NOS3 Glu298Asp Genotype and Blood Pressure Response to Endurance Training
The HERITAGE Family Study

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Abstract—Endothelium-dependent vasodilation is a mechanism that may affect blood pressure response to endurance training. Because NO plays a central role in this process, the endothelial NO synthase gene is a good candidate for the regulation of exercise blood pressure. We investigated the associations between an endothelial NO synthase gene polymorphism (Glu298Asp) and endurance training–induced changes in resting and submaximal exercise blood pressure in 471 white subjects of the HERITAGE Family Study. Two submaximal exercise tests at 50 W were conducted both before and after a 20-week endurance training program. Steady-state exercise blood pressure was measured twice in each test with an automated unit. The Glu298Asp polymorphism was typed with a PCR-based method and digestion with *Ban*II. Both systolic and diastolic blood pressure at 50 W decreased in response to the training program, whereas resting blood pressure remained unchanged. The decrease in diastolic blood pressure at 50 W was greater ($P=0.0005$, adjusted for age, gender, baseline body mass index, and baseline diastolic blood pressure at 50 W) in the Glu/Glu homozygotes (4.4 [SEM 0.4] mm Hg, n=187) than in the heterozygotes (3.1 [0.4] mm Hg, n=213) and the Asp/Asp homozygotes (1.3 [0.7] mm Hg, n=71). The genotype accounted for 2.3% of the variance in diastolic blood pressure at 50 W training response. Both the Glu298 homozygotes and the heterozygotes had a greater ($P=0.013$) training-induced reduction in rate-pressure product at 50 W than the Asp298 homozygotes. These data suggest that DNA sequence variation in the endothelial NO synthase gene locus is associated with the endurance training–induced decreases in submaximal exercise diastolic blood pressure and rate-pressure product in sedentary normotensive white subjects. (*Hypertension*. 2000;36:885-889.)

Key Words: exercise ■ genetics ■ blood pressure ■ endothelial-derived factor ■ nitric oxide

The vascular endothelium plays an important role in the regulation of vasomotor tone and, subsequently, blood pressure (BP). One of the key substances that mediates this process is NO, which is produced from L-arginine by endothelial NO synthase (eNOS, a product of the NOS3 gene). Inhibition of NO synthesis has been shown to increase BP both in humans[1] and in animals.[2] Similarly, NOS3 knockout mice show significantly higher BP levels than wild-type animals,[3] whereas mice that overexpress the NOS3 gene are characterized by hypotension.[4] In addition, transfer of the NOS3 gene has improved endothelial function and vasodilation both in vitro and in vivo.[5,6]

The BP-lowering effect of endurance training in hypertensive subjects is well documented.[7,8] Although the precise mechanisms are unknown, changes in vascular structure and function, and thereby in peripheral resistance, could be involved.[9,10] These changes are at least in part mediated by endothelial NO production. A single bout of exercise has been shown to enhance NOS activity and NO production,[11] and this increase seems to contribute to vasodilation during steady-state exercise.[12,13] In addition to acute responses, endurance training has been reported to increase NOS3 gene expression in coronary resistance arteries and to enhance basal NO production.[14–16] This effect seems to be essential for the beneficial effects of endurance training on BP in both normotensive and hypertensive subjects,[17,18] as well as in patients with chronic heart failure.[19]

Both the physiological role of NOS3 in the regulation of endothelial function and BP and the potential of physical exercise to enhance NO synthesis in vascular endothelium

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make NOS3 an attractive candidate gene for exercise hemo-dynamic phenotypes. Thus, the purpose of the present study was to investigate the associations between the NOS3 Glu298Asp polymorphism and endurance training–induced changes in resting and submaximal exercise BP in normotensive, sedentary white subjects of the HERITAGE Family Study.

Methods

Subjects

A total of 484 white subjects (234 men and 250 women) (mean age 36 years, SD=14.5) from 99 nuclear families completed the 20-week endurance training program. A complete phenotype data set was available for 471 subjects (228 men and 243 women, 99 families). As previously described,[20] participants were required to be sedentary at baseline and in good health to be eligible for the study. Individuals with a resting systolic BP (SBP) of >159 mm Hg and/or diastolic BP (DBP) of >99 mm Hg, as well as those taking antihypertensive medication, were excluded. The study protocol had been approved by each of the institutional review boards of the HERITAGE Family Study research consortium. Written informed consent was obtained from each subject.

Exercise Training Program

The details of the 20-week endurance training program have been published elsewhere.[20,21] Briefly, during the first 2 weeks, training was carried out at a heart rate (HR) that corresponds to 55% of the baseline $\dot{V}O_{2max}$ for 30 minutes per session. Duration and intensity of the training sessions were gradually increased to 50 minutes and 75% of the HR associated with baseline $\dot{V}O_{2max}$, which were then sustained for the last 6 weeks. The average training frequency was 3 times per week, and all training was performed on cycle ergometers under supervision in the laboratory. HR was monitored during all training sessions with a computerized cycle ergometer system (Universal FitNet System), which adjusted the ergometer resistance to maintain target HR.

Hemodynamic Phenotypes

All BP phenotypes were measured using Colin STBP-780 automated units, and recordings were confirmed by technicians wearing head-phones. Resting BP was measured on 2 separate days before 11 AM in the postabsorptive state. Subjects were asked not to use any caffeine-containing or tobacco products for 2 hours before measurements were made. Measurements were taken in a quiet room at a neutral ambient temperature (24° to 26°C) with the lights dimmed. Subjects rested for 5 minutes before the initial measurement in a reclining chair with legs slightly elevated and back support reclined at ≈45° from the ground. After the rest period, ≥4 BP readings were taken at 2-minute intervals between measurements. The first recording was automatically discarded, and 3 valid measurements were kept. SBP and DBP were defined as the mean of all valid readings taken on both days (ie, a maximum of 6).

Submaximal exercise BP was measured during 2 cycle ergometer tests after 8 to 12 minutes at a constant power output (50 W) in relative steady state, both before and after a 20-week endurance training program. BP was recorded twice during each test, and the mean of 4 readings was used for analyses. Steady-state HR was recorded with ECG. Rate-pressure product (RPP), an index of myocardial workload, was calculated by multiplying SBP by HR. Cardiac output (Q) was determined twice at 50 W with the Collier CO_2 rebreathing technique,[22] as described by Wilmore et al.[23] A mean of the 2 measurements was used for the analyses. SV was calculated by dividing Q by HR.

In summary, the following hemodynamic phenotypes were available for the analyses: SBP, DBP, and RPP at rest and during steady-state submaximal exercise at 50 W (SBP50, DBP50 and RPP50, respectively) and Q and SV during submaximal exercise (SV50 and Q50).

Other Phenotypes

Stature was measured to the nearest 0.1 cm with the subject standing erect on a flat surface; the heels, buttocks, and back pressed against the stadiometer; and the head positioned in the Frankfort horizontal plane. Body mass was recorded to the nearest 100 g with a balance scale with subjects clothed in only a lightweight bathing suit. Body mass index (BMI) was calculated by dividing body mass (kg) by stature squared (m²). Body surface area (BSA) was obtained from the following equation: BSA=weight $(kg)^{0.5378} \times$ height $(cm)^{0.3964} \times 0.024265$.[24]

Genotype Determinations

Genomic DNA was prepared from permanent lymphoblastoid cells according to the proteinase K and phenol/chloroform technique. DNA was dialyzed 4 times against 10 mmol/L Tris–1 mmol/L EDTA (pH 8.0) buffer for 6 hours at 4°C and ethanol precipitated.

The Glu298Asp polymorphism of the NOS3 gene was typed with PCR, followed by digestion with *Ban*II, as previously described.[25] The PCR was performed in standard buffer (QIAGEN Inc), and each 20-μL PCR contained 100 ng genomic DNA, 0.2 μmol/L concentration of each primer, 200 μmol/L concentration of each dNTPs, and 0.5 U *Taq* polymerase (QIAGEN Inc). The reactions were incubated at 94°C for 3 minutes, 60°C for 1 minute, and 72°C for 1 minute, followed by 35 cycles of 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds, and finally 1 cycle of 72°C for 10 minutes (model 9600 thermal cycler; Perkin-Elmer Cetus). The PCR product was digested with 5 U *Ban*II (New England Biolabs) at 37°C for 4 hours. The resulting fragments were separated on 2.5% agarose gel and visualized under UV light after ethidium bromide staining.

Statistical Analyses

A χ^2 test was used to confirm that the observed genotype frequencies were in a Hardy-Weinberg equilibrium. Normality of the distributions was checked with the Shapiro-Wilk statistic of the UNIVARIATE procedure of the SAS statistical software package (SAS Institute Inc). The associations between the NOS3 Glu298Asp polymorphism and hemodynamic phenotypes were tested with ANCOVA with the GLM procedure. Baseline BP phenotypes were adjusted for age, gender, and BMI. and BP training response phenotypes, obtained as the difference between pretraining and posttraining values, were adjusted for age, gender, baseline BMI, and baseline value of the BP phenotype. In addition, we tested the effects of training-induced changes in BMI (ΔBMI) on the associations between the NOS3 genotype and BP training responses. However, because ΔBMI showed either no (exercise BP) or only weak (resting BP) correlations with BP responses and because baseline BMI was usually a stronger predictor of BP training responses, the final GLM models included only baseline BMI. Gender-specific associations between the genotype and hemodynamic phenotypes were tested by adding a gender×genotype interaction term into the GLM model. However, no significant interactions were observed, and therefore the analyses were performed with the entire cohort and with gender as a covariate. Values are given as mean and SEM.

Results

The baseline characteristics of the subjects are presented in Table 1. The endurance training program induced significant decreases in resting HR, SBP50, DBP50, HR50, RPP50, and Q50, whereas SV50 increased significantly ($P<0.001$ for all). Resting BP phenotypes were unchanged. The frequencies of the Glu and Asp alleles of the NOS3 Glu298Asp marker were 0.64 and 0.36, respectively, and the observed genotype frequencies were in a Hardy-Weinberg equilibrium.

None of the hemodynamic phenotypes measured in the sedentary state were associated with the NOS3 Glu298Asp polymorphism (Tables 2 and 3). However, DBP50 training

TABLE 1.   Basic Characteristics of the Subjects

Phenotype	Men	Women
n	228	243
Age, y	36.5 (1.0)	35.5 (0.9)
Body weight, kg	84.4 (1.1)	67.0 (0.9)
Height, cm	177.8 (0.4)	163.7 (0.4)
BMI, kg/m^2	26.7 (0.3)	25.0 (0.3)
SBP, mm Hg		
Resting	119.9 (0.7)	112.8 (0.6)
50 W	146.2 (1.2)	142.9 (1.4)
DBP, mm Hg		
Resting	68.0 (0.6)	63.9 (0.4)
50 W	72.1 (0.7)	70.0 (0.7)
HR, bpm		
Resting	62.0 (0.6)	67.1 (0.6)
50 W	106.1 (0.7)	127.8 (1.0)
RPP, mm Hg bpm		
Resting	7448 (88)	7573 (77)
50 W	15 603 (196)	18 352 (250)
SV50, mL/beat	108.7 (1.2)	86.0 (0.9)
Q50, L/min	11.4 (0.1)	10.8 (0.1)

Values are mean (SEM).

response showed a highly significant ($P=0.0005$) association with the NOS3 genotype. The homozygotes for the Glu298 allele showed a 3.1 mm Hg greater reduction in DBP50 than the Asp298 homozygotes, whereas the heterozygotes showed an intermediate response (Table 3). The association was similar in men and women (data not shown) and was

TABLE 2.   Resting Hemodynamic Phenotypes in the Sedentary State and in Response to a 20-week Endurance Training Program According to the NOS3 Glu298Asp genotype in the White Subjects of the HERITAGE Family Study

	NOS3 Glu298Asp Genotype			
Phenotype	Glu/Glu (n=187)	Asp/Glu (n=213)	Asp/Asp (n=71)	P
SBP, mm Hg				
Baseline	115.8 (0.7)	116.6 (0.7)	117.0 (1.2)	0.632
Response	−0.1 (0.4)	+0.4 (0.4)	+0.1 (0.7)	0.628
DBP, mm Hg				
Baseline	66.0 (0.5)	66.0 (0.5)	66.0 (0.8)	0.996
Response	−0.1 (0.4)	+0.2 (0.3)	+0.7 (0.6)	0.482
HR, bpm				
Baseline	64.0 (0.6)	64.8 (0.6)	66.0 (0.8)	0.529
Response	−3.3 (0.4)	−3.1 (0.4)	−2.6 (0.6)	0.616
RPP, mm Hg bpm				
Baseline	7312 (87)	7444 (83)	7535 (144)	0.344
Response	−403 (58)	−337 (55)	−280 (94)	0.496

Values are mean (SEM). Baseline phenotypes are adjusted for age, gender, and BMI and training response phenotypes are adjusted for age, gender, baseline value of the phenotype, and baseline body mass index. SBP and DBP responses (posttraining minus baseline) are statistically nonsignificant; HR and RPP responses are $P<0.001$ in the whole cohort and within all genotypes.

TABLE 3.   Submaximal Exercise (50 W) Hemodynamic Phenotypes in the Sedentary State and in Response to a 20-week Endurance Training Program According to the NOS3 Glu298Asp Genotype in the White Subjects of the HERITAGE Family Study

	NOS3 Glu298Asp Genotype			
Phenotype	Glu/Glu (n=187)	Asp/Glu (n=213)	Asp/Asp (n=71)	P
SBP50, mm Hg				
Baseline	145.7 (1.2)	144.2 (1.2)	142.7 (2.0)	0.388
Response	−7.7 (0.7)	−7.0 (0.6)	−4.8 (1.1)	0.090
DBP50 mm Hg				
Baseline	70.8 (0.7)	69.7 (0.6)	70.2 (1.0)	0.471
Response	−4.4 (0.4)	−3.2 (0.4)	−1.3 (0.7)	0.0005
HR50, bpm				
Baseline	115.0 (1.0)	116.1 (0.9)	116.5 (1.6)	0.599
Response	−11.0 (0.5)	−11.6 (0.5)	−9.2 (0.9)	0.077
RPP50, mm Hg bpm				
Baseline	16 606 (225)	16 625 (211)	16 475 (358)	0.937
Response	−2461 (118)	−2477 (110)	−1857 (191)	0.013
SV50, mL/beat				
Baseline	95.9 (1.7)	95.1 (1.0)	94.0 (1.1)	0.649
Response	+2.9 (0.8)	+3.3 (0.7)	+3.6 (1.2)	0.878
Q50, L/min				
Baseline	11.1 (0.1)	11.1 (0.1)	11.0 (0.2)	0.894
Response	−0.7 (0.1)	−0.7 (0.1)	−0.5 (0.1)	0.440

Values are mean (SEM). Baseline phenotypes are adjusted for age, gender, and BMI (SBP50, DBP50, HR50, RPP50) or body surface area (SV50, Q50). Training response phenotypes are adjusted for age, gender, baseline value of the phenotype, and baseline BMI (SBP50, DBP50, HR50, RPP50) or body surface area (SV50, Q50). All training responses (posttraining minus baseline) are statistically significant ($P<0.001$) in the whole cohort as well as within each genotype, except for DBP50 response in the Asp/Asp genotype group ($P=0.056$).

independent of age, baseline BMI, and initial DBP50 level. The NOS3 genotype explained 2.3% of the variance in DBP50 training response. The Glu298 homozygotes also showed a greater reduction in SBP50 than did the Asp/Asp genotype. However, the association ($P=0.004$) became non-significant ($P=0.090$) after adjustment for the baseline SBP50 values, which tended to be higher in the subjects carrying the Glu/Glu genotype. Both the Glu298 homozygotes and the heterozygotes had a greater training-induced reduction in RPP50 than the Asp298 homozygotes. Training responses in submaximal exercise SV and Q were not associated with the NOS3 genotype.

Discussion

The main finding of the present study is that the Asp allele of the NOS3 Glu298Asp polymorphism is associated with a blunted responsiveness of submaximal exercise DBP and RPP to endurance training in previously sedentary, normotensive white subjects. Although similar findings have not been reported previously, the same allele has been shown to be associated with hypertension,[25] coronary heart disease,[26]

myocardial infarction,[27,28] coronary spasms,[29] and vascular responsiveness to phenylephrine.[30] However, a French study reported a greater frequency of the Glu allele in hypertensives than in control subjects,[31] whereas another Japanese study[32] could not confirm the previously reported[25] association between the Glu298Asp genotype and hypertension.

Both linkage and association studies have shown that DNA sequence variation at the NOS3 locus contributes significantly to the plasma levels of NO metabolites.[33,34] However, there are no data available on the associations between the Glu298Asp polymorphism and plasma NO metabolite levels. Moreover, the biological significance of the Asp-to-Glu substitution in codon 298 of the NOS3 gene locus is still unclear. The codon is located within the amino-terminal oxygenase domain of the endothelial NOS, which includes the binding sites for heme, tetrahydrobiopterin, and L-arginine. The codon 298 falls between the critical residues of the heme domain ($\approx$100 to 200) and the binding sites for L-arginine and tetrahydrobiopterin ($\approx$350 to 450).[35] Whether the Glu298Asp variant has any effect on the binding properties or other functions of the oxygenase domain or whether it is in linkage disequilibrium with another functional mutation remains to be explored in future studies.

Our data suggest that the Glu298Asp polymorphism has a role in the long-term adaptation of hemodynamic phenotypes to endurance training rather than in the short-term response to a single bout of exercise. Previous studies have shown that regular endurance training increases endothelial NO production both in humans[16,18] and in animals[14,15] and that the enhanced NO production capacity mediates several beneficial effects of regular aerobic exercise.[17] Shear stress and cholinergic nerve activity are 2 potential mechanisms that may mediate the greater NO production after endurance training.[36] Increased blood flow during exercise generates greater laminar shear stress on vascular endothelium and thereby activates the expression of several genes, including NOS3.[37] Acetylcholine is a neurotransmitter that induces vasodilation indirectly through an NO-dependent pathway, and endurance training has been reported to enhance cholinergic vasodilation.[36] Further studies are needed to clarify whether the DNA sequence variation in the NOS3 locus affects these pathways and, if so, via which mechanisms.

At this point, we can only speculate on the possible clinical implications of the present findings. However, in consideration of the greater reduction in submaximal exercise DBP and myocardial workload (estimated as RPP50), the Glu allele of the NOS3 Glu298Asp polymorphism could be a marker of normotensive sedentary white individuals, who are most likely to benefit from endurance training in terms of reduction in the hemodynamic load during moderate-intensity physical activity. If this observation, derived from a cohort of normotensive, sedentary subjects, is applicable to resting BP in subjects with elevated BP or hypertension, the NOS3 marker could be useful for the screening of patients who are likely to derive the greatest benefits from regular physical activity. Naturally, the hypothesis itself and whether an endurance training program with different intensity, frequency, and duration conditions could induce greater training

responses in the homozygotes for the Asp allele remain to be tested in future studies.

Another aspect of the NOS3 polymorphism that warrants further studies is the possible interactions with other genetic and environmental factors. NOS has several cofactors, such as tetrahydrobiopterin and calmodulin, that are necessary for the optimal function of the enzyme. The genes that encode these proteins are also potential candidates for themselves and because of their interactions with NOS3. Another interesting possibility involves the level of oxidative stress to which the individual is exposed. Superoxide anions inactivate NO and thereby block its physiological effects.[38] On the other hand, dietary antioxidants, such as vitamin C, have been shown to restore endothelial NO activity in hypertensive subjects.[39] Moreover, oxidized LDL may directly impair endothelial NOS activation.[40] Finally, the suggestion that hypertensives may exhibit a selective defect in endothelial NO synthesis emphasizes the need to explore the interactions with the cholinergic and β-adrenergic pathways of NOS3 stimulation.[41]

In summary, these data from the HERITAGE Family Study cohort suggest that in previously sedentary normotensive adult whites, the DNA sequence variation in the NOS3 locus is associated with the responsiveness of submaximal exercise DBP and RPP to regular endurance training.

Acknowledgments

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