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Endothelial nitric oxide synthase genotype modulates the improvement of coronary blood flow by pravastatin: a placebo-controlled PET study

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Abstract The objective was to study whether coronary blood flow or its response to pravastatin are affected by genetic variation in the endothelial nitric oxide synthase (eNOS) gene. Vascular endothelial nitric oxide maintains endothelium-dependent vasodilatation and also mediates antithrombotic actions. Its formation is catalyzed by eNOS, a constitutive enzyme, which has a polymorphic site in intron 4 (4a/b). Some clinical studies have suggested an association of the rare a-allele of eNOS with coronary artery disease and myocardial infarction. We carried out a double-blind placebo-controlled study involving 43 men (aged 35 ± 4 years), who were randomized to receive either 40 mg/day pravastatin ($n=21$) or placebo ($n=22$) for 6 months. Myocardial blood flow was measured by positron emission tomography (PET) using ^{15}O -labeled water. PET was performed at rest and after stimulation by adenosine infusion. PET and lipid analyses were carried out at baseline and after 6 months.



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eNOS genotyping was done by PCR. At baseline there were no differences in basal or adenosine-stimulated coronary blood flow between subjects with either eNOS bb or ba genotypes. At the end of the study genotypes reacted differently between pravastatin and placebo groups with respect to the change in adenosine-stimulated flow (ANCOVA $P=0.008$). More specifically, after pravastatin treatment the adenosine-stimulated flow increased by 54.5% in men with the eNOS ba genotype, whereas in the men with the bb genotype no significant change in flow was observed ($P=0.002$ for ba versus bb). In the placebo group there were no significant changes in blood flow from the baseline values ($P=0.916$ for ba versus bb). After pravastatin treatment both genotype groups showed a similar decrease in serum total cholesterol and low-density lipoprotein cholesterol ($P<0.00001$ for both). Our results suggest that adenosine-stimulated

myocardial perfusion improves after treatment with pravastatin in subjects with the eNOS ba genotype but not in those with the bb genotype. This effect is not dependent on the decrease of serum cholesterol.

Keywords Positron emission tomography · Endothelium · Atherosclerosis · Endothelial nitric oxide synthase · Genetics

Abbreviations *ANCOVA*: Analysis of covariance · *eNOS*: Endothelial nitric oxide synthase · *BMI*: Body mass index · *CAD*: Coronary artery disease · *LDL*: Low density lipoprotein · *PET*: Positron emission tomography

Introduction

Vascular endothelial cells produce nitric oxide (NO), which is a major contributor to vasodilatation. NO is synthesized from L-arginine by the endothelial isoform of nitric oxide synthase (eNOS). After synthesis, NO diffuses into the arterial wall and causes relaxation of vascular tone through its effects on vascular smooth muscle cells. NO increases intracellular cyclic guanosine-3',5'-monophosphate concentrations by stimulating soluble guanylate cyclase, which results in smooth muscle cell relaxation [1]. The effects mediated by cGMP also include inhibition of smooth muscle cell proliferation, platelet aggregation, monocyte adhesion and leukocyte migration. Thus, loss of endothelium-derived nitric oxide would be expected to promote atherogenesis [2].

Although eNOS is a constitutive enzyme and there is a continuous basal synthesis of NO by the endothelium, its activity and release may be altered. Hemodynamic shear stress is known to enhance NO release [3], and vasorelaxation by certain chemical agents, such as acetylcholine, is mediated by endothelial NO. Impaired vasorelaxation after acetylcholine administration has been shown to occur in atherosclerosis. This correlates with the extent of coronary artery disease (CAD) [4].

Statins appear to have a number of effects beyond their lipid lowering activity. Statin therapy improves coronary function in CAD patients [5, 6, 7]. Statins up-regulate the expression and activity of eNOS [8]. Simvastatin increases coronary blood flow and coronary vasodilatation by acetylcholine in dogs [9]. It is thus possible that some of the clinical benefits of statins may involve NO.

Adenosine has been widely used to measure coronary vasoreactivity [10]. Approximately half of the adenosine-induced vasodilatation is endothelium-dependent [11] and the rest by direct actions on the smooth muscle cells of the arterial wall. Thus, the response to adenosine is an integrating measure of endothelial function and vascular smooth muscle relaxation [12].

Coronary reactivity and myocardial perfusion can be measured with PET. These studies have shown that the classical risk factors of CAD, such as diabetes, hypertension and dyslipidemia, reduce coronary reactivity [13, 14, 15]. Reactivity of the endothelium and susceptibility

to atherosclerosis might also be influenced by genetic factors controlling the function of the endothelial cell. One candidate for explaining endothelial dysfunction is the eNOS gene. The eNOS gene has a common polymorphism in intron 4 (4a/b), and some clinical studies have suggested an association of the a-allele with CAD and myocardial infarction (MI) [16, 17, 18], but also opposite results have been reported [19, 20, 21, 22].

If statins involve endothelial NO synthesis in their mode of action, it is possible that their effect on coronary reactivity could be influenced by the genotype of eNOS. The purpose of the present study was to relate eNOS genotype to myocardial blood flow indices as measured by PET in healthy male subjects and to study whether the response of coronary function to pravastatin treatment is related to the eNOS genotype.

Methods

Subjects and study design

Fifty-one men undergoing routine physical examination were invited to participate in the study. The inclusion criteria were: (1) age 25–40 years, (2) clinically healthy, and (3) no continuous medication or use of antioxidant vitamins. The study was randomized, double blind and placebo controlled (placebo, $n=26$ and pravastatin 40 mg/day, $n=25$, for 6 months). Pravastatin (Pravachol) tablets and matching placebos were provided by Bristol-Myers Squibb (Espoo, Finland). Family history of CAD, alcohol and caffeine consumption, medications, smoking and exercise habits were recorded using a questionnaire. There were three recent smokers and six ex-smokers. Study participants were instructed to adhere to their normal diet during the study. The study protocol was approved by the Joint Ethics Committee of the Turku University and the Turku University Central Hospital. Each subject gave written informed consent.

Laboratory measurements

The subjects fasted overnight before collection of the blood samples for biochemical analyses before and after the treatment period. Plasma triglycerides and total and high-density lipoprotein (HDL) cholesterol concentrations were analyzed by a Cobas Integra 700 automatic analyzer with reagents and calibrators recommended by the manufacturer (Hoffmann-La Roche, Basel, Switzerland). LDL concentration was calculated using Friedewald's formula [23]. All samples were collected and stored in a similar way and analyzed in a randomized order.

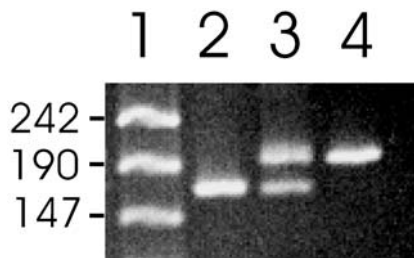


Fig. 1 Electrophoresis picture of the eNOS 4 a/b PCR genotyping visualized by ethidium bromide staining on a 3% Metaphor agarose gel. Lane 2: aa genotype; lane 3: ba genotype; lane 4: bb genotype. Lane 1: *MspI* digest of pUC19 DNA. Numbers on the left refer to bp (eNOS endothelial nitric oxide synthase)

DNA extraction and eNOS genotyping

eNOS genotyping was done without any knowledge of the patient's treatment group or PET measurements. DNA was isolated from leukocytes using a commercial kit (Qiagen, Calif.). Primers for DNA amplification were designed according to published sequence of the human NOS3 gene (NCBI/ D26607). The predicted length of the repeats was 167 bp for the aa genotype and 194 bp for the bb genotype. DNA was amplified in a 50- μ l reaction consisting of 50 pmol of both primers (5'-TTA TCA GGC CCT ATG GTA GT-3'; forward; 5'-AAC TCC GCT CAG CTG TCC T-3'; reverse), 200 μ M each of NTPs and 1.0 U Dynazyme II DNA polymerase in 1x reaction buffer (Finnzymes Oy, Finland). PCR conditions were: 5 min of denaturation at 94°C followed by 41 cycles of 30 s at 94°C, 30 s of annealing at 50°C and 1 min of extension at 72°C and a final extension time of 7 min at 72°C. The PCR products were resolved in 3% Metaphor agarose gel (FMC BioProducts, Denmark) using *MspI* digest of pUC19 DNA as bp marker (MBI Fermentas, Germany) (Fig. 1).

PET protocol and calculation of blood flow

PET studies were done at baseline and at the end of the treatment period. All PET studies were performed after the subject had fasted for 6 h. Alcohol and caffeine were prohibited for 12 h before the study. The detailed protocol has been described in detail previously [13]. In short, myocardial perfusion was measured twice: once at rest and once after administration of adenosine. Heart rate and blood pressure, as well as electrocardiogram, were monitored throughout the studies to calculate the rate-pressure product; $1,630 \pm 115$ MBq [^{15}O]H₂O was injected intravenously over 2 min. To determine blood flow, large regions of interest (ROI) were placed over representative transaxial images of the left ventricle and values of regional myocardial blood flow (expressed as $\text{ml} \times \text{g}^{-1} \times \text{min}^{-1}$) were calculated according to a previously published method employing the single compartment model [24, 25]. Since there were no differences of myo-

cardial blood flow in the different regions, global left ventricular blood flow was used for further analyses.

Statistical methods

Data analysis was carried out by analysis of covariance (ANCOVA). The association between eNOS gene genotype (eNOS 4a/b) and myocardial blood flow parameters was analyzed with ANCOVA adjusted for age, body-mass index (BMI) and LDL cholesterol. The effect of pravastatin treatment was calculated from the change in myocardial blood flow values between the two time points (baseline and after 6 months of treatment). The change was used as the dependent variable in the ANCOVA model, in which the eNOS genotype and treatment group (pravastatin or placebo) were used as factors, and age, BMI and the change of LDL cholesterol as covariates. In case of a significant interaction, least significant difference (LSD) post-hoc tests were performed. The power of the test measuring the differences in adenosine stimulated blood flow according to the eNOS genotypes was 78%. To analyze the change in serum lipids, ANCOVA for repeated measurements was performed separately for the pravastatin and placebo groups. Differences in clinical characteristics between different eNOS genotype and treatment groups were tested by ANOVA or Pearson's χ^2 test. Values are mean \pm standard deviation (SD). The level of significance was set at $P < 0.05$.

Results

The final statistical analyses were carried out from the results of 43 men (22 in the placebo and 21 in the pravastatin group) out of the 51 men initially selected for the study. Three men in the placebo group and 4 men in the pravastatin group had to be rejected because of technical problems with PET measurements. After that, there was only one man with the aa genotype, who was therefore rejected from the placebo group. The mean age of the remaining 43 subjects was 35.1 ± 4.0 years and their mean BMI was 24.9 ± 2.4 kg/m². At baseline serum total cholesterol levels were normal or mildly elevated (5.5 ± 0.8 mmol/l). All subjects had normal electrocardiograms both at rest and during adenosine infusion. All blood flow measurements were considered normal, suggesting that study subjects were free of coronary atherosclerosis detectable with the PET technique.

eNOS genotype, clinical characteristics and serum lipids

The overall allele frequencies in the initial study population were 0.70 for bb, 0.27 for ba, and 0.03 for aa, which are comparable to the frequencies in Finnish population studies [21]. Because of the low frequency for the aa genotype, only the ba and bb groups were compared.

Table 1 Clinical characteristics of eNOS genotype groups (eNOS endothelial nitric oxide synthase)

	Pravastatin		Placebo		P value
	bb (n=16)	ba (n=5)	bb (n=15)	ba (n=7)	
Age (years)	35.5±3.8	36.2±2.8	35.4±4.1	32.3±4.4	0.24*
Body mass index (kg/m ²)	25.4±3.1	25.0±1.4	24.1±1.4	25.7±2.1	0.38*
Systolic blood pressure (mmHg)	131±16	135±15	122±8	130±11	0.14*
Diastolic blood pressure (mmHg)	76±8	79±9	73±5	73±8	0.27*
Use of coffee (cups/day)	4.4±2.8	6.0±2.3	4.5±2.5	3.3±1.4	0.32*
Smokers (yes/ex-smoker/no)	2/3/11	0/2/3	1/1/13	0/1/6	0.68**
Alcohol consumption (drinks/week)	4.1±5.1	4.7±2.9	3.5±2.5	4.5±5.4	0.93*
Exercise (times/week)	2.6±1.3	2.1±1.0	3.2±1.9	2.2±1.6	0.39*
Family history of CAD (yes/no)	4/12	2/3	2/13	1/6	0.95**

*One-way analysis of variance or ** Pearson χ^2 test was used

Table 2 Basal and adenosine-stimulated myocardial blood flow by treatment and eNOS genotype. Values are mean ±SD

	Pravastatin			Placebo		
	bb	ba	P values*	bb	ba	P values*
	(n=16)	(n=5)	eNOS Time eNOS*Time	(n=15)	(n=7)	eNOS Time eNOS*Time
Total cholesterol (mmol/l)			0.34 <0.00001 0.84			0.09 0.62 0.17
Baseline	5.59±0.98	5.95±0.40		5.51±0.59	5.02±0.69	
6 months	4.35±0.79	4.78±0.99		5.37±0.75	5.30±0.67	
LDL cholesterol (mmol/l)			0.17 <0.00001 0.68			0.13 0.90 0.36
Baseline	3.70±0.81	4.08±0.32		3.53±0.50	3.24±0.67	
6 months	2.42±0.63	2.94±0.90		3.44±0.60	3.36±0.76	
HDL cholesterol (mmol/l)			0.51 0.19 0.54			0.97 0.65 0.44
Baseline	1.32±0.26	1.23±0.34		1.46±0.36	1.39±0.22	
6 months	1.36±0.29	1.34±0.24		1.44±0.31	1.45±0.19	
Triglycerides (mmol/l)			0.78 0.10 0.18			0.39 0.31 0.059
Baseline	1.27±0.80	1.40±0.37		1.14±0.56	0.85±0.24	
6 months	1.25±0.88	1.10±0.29		1.07±0.54	1.07±0.36	

*Two-way repeated measures ANCOVA with eNOS genotype and time as factors, adjusted for age and BMI. Analyses were performed separately for the pravastatin and placebo groups

There were no significant differences in clinical characteristics between the subjects with different eNOS genotypes or between different treatment groups (Table 1). Treatment with pravastatin for 6 months reduced serum total cholesterol and LDL cholesterol significantly ($P<0.00001$ for both) and independently of the eNOS genotype (Table 2). Pravastatin had no significant effect on serum HDL cholesterol or triglyceride concentrations. Placebo did not affect serum lipids.

eNOS genotype and myocardial blood flow before treatment

At baseline, there was no significant association between eNOS genotype and basal ($P=0.28$) or adenosine-stimulated blood flow ($P=0.30$) among all 43 subjects. The values of adenosine-stimulated flow were similar in men with bb (3.5 ± 0.9 , $n=31$) and in men with ba genotypes

(3.1 ± 0.7 , $n=12$). The results were adjusted for the effects of age, BMI and LDL cholesterol.

eNOS genotype and the response of myocardial blood flow to pravastatin

At the end of the study genotypes reacted differently between pravastatin and placebo groups with respect to the change in adenosine-stimulated flow (ANCOVA $P=0.008$) (Table 3). Pravastatin did not induce any significant change in basal myocardial blood flow but enhanced adenosine-stimulated flow genotype dependently. After pravastatin treatment the adenosine-stimulated flow increased by 54.5% in men with the eNOS ba genotype, whereas in the men with the bb genotype no significant change in flow was observed ($P=0.002$ for ba versus bb). In the placebo group there were no significant changes in blood flow from the baseline values ($P=0.916$ for ba versus bb).

Table 3 Basal and adenosine-stimulated myocardial blood flow by treatment and eNOS genotype. Values are mean \pm SD

	Pravastatin		Placebo		ANCOVA <i>P</i> values*		
	bb (<i>n</i> =16)	ba (<i>n</i> =5)	bb (<i>n</i> =15)	ba (<i>n</i> =7)	eNOS	Group	eNOS*Group
Basal flow (ml \times g ⁻¹ \times min ⁻¹)							
Baseline	0.89 \pm 0.28	0.88 \pm 0.12	0.81 \pm 0.16	0.66 \pm 0.07			
6 months	0.88 \pm 0.21	0.96 \pm 0.36	0.72 \pm 0.15	0.68 \pm 0.19			
Δ	-0.01 (-1.1%)	0.08 (9.1%)	0.09 (-11.1%)	0.02 (0.3%)	0.22	0.57	0.870
Adenosine-stimulated flow (ml \times g ⁻¹ \times min ⁻¹)							
Baseline	3.76 \pm 1.06	3.10 \pm 0.56	3.22 \pm 0.71	3.10 \pm 0.75			
6 months	3.47 \pm 1.15	4.79 \pm 1.43	3.13 \pm 0.78	3.08 \pm 0.81			
Δ	0.29 (-7.6%)	1.69 (54.5%)	0.09 (-2.8%)	0.02 (-0.6%)	0.051	0.017	0.008

*ANCOVA with eNOS genotype and treatment group as factors and change as dependent variable, adjusted for age, BMI and the change of LDL cholesterol

Discussion

This randomized placebo-controlled double-blind study shows that the effect of pravastatin on coronary artery reactivity in young adults is eNOS genotype dependent. We show that adenosine-stimulated myocardial blood flow increased in men with the ba genotype but not in men with the bb genotype. The validity of the present findings is strengthened by the fact that the study population was homogeneous. All subjects were male with age range of 26–40 years and had no clinical symptoms of any disease. Changes in lipid levels did not explain the differences.

In the myocardium, adenosine is released in small amounts at constant basal rate during normoxia. During ischemia the production of endogenous adenosine increases several fold, due to breakdown of adenosine triphosphate, and leads to coronary vasodilatation [26]. We report that subjects with the eNOS a-allele that have been treated with pravastatin show significantly improved coronary vasodilation by exogenously administered adenosine compared to subjects with the bb genotype. This potentiating effect on vascular relaxation may also have clinical significance in subjects with myocardial ischemia and increased endogenous adenosine.

Statin therapy has been shown to improve coronary function in CAD patients [5, 6, 7]. This may be partly explained through an increase in NO production, independent of the cholesterol-lowering effect [27]. The regulation of eNOS activity has been demonstrated to involve complex mechanisms at the transcriptional, post-transcriptional and posttranslational level [3]. Caveolae are invaginations of the plasma membrane and the localization of eNOS within caveolae renders the enzyme inactive. Statins may increase eNOS activity by decreasing caveolin-1 and its heterocomplex with eNOS [28]. Statins have also been suggested to up-regulate eNOS expression and activity through inhibiting a small GTP-binding protein, Rho [29]. However, the precise mechanisms of how statins regulate eNOS are likely to be even more complex. The mechanism by which pravastatin en-

hances adenosine-stimulated vasodilatation remains unclear.

The a-allele of the eNOS gene has been reported to be related to the severity of CAD and risk for MI [16, 17, 18], but these associations have not been confirmed in some other studies [19, 20, 21, 22]. In fact, we have recently found that the a-allele has an association with reduced risk of coronary artery disease and myocardial infarction [30]. The a-allele may also somehow mediate positive actions of pravastatin on coronary function, since in our present study population of healthy males, the ba-genotype carriers seemed to respond to pravastatin, while no effects were detected in the bb-genotype carriers.

In addition to the 4 a/b polymorphism, there are two other widely analyzed genetic variations in the eNOS gene, namely the Glu298Asp polymorphism of exon 7 and the T(-786)C mutation in the 5'-flanking region. A number of studies on these variants in different populations have also yielded contradictory results, either confirming their detrimental role in vascular diseases or failing to detect an association. There is presently no clear evidence about the functionality of any of the eNOS polymorphisms in vivo [31]. Although the 4ab repeat is located in an intron, it does not rule out its functional relevance, since among healthy populations the a-allele carriers had increased amount of NO as measured by its metabolites (nitrite and nitrate) [32]. This finding further supports the protective role of the a-allele in vascular diseases.

In conclusion, we found that pravastatin treatment improves coronary reactivity by a mechanism that is eNOS genotype dependent. The eNOS a-allele together with pravastatin treatment can modulate coronary reactivity by increasing NO production by the endothelium or by affecting some other mechanism in the coronary artery wall itself.

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