Exercise training improves muscle vasodilatation in individuals with T786C polymorphism of endothelial nitric oxide synthase gene

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Negrao MV, Alves CR, Alves GB, Pereira AC, Dias RG, Laterza MC, Mota GF, Oliveira EM, Bassanez V, Krieger JE, Negrao CE, Rondon MU. Exercise training improves muscle vasodilatation in individuals with T786C polymorphism of endothelial nitric oxide synthase gene. Physiol Genomics 42A: 71–77, 2010. First published July 6, 2010; doi:10.1152/physiolgenomics.00145.2009.—Allele T at promoter region of the eNOS gene has been associated with an increase in coronary disease mortality, suggesting that this allele increases susceptibility for endothelial dysfunction. In contrast, exercise training improves endothelial function. Thus, we hypothesized that: 1) Muscle vasodilatation during exercise is attenuated in individuals homozygous for allele T, and 2) Exercise training improves muscle vasodilatation in response to exercise for TT genotype individuals. From 133 preselected healthy individuals genotyped for the T786C polymorphism, 72 participated in the study: TT (n = 37; age 27 ± 1 yr) and CT + CC (n = 35; age 26 ± 1 yr). Forearm blood flow (venous occlusion plethysmography) and blood pressure (oscillometric automatic cuff) were evaluated at rest and during 30% handgrip exercise. Exercise training consisted of three sessions per week for 18 wk, with intensity between anaerobic threshold and respiratory compensation point. Resting forearm vascular conductance (FVC, P = 0.17) and mean blood pressure (P = 0.70) were similar between groups. However, FVC responses during handgrip exercise were significantly lower in TT individuals compared with CT + CC individuals (0.39 ± 0.12 vs. 1.08 ± 0.27 units, P = 0.01). Exercise training significantly increased peak VO2 in both groups, but resting FVC remained unchanged. This intervention significantly increased FVC response to handgrip exercise in TT individuals (P = 0.03), but not in CT + CC individuals (P = 0.49), leading to an equivalent FVC response between TT and CT + CC individuals (1.05 ± 0.18 vs. 1.59 ± 0.27 units, P = 0.27). In conclusion, exercise training improves muscle vasodilatation in response to exercise in TT genotype individuals, demonstrating that genetic variants influence the effects of interventions such as exercise training.

NITRIC OXIDE (NO) is synthesized in the endothelium by the endothelial isof orm of nitric oxide synthase (eNOS) (27), a protein encoded in the long arm of chromosome 7 (19). NO is responsible for the maintenance of vascular tone due to its capability of inducing vasodilatation in response to shear stress (9, 26). A disruption of eNOS function or of its production can decrease NO bioavailability and, consequently, lead to deleterious effects on endothelial and vasomotor function. Recent findings suggest that, apart from classic risk factors such as smoking and diabetes, genetic variants are capable of altering eNOS expression and enzymatic activity (21, 23, 36). Among the genetic variants present in the eNOS gene, considerable attention has been focused on the T786C polymorphism, which is located on the 5’-flanking region of the promoter region of the eNOS gene. The C allele of this polymorphism has been associated with coronary spasm (23) and blunted vasodilator response to acetylcholine infusion, an endothelial-mediated vasodilator agent (11, 31). Contrasting with these results, two meta-analyses revealed no association between development of cardiovascular disease and the T786C polymorphism of the eNOS gene (6, 28). In an attempt to clarify the mechanisms underlying T786C polymorphism effect on vasomotor function, Rossi et al. (30) measured serum nitrotyrosine levels, a biomarker for oxidative stress, in coronary artery disease patients who were homozygous for the T and the C allele. This study revealed conflicting results because TT individuals presented a lower survival rate while possessing lower oxidative stress, as shown by the lower serum nitrotyrosine concentration in these patients. Molecular studies also show conflicting results when determining which of these two alleles leads to a decline in eNOS expression. Nakayama et al. (23) and Miyamoto et al. (21) have shown that allele C at locus 786 of the promoter region of the eNOS gene led to a decrease in promoter region activity and eNOS expression. However, while using a similar methodology, Wang et al. (36) showed that allele C was associated with an increase in eNOS transcription efficiency. The main difference between these studies is that Wang et al. determined the effect of a haplotype that contained locus 786 of the eNOS promoter region. Therefore, the functionality of the T786C polymorphism is unclear. Also, it remains unknown whether this polymorphism alters muscle vascular function during physiological maneuvers.

Accumulated evidences show that exercise training plays a role in endothelial function. Exercise training repairs endothelial lesions (17, 39), decreases neointima formation, and reduces atheromatous lesions (14–17), as well as increasing angiogenesis and vascular irrigation (17). This intervention is also responsible for an increase in the concentration of circulating endothelial progenitor cells (17) and for an increase in eNOS activity (13). In addition, exercise training reduces oxidative stress (12, 18, 32) through a decrease in angiotensin II production and NADPH oxidase expression (1). These alterations result in a reduction in eNOS uncoupling and an increase in NO bioavailability (34), key elements for the maintenance of vascular function. In light of these findings, a study shows that patients with coronary artery disease submitted to an exercise training protocol presented an increase in
their vasodilatory response to a physiological maneuver (10). Despite this knowledge, the effects of exercise training on the vascular function in response to exercise in healthy individuals carrying the T786C polymorphism of the eNOS gene are yet to be demonstrated.

In the present study, we tested two hypotheses: 1) The presence of T786C polymorphism of the eNOS gene would lead to a decreased muscle vasodilatory response to exercise; and 2) Exercise training would restore muscle vasodilatory response to exercise in individuals carrying the T786C polymorphism of the eNOS gene.

MATERIALS AND METHODS

Study Population

From 133 preselected individuals, all recruits from the Military Police Academy of São Paulo, 72 participated in the study. These 72 individuals were male, with age varying between 18 and 35 yr, and were not involved in regular physical activity for a minimum period of 6 mo. They were screened for cardiovascular, metabolic, and endocrine diseases through careful clinical examination, laboratory testing, echocardiogram, and cardiopulmonary stress testing. Caffeine, alcohol, and saturated fat acid ingestion were prohibited 1 day prior to the studies, and these were performed at least 3 h in the postabsorptive state. Genotyping for locus 786 of the promoter region of the eNOS gene was performed to create two study groups: the TT group (n = 37) and the CT + CC group (n = 35). The Human Subject Protection Committee of the Heart Institute (InCor) and of the Clinical Hospital of University of São Paulo’s Medical School, the ethics committee of the institution where this project was developed, approved the study protocol. Written informed consent was provided by each of the 133 individuals that participated in our study protocol.

Measurements and Procedures

Assessment of the T786C eNOS polymorphism genotype. Genomic DNA was extracted from blood leukocytes, and the polymerase chain reaction (PCR)–restriction fragment length polymorphism method was used to determine the genotype of all subjects. PCR primers were generated to amplify the 180 bp fragment encompassing the T786C polymorphism (sense and antisense primers were 5'-TGG AGA GTG CTG TAC CCC A-3' and 5'-GCC TCC ACC CCC ACC CTG TC-3', respectively). The PCR product was submitted to digestion by MspI restriction enzyme, which produces two fragments of 140 and 40 bp, respectively. The allele sequences (sense and antisense primers were 5'-CTT TCG TCC TGT GAG GGT TCC ACC ACC ACC CTG TC-3', respectively). The PCR product was submitted to digestion by MspI restriction enzyme, which produces two fragments of 140 and 40 bp, respectively. The allele sequences were: (T786C: 140/40; T786T: 180). The PCR product was submitted to digestion by MspI restriction enzyme, which produces two fragments of 140 and 40 bp, respectively. The allele sequences were: (T786C: 140/40; T786T: 180).

Statistical Analysis

Data are presented as means ± SE. The demographic characteristics and comparisons between groups at baseline were analyzed using non-paired Student’s t-test. The responses of forearm blood flow, forearm vascular conductance, mean blood pressure, and heart rate (absolute change) were subjected to two-way ANOVA with repeated measures. Forearm blood flow, forearm vascular conductance, mean blood pressure, heart rate (absolute change), and serum nitrotyrosine concentration before and after exercise training were also subjected to two-way ANOVA with repeated measures. When significance was found, Scheffé’s post hoc comparisons were performed. Significant differences were considered to be at P < 0.05.

RESULTS

Allelic Distribution and Baseline Measurements

Genotype and allele frequencies were consistent with Hardy-Weinberg equilibrium for the study population. The frequency measured at different time interval in the same individual expressed as ml/kg/min in our laboratory is r = 0.95.

Forearm blood flow. Forearm blood flow was measured by venous occlusion plethysmography (24). The nondominant arm was elevated above heart level to ensure adequate venous drainage. A mercury-filled silastic tube attached to a low-pressure transducer was placed around the forearm and connected to a plethysmograph (model EC-6; Hokanson, Bellevue, WA). Sphygmomanometer cuffs were placed around the wrist and upper arm. At 15-s intervals, the upper cuff was inflated above venous pressure for 7–8 s. Forearm blood flow (ml-min⁻¹·100 ml tissue⁻¹) at baseline and during each minute of exercise was determined based on a mean of three or four plethysmographic measures. Forearm vascular conductance was calculated by dividing forearm blood flow by mean arterial pressure. The reproducibility of forearm blood flow measured at different time intervals in the same individual expressed as milliliters per minute per 100 milliliters tissue in our laboratory is r = 0.93.

Blood pressure and heart rate. Blood pressure was monitored noninvasively and intermittently with an automatic and oscillometric cuff (Dixtal, DX 2710; Manaus, Brazil) placed on the ankle with a cuff width adjusted to ankle circumference (24, 29). The cuff inflated every 30 s. Heart rate was monitored continuously through lead II of EKG.

Handgrip exercise. After the maximum voluntary contraction (MVC; average of three trials) was obtained, handgrip isometric exercise was performed at 30% of MVC with the dominant arm using a handgrip dynamometer. It has been shown that this strategy causes substantial nonexercise muscle vasodilatation in humans (3, 29, 35). The individuals were instructed to breath normally during exercise and to avoid inadvertent performance of Valsalva maneuver.

Exercise training protocol. Exercise training was performed under supervision and consisted of three 60-min sessions per week for a period of 18 wk. Exercise intensity was graded individually according to the heart rate in correspondence to ventilatory thresholds. During the first half of the training protocol, running intensity was moderate with heart rate between the ventilatory thresholds, and, in the second half, heart rate was kept slightly above the respiratory compensation point.

Experimental Protocol

After instrumentation, the individuals rested for a 15-min period. Afterward, a 3 min baseline period was initiated followed by a 3 min period of isometric handgrip exercise which was performed by the dominant arm with a workload of 30% MVC. Heart rate, blood pressure, and forearm blood flow were monitored for the entire duration of the protocol.
Baseline measurements of individuals with TT and CT + CC genotypes of the eNOS gene

<table>
<thead>
<tr>
<th>n</th>
<th>TT (37)</th>
<th>CT + CC (35)</th>
<th>P</th>
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<tr>
<td></td>
<td>Demographic Characteristics</td>
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<tr>
<td></td>
<td>Age, yr</td>
<td>27 ± 1</td>
<td>26 ± 1</td>
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<tr>
<td></td>
<td>Weight, kg</td>
<td>75.7 ± 1.7</td>
<td>74.2 ± 1.7</td>
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<tr>
<td></td>
<td>Height, cm</td>
<td>176 ± 1</td>
<td>176 ± 1</td>
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<td></td>
<td>BMI, kg/m²</td>
<td>24.4 ± 0.4</td>
<td>23.8 ± 0.4</td>
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<td>Metabolic Measurements</td>
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<tr>
<td></td>
<td>Glucose, mg/dl</td>
<td>89 ± 3</td>
<td>91 ± 4</td>
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<tr>
<td></td>
<td>Total cholesterol, mg/dl</td>
<td>168 ± 6</td>
<td>166 ± 10</td>
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<tr>
<td></td>
<td>LDL cholesterol, mg/dl</td>
<td>117 ± 5</td>
<td>120 ± 9</td>
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<td></td>
<td>HDL cholesterol, mg/dl</td>
<td>40 ± 2</td>
<td>41 ± 2</td>
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<td></td>
<td>Triglycerides, mg/dl</td>
<td>106 ± 13</td>
<td>102 ± 14</td>
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<td></td>
<td>*Nitrotyrosine, μM</td>
<td>0.011 ± 0.001</td>
<td>0.150 ± 0.080</td>
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<td></td>
<td>Vascular Measurements and VO₂peak</td>
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<tr>
<td></td>
<td>FBF, ml·min⁻¹·10⁻¹·10³ ml⁻¹</td>
<td>2.37 ± 0.11</td>
<td>2.71 ± 0.20</td>
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<tr>
<td></td>
<td>FVC, units</td>
<td>2.67 ± 0.14</td>
<td>3.02 ± 0.21</td>
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<tr>
<td></td>
<td>HR, beats/min</td>
<td>64 ± 2</td>
<td>62 ± 3</td>
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<tr>
<td></td>
<td>MBP, mmHg</td>
<td>90 ± 2</td>
<td>89 ± 1</td>
</tr>
<tr>
<td></td>
<td>VO₂peak, ml·kg⁻¹·min⁻¹</td>
<td>43.7 ± 1.5</td>
<td>46.6 ± 1.1</td>
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</table>

Values are means ± SE. eNOS, endothelial nitric oxide synthase; BMI, body mass index; FBF, forearm blood flow; FVC, forearm vascular conductance; HR, heart rate; MBP, mean blood pressure; VO₂peak, peak oxygen consumption. *n = 21 (TT 10; CT + CC 11).

Effect of T786C Polymorphism of the eNOS Gene on Vasomotor Function

During handgrip exercise, heart rate, and mean blood pressure increased significantly and similarly in TT and CT + CC individuals (Table 2). Forearm blood flow significantly increased in both TT and CT + CC individuals. However, forearm blood flow responded (delta change) was significantly lower in TT individuals when compared with CT + CC individuals (Fig. 1). The results for forearm vascular conductance reveal further differences between groups. While the TT group presented no change in forearm vascular conductance during handgrip exercise compared with baseline, the CT + CC individuals presented a significant increase in this hemodynamic parameter throughout the three min of handgrip exercise (Fig. 1).

**Effect of Exercise Training**

Exercise training significantly increased VO₂peak levels in both TT and CT + CC individuals (Table 3). Also, the training regimen provoked a significant decrease in baseline heart rate for the TT and the CT + CC groups (Table 3). Body weight remained unchanged in the TT (P = 0.12) and CT + CC (P = 0.68) groups after exercise training. Exercise training did not significantly change nitrotyrosine levels in the TT and CT + CC groups (Table 3). Also, exercise training caused no significant change in resting mean blood pressure, forearm blood flow, and forearm vascular conductance (Table 3). Exercise training did not change heart rate or mean blood pressure responses to
Handgrip exercise (Table 4). However, exercise training significantly increased forearm blood flow responses in the TT group, while the CT+CC group presented no such changes. Thus, the difference between groups was no longer observed after exercise training (Fig. 3). Similar data were observed for forearm vascular conductance after exercise training, which did not occur with CT + CC individuals. Therefore, forearm vascular conductance response was equivalent between TT and CT+CC groups after exercise training (Fig. 3).

**DISCUSSION**

The main and new findings of the present study are: 1) Homozygous individuals for the T allele in position 786 of the promoter region of the eNOS gene have reduced muscle vasodilatory response to exercise compared with individuals possessing the CT and CC genotypes for this same genetic region; 2) Exercise training restores exercise-induced muscle vasodilatory response in individuals with the TT genotype; and 3) The differences in vascular function observed between groups are not associated with a decrease in serum nitrotyrosine concentration.

**Effect of T786C Polymorphism**

Muscle vasodilatation in response to exercise is a well-characterized reflex control in mammals (25). This response reflects endothelial- and sympathetic-mediated vascular function (8), which guarantees adequate blood supply to the skeletal muscle during the entire continuation of muscle contraction. The present study confirms this muscle reflex control since moderate handgrip exercise provoked a significant increase in the contralateral forearm blood flow in young individuals.

The G894T polymorphism is one of the many polymorphisms included in the eNOS gene, which is located in the long arm of chromosome 7 and is composed by 26 exons spanning 21 Kb of genomic DNA (19). This genetic variant leads to the substitution of a glutamate (Glu) for an aspartate (Asp) amino acid, and, because of this alteration in the eNOS protein chain, which could lead to an alteration in enzymatic function, has been the focus of many studies involving the eNOS gene. A previous study comparing forearm muscle blood flow in individuals homozygous for alleles G and T of this polymorphism determined that the TT genotype was associated with a reduction in vasodilatory response (8). Our study extends knowledge regarding the effect of the eNOS gene on vascular function by showing that the TT genotype for position 786 of the promoter region of the eNOS gene attenuates muscle vasodilatation in response to exercise in young male individuals. There are many mechanisms that may underlie this response. Previous studies show that the TT genotype is associated with an increased production of myeloperoxidase, an enzyme involved in the synthesis of reactive oxygen species (ROS) (30). Thus, carriers of the TT genotype might be submitted to an increase in oxidative stress, which leads to the degradation of NO and, in consequence, impaired endothelial function during exercise. However, this does not seem to be the case in our study since nitrotyrosine, a biomarker of oxidative stress, presented similar levels between the studied groups (Table 1). Another possibility is that C allele decreases promoter region activity and, consequently, eNOS expression (21, 23). However, the production of NO in the endothelium is redundant, which leads to the production of this gas by a manner other than eNOS (5, 33). Thus, eNOS deprivation would upregulate other vasodilatory pathways, such as those mediated by prostaglandins and hyperpolarizing endothelium factor, and downregulate vasoconstrictor pathways, such as those linked to angiotensin II.

**Table 3. Hemodynamic and respiratory parameters before and after exercise training for groups TT and CT+CC of the eNOS gene**

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<th>TT</th>
<th>CT+CC</th>
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<tr>
<td></td>
<td>Pretraining</td>
<td>Posttraining</td>
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<tr>
<td>HR, beats/min</td>
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<tr>
<td></td>
<td>64 ± 2</td>
<td>60 ± 2*</td>
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<tr>
<td>MBP, mmHg</td>
<td>90 ± 2</td>
<td>91 ± 2</td>
</tr>
<tr>
<td>FBF, ml·min⁻¹·100 g⁻¹</td>
<td>2.37 ± 0.14</td>
<td>2.70 ± 0.20</td>
</tr>
<tr>
<td>FVC, units</td>
<td>2.67 ± 0.14</td>
<td>3.00 ± 0.24</td>
</tr>
<tr>
<td>VO₂peak, ml·kg⁻¹·min⁻¹</td>
<td>43.7 ± 1.5</td>
<td>51.7 ± 1.5*</td>
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<tr>
<td>Nitrotyrosine, µM</td>
<td>0.011 ± 0.001</td>
<td>0.010 ± 0.001</td>
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Values are means ± SE. *Pre vs. post, P < 0.05.

**Table 4. Baseline and actual values for HR and MBP during 30% maximal voluntary contraction handgrip exercise before and after exercise training of groups TT and CT+CC of the eNOS gene**

<table>
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<th>TT</th>
<th>CT+CC</th>
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<tr>
<td></td>
<td>Baseline</td>
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<tr>
<td>HR, beats/min</td>
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<tr>
<td>Pre</td>
<td>64 ± 2</td>
<td>9 ± 1*</td>
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<tr>
<td>Post</td>
<td>60 ± 2†</td>
<td>8 ± 1*</td>
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<tr>
<td>MBP, mmHg</td>
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</tr>
<tr>
<td>Pre</td>
<td>90 ± 2</td>
<td>9 ± 1*</td>
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<tr>
<td>Post</td>
<td>91 ± 2</td>
<td>6 ± 1*</td>
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Values are means ± SE. *Vs. baseline, P < 0.05; †Pre vs. Post, P < 0.05.
allowing for an increased vasodilatory response to exercise in the CT and CC individuals.

Effects of Exercise Training

Recent studies have focused on the effect that genetic variants exert over exercise training and its physiological effects. Montgomery et al. (22) reported that 10 wk of aerobic training caused greater cardiac hypertrophy in Caucasian military recruits homozygous for the 287 bp deletion allele (allele D) of the ACE gene than in those not carrying the deletion (allele I) for the ACE gene. In a more recent study, other investigators showed no association between the I allele of the ACE gene and aerobic capacity in young healthy individuals after long-term exercise training (2). These studies show conflicting result as to if the response to exercise is indeed modulated by genetic variants of the ACE gene. In a study regarding eNOS polymorphisms, Erbs and colleagues (10) elegantly demonstrated that 4 wk of exercise led to a significant increase in FBF response after exercise training that was not observed for the CT+CC group. This allowed for an equivalent FBF response between these groups after exercise training. *Vs. baseline, $P < 0.05$. +Pre- vs. postraining, $P < 0.05$. 

![Fig. 2. FBF responses during exercise before and after exercise training for groups TT and CT+CC for the promoter region of the eNOS gene. TT, pretraining vs. posttraining (A); CT+CC, pretraining vs. postraining (B); TT vs. CT+CC postraining (C). Note that individuals of the TT group had an increase in FBF response after exercise training that was not observed for the CT+CC group. This allowed for an equivalent FBF response between these groups after exercise training. *Vs. baseline, $P < 0.05$. +Pre- vs. postraining, $P < 0.05$.](http://physiolgenomics.physiology.org/)

![Fig. 3. FVC responses during exercise before and after exercise training for groups TT and CT+CC for the promoter region of the eNOS gene. TT, Pretraining vs. posttraining (A); CT+CC, pretraining vs. postraining (B); TT vs. CT+CC postraining (C). Note that individuals with the T/T genotype had an increase in FVC response after exercise training that was not observed for those with the C/C genotype. This allowed for an equivalent FVC response between these individuals after exercise training. *Vs. baseline, $P < 0.05$. +Pre- vs. postraining, $P < 0.05$.](http://physiolgenomics.physiology.org/)
increase in acetylcholine-induced changes in average peak velocity in coronary and left internal mammary artery in patients with coronary artery disease carrying the T786C promoter region polymorphism of the eNOS gene and individuals carrying the G894T exon 7 polymorphism of the eNOS gene. However, these effects of exercise were lower in patients with the T786C promoter region polymorphism compared with TT individuals for T786C polymorphism and GT individuals for G894T polymorphism. These findings led to the conclusion that promoter region T786C polymorphism of the eNOS gene affects training-induced correction of endothelial function. The novelty of our study is the significant improvement in muscle blood flow during a physiological maneuver in exercise-trained healthy individuals carrying the TT genotype, in which the vasodilatory response is lower when compared with individuals carrying the CT and CC genotype. In addition, since exercise training did not affect serum nitrotyrosine concentration in the TT and CT+CC group, the lower vasodilatory response in the TT group appears to have no association with oxidative stress.

The present study provides information regarding the influence of an environmental factor, such as exercise training, in suppressing the effect of a genetic variant on a complex phenotype represented by the improvement of muscle vasodilatation in response to exercise. However, it still remains unknown if the T786C polymorphism is functional in the eNOS gene or if this region is in linkage disequilibrium with other polymorphisms that are able to interfere with eNOS expression. In our study population, we observed that position 786 of the promoter region of the eNOS gene was not in linkage disequilibrium with the exon 7 G894T marker or the 27 bp variable number of tandem repeats marker in intron 4. Thus, if there is indeed another marker in linkage disequilibrium with the T786C polymorphism that proves to be functional in eNOS expression it is still to be discovered. Future studies should use a haplotypic approach to obtain further information on this matter.

**Limitations**

We studied recruits initiating their training at the Military Police Academy of Sao Paulo. Thus, it is likely that are our study sampling is not representative of the Brazilian population because it included only male individuals of a similar socioeconomic background and age group. This is evidenced by the difference in alleleic and genotypic frequencies obtained in our study compared with those previously reported in another Brazilian population (20). On the other hand, these characteristics strengthened the present study, since all individuals were subjected to the same routine, physical activities, and feeding habits during the study. Furthermore, their similar demographic characteristics may eventually reduce bias regarding the influence of the T786C polymorphism of the eNOS gene on muscle vasodilatation and the effects of exercise training on muscle vasodilatory response to exercise. We note that an untrained group was not included in the present study. Although the inclusion of an untrained group as a control would add further data to our study, the low frequency of allele C in the study population limited the inclusion of a control group. Recent studies show that the influence of one genetic marker on a given phenotype is discrete (7). Despite this fact, our study demonstrated differences on vasodilatory response to exercise in the presence of T786C polymorphism. Exercise training did not improve vasodilatory responses in individuals carrying the CT and CC genotypes. One possible explanation is that carriers of the C allele already have a near-maximum vasodilatory capacity, which limits the amelioration caused by exercise training on muscle blood flow response in these individuals.

**Conclusion**

Exercise training improves muscle vasodilatation in response to exercise in individuals homozygous for the T allele of polymorphism T786C. Therefore, this finding suggests that genetic variants influence vascular function as well as the response that interventions such as exercise training might have on this complex phenotype.

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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