Promoter but Not Exon 7 Polymorphism of Endothelial Nitric Oxide Synthase Affects Training-Induced Correction of Endothelial Dysfunction

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Background—Polymorphisms in the promoter (T/H11002 786C) and exon 7 (G894T) of the endothelial nitric oxide synthase (eNOS) gene were shown to be associated with reduced vascular NO production or increased proteolytic cleavage of eNOS. Therefore, we aimed to determine the effects of these polymorphisms on endothelial function and endothelial response to physical exercise in patients with coronary artery disease (CAD).

Methods and Results—Sixty-seven patients were randomized to either a training or a control group. At the beginning and after 4 weeks, acetylcholine-induced changes in average peak velocity (APV) of a coronary or mammary artery were invasively assessed by Doppler velocimetry. Polymorphisms were detected by polymerase chain reaction–restriction fragment length polymorphism. At the beginning, in subjects with the wild-type (WT) variant, APV increased by 88% in response to acetylcholine. This response was significantly blunted in patients who were positive for the promoter (44%) or the exon 7 (62%) polymorphism. Four weeks of exercise training resulted in augmentation of an endothelium-dependent increase in APV by +36±12% in promoter polymorphism–positive patients but by +81±18% and +91±15% in WT variant– and exon 7 polymorphism–positive subjects, respectively.

Conclusions—These results suggest that the presence of either one of the polymorphisms attenuates endothelium-dependent vasodilatation in CAD patients. Only the promoter polymorphism might have an adverse effect on training-induced improvement in endothelial function. (Arterioscler Thromb Vasc Biol. 2003;23:1814-1819.)

Key Words: endothelial dysfunction ■ gene polymorphisms ■ endothelial nitric oxide synthase ■ exercise training ■ coronary artery disease

The maintenance of regular vascular tone substantially depends on the bioavailability of endothelium-derived nitric oxide (NO) synthesized by the endothelial isoform of nitric oxide synthase (eNOS). Apart from the classic risk factors for coronary artery disease (CAD), eg, diabetes mellitus, hypertension, hyperlipidemia, or smoking, numerous previous studies have assumed that genetic factors, such as gene polymorphisms of eNOS, might contribute to the development of CAD.1,2

Considerable attention has been focused on polymorphisms of the eNOS gene in its promoter region (T/H11002 786C) and in exon 7 (G894T). The promoter (T/H11002 786C) polymorphism, a mutation located in the 5’-flanking region of the eNOS gene, was not only shown to reduce eNOS protein expression but also found to be an independent predictor of coronary artery spasm.3,4 The exon 7 polymorphism, which results in an amino acid exchange at position 298 of the eNOS protein from glutamate to aspartate, was associated with the development of CAD, myocardial infarction, hypertension, and endothelial dysfunction in healthy, young adults, but only in the presence of the risk factor smoking.5,6

During the past several years, correction of endothelial dysfunction has become a therapeutic target. Besides pharmacologic approaches, regular physical exercise training represents a powerful intervention not only in correcting endothelial dysfunction in patients with CAD or chronic heart failure but also in improving their quality of life and survival.7-9 However, in those previous studies, we observed that some of the patients benefited more than others from the training program. Therefore, the question raised as to whether these differences in endothelial response to exercise training might be influenced by the presence of eNOS gene polymorphisms.

The aim of the present study was to determine the effects of the promoter (T/H11002 786C) and exon 7 (G894T) polymorphisms of the eNOS gene on endothelial function in patients with CAD. Furthermore, we investigated whether the presence of these polymorphisms diminished the response of
the endothelium to regular physical exercise training compared with normal-allele carriers.

**Methods**

Participants from 2 randomized studies that examined the effects of a 4-week exercise training program on endothelial function in patients with stable CAD were reassessed in this investigation. Endothelial function was measured in the coronary artery in 24 patients (coronary study group) and in the left internal mammary artery in 43 patients (LIMA study group).

**Patient Selection (Coronary and LIMA Study Groups)**

Sixty-seven male patients aged <70 years with stable CAD, preserved left ventricular function (left ventricular ejection fraction >50%), and a physical work capacity of at least 50 W were studied. Exclusion criteria were recent myocardial infarction (within the last 4 weeks), significant left main coronary artery stenosis, and coronary risk factors known to affect endothelial function, such as untreated hypertension (>160 mm Hg systolic and >90 mm Hg diastolic blood pressure), insulin-dependent diabetes mellitus, smoking, and untreated hypercholesterolemia (LDL >165 mg/dL [>4.3 mmol/L]).

Patients were eligible for the coronary study group if they had a hemodynamically relevant coronary artery stenosis that required nonsurgical revascularization (percutaneous, transluminal coronary angioplasty) and a noncritical stenosis in another vessel, which could therefore be used for testing (target vessel). To be suitable for testing, the target vessel had to have signs of endothelial dysfunction, defined as either vasoconstriction (decrease of ≧5% in mean luminal diameter) or no change (decrease of <5% or no decrease in mean luminal diameter) in response to acetylcholine.

Patients were eligible for the LIMA study group if they had a hemodynamically relevant coronary artery stenoses that required surgical revascularization and that also used the LIMA as a bypass graft. The LIMA was chosen as a target vessel to assess the effects of exercise training on endothelial function of a vessel not prone to overt atherosclerosis in patients with CAD.

**Study Program**

The study protocol was approved by the ethics committee of the University of Leipzig, and written, informed consent was obtained from all patients. After baseline measurements were taken, patients were randomly assigned to a training or a control group. Patients in the control group were encouraged to continue their sedentary lifestyle and were supervised by their private physicians. Patients allocated to the training group stayed in hospital during the entire 4-week study period and exercised 6 times daily for 10 minutes, either on a bicycle ergometer only (coronary study group) or on a bicycle (3 times daily) and a rowing ergometer (3 times daily) (LIMA study group) at 80% of the target heart rate that they had reached during the initial exercise test.

**Assessment of Endothelial Function**

Cardiovascular medications remained unchanged during the study period in all patients but were discontinued for at least 24 hours before measurement of endothelial function. At the beginning and after 4 weeks, endothelial function of the respective vascular bed (coronary or mammary artery) was invasively assessed. An 8F or a 6F guiding catheter was used to cannulate the target coronary artery or the LIMA, respectively. A 2.5F infusion catheter (Transit Infusion Catheter, Cordis) was then advanced over a 0.014-in. (0.036-cm) guidewire into the target vessel. The guidewire contained a 12-MHz, pulsed Doppler ultrasound velocimeter (FlowMAP, Cardiometrics, Endosonics). The tip of the guidewire was positioned 1 cm distal to the end of the infusion catheter, close to an anatomic landmark to facilitate its precise positioning at follow-up. The average peak flow velocity (APV) measured by the Doppler velocimeter was continuously recorded throughout the test protocol and drug administration.

**Results**

**Genotype Data**

Fifteen patients (25.8%) had 2 normal WT alleles, 18 (31.6%) patients were positive for the promoter (T-786C) polymorphism (all heterozygous), and 25 (43.1%) were positive for the exon 7 polymorphism (22 heterozygous and 3 homozygous). Nine patients (13.5%) were found to be positive (all heterozygous) for both the promoter and the exon 7 polymorphisms (Table 1).

**Baseline Characteristics and Clinical Follow-Up**

A total of 34 patients was assigned to the training group (LIMA/coronary study group, 20:14 patients) and 33 to a
control group (LIMA/coronary study group, 23:10 patients). The groups did not differ with respect to cardiovascular medical treatment at baseline. Furthermore, patients were comparable with respect to age, left ventricular ejection fraction, left ventricular end-diastolic diameter, body weight, and serum glucose and LDL cholesterol levels (Table 2). Baseline characteristics were also not significantly different between the different genotypes (Table 3).

In all patients, body weight remained essentially unchanged during the study period of 4 weeks, as did metabolic variables like total cholesterol, HDL, LDL, serum triglyceride levels, and serum glucose (data not shown). Furthermore, systolic and diastolic blood pressures were comparable between control and exercise training groups within the LIMA and the coronary study groups at baseline and did not change significantly during the 4 weeks of study (data not shown).

**Effect of Promoter (T−786C) and Exon 7 (G894T) Polymorphisms on Endothelial Function at Baseline**

**Change in APV**

Because acetylcholine administration resulted in comparable increases in APV in both the coronary and the LIMA group, circulation data were pooled and analyzed together. Acetylcholine administration resulted in augmentation of APV by 23.7±2.2 cm/s (88±7%) versus saline infusion in WT-variant patients. In contrast, the acetylcholine-mediated increase in APV was significantly blunted in patients positive for the promoter polymorphism (13.3±2.7 cm/s, 44±7%; \( P<0.05 \) vs WT) as well as in patients positive for the exon 7 polymorphism (16.2±2.1 cm/s, 62±9%; \( P<0.05 \) vs WT). The simultaneous occurrence of both polymorphisms in 1 individual did not further deteriorate the response to acetylcholine (16.2±3.3 cm/s, 57±11%; \( P<0.05 \) vs WT; Figure 1).

**Change in Vessel Diameter**

Administration of 7.2 \( \mu \)g acetylcholine resulted in comparable vasoconstrictive responses of the coronary arteries: by \(-9±1\% \) in WT-variant patients, \(-16±4\% \) in patients positive for the promoter polymorphism, \(-12±2\% \) in exon 7 polymorphism–positive patients, and \(-8±6\% \) in patients positive for both polymorphisms (\( P=NS; \) Table 4). To our surprise, the LIMA of patients homozygous for the exon 7 polymorphism (\( n=3 \)) was characterized by a vasoconstriction of \(-0.13±0.01 \) mm (\(-5±2\% \)) in response to acetylcholine.

**Effect of Promoter (T−786C) and Exon 7 (G894T) Polymorphisms on Exercise Training Effects on Endothelial Function**

Exercise training for a period of 4 weeks led to a significant improvement in coronary and LIMA APV from baseline (again, data were pooled for both investigated vascular beds). In the training group, the increase in APV after infusion of acetylcholine (7.2 \( \mu \)g/min) was \(+81±18\% \) in WT-allele carriers and \(+91±15\% \) in patients positive for the exon 7 polymorphism (\( P<0.05 \) for comparison with the change in
the control group and versus at the beginning). However, the 3 patients in the training group who were homozygous for the exon 7 polymorphism showed a blunted training response, as reflected by an impaired increase in APV by only +24±14% after acetylcholine stimulation.

In patients having the promoter polymorphism, exercise training also led to an augmentation of APV in response to acetylcholine infusion, by +36±12%, but interestingly, this training-induced improvement in endothelium-dependent vasodilatory capacity was significantly blunted compared with that in WT-allele patients and in patients positive for the exon 7 polymorphism (P<0.05 for comparison with the change in the control group and versus at the beginning, P<0.05 vs WT- and exon 7 mutation–positive patients; Figure 2). In patients in the control group, the changes in APV in response to acetylcholine infusion at 4 weeks were not significantly different from those in the initial study (Figure 2). Because the patients who were positive for both polymorphisms were underrepresented in the training group (only 1 in the training group vs 8 in the control group), the influence on the endothelial response to exercise training could not be reasonably assessed.

**Endothelium-Independent Vasodilatation**

The vasodilatory response of epicardial coronary arteries and the LIMA in response to the endothelium-independent vasodilator nitroglycerin was not affected by the presence of the promoter or the exon 7 polymorphism. At the beginning, APV increased in WT-allele patients by 150±26%; in promoter-positive patients, by 169±45%; and in patients having the exon 7 polymorphism, by 136±13%; these values were essentially unchanged after 4 weeks of exercise training (P=NS).

**Discussion**

Three major findings emerge from this study that investigated the influence of the promoter (T−786C) and the exon 7 (G894T) polymorphisms of the eNOS gene on baseline endothelial function and on endothelial function in response to exercise training in patients with CAD: (1) In patients positive for the promoter or the exon 7 polymorphism, agonist-mediated, endothelium-dependent vasodilatation was blunted compared with that in WT-allele patients. (2) The presence of the promoter polymorphism of the eNOS gene was associated with remarkable impairment of endothelial function in response to exercise training in comparison with WT-allele patients. (3) In contrast, patients heterozygous for the exon 7 polymorphism showed an improvement in endothelial function in response to exercise training similar to that of WT-allele carriers.

**Promoter (T−786C) Polymorphism and Endothelial Function**

Because previous studies had suggested that the eNOS promoter (T−786C) polymorphism predisposes patients to coronary spasm and accounts for impaired cerebral blood flow in the presence of additional risk factors, we were interested in whether this polymorphism had an impact on endothelial function in patients with CAD.1,10 The increase of APV in the coronary and LIMA circulations in response to acetylcholine stimulation was significantly diminished in patients having the promoter polymorphism in comparison with WT-allele patients. However, the coronary arteries from WT- and T−786C mutation–positive patients showed a similar vasoconstrictive response to acetylcholine infusion, whereas the LIMAs of those patients were characterized by comparable acetylcholine-induced vasodilatation.

Because all of our patients in the coronary study group had overt atherosclerosis with severe damage of the endothelial cell layer, the vasoconstrictive response of the coronary target vessel to acetylcholine is not surprising and is most likely related to damage of the endothelial cell layer rather than to the polymorphism of the promoter. The severe arteriosclerosis of large, epicardial coronary arteries might offset the effects of the polymorphism on endothelial function, making it impossible to detect small differences between the groups.

Irrespective of the vascular bed studied, changes in APV in response to acetylcholine were significantly affected by the promoter polymorphism. In our understanding, the APV mainly reflects dilatation of the downstream resistance vasculature. Because acetylcholine infusion also resulted in an increase in APV from baseline (saline infusion) in the coronary circulation, downstream vessels might not exhibit overt arteriosclerosis and therefore, still possess a vasodila-

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<th>TABLE 4. Change in Vessel Diameter at Baseline</th>
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Change in vessel diameter in response to acetylcholine administration 7.2 μg/min vs saline infusion at baseline (mm). In the LIMA study group, the respective data only of the patients who are heterozygous for the exon 7 polymorphism (n=22) are shown. P=NS.

![Figure 2. Percent change in APV vs baseline (saline infusion) at 4 weeks minus percent change in APV vs baseline at the beginning of the study. Exon 7 indicates patients who were positive for the exon 7 polymorphism only; and promoter, patients who were positive for the promoter polymorphism only. *P<0.05 vs WT- and exon 7–positive patients; †P<0.05 vs beginning and control.](image-url)
tory capacity. On the basis of the assumption that the APV is mainly affected by the microcirculation, which is not characterized by such severe atherosclerosis, one would expect to detect changes in response to acetylcholine more exactly. This was the case in our study, and we were able to establish that patients with the promoter polymorphism were characterized by an impaired acetylcholine-mediated increase in APV in the coronary circulation. In the LIMA, which is not prone to atherosclerosis, the differences in acetylcholine-induced changes in APV between WT- and polymorphism-positive patients were similar to those found in the coronaries.

The demonstrated effects on APV might originate from alterations in eNOS promoter activity due to the polymorphism. In fact, Nakayama et al. discovered that the T→786C mutation in the eNOS gene decreases promoter activity by 50%, thereby leading to suppressed eNOS protein expression, a mechanism probably accounting for the development of coronary vasospasm.

From a subgroup of patients, LIMA tissue samples were obtained during coronary artery bypass grafting, and eNOS protein expression was determined by Western blotting techniques. We were able to confirm that the presence of the promoter polymorphism is associated with a decrease in eNOS protein expression by 37% compared with WT-allele carriers (data not shown). Furthermore, the presence of 1 or 2 mutated alleles was also significant when patients had a smoking habit, and this led to reduced cerebral perfusion compared with that of WT-allele carriers. These data suggest that the reduced eNOS promoter activity due to the T→786C polymorphism affects eNOS protein expression and partially contributes to endothelial dysfunction in the LIMA and coronary circulations in our clinical setting. Furthermore, additional factors like oxidative stress are believed to aggravate the alterations due to the eNOS promoter polymorphism and play a major role in CAD.

Exon 7 (G894T) Polymorphism and Endothelial Function

In the present study, patients with CAD recruited for our training trials were analyzed for the impact of the exon 7 polymorphism of the eNOS gene on coronary and LIMA endothelial function. However, acetylcholine infusion resulted in nearly similar vasoconstrictive responses of the coronary arteries in all patients, independent of the G894T mutation of the eNOS gene. The LIMA, a vessel usually unaffected by atherosclerosis, showed a vasodilatory response to acetylcholine infusion in all patients heterozygous for the exon 7 polymorphism. Surprisingly, the 3 exon 7 polymorphism–homozygous patients were the only ones who developed vasoconstriction of the LIMA as a result of acetylcholine stimulation.

The molecular effects of the exon 7 polymorphism on eNOS enzyme function and activity are still under debate. The kinetics of mutated and WT eNOS do not seem to differ significantly, at least under in vitro conditions. However, the eNOS protein derived from the homozygous genotype was discovered to be more susceptible to proteolytic cleavage than was the WT isoform. The steady-state levels of eNOS protein in carriers of 2 mutated alleles might therefore be lower than in WT-allele patients, leading to reduced NO production. At the moment, we can only speculate about the mechanisms responsible for vasoconstriction of the LIMA in patients homozygous for the exon 7 polymorphism. Even if the steady-state levels of eNOS protein were lower in carriers of 2 mutated alleles, stimulation with acetylcholine should result in NO release and finally, vasodilatation. One might speculate that the mutated eNOS possibly produces reactive oxygen species instead of NO under in vivo conditions in patients with CAD, thus contributing to a vasoconstrictive reaction. Many partially unknown cofactors that are pathophysiologically relevant only in a disease state (e.g., CAD) might give the polymorphism its impact and affect eNOS function in vivo.

However, despite the differences in dilatory response of the LIMA in patients heterozygous and homozygous for the exon 7 polymorphism, augmentation of APV in response to acetylcholine infusion was significantly diminished in the coronary and LIMA circulations of all patients positive for the exon 7 polymorphism. Recently, Leeson et al. did not find any differences in flow-mediated vasodilatation of the forearm between healthy, young adults heterozygous, homozygous, or WT for the exon 7 polymorphism. On the other hand, in males, smoking was associated with a blunted flow-mediated vasodilatation in exon 7 mutation–positive subjects but not in normal-allele (WT) carriers. These data suggest that the G894T mutation in exon 7 has an influence on endothelial function in the presence of environmental cofactors, such as elevated levels of reactive oxygen species. However, because CAD is also associated with increased oxidative stress, leading to the premature breakdown of NO, the eNOS genotype might become significant under those circumstances, as was the case in our study.

Impact of Both Promoter (T→786C) and Exon 7 (G894T) Polymorphisms on Endothelial Function

Patients having a mutation in the promoter and exon 7 of the eNOS gene were not characterized by further deterioration of endothelial function in response to acetylcholine. In the present study, we were unable to determine whether only 1 eNOS gene was affected by both polymorphisms or whether these polymorphisms were localized on 2 different eNOS genes. If both polymorphisms are localized on the same gene, then the organism should be able to transcribe normal eNOS protein from the second, unaffected gene. In the other case, in which 1 eNOS gene is affected by the promoter polymorphism and the other by the exon 7 polymorphism, we would expect further deterioration of endothelial function, because 1 eNOS gene is transcribed less and the eNOS protein derived from the other gene undergoes earlier proteolytic cleavage. Further studies involving a larger number of patients and with the goal of analyzing the exact localization of the eNOS polymorphisms are therefore necessary.

Impact of Promoter (T→786C) and Exon 7 (G894T) Polymorphisms on Exercise-Induced Improvement of Endothelial Function in Patients With CAD

This is the first study to investigate the effect of the promoter and exon 7 polymorphisms on endothelial function in re-
response to physical exercise training in CAD patients. Exercise training resulted in comparable improvements of endothelial function in CAD patients who were either positive for the exon 7 polymorphism or WT-allele carriers. However, CAD patients positive for the promoter polymorphism had a blunted increase in APV during acetylcholine infusion after 4 weeks of regular physical exercise compared with WT- and exon 7 mutation–positive patients. Our data suggest that the promoter polymorphism is of major significance for improvement of endothelial function in response to exercise training, at least in patients with CAD.

In previously published studies, improvement of endothelial function during exercise training in CAD or chronic heart failure patients was primarily attributed to an increase in eNOS expression. Expression of the eNOS protein is mainly regulated by the activity of the promoter. However, the physiologic effect of the T→786C mutation in the eNOS gene was discussed controversially, despite the fact that this mutation was discovered to decrease promoter activity by 50%, thereby leading to suppressed eNOS protein expression. Here, we show for the first time that the T→786C mutation in the eNOS gene has a critical impact on the physiologic response of the endothelium to physical exercise: patients positive for the promoter polymorphism were characterized by a blunted correction of endothelial dysfunction as a result of regular physical activity compared with WT-allele carriers.

In contrast, CAD patients heterozygous for the exon 7 polymorphism seemed to benefit from the exercise training program, as did patients with the WT allele. Despite the fact that the eNOS protein derived from the mutated allele undergoes proteolytic cleavage earlier than does the WT form, the specific activity of mutated eNOS protein and its regulation by phosphorylation appear to be unaltered. This might explain why the endothelium of CAD patients heterozygous for the exon 7 eNOS polymorphism responded to regular physical exercise like the endothelium of WT-allele carriers. Because oxidative stress was also shown to contribute to a functional phenotype of the exon 7 mutation in healthy individuals, the reduction in circulating reactive oxygen species during regular exercise training in CAD patients might additionally account for the abolition of a functional phenotype of the exon 7 polymorphism after regular physical activity.

Clinical Implications

Despite impaired endothelial function at the beginning of the study, the response to exercise training at the endothelial level in patients with CAD was equal in exon 7 polymorphism–positive (heterozygous) and WT-allele patients. In contrast, the endothelium of CAD patients positive for the promoter (T→786C) polymorphism seemed to benefit less from regular physical exercise. However, endothelial function improved in all patients with CAD, emphasizing the importance of exercise training for at least a partial correction of endothelial dysfunction, even in the presence of a promoter (T→786C) mutation in the eNOS gene. Whether a homozygous genotype of the exon 7 polymorphism abolishes the exercise-induced changes in endothelial function, as our data of 3 patients suggest, deserves additional investigation.

References


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