Shear Stress Insensitivity of Endothelial Nitric Oxide Synthase Expression as a Genetic Risk Factor for Coronary Heart Disease

Marco Cattaruzza, Tomasz J. Guzik, Wojciech Słodowski, Ayşegül Pelvan, Jürgen Becker, Martin Halle, Arnd B. Buchwald, Keith M. Channon, Markus Hecker

Abstract—Coronary heart disease (CHD) is based on the development of atherosclerosis in coronary arteries. Shear stress-induced endothelial nitric oxide (NO) release not only contributes to local blood pressure control but also effectively helps to retard atherosclerosis. Therefore, functionally relevant polymorphisms in the endothelial NO synthase (NOS-3) gene may contribute to the development of CHD. NOS-3 expression was analyzed in endothelial cells isolated from umbilical cords genotyped for the −786C/T single nucleotide polymorphism (SNP) of the human nos-3 gene. Moreover, NO-dependent relaxation was examined in segments of saphenous vein isolated from genotyped patients undergoing aortocoronary bypass surgery, and patients subjected to quantitative coronary angiography were genotyped to verify an association between this SNP and CHD. Shear stress-induced NOS-3 mRNA and protein expression was present in TT and CT genotype cells but absent in cells with CC genotype. Pretreatment of these cells with a decoy oligonucleotide comprising position −800 to −779 of the C-type nos-3 promoter reconstituted shear stress-induced NOS-3 expression. These results were confirmed by reporter gene analysis with the corresponding nos-3 promoter luciferase constructs. In addition, the NO-mediated relaxant response of vein grafts from CC genotype patients was significantly attenuated as compared with the CT or TT genotype, and in CHD-positive patients, the CC genotype was significantly more frequent (19.0%) than in CHD-negative patients (4.4%). The −786C/T SNP of the nos-3 gene thus constitutes a genetic risk factor for CHD, presumably due to binding of an inhibitory transcription factor to the C-type promoter blocking shear stress-dependent maintenance of NOS-3 expression. (Circ Res. 2004;95:841-847.)

Key Words: coronary heart disease ■ shear stress ■ atherosclerosis ■ endothelial dysfunction ■ nitric oxide synthase ■ single nucleotide polymorphism ■ decoy oligonucleotide

Clinical Research

Coronary heart disease (CHD) is the leading cause of death in industrialized nations. Although cellular events leading to the formation of coronary atherosclerotic lesions are not yet fully characterized, persistent dysfunction of the endothelium in affected arteries is an important aspect of this chronic inflammatory disease. Apart from environmental factors influencing endothelial function, intrinsic impairment of the expression of endothelial gene products involved in the maintenance of vascular homeostasis may predispose to atherosclerosis and thus CHD. Consequently, characterizing polymorphisms in genes encoding such proteins may help to define genetic risk factors for CHD.

The endothelial isoform of nitric oxide (NO) synthase (NOS-3) is such a gene product, since NO plays a crucial role not only in the regulation of vascular tone but also in maintaining the functional and structural integrity of the vessel wall. Endothelial cells exposed to elevated levels of shear stress respond with a compensatory increase in NOS-3 activity. Moreover, expression of the enzyme is up-regulated by this and other stimuli. This ability to dynamically adapt both NOS-3 activity and expression to the demand is a decisive factor for maintaining the anti-inflammatory state of the endothelium. Therefore, reduced activity or expression of NOS-3 due to common genetic alterations might predispose to or accelerate CHD.

Although many studies have addressed possible associations between cardiovascular disease and nos-3 gene polymorphisms, their significance still is a matter of debate, primarily due to different study designs. Hitherto, three potentially functional nos-3 gene polymorphisms have been studied in more detail. Besides a 27 bp tandem repeat in intron 4 possibly affecting basal NOS-3 expression, two single nucleotide polymorphisms (SNPs) have been focused on. One, the G to T transition at position 894 of the nos-3 promoter blocking shear stress-dependent maintenance of NOS-3 expression.
gene results in an exchange of glutamate 298 to aspartate and is thought to alter protein stability. The other, a T to C transition at position −786 in the promoter of the nos-3 gene, is unlikely to affect the function of the mature enzyme. The controversial association between this SNP and CHD or myocardial infarction has prompted us to systematically investigate its functional consequences both in cultured and native endothelial cells derived from genotyped individuals. In addition, we studied allele frequencies for this SNP in age-matched patients with and without angiographically confirmed CHD as compared with the general population.

### Materials and Methods

#### Sample Collection and Diagnosis of CHD

Umbilical cords were collected from various hospitals in Göttingen and the Southern part of Lower Saxony. Over the last 55 years the population of Lower Saxony experienced no major changes and, therefore, can be regarded as genetically stable. Umbilical cords were employed not only for isolation of endothelial cells, but also to analyze genotype distributions in the general population.

Blood samples were collected in a blinded fashion from randomly selected patients undergoing quantitative coronary angiography (Hospital NeuBethlehem and University Clinics, Göttingen). They were considered CHD-positive when the luminal diameter in at least one coronary artery was reduced by >50% (typically 75% to 90%). The number of atherosclerotic lesions, their distribution, length and degree of stenosis monitored during the procedure were found in one coronary artery was reduced by >50% (typically 75% to 90%).

Blood samples were collected in a blinded fashion from randomly selected patients undergoing quantitative coronary angiography (Hospital NeuBethlehem and University Clinics, Göttingen). They were considered CHD-positive when the luminal diameter in at least one coronary artery was reduced by >50% (typically 75% to 90%).

#### Analysis of NOS-3 Expression

Isolation of RNA and conventional semi-quantitative RT-PCR analysis were done as described previously. Standard-calibrated quantitative real-time PCR analysis was performed according to the method of Wagner et al.

In brief, 5 μL of cDNA (M-MLV reverse transcriptase, Promega, Mannheim, Germany), the equivalent of 10 ng of total RNA, was added to each PCR reaction. The fluorescent QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) and a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) were used for PCR. Nos-3 mRNA fragments (GenBank™ accession No. NM_000603; 129 bp, position 1151 to 1279) were amplified using 5′-GGAGTGGCCTGGCATGGG-3′ as forward and 5′-TCGTTCAACTGGTGCTTGTTTGG-3′ as reverse primer. To normalize sample load, cDNA coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank™ accession No. BT006893; 138 bp, position 525 to 662) was employed (forward primer 5′-GACCACAAGTCGTAGCCACTGC-G-3′, reverse primer 5′-AGACCTTGGCACAACGCCTGG-3′). The program used for amplification of both gene products was 40 cycles of 3 s at 95°C, 25 s at 58°C and 10 s at 72°C, preceded by 15 minutes at 95°C for enzyme activation. The melting curve was programmed to rise from 65°C to 95°C with a slope of 0.2°C/s. Fluorescence intensity was monitored at the end of each cycle. Melting curve analysis (LDCA software version 3.5.28, Roche Diagnostics) and exemplary sequencing were used to confirm assay homogeneity and product identity, respectively.

#### Transfection of the Human Endothelial Cell Line HUE With T-Type and C-Type Reporter Gene Constructs

For reactivity analysis of the T and C-type promoters to laminar shear stress a pGL2 plasmid containing 1600 bp of the human nos-3 promoter, kindly provided by Dr. William C. Sessa (Yale University, New Haven, CT), was used. As the promoter construct provided was of the C-type, the cytosine at position −786 was replaced by thymidine using the PCR-based QuickChange mutagenesis kit (Stratagene, Amsterdam, The Netherlands) according to the manufacturer’s instructions. HUE cells were transfected in a 75 cm² Petri dish at 70% confluence using lipofectamid (Invitrogen, Karlsruhe, Germany) and 5 μg of the pGL2 plasmids, respectively, according to the manufacturer’s instructions. After 24 hours, the transfected cells were trypsinized and equal numbers of cells (1×10⁵ cells per well) transferred to 60-mm diameter Petri dishes. Twenty four hours later the passaged cells were subjected to laminar shear stress as described. Due to this procedure, normalization for transfection efficiency was unnecessary.

#### Organ Bath Experiments

Human saphenous vein specimens were obtained from patients (mean age 62.0±1.2 years) undergoing aortocoronary bypass (ACVB) surgery (John Radcliffe Hospital, Oxford, UK) immediately after excision of the blood vessel. Three mm segments were mounted in organ baths. Optimal resting tension and concentration-response curves to phenylephrine, acetylcholine and sodium nitroprusside were determined as previously described.

All experiments were performed in the presence of indomethacin (10 μmol/L) to inhibit vascular prostacyclin synthesis. Contractions and relaxations were expressed as a percentage of the level of pre-constriction or the maximum level of contraction, respectively.
30 s at 60°C and 1 minute at 72°C; forward primer 5'-GAGTCTGGCCAACACAAATCC-3', reverse primer 5'-GACCTCTAGGGTCATGCAGGT-3'. The genotyping procedure took advantage of the fact that the C-type promoter of the nos-3 gene contains an additional restriction site for HpaII (Figure 1). For analysis of the 894 G/T SNP, a 967 bp fragment of the coding region (GenBank™ accession No. D26607; position 6356 to 7322) was amplified by PCR as described before (forward primer: 5'-CAGGTCTGCACAGGAAATGTTC-3', reverse primer 5'-AGGGTCACACAGGTTCCTCG-3'). Here, the T-variant yields a new restriction site for DpnII, which was employed for genotyping (Figure 1).

Statistical Analysis
Allele frequencies among the umbilical artery specimens and blood samples were confirmed to be in the Hardy-Weinberg equilibrium by using Pearson’s chi-square test with Yates’s continuity correction. Fisher’s exact test and odds ratio calculation were used to analyze differences between the different study groups.

NOS-3 expression data are presented as mean±SEM of n observations with cells obtained from individual umbilical cords. Kruskal-Wallis non-parametric test followed by Dunn’s post test (shear stress dependence of NOS-3 mRNA expression) or Student t test (Western blot and organ bath experiments) were used to determine statistically significant differences between the means. The software used was GraphPad InStat Version 3.00 (GraphPad Software Inc., San Diego, CA, USA). A value of P<0.05 was considered significant.

Results
-786C/T SNP and Shear Stress-Induced NOS-3 Expression
The basal amount of NOS-3 mRNA, was moderately higher in cells derived from TT individuals as compared with CC cells whereas cells derived from CT donors revealed an intermediate NOS-3 mRNA content under static conditions (Figure 2). When exposed to laminar shear stress (30 dyn/cm²) for 24 hours (mRNA level) or 36 hours (protein level), however, a striking difference was noted. In contrast to TT or CT genotype cells, both NOS-3 mRNA and protein expression failed to increase in response to shear stress in cells derived from CC genotype donors (Figure 2). Pretreatment of the CC genotype cells with a decoy oligonucleotide mimicking part (800 to 452) of the C-type but not T-type promoter of the nos-3 gene, on the other hand, led to a reconstitution of the shear stress sensitivity of the nos-3 gene (Figure 3A).

To independently analyze the effects of the -786C-variant of the nos-3 gene promoter, the human endothelial cell line HUE was transfected with a 1600 bp C- or T-type fragment of the nos-3 gene promoter luciferase-reporter-gene construct. The genotype of the HUE cells is CT and they respond to laminar shear stress with a 2-fold increase in NOS-3 mRNA expression (not shown). The T-type but not the C-type reporter gene construct revealed a similar reactivity to laminar shear stress in the HUE cells as the endogenous nos-3 gene promoter (Figure 3B). Moreover, pre-incubation of the
C-type reporter gene construct-transfected cells with the C-type decoy oligonucleotide fully restored shear stress-induced reporter gene expression. In T-type reporter gene construct-transfected cells, on the other hand, the decoy oligonucleotide had no further stimulatory effect (Figure 3B).

786C/T SNP and NO-Mediated Relaxation

To test whether the impaired shear stress inducibility of NOS-3 expression in genotype endothelial cells observed in vitro results in a decreased NO-synthesizing capacity in situ, organ bath experiments were performed with freshly isolated saphenous vein segments obtained from genotyped ACVB patients. The endothelium-dependent NO-mediated relaxant response to acetylcholine (ACh) was significantly attenuated in segments derived from CC genotype donors as compared with CT or TT genotype segments (Figure 4A). In contrast, neither phenylephrine-induced vasoconstriction (maximum tension: CC, 7.5±1.3 g, n=13; CT, 8.9±0.6 g, n=45; TT, 8.5±0.7 g, n=41) nor the endothelium-independent relaxant response to the NO donor sodium nitroprusside (Figure 4B), differed with respect to either genotype. Analysis of NO-mediated relaxations in comparison with the primary CHD risk factors (diabetes, hypercholesterolemia, hypertension, and smoking) revealed strong inverse associations, but the CC genotype remained significantly associated with a decreased relaxant response to ACh, independently of these risk factors (Table 1).

Genotype Distribution in Patients With CHD

To establish general allele frequencies in the population of Southern Lower Saxony, without the potential bias of case/control allocation according to disease phenotype, 662 randomly collected umbilical cords were analyzed. The results are shown in Table 2. To minimize the confounding effects of a greatly increased CHD prevalence in humans over 65 years of age, younger patients undergoing quantitative coronary angiography were genotyped (Table 2). Average allele frequencies among these patients closely matched those in the general population and did not deviate from the Hardy-Weinberg equilibrium, predicting approximately 13%, 46% and 41% for the CC, CT and TT genotype, respectively ($\chi^2=0.25$ with CI<0.99 in both groups). Moreover, these allele frequencies of the German population closely matched those recently reported for a British" and an Italian cohort.24

In contrast, subgroup analysis of the CHD-positive and negative patients revealed a significant deviation from the Hardy-Weinberg equilibrium. Thus, the CC genotype was

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>No. With RF</th>
<th>No. Without RF</th>
<th>ACh Maximal Relaxation, %±SEM</th>
<th>With RF</th>
<th>Without RF</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC genotype</td>
<td>13</td>
<td>85</td>
<td>18.0±2.8</td>
<td>27.3±1.0</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>HT</td>
<td>70</td>
<td>28</td>
<td>24.5±1.7</td>
<td>29.7±2.4</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>HC</td>
<td>64</td>
<td>34</td>
<td>22.3±1.4</td>
<td>33.5±2.4</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>NCT</td>
<td>41</td>
<td>57</td>
<td>21.6±1.8</td>
<td>29.1±1.9</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>DB</td>
<td>25</td>
<td>73</td>
<td>17.5±1.6</td>
<td>29.1±1.7</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

ACh indicates acetylcholine; DB, diabetes; HC, hypercholesterolemia; HT, hypertension; NCT, smoking.

Table 1. Association Between the 786C/T SNP and Clinical Risk Factors and Endothelial Function

C-type decoy oligonucleotide effects on shear stress-induced NOS-3 mRNA expression in CC genotype endothelial cells. Statistical summary (n=4) of mRNA levels following 24-hour exposure to laminar shear stress (LSS). Cells were pretreated with the C-type (C-ODN, right panel) or T-type (T-ODN; left panel) decoy oligonucleotide (10 μmol/L each) for 4 hours (cf Buchwald et al23) before placing them into the cone-and-plate viscometer (P<0.05 vs control). (B) Reporter gene analysis of the T-variant (left panel) and C-variant (right panel) nos-3 gene promoters in HUE cells. Statistical summary (n=7) of luciferase activity (relative light units (RLU) per 50 μg of protein) following 24-hour exposure to laminar shear stress (LSS). Cells were pretreated with the C-type (C-ODN) or T-type (T-ODN) decoy oligonucleotide (10 μmol/L each) for 4 hours before placing them into the cone-and-plate viscometer (P<0.05 vs control).
Table 2 shows the allele frequencies in the general population and in patients with and without CHD. The table includes patients with CHD, patients without CHD, and all patients. The data is presented in a contingency table format with percentages and odds ratios. The table also includes a note on the significance of the differences observed.

The Discussion section highlights the importance of the NOS-3 gene and its genotype in CHD. It is noted that the CC genotype is associated with a reduced NO-mediated vasomotor function in vivo. The finding is supported by the trinity of endothelial dysfunction seen in CHD-positive patients: (1) a deficit in NOS-3 expression in human endothelial cells on exposure to laminar shear stress, with (2) a reduced NO-mediated vasodilatory response in freshly isolated human blood vessels, and (3) an increase in the presence of CHD in patients undergoing quantitative coronary angiography. These findings identify the 786C/T SNP of the nos-3 gene as a biologically functional and clinically important genetic factor in CHD risk.

NOS-3 Expression and the CC Genotype

As laminar shear stress is the most important stimulus controlling NOS-3 expression in endothelial cells in vivo, we determined its effects on NOS-3 expression in cultured cells using a cone-and-plate viscometer set to an arterial level of shear stress. Basal NOS-3 mRNA expression, as judged by quantitative real-time PCR analysis was 2-fold higher in cells from TT genotype donors as compared with CC genotype donors. Expression of the C-variant promoter reporter gene construct in the HUE cells also tended to be lower than that of the T-variant (for example, see Miyamoto et al). This apparent genotype difference in basal transcription, however, did not seem to be reflected on the protein level. Exposure to laminar shear stress, on the other hand, revealed a striking defect of the C-type promoter. Whereas TT or CT genotype cells responded to shear stress with the expected rise in NOS-3 mRNA and protein, CC genotype cells showed no increase at all over the 24 to 36-hour observation period. Moreover, transfection of the endothelial cell line HUE with either a T-type or C-type promoter reporter gene construct fully corroborated the shear stress-insensitivity of the C-type nos-3 gene promoter.

Acetylcholine-induced NO-mediated relaxation/vasodilation is frequently used to evaluate endothelial dysfunction in vitro and in vivo that is related to an alteration in NO synthesis. We found that in phenylephrine-preconstricted segments of the saphenous vein derived from ACVB patients with CC genotype, the relaxant response to ACh is significantly impaired, suggesting that the NO-synthesizing capacity of the endothelium is reduced even in apparently non-diseased vessels of individuals homozygous for this SNP. As in arteries, shear stress-dependent maintenance of NOS-3 expression also plays a crucial role for the capacity of venous endothelial cells to generate NO.

The C-Type Promoter: Impaired Activation or Active Inhibition?

What is the molecular basis for the impaired sensitivity of the C-type promoter to laminar shear stress? The potential binding sites, ie, 5’-TGGCCGGCT-3’ or 5’-TGGCTGGCT-3’ do not resemble the binding motif of any known transcription factor according to database analysis (TRANSFAC). In principle, there are two possibilities: (1) The promoter variant can no longer interact with a stimulatory transcription factor mediating the shear stress effect or (2) binding of an inhibitory DNA-binding protein is enabled which prevents the aforementioned interaction.

To test for the latter hypothesis without knowing the protein potentially binding to the C-type promoter, the decoy oligonucleotide technique was employed. This technique

The table below shows the primary risk factor profile in the Göttingen group of CHD-positive patients genotyped for the 786C/T SNP.

<table>
<thead>
<tr>
<th>HT, %</th>
<th>HC, %</th>
<th>NCT, %</th>
<th>DM, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>44.1*</td>
<td>38.2†</td>
<td>26.4†</td>
</tr>
<tr>
<td>CT</td>
<td>65.2</td>
<td>70.7</td>
<td>42.7</td>
</tr>
<tr>
<td>TT</td>
<td>59.6</td>
<td>51.9</td>
<td>42.3</td>
</tr>
</tbody>
</table>

DB indicates diabetes; HC, hypercholesterolemia; HT, hypertension; NCT, smoking.

Odds ratios (OR) for risk factors of 786C/T genotype patients vs patients with other genotype: *OR=0.71, P=0.33; †OR=0.60, P=0.18; ‡OR=0.62, P=0.27; #OR=1.73, P=0.25.
takes advantage of the fact that short double-stranded DNA molecules (12 to 24 bp), typically mimicking the DNA-binding site of a given transcription factor, readily enter cells and neutralize their target transcription factors binding to the decoy oligonucleotide instead of their natural DNA-binding site. Pre-treatment of CC genotype endothelial cells with a decoy oligonucleotide mimicking the C-type but not the T-type binding motif effectively restored shear stress-dependent NOS-3 expression in these cells. The same effect of the decoy oligonucleotide was observed in the HUE cell line transfected with the C-type nos-3 gene promoter reporter gene construct. This indicates that in principle the C-type promoter of the nos-3 gene is shear stress-responsive, ie, the shear stress response element is not destroyed by the T to C transition, but blocked by an inhibitory DNA-binding protein.

In this context, replication protein A1 has been suggested to interact with the C-type promoter. This hypothesis, however, is somewhat precarious for several reasons, eg, replication protein A1 typically binds to single-stranded DNA, so that in our opinion further work is necessary to identify the inhibitory factor binding to the C-type promoter of the nos-3 gene.

Clinical Consequences of the CC Genotype

The patient groups studied were age and sex matched. As all patients were randomly recruited from the two major centers performing angiographic examinations in Göttingen, no unusual accumulation of diseases or risk factors occurred in one of the sub-groups. As it was crucial for statistical reasons to compare allele frequencies in the patient groups with those of the general population in the area of Göttingen, umbilical cord specimens randomly collected in the course of endothelial cell preparation were genotyped. In our opinion, this is a valid population control for the following reasons: (1) In the period of interest reflecting the birth dates of the patients analyzed (1940s to 1960s) no major changes in the population, eg, due to mass immigrations, occurred. (2) Distribution of the $^{786}C/T$ SNP both in the control (umbilical cords) and in the patient group is virtually identical to that noted in Caucasians in the U.K., ITaly or Canada. (3) By genotyping umbilical cords a possible bias associated with the recruitment of age-matched volunteers, ie, a potentially confounding “social drift” in the control group, is avoided.

In patients below 65 years of age undergoing quantitative coronary angiography, we found that individuals homozygous for the C-type nos-3 gene have a higher risk of developing CHD than individuals with the two other genotypes, independently of the presence of other conventional risk factors. This finding is in line with two recent studies from Italy in which the same association has been found. Myocardial infarction, a frequent consequence of CHD, on the other hand, seems to be associated with the presence of the C allele in Japan but this is less certain in Caucasians. In this context, confounding by patient selection may be a decisive factor, because ‘control’ patients could have prominent atherosclerosis in their coronary arteries in the absence of myocardial infarction or other symptoms of CHD. Moreover, patients with CHD and/or a history of myocardial infarction have been excluded from previous association studies.

In the present study, the marked difference in CC genotype distribution between patients without and with CHD could be observed only by determining CHD status using quantitative coronary angiography. As manifest CHD does not necessarily lead to an acute coronary syndrome, it cannot be decided at present whether the CC genotype also predisposes to myocardial infarction in addition to CHD. The only appropriate way to finally address this question may be a prospective long-term analysis of angiographically controlled volunteers.

Interestingly, analysis of the second SNP of the nos-3 gene frequently reported to be associated with CHD and myocardial infarction, the G to T transition at position 894 in the coding region of the gene, did not reveal a significant accumulation of the T-allele in CHD-positive patients. In our study population, therefore, the $^{986}G/T$ SNP of the nos-3 gene does not seem to play a major role in the development of CHD. Moreover, the lack of effect of the T-allele on NO-dependent relaxation ex vivo argues against a potential linkage between the $^{786}C/T$ and the $^{986}G/T$ SNP in this regard.

Conclusions

The observed association of the $^{786}CC$ genotype of the nos-3 gene with endothelial dysfunction and increased CHD risk suggests that shear stress-dependent maintenance of NOS-3 expression in vivo is a key determinant for the development of cardiovascular complications. The decreased capacity of the endothelium of CC carriers to generate NO is likely to promote the early phase of atherosclerosis and, as a consequence, accelerate plaque formation not only in the heart but also at other clinically important sites.

The molecular mechanism underlying this insensitivity of NOS-3 expression to shear stress likely comprises binding of an inhibitory DNA-binding protein to the C-type promoter of the nos-3 gene. Since about 12% of the Caucasian population is homozygous for this defect, genotyping of young patients may provide an option for a more effective health management by increasing the awareness and improving the conventional risk factor profile in affected individuals.

Acknowledgments

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