
Family History*	20.2%	23.5%	NS
Glucose	86 ± 9	85 ± 9	NS
Total Cholesterol	172 ± 35	166 ± 27	NS
HDL	41 ± 9	51 ± 12	< 0.001
LDL	103 ± 28	96 ± 23	0.024
Triglyceride	139 ± 70	92 ± 47	< 0.001
Uric acid	4.9 ± 1.0	3.6 ± 0.8	< 0.001
BMI	25.2 ± 3.1	22.9 ± 3.9	< 0.001

antisense primers, respectively. The reaction conditions were: 2 ng/(L of genomic DNA, 0.75 (mol/L of each oligonucleotide, 75 (mol/L of each dNTP, 1.5 mmol/L of $MgCl_2$, 75 mmol/L Tris-HCl (pH 9.0), 5 mmol/L KCl, 20 mmol/L (NH4)2SO4, 0.2 U Taq DNA polymerase (MBI Fermentas Inc., New York, NY, USA), in a final volume of 10 (L DNA was amplified with an initial denaturation step at 94^0C for 3 min, followed by 40 cycles of 940C for 30 sec, 60^0C for 30 sec, 72°C for 90 sec, and a final elongation step at 72^0C for 5 min. The A1166C polymorphism was detected by digestion of the PCR product with Ddel (Dopachrome Tautomerase Distal Enhancer 1), which cuts at positions 1167, when C is present at 1166 and 1023.

Endothelial Function Assessment

Brachial artery was imaged on a commercially available ultrasound system (VINGMED Technology, System Five, Horten, Norway) using a 10.0 MHz linear phased-array ultrasound transducer longitudinally just above the antecubital fossa as previously described[15,16]. Two hundred and thirty-two subjects were assessed for endothelial function. Blood pressure cuff was wrapped around the upper arm[16], inflated to 250 mmHg and held for 5 minutes to induce ischemia. The cuff was released and brachial artery diameter was measured every minute for 5 minutes to assess maximal endothelium dependent vasodilation (EDD) in response to reactive hyperemia. After vessel diameter returned to baseline values (~ 7-10 minutes), endothelium independent vasodilation (EID) was assessed after 0.5 mg sublingual nitroglycerine, every minute for 5 minutes. Vessel diameters were measured at the end diastole coincident with the onset of the R-wave of the simultaneously obtained ECG trace. During the measurements particular attention was paid to the temperature of the laboratory, menstrual cycle, exercise, drugs, food, and sympathetic stimuli as recommended by the guidelines[17]. The percent vasodilation was calculated with the following formula:

$$\text{Percent EDD or EID} = 100 \times \frac{(\text{pBAD} - \text{bBAD})}{(\text{bBAD})}$$

pBAD: Peak brachial artery diameter after intervention
bBAD: Baseline brachial artery diameter

The intra and inter-observer variability of the measurements in our laboratory was 1-3%. Brachial artery ultrasound evaluation was done by an experienced physician.

Statistical Analysis

The statistical analysis was done with a commercially available Statistical Package for Social Sciences for Windows version 10.0 (SPSS Inc, Chicago, IL, USA). The means of two groups of numerical variables were compared with independent samples Student's *t* test. The variables were log-transformed if the assumptions for normal distribution were violated. Ordinal variables were analyzed with χ^2 test. For 2↔2 contingency tables Yates correction was done. If expected frequencies in the cells of 2↔2 table were less than

Table III. The correlation of risk factors with endothelium dependent (EDD) and independent (EID) vasodilation

	EDD		EID	
	r	*p* value	r	*p* value
Age	-0.34	<0.001	-0.37	<0.001
Sex	0.33	<0.001	0.30	<0.001
Glucose levels	-0.16	0.016	-0.16	0.015
Uric acid levels	-0.30	<0.001	-0.31	<0.001
Body mass index	-0.26	<0.001	-0.25	<0.001
Creatinine levels	-0.21	0.001	-0.19	0.004
Hemoglobin levels	-0.28	<0.001	-0.31	<0.001
AT1R	-0.13	0.041	-0.09	NS

5, Fisher's exact test was used. If tables examined were greater than 2×2 then appropriate rows or columns were combined to obtain expected frequencies of more than 5. Correlation between two numerical variables with normal distribution was sought with Pearson's bivariate correlation test, else correlation was done with Spearman's correlation test. The patients were divided into lower, mid and higher thirds for age, hemoglobin, uric acid, glucose, body mass index (BMI), creatinine and analyzed with one-way ANOVA to examine the effect of the variability of the parameters on EDD and EID. Factors that were significantly related to EDD and EID in univariate testing were examined with multivariate linear regression analysis with forward inclusion to identify the principal causes of the variability observed in EDD and EID. p value < 0.05 was accepted as statistically significant.

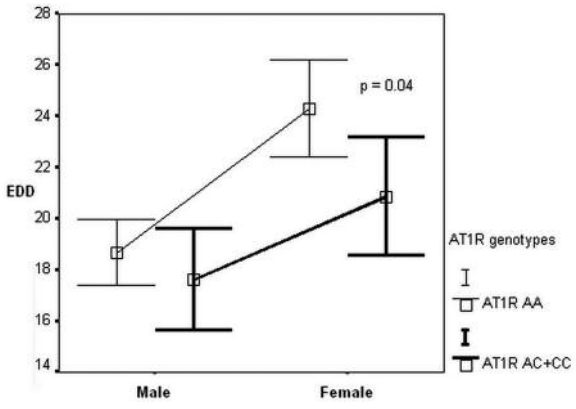

Figure 1. Female subjects with AT1RAC+ CC genotype had significantly lower EDD compared to female subjects with AT1RAA genotype. No significant difference was detected in males in terms of AT1R gene polymorphism.

Results

The frequencies of genotypes and alleles of ACE, AT1R, and ecNOS genes were presented in Table I. The distributions of the genotypes were in Hardy-Weinberg equilibrium. The presence of ecNOS and ACE gene polymorphisms had no significant effect on EDD and EID. However, female subjects with AT1RAC+ CC genotype had significantly lower EDD compared to female subjects with AT1RAA genotype (19.4±6.6% vs 21.5 ±7.8%, $p = 0.041$) (Figure 1).

The comparison of common cardiovascular risk factors are shown according to sex in Table II. Accordingly, females had a more favorable cardiovascular risk profile compared to males.

EDD and EID were found to be strongly correlated with each other ($r = 0.88$, $p < 0.001$). The correlations found with biochemical parameters and other risk factors are listed in Table III. Accordingly EDD and EID showed significant correlations with age, sex, BMI, hemoglobin, serum creatinine, uric acid and glucose. The EDD and EID were significantly related to sex with males having lower EDD (18.4 ± 5.7% vs 23.2 ± 8.2%, $p < 0.001$) and EID (21.7 ± 6.6% vs 27.1 ± 9.4%, $p < 0.001$) compared to females. When females were compared with age matched male controls for EDD and EID in premenopause (EDD 18.9 ± 5.3% vs 24.0 ± 7.9%, $p < 0.001$; EID 22.6 ± 6.2% vs 28.1 ± 8.8%, $p < 0.001$) and in postmenopause (EDD 16.3 ± 6.9% vs 16.7 ± 8.3%, p = NS; EID 18.2 ± 7.1% vs 19.3 ± 10.1%, p = NS), the difference between sexes were apparent only in premenopause.

When the data on age, uric acid, BMI, glucose, creatinine, and hemoglobin were split into 3 (low-1/3, mid-1/3 and high 1/3), there was significant graded decrease in EDD and EID with

Table IV. The effect of the distribution of biochemical parameters on endothelium dependent (EDD) and independent (EID) vasodilatation.

		EDD %	EID %
Age	*p* value	<0.001	<0.001
	Low 1/3	23.2 ± 8.2	27.3 ± 9.2
	Mid 1/3	20.6 ± 6.6	24.1 ± 7.4
	High 1/3	18.4 ± 6.6	21.6 ± 8.0
Uric acid	*p* value	<0.001	<0.001
	Low 1/3	23.6 ± 8.5	27.5 ± 9.5
	Mid 1/3	20.4 ± 7.7	24.1 ± 8.8
	High 1/3	18.4 ± 5.0	21.8 ± 6.1
Body mass index	*p* value	<0.001	<0.001
	Low 1/3	23.6 ± 8.3	27.4 ± 8.8
	Mid 1/3	19.6 ± 6.9	23.2 ± 8.5
	High 1/3	19.2 ± 6.4	22.7 ± 7.6
Serum creatinine	*p* value	0.029	0.025
	Low 1/3	22.5 ± 8.9	26.6 ± 10.1
	Mid 1/3	21.2 ± 7.0	24.6 ± 8.1
	High 1/3	18.9 ± 6.8	22.4 ± 7.5
Hemoglobin	*p* value	0.002	<0.001
	Low 1/3	22.7 ± 8.4	27.1 ± 9.5
	Mid 1/3	21.3 ± 7.2	24.9 ± 8.0
	High 1/3	18.6 ± 6.2	21.4 ± 7.1
Glucose	*p* value	0.005	0.008
	Low 1/3	23.2 ± 9.4	27.0 ± 10.4
	Mid 1/3	19.8 ± 6.3	23.7 ± 7.6
	High 1/3	19.8 ± 6.2	22.9 ± 7.1

increasing age, glucose, BMI, creatinine, hemoglobin and uric acid levels (Table IV).

When factors found significantly related to EDD in univariate testing included in multiple regression test, only age, sex and presence of AT1RAC+CC genotypes were retained as significant (multiple R = 0.461, adjusted $R^2 = 0.202$, F = 20.47, $p < 0.001$). When the same analysis was performed for EID, age, hemoglobin levels and presence of AT1RAC+CC genotype were retained as significant (multiple R = 0.484, adjusted $R^2 = 0.224$, F = 23.202, $p < 0.001$).

Discussion

In this study, possible influences of ecNOS, ACE and AT1R gene polymorphisms on endothelial functions were investigated in healthy individuals. AT1RAC+CC genotype was found to adversely affect the endothelial functions as both endothelium dependent and independent vasodilatation in healthy individuals. Reports assessing the effect of the genes on endothelial function in healthy humans are scarce in the literature[6-9,18-22]. In a study that endothelial functions were measured non-invasively, no significant influence of ACE genotypes on EDD and EID was found[18]. However, Butler et al[21] (n = 68) found that both, EDD and EID were impaired in young healthy men with D allele. Later Rossi et al[22] investigated another polymorphism and showed that the T-786C promoter polymorphism and its interaction with exon 7 Glu298Asp affected EDD in healthy normotensive Caucasian subjects. In the study by Gururajan et al[6], ecNOS gene polymorphism was investigated in 106 patients with acute coronary syndrome and 100 healthy controls. ecNOS gene polymorphism was significantly associated with acute coronary syndrome. In another study[7], the ecNOS4a allele was found to be related to carotid atherosclerosis in type 2 diabetic patients. In our work, we evaluated 3 gene polymorphisms and AT1RC allele was shown to have deleterious effect on EDD in women. As reported above, our findings about ACE gene are in agreement with the literature. In contrast to Rossi et al study[22] we investigated ecNOS gene polymorphism in intron 4 and found no effect on EDD and EID. Studies performed with AT1R antagonists have shown that AT1R blockage may improve endothelial function endothelial function. The putative mechanism involved was the prevention of the inactivation of NO by superoxide anions. This hypothesis was supported by the finding that NOS inhibitor, L-N-monomethyl-arginine prevented the improvement in endothelial function seen with losartan[23] and AT1R antagonists increased the antioxidative potential of the vessel wall by increasing the activity of endothelial superoxide dismutase[24].

In this study, we observed that women had better EDD compared to men. This difference in EDD disappeared after menopause as reported elsewhere[25-27]. These findings may imply the importance of sex hormones in protecting the endothelium. All these studies are in line with our findings.

We also found that increasing glucose levels adversely affected endothelial functions as reflected by impaired EDD and EID. These findings were in parallel to the findings of the study by Sarabi et al[27].

In this report only 20% of the variability of EDD and EID was explained by the parameters collected in the study which implies that the greater part of variability in EDD and EID remained unexplained. Further studies with novel parameters are required to explain the remaining variability in EDD and EID.

Conclusions

Gene polymorphisms of endothelial constitutive nitric oxide synthase and angiotensin converting enzyme had no effect on endothelial functions. However, the presence of angiotensin II type 1 receptor polymorhism (AT1RAC+CC genotype) seemed to adversely affect the endothelial functions as reflected by impaired endothelium dependent and independent vasodilatation in healthy individuals.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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