Acta Biochimica Polonica

Vol. 53 No. 2/2006, 299–302

on-line at: www.actabp.pl

Regular paper

Allelic polymorphism of endothelial NO-synthase gene and its functional manifestations

Victor E. Dosenko[1]✉, Vyacheslav Y. Zagoriy[1], Nikolay V. Haytovich[2], Olga A. Gordok[2] and Alexey A. Moibenko[1]

[1]*Department of Experimental Cardiology, Bogomoletz Institute of Physiology, Kiev, Ukraine;* [2]*Department of Pediatrics, Bogomoletz National Medical University, Kiev, Ukraine;*
✉*e-mail: dosenko@biph.kiev.ua*

Received: 18 May, 2005; revised: 07 April, 2006; accepted: 05 May, 2006
available on-line: 30 May, 2006

Investigation of the mechanisms of phenotypic realization of allelic polymorphism of the eNOS gene has shown that the level of eNOS mRNA and activity of this enzyme in platelets depends from genotype. We identified a $T^{-786}\rightarrow C$ polymorphism in the promoter region, a variable number of tandem repeats (4a/4b) in intron 4 and the $G^{894}\rightarrow T$ polymorphism in exon 7 of the eNOS gene in isolated human platelets. We measured eNOS mRNA in isolated platelets by reverse transcription-PCR and eNOS enzyme activity by fluorimetric detection system FCANOS-1 using diaminofluorescein diacetate (DAF-2A). It was shown that the level of eNOS mRNA is the lowest for the –786C/C promoter genotype. In exon 7 homozygotes (894T/T) the level of RNA is lower than in normal homozygotes (894G/G), but higher than in heterozygotes (894G/T). The eNOS activity in platelets is lower in carriers of the 786C/C promoter genotype than in normal homozygotes (2.1 times; $P=0.03$), and lower comparing to heterozygotes (2.9 times; $P>0.05$). The eNOS activity accompanying the 894T/T variant of exon 7 is also lower than in normal homozygotes ($P>0.05$). Regarding the polymorphism in intron 4 – the enzyme's activity is lower in carriers of the 4a/4a genotype comparing to normal homozygotes (1.7 times; $P>0.05$) and lower than in heterozygotes (1.9 times; $P>0.05$). These results allow one to conclude that the $T^{-786}\rightarrow C$ polymorphism of the eNOS gene promoter most significantly affects the gene expression and eNOS activity.

Keywords: endothelial nitric oxide synthase, allelic polymorphism, RNA expression, activity of nitric oxide synthase, platelets

INTRODUCTION

Allelic polymorphism of endothelial NO-synthase (eNOS) increases the risk of development of cardio-vascular diseases (Hingorani *et al.*, 1999; Yoshimura *et al.*, 2000; Dosenko *et al.*, 2002; 2005; Wang *et al.*, 2002; Casas *et al.*, 2004). But it is not clearly elucidated how the gene polymorphism affects the gene expression and enzyme activity in the cells. Studies dealing with this problem using different methodological approaches have not clarified the question (Yoon *et al.*, 2000; Golser *et al.*, 2003, Song *et al.*, 2003). What mechanism of the phenotypic expression of pathologic allelic variants of the eNOS gene is the major cause of NO production decrease is not known. It may be explained either in terms of the NO-synthase functional or quantitative deficiency (abnormalities of transcription, mRNA stability, formation of catalytically defective protein) or by enhanced degradation of the enzyme. We therefore analyzed eNOS gene expression and NO-synthase activity in platelets isolated from individuals with genotyped promoter ($T^{-786}\rightarrow C$ polymorphism), exon 7 ($G^{894}\rightarrow T$ polymorphism) and intron 4 (4a/4b polymorphism) to elucidate these parameters in conditions similar to physiological.

MATERIALS AND METHODS

Blood platelet isolation. Venous blood was taken from 30 teenagers with essential hyperten-

Abbreviations: DAF-2A, 4,5-diaminofluoresceine diacetate; NOS, nitric oxide synthase; NO, nitric oxide; NO_X, NO_2^- and NO_3, nitrate and nitrite; RT-PCR, reverse transcription PCR.

sion, who were treated at the clinics of Pediatric Department No. 4 of Bogomoletz National Medical University. The blood was taken in sterile conditions into 2.7 ml monovettes with EDTA potassium salt (11.7 mM) as an anticoagulant (Sarstedt), and for preventing adhesion and aggregation of platelets apirase (1 U/ml) was used. Isolation of platelets was performed in three stages: centrifuging (100 × *g*) of whole blood for 5 min (supernatant contained platelets and monocytes); centrifuging (400 × *g*) for 2 min (monocytes precipitate on the bottom, and platelets remain in suspension); centrifuging (900 × *g*) for 6 min with further resuspension in Tyrode buffer containing: 137 mM NaCl, 12 mM $NaHCO_3$, 2 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 5.5 mM glucose, 5 mM Hepes (pH 7.3), containing 0.35% bovine serum albumin (Oury *et al.*, 2002). Counting of platelets was performed using a Goryaev chamber.

Procedures with human subjects were approved by the regional bioethics committee at the Government of Ukraine.

DNA isolation and PCR. DNA was isolated from whole stabilized blood using a DNA-prep kit (Isogene). Using PCR with following analysis of the length of restriction fragments the $T^{-786}\rightarrow C$ polymorphism of the promoter, the $G^{894}\rightarrow T$ polymorphism of exon 7 and the 4a/4b polymorphism of intron 4 of eNOS gene were determined as described before (Hibi *et al.*, 1998; Wang *et al.*, 2000; Dosenko *et al.*, 2002). PCR was carried out using a GeneAmp System 2700 thermocycler (Applied Biosystems).

RNA isolation and measurement of eNOS mRNA. RNA isolation from platelets was performed using a Trizol RNA-prep kit (Isogen). Reverse transcription was performed using a RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas), using 500 μg of total RNA and a $(dT)_{18}$ primer. The single-strand DNA obtained was used for PCR using the following primers: upstream (sense)-5′-TCC CTG AGG AGG GCA GGC-3′ and down-stream (antisense)-5′-TGA GGG TCA CAC AGG TTC CT-3′. Amplification mixture contained 5 μl of 5 × PCR-buffer, 2.5 mM of magnesium sulfate, 200 μM mixture of four nucleotide triphosphates, 20 pM of each primer and 0.5 U of *Taq* polymerase (AmpliSense), the volume was brought up to 25 μl with deionized water. Amplification of the fragment of exon 7 consisted of 35 cycles: denaturation 94°C, 1 min, primer annealing 64°C, 1 min, and elongation 74°C, 1 min. For controlling the quality of RNA isolation and comparison of intensity of the eNOS gene expression, a fragment of β-actin gene was amplified according to (Øvstebø *et al.*, 2003) as an internal control. Amplified fragments were separated in 2.5% agarose gel containing ethidium bromide. Visualization and estimation of the brightness of amplified fragments after horizontal electrophoresis (160 V during 40 min) was performed using transilluminator and Vitran software (Biocom).

eNOS activity. For an assay of eNOS activity we used a fluorimetric detection system FCANOS-1 (Sigma), based on the principle of fluorescence of triazolofluoresceine, which is formed after interaction of NO with 4,5-diaminofluoresceine, which is formed from 4,5-diaminofluoresceine diacetate (DAF-2A) under the action of intracellular esterases. The wavelength of excitation/emission was 492/515 nm. The NOS inhibitor diphenyliodonium chloride (100 μM) inhibited the reaction and this confirms the specificity of the NOS activity assay. Enzyme activity was evaluated in units of fluorescence (UF) per min per 10^6 cells. In mammals there are few cell types, which express only one isoform of NO-synthase. In platelets under normal conditions only endothelial NOS is expressed (Jayachandran & Miller, 2002; Randriamboavonjy *et al.*, 2004), which ensures that only the activity of this particular isoform was assayed.

Statistical analysis. Statistical analysis was performed with Excel 2000 and Origin 7.0. All data was expressed as the mean (±S.E.) of replicate experiments performed in each assay. Statistical differences were evaluated using the Student's *t*-test. $P<0.05$ was taken to indicate a statistically significant difference.

RESULTS

eNOS polymorphism frequency

In the case of the $T^{-786}\rightarrow C$ promoter polymorphism it turned out that 11 of 30 individuals had the T/T genotype, 13 had the T/C genotype and 6 had the C/C genotype. The $G^{894}\rightarrow T$ exon 7 polymorphism showed the following allelic distribution: 11 G/G, 14 G/T and 5 T/T. Intron 4 VNTR had the following distribution: 18 of 30 individuals had the 4b/4b genotype, 9 had the 4b/4a genotype and 3 had the 4a/4a genotype. The observed ratio of the frequencies of the allelic variants in general corresponds to such a ratio in the Ukrainian population (Dosenko *et al.*, 2005).

eNOS mRNA quantification by RT-PCR

We observed a dependence of eNOS mRNA quantity on the genotype (Figs. 1, 2). The eNOS mRNA level was the lowest in persons with the C/C promoter genotype. The pathologic homozygotes (T/T) of exon 7 had a lower eNOS mRNA level than normal homozygotes (G/G), but higher than the het-

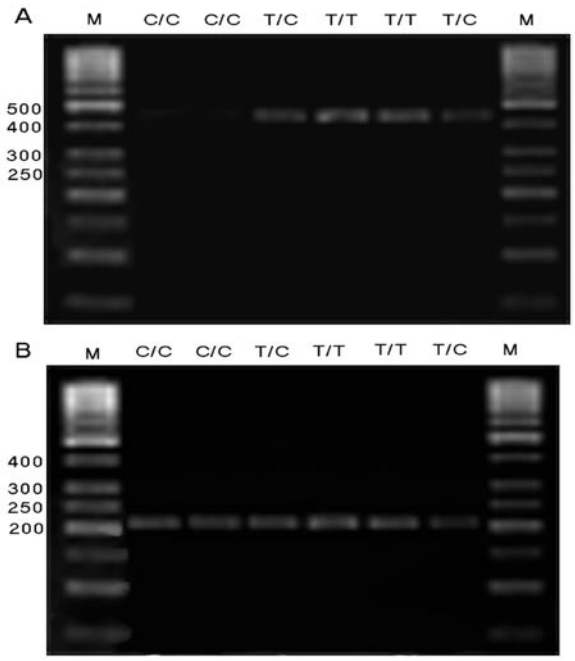

Figure 1. **Results of electrophoresis of amplified fragments of eNOS gene exon 7 (A) and β-actin gene (B)**.
The data show dependence of eNOS gene expression on the allelic variant of the $T^{-786}\rightarrow C$ promoter polymorphism. M, marker of molecular size (500, 400 etc. base pairs); T/T, normal homozygotes; T/C, heterozygotes; C/C, pathologic homozygotes.

erozygotes (G/T). eNOS mRNA concentration was higher in subjects with the 4a/4a genotype than in normal homozygotes and heterozygotes.

eNOS enzyme activity

The eNOS activity in platelets according to genotype is summarized in Fig. 3. The C/C promoter genotype gave a 2.1 times lower NO-producing activity than in normal homozygotes ($P=0.03$) and 2.9 lower than in heterozygotes ($P>0.05$). So, the data about gene expression entirely corresponds to the level of eNOS activity in relation to the promoter polymorphism. The pathologic genotype of exon 7 exhibits a lower eNOS activity than normal homozygotes, but the difference is not significant. The subjects with the 4a/4a genotype of intron 4 VNTR have a 1.7 times lower NO-producing activity comparing to normal homozygotes ($P>0.05$) and 1.9 times lower comparing to heterozygotes ($P>0.05$). There was no positive correlation between the eNOS gene expression and eNOS activity for intron 4 polymorphism. On the contrary, while eNOS expression was high in the 4a/4a genotype, the enzyme activity was low.

DISCUSSION

Different approaches used by investigators have permitted the establishing of main rules of expression, translation and catalytic activity of NOS depending on the NOS gene polymorphism. However, evident controversies remain: in the work of Golser *et al*. (2003) it was shown that the Glu/Asp polymorphism does not affect the protein's catalytic activity. The same conclusion was made by Song *et al*. (2003). But in both those studies investigation of pathologic Asp/Asp variant was absent, while it is this allelic variant, according to Casas *et al*. (2004), that is the most significant predictor of cardio-vascular diseases. According to our data, NOS gene expression is lower in subjects with the Glu/Asp and Asp/Asp genotype compared to the Glu/Glu one, while the enzyme's activity is not significantly changed. In any case, for elucidating the role of this polymorphism additional studies are needed. Nakayama *et al*. (1999) were the first to report about the loss of promoter activity upon a T→C substitution in position –786. But the results of population studies were ambiguous (Nakayama *et al*., 1999; Yoshimura *et al*., 2000; Casas *et al*., 2004). Using isolated platelets we managed to show that eNOS gene expression with the C/C genotype is significantly lower than in the case of the T/T and T/C allelic variants and the enzyme activity is significantly decreased, too.

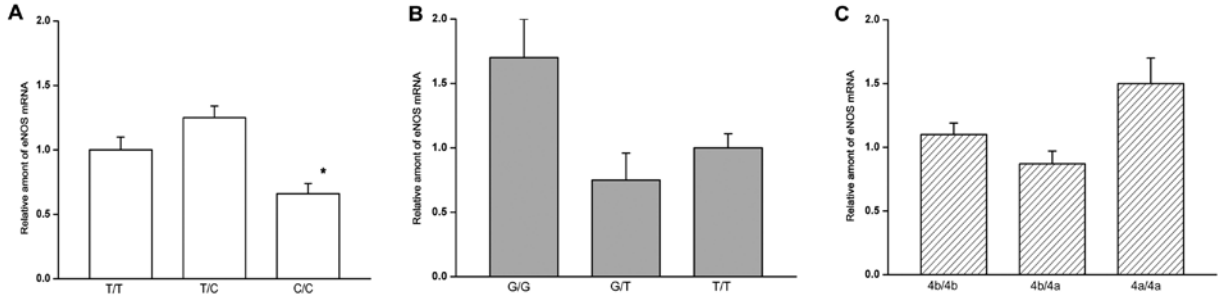

Figure 2. **Level of eNOS mRNA in isolated platelets at different allelic variants of the promoter (A), exon 7 (B) and intron 4 (C) of eNOS gene**.
Results are expressed as means (±S.E.; bars). Values of eNOS mRNA are given as the ratio of the intensity of the eNOS band to the β-actin band. *$P<0.05$ comparing to eNOS gene expression at the T/T promoter variant, obtained by the Student's *t*-test.

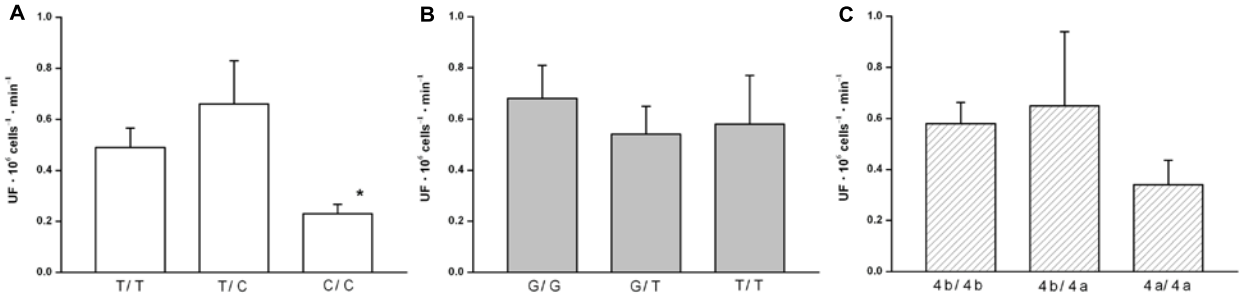

Figure 3. eNOS activity in isolated platelets at different allelic variants of the promoter (A), exon 7 (B) and intron 4 (C) of eNOS gene.
Results are expressed as means (±S.E.; bars). *$P<0.05$ comparing to eNOS activity at T/T promoter variant, obtained by the Student's *t*-test.

The obtained data explain to a certain extent the pathogenic meaning of eNOS gene promoter polymorphism. In case of disturbances of coronary blood flow in individuals with the pathologic variant of the promoter, eNOS gene expression is activated to lower extent, insufficient number of eNOS molecules are produced, thus resulting in an inability to produce additional NO. As a consequence, the vessels are dilated to a lesser extent, platelet adhesion and aggregation is not prevented and other mechanisms of the angio- and cardioprotective action of NO are inoperative as well.

REFERENCES

Casas JP, Bautista LE, Humphries SE, Hingorani AD (2004) Endothelial nitric oxide synthase genotype and ischemic disease: meta-analysis of 26 studies involving 23028 subjects. *Circulation* **109**: 1359–1365.

Dosenko VIe, Zahorii VIu, Moibenko OO, Parkhomenko OM (2002) Pathophysiologic aspects of endothelial NO-synthase genetic polymorphism. *Fiziol Zh* **48**: 86–102 (in Ukrainian).

Dosenko VIe, Lutay YaM, Parkhomenko OM, Moibenko OO (2005) Endothelial NO-synthase allelic polymorphism frequency in patient with acute coronary syndrome in Ukrainian population. *Tsytol Genet* **2**: 48–54 (in Ukrainian).

Golser R, Gorren ACF, Mayer B, Schmidt K (2003) Functional characterization of Glu298Asp mutant human endothelial nitric oxide synthase purified from a yeast expression system. *Nitric Oxide* **8**: 7–14.

Hibi K, Ishigami T, Tamura K, Mizushima S, Nyui N, Fujita T, Fujita T, Ochiai H, Kosuge M, Watanabe Y, Yoshii Y, Kihara M, Kimura K, Ishii M, Umemura S (1998) Endothelial nitric oxide synthase gene polymorphism and acute myocardial infarction. *Hypertension* **32**: 521–526.

Hingorani AD, Liang CF, Fatibene J, Lyon A, Monteith S, Parsons A, Haydock S, Hopper RV, Stephens NG, O'Shaughnessy KM, Brown MJ (1999) A common variant of the endothelial nitric oxide synthase (Glu298→Asp) is a major risk factor for coronary artery disease in the UK. *Circulation* **100**: 1515–1520.

Jayachandran M, Miller VM (2002) Ovariectomy upregulates expression of estrogen receptors, NOS, and HSPs in porcine platelets. *Am J Physiol Heart Circ Physiol* **283**: H220–H226.

Nakayama M, Yasue H, Yoshimura M, Shimasaki Y, Kugiyama K, Ogawa H, Motoyama T, Saito Y, Ogawa Y, Miyamoto Y, Nakao K (1999) T-786→C Mutation in the 5'-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. *Circulation* **99**: 2864–2870.

Oury C, Toth-Zsamboki E, Vermylen J, Hoylaerts MF (2002) P2X(1)-mediated activation of extracellular signal-regulated kinase 2 contributes to platelet secretion and aggregation induced by collagen. *Blood* **100**: 2499–2505.

Øvstebø R, Haug KBF, Lande K, Kierulf P (2003) PCR-based calibration curves for studies of quantitative gene expression in human monocytes: development and evaluation. *Clin Chem* **49**: 425–432.

Randriamboavonjy V, Schrader J, Busse R, Fleming I (2004) Insulin induces the release of vasodilator compounds from platelets by a nitric oxide–G kinase–VAMP-3–dependent pathway. *J Exp Med* **199**: 347–356.

Song J, Yoon Y, Park KU, Park J, Hong YJ, Hong SH, Kim JQ (2003) Genotype-specific influence on nitric oxide synthase gene expression, protein concentration, and enzyme activity in cultured human endothelial cells. *Clin Chem* **49**: 847–852.

Wang J, Dudley D, Wang XL (2002) Haplotype-specific effects on endothelial NO synthase promoter efficiency: modifiable by cigarette smoking. *Arterioscler Thromb Vasc Biol* **22**: 1–4.

Wang XL, Sim AS, Wang MX, Murrell GA, Trudinger B, Wang J (2000) Genotype dependent and cigarette specific effects on endothelial nitric oxide synthase gene expression and enzyme activity. *FEBS Lett* **471**: 45–50.

Yoon Y, Song J, Hong SH, Kim JQ (2000) Plasma nitric oxide concentrations and nitric oxide synthase gene polymorphisms in coronary artery disease. *Clin Chem* **46**: 1626–1630.

Yoshimura M, Yasue H, Nakayama M, Shimasaki Y, Ogawa H, Kugiyama K, Saito Y, Miyamoto Y, Ogawa Y, Kaneshige T, Hiramatsu H, Yoshioka T, Kamitani S, Teraoka H, Nakao K (2000) Genetic risk factors for coronary artery spasm: significance of endothelial nitric oxide synthase gene T–786→C and missense Glu298Asp variants. *J Investig Med* **48**: 367–374.