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Endothelial Nitric Oxide Synthase Glu298Asp Gene Polymorphism is Associated with Hypertensive Response to Exercise in Well-Controlled Hypertensive Patients

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Purpose: Hypertensive response to exercise (HRE) is known to be an adverse prognostic factor for future cardiovascular events and may be associated to endothelial dysfunction. Previous studies regarding endothelial nitric oxide synthase (eNOS) Glu298Asp polymorphism focused upon its relation to hypertension. In this study, we hypothesize that the polymorphism may be associated with inherent difference in endothelial response to exercise. **Patients and Methods:** Two hundred sixty nine patients who underwent treadmill test were enrolled in this study; 77 patients (mean age 55.8 $\pm$ 9.4 years) had hypertensive response (peak systolic BP of $\geq$ 210 mmHg in men and $\geq$ 190 mmHg in women). Pulse wave velocity (PWV) was measured on 153 patients of them. The Glu298Asp exchange in exon 7 was determined by the methods of single base extension with amplifying primers and probes for TaqMan. **Results:** The percentages of the GG, GT and TT genotypes were 81.0, 18.6 and 0.4 %, respectively. The presence of GT or TT genotype was independently associated with prevention of HRE when controlled for age, sex, baseline systolic BP and homeostatic model assessment (HOMA) index (OR = 0.35, p = 0.016). Subgroup analysis showed that preventive effect for HRE of T allele was significant in females (p < 0.001) and patients without insulin resistance (p = 0.009). **Conclusion:** In our study, eNOS Glu298Asp polymorphism was significantly associated with HRE. This result suggests that the presence of T allele of the Glu298Asp polymorphism may be a favorable factor to in preventing HRE, especially in female and patients without insulin resistance.

Key Words: Endothelial nitric oxide synthase, gene polymorphism, hypertension, hypertensive response to exercise

INTRODUCTION

Hypertensive response to exercise (HRE) can be an adverse clinical factor for cardiovascular events.[1-3] Excessive sympathetic stimulation and activation of the renin-angiotensin-aldosterone system, endothelial dysfunction, and capillary rarefaction may all contribute to the hypertensive response to exercise.[4-9] Previous studies have shown that hypertensive response during exercise in hypertension is related with increased morbidity from myocardial infarction and adverse left ventricular remodeling.[10,11] Among the possible causes of hypertensive response to exercise, endothelial dysfunction may play an important role in impairment of peripheral vasodilation during exercise which may contribute to hypertensive response to exercise in this study.[12] Genetic factors associated with endothelial nitric oxide synthase activity may influence peripheral vasodilation response during exercise. Although previous studies done regarding endothelial nitric oxide synthase (eNOS) Glu298Asp (G894T) gene polymorphism focused upon its association with hypertension, there were no studies regarding its relationship

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with hypertensive response.[12-14] In this study, we hypothesize that the polymorphism may be associated with inherent difference in endothelial response to exercise. Therefore we investigated the association of eNOS Glu298Asp gene polymorphism with hypertensive response to exercise in hypertensive patients.

MATERIALS AND METHODS

Study population

Two hundred nine non diabetic hypertensive patients were enrolled in this study (treated blood pressure of < 160/100 mmHg) at Yonsei Cardiovascular Hospital from January of 2004 to April of 2005. At the time of initial enrollment, patients underwent a complete physical examination, a baseline electrocardiogram, and laboratory assessment. Hypertensive response to exercise was defined as systolic BP > 210 mmHg in men and systolic BP > 190 mmHg in women as described previously.[3] The treadmill test was performed all patients and pulse wave velocity (PWV) was measured on 153 patients of them.

Protocol

Fasting insulin level was assessed by the immunoradiometric assay using gamma counter (Hewlett Packard, Palo Alto, CA, USA). Insulin resistance was calculated as the upper quartile level of the homeostatic model assessment (HOMA) index (2.20), which is defined as [fasting blood glucose (mg/dL) X serum fasting insulin (mIU/mL)/405].[15] Prior to the study, all participants submitted written informed consent. The study was approved beforehand by the institutional ethics committee and the procedures were followed in accordance with the institutional guidelines. After at least 5 minutes of rest in sitting position, resting blood pressure (BP) was measured using a sphygmomanometer using the appropriate cuff size. Two blood pressures at least 5 minutes apart were measured and the mean BP was used for analysis as baseline BP. Symptom limited exercise test was performed using the Bruce protocol (3 minutes per every 4 stages). Sphygmomanometric blood pressure was measured at the end of each 3 minute stage.

PWV was determined by measuring baPWV using the VP-1000 pulse wave unit (Nippon Colin Ltd, Komaki City, Japan). After an overnight fast, the PWV was measured in a supine position after 5 minutes of rest. Pressure waveforms of the carotid and femoral arteries were recorded using multielement tonometry sensors placed at the left carotid and the left femoral arteries. Pressure waveforms of the brachial and tibial arteries were measured by an oscillometric method.

Gene assay

The eNOS Glu298Asp exchange (G298T) in exon 7 was determined by the methods of single base primer extension technology. Genomic DNA was prepared from peripheral blood samples using a Puregene® DNA purification kit (Gentra, Minneapolis, MN, USA). SNP genotyping was performed by SNP-ITTM assays using SNPstream 25K® System (Orchid Biosciences, Princeton, NJ, USA). Briefly, the genomic DNA region spanning the polymorphic site was PCR-amplified using one phosphothiolated primer and one regular PCR primer. The amplified PCR products were then digested with exonuclease (Amersham Biosciences, Uppsala, Sweden). The 5′ phosphothiolates were used in this study to protect one strand of the PCR-product from exonuclease digestion. The single-stranded PCR template generated from exonuclease digestion was overlaid onto a 384 well plate that precoated covalently with the primer extension primers, SNP-ITTM primers. These SNP-ITTM primers were designed to hybridize immediately adjacent to the polymorphic site. After hybridization of template strands, SNP-ITTM primers were then extended by a single base with DNA polymerase at the polymorphic site of interest. The extension mixtures contained two labeled terminating nucleotides (one FITC, one biotin) and two unlabeled terminating nucleotides. The final single base incorporated was identified with serial colorimetric reactions with anti-Fluorescein-AP (Roche, Basel, Switzerland) and streptavidin-HRP (Pierce, Rockfold, IL, USA), respectively. The results of blue and/or yellow color developments were

analyzed with an ELISA reader and the final genotyping (allele) calls were made with the QCReview™ program.

Statistical analysis

Values were expressed as mean ± SD. Chi-square test for goodness of fit was used to verify the agreement with Hardy-Weinberg equilibrium. Comparison of the discrete variables was performed using the Chi-square test. Comparison of continuous variables with a normal distribution between the two study groups was performed using the student's t-test. If the distribution were skewed, non-parametric test was used. The association of genotype with hypertensive response to exercise was performed using Chi-square test. Independent predictors of hypertensive response to exercise were determined using the logistic regression analysis. Statistical analysis was performed with SPSS 11.0 (SPSS Inc, Chicago, IL, USA).

RESULTS

There were 77 patients with HRE (55.8 ± 9.4, M:F = 38:39) and 192 patients without HRE (53.9 ± 8.9, M:F = 104:88). There were no significant difference in clinical variables such as age, gender, smoking, total cholesterol, HDL cholesterol, triglyceride and body mass index (BMI) (Table 1). The peak SBP, DBP and the magnitude of pressure increase of SBP, DBP were all significantly higher in patients with HRE (Table 1). Also, the HOMA index and baPWV was significantly higher in patients with HRE (2.61 ± 1.56 vs. 1.76 ± 0.85 and 1578 ± 318 vs. 1451 ± 181, $p < 0.05$, respectively). But metabolic equivalents (METs) was lesser and total exercise time was shorter in patient with

Table 1. Baseline Characteristics according to the Hypertensive Response and Genotype

	HRE group			G894T genotype		
	HRE(+) (n = 77)	HRE (-) (n = 192)	p value*	GG (n = 218)	GT or TT (n = 51)	p value*
Age (yrs)	55.8 ± 9.4	53.9 ± 8.9	0.123	52.5 ± 9.2	54.9 ± 8.9	0.094
Sex (M : F)	38 : 39	104 : 88	0.474	115 : 103	27 : 24	0.981
Baseline SBP (mmHg)	137.3 ± 13.9	130.2 ± 14.4	< 0.001	131.9 ± 14.3	133.5 ± 15.6	0.487
Peak SBP (mmHg)	213.8 ± 17.6	175.1 ± 18.3	< 0.001	186.5 ± 24.3	184.8 ± 28.8	0.672
SBP increase (mmHg)	76.5 ± 20.2	45.0 ± 20.1	< 0.001	54.7 ± 23.9	51.3 ± 27.9	0.379
Exercise time (sec)	610 ± 121	652 ± 96	0.022	641 ± 104	641 ± 107	0.982
% of target Heart tate	96.9 ± 13.1	93.6 ± 15.8	0.175	94.0 ± 14.7	96.5 ± 16.9	0.340
METs	11.2 ± 2.1	12.1 ± 1.6	0.07	11.8 ± 1.8	11.9 ± 1.7	0.571
Smoking (%)	35 (45.5%)	77 (40.1%)	0.421	92 (42.2%)	20 (39.2%)	0.697
T. chol (mg/dL)	202.3 ± 38.9	193.8 ± 37.4	0.096	195.7 ± 38.6	198.5 ± 35.2	0.647
TG (mg/dL)	158.7 ± 124.8	163.5 ± 104.5	0.743	158.2 ± 96.2	179.1 ± 157.6	0.225
HDL (mg/dL)	47.1 ± 10.8	45.4 ± 10.9	0.249	46.1 ± 11.5	45.1 ± 8.3	0.597
HOMA index	2.61 ± 1.56	1.76 ± 0.85	< 0.001	2.00 ± 1.15	2.00 ± 1.24	0.997
baPWV (n = 153)	1578 ± 318	1451 ± 181	0.027	1489 ± 235	1432 ± 152	0.307

Values are n (%) or mean ± SD.
HRE, hypertensive response to exercise; SBP, systolic blood pressure; SBP increase, difference between maximal and baseline SBP; T. chol, total cholesterol; TG, triglyceride; HDL, high density lipoprotein; HOMA, homeostatic model assessment; METs, metabolic equivalents; baPWV, brachial to ankle pulse wave velocity.
*$p < 0.05$ is considered significant.

Table 2. Multiple Logistic Regression Analysis for Independent Determinants of Hypertensive Response to Exercise

	Odd ratio	*p* value*
Elevated HOMA index (≥ 2.20)	3.15 (1.76 - 5.64)	< 0.001
Baseline SBP	1.04 (1.02 - 1.06)	< 0.001
Presence of T allele	0.35 (0.15 - 0.83)	0.016
Female gender	1.34 (0.76 - 2.37)	0.316

SBP, indicates systolic blood pressure; HOMA, homeostatic model assessment.
*$p < 0.05$ is considered significant.

Table 3. Association of eNOS Polymorphism with Hypertensive Response according to Gender and Insulin Resistance

	G894T genotype	HRE (+)	*p* value*	SBP increase	*p* value*
Total	GG	218 (81.1%)	0.036	54.7 ± 23.9	0.148
	GT or TT	51 (18.9%)		51.3 ± 27.9	
Female	GG	39/103 (37.9%)	< 0.001	48.8 ± 23.3	< 0.001
	GT or TT	0/24 (0.0%)		34.4 ± 13.0	
Male	GG	30/115 (26.1%)	0.708	59.9 ± 23.2	0.220
	GT or TT	8/27 (33.3%)		66.3 ± 29.3	
HOMA index < 2.20	GG	38/150 (25.3%)	0.009	51.4 ± 23.9	0.019
	GT or TT	2/36 (5.6%)		42.1 ± 21.9	
HOMA index ≥ 2.20	GG	31/68 (45.6%)	0.693	61.7 ± 23.9	0.246
	GT or TT	6/15 (40.0%)		73.3 ± 29.3	

Values are n (%) or mean ± SD.
HOMA, indicated homeostatic model assessment; HRE, hypertensive response during exercise; SBP increase, magnitude systolic blood pressure increase.
*$p < 0.05$ is considered significant.

HRE. There were no significant difference in terms of HOMA index, age, lipid profiles, baseline SBP, METs, total exercise time, % of achievement of predictive heart rate, peak SBP, SBP increase between patients and baPWV with GG genotype and GT or TT genotype (Table 1). Although there was statistically insignificant, baPWV had a higher tendency in GG genotype than GT or TT genotype (1489 ± 235 vs. 1432 ± 152, $p = 0.307$). The percentages of the GG, GT and TT genotypes were 81.0, 18.6 and 0.4%, respectively. The allele frequency did not deviate from those expected under the Hardy-Weinberg equilibrium (G : T = 90.5%:9.5%). The presence of GT or TT genotype, assuming co-dominant effect of the T allele, was independently associated with prevention of hypertensive response when controlled for sex, baseline systolic blood pressure and insulin resistance (HOMA IR ≥ 2.20) [Table 2, OR = 0.35 (0.15 - 0.83), $p = 0.016$]. The multiple logistic regression revealed increased insulin resistance [OR = 3.15 (1.76 - 5.64), $p < 0.001$] and baseline SBP [1.04(1.02 - 1.06), $p < 0.001$] as the other independent predictors of HRE. Subgroup analysis showed that this effect was not significant in males, but in female patients with GT or TT genotypes, significant association with absence of hypertensive response [Table 3, GG : GT or TT = 64/103(60.9%) : 0/24(0.0%), $p < 0.001$] and significantly lower increment of systolic blood pressure response during exercise was observed compared to the GG genotype (Table 3, 48.8 ± 23.3 vs. 34.4

± 13.0, $p < 0.001$). Also, subgroup analysis according to the presence of insulin resistance (upper quartile of HOMA index, ≥ 2.20) revealed that the protective effect of T allele was observed only for patients without insulin resistance [Table 3, GG : GT or TT = 38/112 (33.9%) : 2/34 (5.9%), $p = 0.009$].

DISCUSSION

Our study showed eNOS Glu298Asp gene polymorphism was functionally associated with hypertensive response to exercise in hypertension patients. Subgroup analysis shows that this finding was prominent in female and patients without insulin resistance. To our best knowledge, this is the first study to demonstrate the association of Glu298Asp polymorphism with change of blood pressure during exercise.

Endothelial dysfunction may play an important role in impairment of peripheral vasodilation during exercise which may contribute to hypertensive response to exercise in this study.[12] Increased production of nitric oxide (NO) by eNOS during exercise may be important in modulating the blood pressure response with increased peripheral vasodilation. Genetic determinants that influence the NO production during exercise may have a significant influence on the hypertensive response. We analyzed eNOS Glu298Asp polymorphism because the functional changes associated with the single nucleotide polymorphisms (SNPs) may possibly alter the eNOS activity. The Glu298Asp polymorphism occurs at exon 7 resulting in amino acid substitution and may induces functional changes of the eNOS due to increased susceptibility to proteolytic cleavage.[13,14] However, these findings have been challenged by recent data that suggest that the increased cleavage was the result from sample preparation methods.[16] Previous studies regarding the possible association of eNOS gene polymorphism of Glu 298Asp with hypertension have resulted in mixed results.[17-20] Recently, Sandrim et al report eNOS haplotype are associated with hypertension, independently of ethnity or presence with diabetes.[21,22] But, these studies have a limitation for relating with multifactorial factors involved with human genetics in cardiovascular diseases. Therefore, difference in genetic backgrounds among different ethnicity as well as gender and the low statistical power of the small scale association study could be attributed to the disparity of result.[20,23] Perhaps, linkage disequilibrium of Glu298Asp allele with other functional alleles in the eNOS gene may influence the association of the Glu298Asp polymorphism with cardiovascular disease. The contradictory findings of our study from previous reports, the protective effect against hypertensive response of T allele, may be due to linkage of different functional alleles with the Glu298Asp allele according to ethnicity and gender. Another possible explanation is that Glu298Asp polymorphism may manifest functional difference in eNOS activity during exercise compared to resting state. This finding can be supported by having a higher tendency of PWV value on GG genotype than GT or TT genotype (1489 ± 235 vs. 1432 ± 152), which was not significant but it may be due to measure the PWV only half of all patient. Lastly, although we investigate the other gene analysis, gene to gene interaction and gene to environment interplay are possible explanation because recent studies showed the interactions of unfavorable common genetic mutations and environment may play a role in circulatory disorder.[24-26]

Insulin resistance are significantly related with endothelial dysfunction and modification of insulin resistance is associated with improvement of endothelial function.[27] Because HOMA values to define insulin resistance in Koreans were not available, we defined insulin resistance as the upper quartile level of HOMA index in normal control subjects who were enrolled in the Cardiovascular Genome Center database. The presence of insulin resistance was independently associated with hypertensive response to exercise in this study. It was interesting to note that subgroup analysis revealed that the T allele was protective from hypertensive response only in patients without insulin resistance (Table 3). Perhaps, the adverse influence of insulin resistance on endothelial function regardless of genotype may dilute any influence the polymorphism and have on endothelial function during exercise.

Also, it was interesting to note that the hypertensive response was modulated only in females.

Previous studies have reported gender specific difference in vascular reactivity and endothelial function which may be due to sex hormones or difference in intrinsic genetic phenotypes.[28,29] Because predominant women in the study population were over the age of 50 [104/127(81.9%)] difference in gender specific genetic expression rather than sex hormones may have influenced the result in this study.

The allele frequency of this study (G : T = 90.5 : 9.5) was similar to previous reports regarding the allele frequency in Asians.[13] The relatively rare allele frequency compared to Caucasians and the possible ethnic difference in genetic linkage suggests the need to assess the association of hypertensive response to exercise in different ethnic groups.

The proportion of patients taking each class of drugs and the average number of antihypertensive medications were not different among the patients with or without HRE. Also exercise duration and METs was rather shorter in patients with HRE than those without HRE. Therefore, we believe that blood pressure medications including beta-blocker and exercise duration did not confound the analysis of data in this study.

This is the first study, to our knowledge, that assessed the association of eNOS Glu298Asp gene polymorphism with hypertensive response to exercise. This result suggests that the presence of T allele of the Glu298Asp polymorphism may be a favorable factor to in preventing HRE, especially in female and patients without insulin resistance. Since hypertensive response to exercise is associated with adverse cardiovascular events in hypertensive patients, the present findings may have important implications to predict the future cardiovascular disease risk in hypertensive patients. Although the results from this study may be contradictory to some other previous reports, previous studies regarding the possible association of eNOS gene polymorphism of Glu298Asp with hypertension have resulted in mixed results at best. The disparities in results have been attributed to difference in genetic backgrounds among different ethnicity, gender and the low statistical power of the small scale association study. Further studies to demonstrate this result in normotensive population and people of different ethnic background is needed.

Study limitation

We suggested the new information about the functional activity of eNOS related to Glu298Asp polymorphism during exercise. But we didn't conduct to measure the functional activity directly and other gene analysis to find out the gene to gene interaction. Therefore functional correlation during exercise with eNOS polymorphism will be investigated and large-scaled study will be needed to clarify the mechanism.

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