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Genotype-Dependent Expression of Endothelial Nitric Oxide Synthase (eNOS) and Its Regulatory Proteins in Cultured Endothelial Cells

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Abstract

DNA polymorphisms in endothelial nitric oxide synthase (eNOS) gene have been shown to be associated with constitutive eNOS expression and coronary artery disease (CAD). In the present study we explored the hypothesis whether genotype-dependent effects can be maintained *in vitro* during replication, or the effect is conditional on *in vivo* biological environments. Human umbilical vein endothelial cells (HUVEC) were collected and cultured from 89 normal deliveries of Mexican Americans. The cells were treated with or without cigarette smoking extracts (CSE) and genotypes of eNOS polymorphisms were determined by PCR. We measured the levels of eNOS by ELISA and its binding proteins including heat-shock protein 90 (Hsp-90) and caveolin-1 by Western blotting. The rare C allele for the promoter T786C polymorphism (0.2), and the rare 4 × 27-bp repeat allele in the intron 4 (0.30) were different from those reported in other populations. Yet, the rare T allele in the exon 7 (G894T polymorphism) was similar as others. After four passages *in vitro*, both the intron 4 and promoter polymorphisms maintained significant effects on eNOS mRNA levels in HUVECs ($P < 0.05$). However, the effects on eNOS protein and enzyme activity were less consistent. Although primary smokers had significantly lower eNOS protein levels ($P < 0.05$), the *in vitro* CSE treatment on cultured HUVECs only resulted in a significant reduction in NO levels as measured by the stable metabolites of nitrite/nitrate ($P < 0.001$). Neither Hsp-90 nor caveolin-1—important eNOS regulators—appears to mediate the genotype-smoking effects on eNOS expression although HUVECs did produce more Hsp-90 when exposed to CSE. Our study demonstrates that endothelial cells maintain genotype-dependent expression even after the deprivation of *in vivo* environment. However, the cigarette smoking–genotype interaction may require such *in vivo* conditions to be manifested.

INTRODUCTION

NITRIC OXIDE (NO) is an endothelial vasodilator synthesized from L-arginine by the endothelial isoform of nitric oxide synthase (eNOS). Deficient eNOS has been implicated in the pathogenesis of hypertension, atherosclerosis, and preeclampsia (Celermajer *et al*., 1992, 1994; Cayatte *et al*., 1994; Seligman *et al*., 1994; Cockell and Poston, 1997; Forte *et al*, 1997). The eNOS-dependent NO release is regulated by a number of processes including cofactor association, phosphorylation, protein–protein interactions, and substrate availability. Furthermore, numerous studies at population and tissue levels have demonstrated that certain

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DNA variants at the *eNOS* gene may have constitutively low eNOS expression, and hence, render the carrier endothelial cells more susceptible to environmental insult.

A point mutation at nucleotide −786 bp (T-786-C), has been identified in the 5′-flanking region of the eNOS gene (Sim *et al*., 1998; Nakayama *et al*, 1999). This variant, which results in a significant reduction in the eNOS gene promoter activity, has been associated with an increased risk for coronary spasm in a Japanese population (Nokayama *et al*., 1999). However, its association with CAD in other populations, such as Caucasians, is less consistent (Sim *et al*, 1998; Wang and Wang, 2000). Another variant, a 27-bp repeat polymorphism in intron 4 of the eNOS gene, has been associated with variations in plasma levels of nitrite and nitrate (NOx)-stable metabolites of NO (Wang *et al*., 1997; Tsukada *et al*., 1998). This polymorphism is associated with smoking-dependent risk of CAD in some studies but not others (Wang *et al*, 1996; Wang and Wang, 2000; Gardemann *et al*., 2002; Hwang *et al*., 2002). In addition, a G894T mutation in exon 7 of the eNOS gene, which leads to an amino acid change from Glu to Asp (Glu298Asp), is associated with reduced basal NO production (Veldman *et al*., 2002). The prevalence of the G894T polymorphism has been investigated in patients with CAD with controversial results (Yoshimura *et al*., 1998; Hingorani *et al*, 1999; Wang and Wang, 2000; Granath *et al*., 2001). Our previous studies point out that the T-786C and the 27-bp repeat in intron 4 could be functional in quantitative eNOS regulation, which is further modifiable by cigarette smoking (Wang *et al*., 2002).

In the present study we have explored the hypothesis of whether the genotype-dependent effect can be maintained *in vitro* during cellular replication, or the effect is conditional on *in vivo* biological environments. We have further investigated the possible roles of other eNOS regulatory proteins, such as heat-shock protein 90 (Hsp-90) and caveolin-1, in this genotype-dependent eNOS regulation.

MATERIALS AND METHODS

Culture of human umbilical venous endothelial cells (HUVEC)

We collected human umbilical cords from 89 normal deliveries, either vaginal or Caesarian section. A signed consent was obtained, and histories of diabetes, hypertension, and smoking were recorded. The cord was then assigned a lab code before transported in cold DMEM to the laboratory within 4–6 h. Venous endothelial cells were isolated from human umbilical veins by collagenase treatment of the vessels in a method described previously (Wang *et al*., 2001). HUVECs were cultured in DMEM containing 20 mg/l bovine endothelial cell growth factor (bECGF, Roche, Indianapolis, IN)), 100 mg/l heparin, 15 mmol/l HEPES, penicillin (50 IU/l), streptomycin (50 mg/l), $NaHCO_3$ (44 mmol/l), and 20% fetal calf serum, in a 5% CO_2/air atmosphere. Cells were cultured up to fourth passage before they were exposed to 0.01 cigarette equivalent/ml culture medium of cigarette smoke extracts (CSE) as described by Wang *et al*. (2001). After 4-h exposure, both CSE-treated and nontreated HUVECs were collected for DNA, RNA, and protein extraction. The culture medium was collected for NOx measurements. The study was approved by the IRB of the University of Texas Health Science Center at San Antonio and Baylor College of Medicine, Houston, TX.

DNA extraction and genotyping

Genomic DNA was extracted from the control and CSE-treated HUVEC by genomic DNA isolation reagent–DNAzol (Invitrogen Life Technologies, Carlsbad, CA) and used for the detection of following genotypes.

T786 → C polymorphism in the promoter region—T786 → C polymorphism was determined by polymerase chain reaction (PCR)-restriction fragment length polymorphism as

described previously (Sim *et al*., 1998). PCR was performed with primer pairs designed to amplify a 495-bp fragment containing the promoter (T786 → C) variant (sense, 5′-GAGGTCTCGAAAT-CACGAGG-3′; antisense, 5′-ATACAAGAACTCCTG-GATCC-3′). The PCR product was digested with *Msp*I, which cuts the PCR product when the T at position −786 bp is replaced by a C. Digested samples were separated on 8% non-denaturing polyacrylamide gel and visualized by silver staining.

27-bp repeat polymorphism in intron 4—We determined the 27-bp repeat polymorphism as described previously (Wang *et al*., 1996). We used primers that flank the region of the 27-bp repeat in intron 4 of the eNOS gene (located from 5111 to 5130 bp). The forward primer was 5′-AGGCCCTATGGTAGTGC-CTTT-3′ and the reverse primer was 5′-TCTCTTAGTGCT-GTGGTCAC-3′. The PCR products were separated by electrophoresis in 8% nondenaturing polyacrylamide gel and visualized by silver staining.

Glu-298-Asp mutation at exon 7—For the Glu-298-Asp mutation at exon 7, we amplified the region of the eNOS gene with the forward and reverse primers as 5′-AAGGCAGGA-GACAGTGGATG-3′ and 5′-CAGTCAATCCCTTTGGT-GCT-3′ as described by Cai et al. (1999). The amplification was performed in 25-μl volume containing 100 ng DNA, 20 pmol of each primer, 2.1 mmol/l $MgCl_2$, 50 mmol/l KCI, 25mmol/l dNTP, 5 mmol/l Tris-HCI (pH 8.3), and 1 U Taq polymerase. Samples were subjected to denaturing at 94°C for 5 min, 30 cycles of denaturing at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1.5 min, and a final extension at 72°C for 5 min. The 246-bp PCR products were digested with a *Dpn*II restriction enzyme (recognition site: 5′ . . . ↓GATC . . . 3′) and electrophoresed in 8% poly-acryl-amide gel for 1 h before the gel was silver stained. This resulted in two fragments (158 and 88 bp) when the restriction site created by the 894G-T transition (5′-GAG/TC-3′) was present. The genotypes were identified as GG, TG, and TT.

eNOS activity measurements

After treatment, the cells were collected and lysed using Tris-EDTA buffer with protease inhibitor and the cell lysates were used for the estimation of total eNOS activity as we described previously (Wang *et al*., 2000). The final result was expressed as cpm/μg total protein. The protein concentration of cell lysate was determined using the Bradford method (Sigma, St. Louis, MO; Cat# B-6916).

Determination of eNOS protein levels

We used ELISA to determine the protein levels of eNOS from the protein extracts of the endothelial cells treated by various conditions (Wang *et al*., 2003). The method was based on a sandwich antibody detection mechanism (R&D systems, Minneapolis, MN). The eNOS protein levels were expressed as pg/μg of total protein in endothelial extracts. We also confirmed that the anti-eNOS antibody used in the ELISA only reacted with a single eNOS band from the endothelial cell extracts.

Measurement of eNOS mRNA

After the experimental period total RNA was isolated using the Trizol reagent (Invitrogen). Gene expression quantification was performed using a two-step real-time quantitative RT-PCR in which the PCR step is coupled with a 5′ fluorogenic nuclease assay (Aida *et al*., 2004). An Assays-on-Demand kit specifically designed for human eNOS mRNA (Applied Biosystems, Faster City, CA) was used following the manufacturer's protocol in an ABI 7700 analyzer. The mRNA levels were expressed as an arbitrary unit according to standard RNA preparation with known RNA levels. Beta actin was used as a housekeeping gene.

Determination of nitrite and nitrate (NOx) levels in the culture medium

At the end of each experimental period the culture media were collected and the amount of NOx metabolites (NOx) was determined by a modified Griess reaction (Cheang *et al*., 1999). Briefly, the nitrate in the culture media was first converted to nitrite by NADPH-dependent nitrate reductase. The Griess reaction was initiated by the addition of 1% (w/v) sulfanilamide and 0.1% (w/v) N-(1-naphthyl)-ethylenediamine. The absorbance of the reaction mixture was measured at 540 nm and the NOx levels were expressed as μM/μg of total cellular protein where the medium was collected for NOx levels.

Measurement of caveolin-1 and heat-shock protein-90 (Hsp-90) in HUVEC

Endothelial cells treated with or with out CSE for 4 h were lysed in RIPA buffer consisting of 20 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.1 mM sodium *ortho*-vanadate, 1 mM PMSF, 0.1 % aprotinin, and 1% Nonidet P-40. Equal amounts of homogenates were separated by 8.0% SDS-PAGE gel and transferred to a PVDF membrane (Amersham-Pharmacia Biotech, Piscataway, NJ) overnight. The membrane was probed with 1:2000 dilution of antibody against caveolin-1 or Hsp-90 (Santa Crutz Biotechnology, Santa Crutz, CA). The latter was detected by antirabbit IgG–horseradish peroxidase conjugate. The reactive bands were detected by chemiluminescence reagent. The membrane was stripped and immunoblotted with the antibody against actin to assess the loading of samples. The protein band densities were analyzed by NIH Image software and the values were corrected by actin.

Statistical analysis

Quantitative measures of eNOS, Hsp-90, and caveolin-1 were presented as mean ± SEM. The genotype-dependent effects on these levels were analyzed using ANOVA. We used a paired Student's *t*-test for effects of CSE treatment on endothelial cells. Pearson correlation coefficients were reported for the prior and post-CSE treatments on quantitative measures. Univariate ANOVA model was applied for interactive effects among genotypes. Two-tailed $P < 0.05$ was regarded as statistically significant.

RESULTS

Characteristics of the studied population

HUVECs from mothers of Hispanic origin were included in the final analyses. While the frequency distributions for exon 7 G894 → T polymorphism was similar as reported previously, the rare C allele in the promoter T786-C polymorphism was much less frequent than that in Caucasians (Table 1). Yet, the rare 4 × 27-bp allele in intron 4 was much more frequent (Table 1). Furthermore, none of these three polymorphisms were in linkage disequilibrium ($P > 0.05$), which is different from what we reported previously in Caucasians among whom the intron 4 polymorphism was in close linkage with the promoter T786 → C variant (Sim *et al*., 1998;Wang and Wang, 2000). In those pregnant mothers, five of them had been smoking until early term of the pregnancies (5.4%), and they had significantly reduced eNOS protein levels (Table 2). This finding should be interpreted with caution, since only five smokers were included in the population—a number that is too small to have sufficient statistical power. None of the other medical conditions, including gestational diabetes and hypertension, were associated with eNOS expression.

Relationships between eNOS polymorphisms and eNOS expression

As shown in Table 3a, the C/C homozygotes at the promoter region had very low levels of eNOS mRNA ($P < 0.05$). Levels of eNOS protein and enzyme activity, on the other hand, tended to be similar. This effect was not altered by the CSE treatment. Since there were only three C/C homozygotes, this comparison may not be statistically valid. Combining with the T/

C heterozygotes may not be a justified regrouping, since it would carry the assumption of the dominant allelic effect. The effects of the promoter polymorphism on eNOS expression are consistent with that the rare allele being associated with reduced eNOS expression and increased risk of vascular diseases (Wang and Wang, 2000;Bilsborough *et al*., 2003).

For the 27-bp repeat polymorphism at intron 4, the rare allele 4 repeat homozygotes had the highest eNOS mRNA level ($P < 0.05$) (Table 3b), which was, however, associated with lower eNOS protein levels and enzyme activities. The effect of CSE on eNOS expression appeared to be consistent for all three different genotypes. The reduced eNOS protein levels in rare 4-repeat allele homozygotes are consistent with the homozygotes being associated with increased risk of vascular disease (Wang *et al*, 1996;Pulkkinen *et al*., 2000). However, it is difficult to reconcile the paradoxically elevated mRNA levels in those homozygote cells, which had reduced protein levels. We also noted that the smoking–genotype interaction was not present in these cultured endothelial cells.

For the G894 → T transition at exon 7, we observed a low eNOS protein levels in rare allele T/T homozygotes but relatively high mRNA levels in both control and CSE-treated endothelial cells (Table 3c). The reduced eNOS protein levels and enzyme activities are in agreement with the rare allele being associated with increased vascular risk. The relatively higher eNOS mRNA in the same cells could be a compensatory up-regulation in transcription, since the mutation at exon 7 could result in accelerated protein degradation (TeSauro *et al*., 2000).

Effects of cigarette smoking on eNOS expression

To explore whether CSE treatment to these cells would cause altered eNOS expression and activity, we exposed these cells to 0.01 cigarette equivalent CSE for 4 h before eNOS was measured. As shown in Table 4, eNOS levels and enzyme activities were highly correlated before and after CSE treatment. However, the correlation for the NOx levels was only marginally significant ($P = 0.026$). Using a paired *t*-test, we found that CSE had no effect on eNOS mRNA and protein levels. However, CSE induced a 40% reduction in NO production as measured by NOx, although its impact on eNOS enzyme activity was only marginal (Table 4). Using a subgroup analyses, we found no CSE–eNOS genotype interactive effects on eNOS expression or enzyme activity, as was demonstrated in Table 3.

Roles of Hsp-90 and caveolin-1 in genotype-dependent eNOS expression

A significant correlation was observed between levels of caveolin-1 and eNOS protein in both control ($r = 0.559$, $P = 0.0001$) and CSE-treated ($r = 0.458$, $P = 0.0001$) endothelial cells. While there was no association between caveolin-1 and eNOS enzyme activity among all samples, the association was significant in wild-type 5 × 27-bp homozygotes ($r = 0.405$, $P < 0.01$ for control and $r = 0.403$, $P < 0.01$ for CSE treatment, $n = 39$). We found no relationship between eNOS DNA variants and levels of caveolin-1. Although smokers tended to have a lower Hsp-90 levels *in vitro*, CSE treatment increased Hsp-90 levels significantly (1.06 ± 0.19 versus 2.22 ± 0.68). This increase was only present in wild-type 5 × 27 bp homozygotes (0.95 ± 0.16 versus 2.62 ± 1.20, $P < 0.01$), but not the other genotypes. For example, the Hsp-90 level in the rare 4 × 27-bp allele homozygotes (2.75 ± 1.64, $n = 7$) was already high prior to CSE treatment compared to the levels after CSE exposure (2.31 ± 1.41). Levels of Hsp-90 were also highly correlated between control and CSE treated cells (Table 4). No association between Hsp-90 and eNOS expression, enzyme activity and NOx production were observed among these endothelial cells.

DISCUSSION

Previous studies of ours and others have suggested that both the promoter T786-C variant and the 27-bp repeat polymorphism can quantitatively regulate eNOS expression. The genotype-dependent regulation of these polymorphisms is associated with various forms of vascular diseases including myocardial infarction, hypertension, renal vascular disease, and cerebral vascular disease (Wang and Wang, 2000). On the other hand, the G894T mutation in exon 7 could also affect bioavailable eNOS by reducing protein stability. However, none of these polymorphisms are associated with vascular diseases in all studied populations. If we take the biased unpublished nonsignificant findings into consideration, at least 50% of the population studies show nonsignificant associations with these polymorphisms. While there is little doubt that dysfunctional eNOS is involved in pathogenesis of vascular diseases, several reasons can explain the apparent inconsistent population findings. First, significant associations between these polymorphisms and vascular diseases in some populations may represent false-positive statistical results. Second, none of these studied polymorphisms are functional in regulating eNOS expression. Their significant associations with vascular diseases are mediated through linkage with other functional variant site(s). The putative functional site(s) may only be polymorphic in some populations but not in others. Last, the putative function of these polymorphisms or possibly linked variants at other sites may be conditional on specific environmental factor(s). These environmental factors could either be local, such as hemodynamic conditions, or systemic, such as cigarette smoking.

Given the number of significant associations in various populations is so large that the first possibility is less likely. The second possibility is a popular hypothesis. In fact, we discovered the T786C base variant through the upstream search of the hot spots at the promoter region (Sim *et al*., 1998). Indeed, this T786C is in close linkage with the 27-bp repeat polymorphism. Later studies heralded by the Japanese group have shown that the T/C variant at the promoter region could be associated with reduced eNOS promoter activity and vascular disease (Nakayama *et al*., 1999). Although the same promoter variant was not shown to be associated with vascular diseases in an Australian population, our *in vitro* transfection experiment shows that the promoter variant may affect transcription efficiency (Wang *et al*., 2002). We have further demonstrated that the 27-bp repeats in intron 4 could also be potentially functional, since it not only binds to certain nuclear proteins but also affects promoter efficiency (Wang *et al*., 2002).

We therefore, hypothesized that variants at both sites could be functional at genomic level by affecting the transcription efficiency. Findings of our current study clearly demonstrate that even after 4 *in vitro* passages for approximately 12 generations of cell replication, when *in vivo* hemodynamic and biological environment are no longer present, the genotype dependent eNOS expression is still statistically significant. Our data show that DNA variants at the promoter and intron 4 may directly affect the transcription efficiency that is transmitted with cell divisions and DNA replication. On the other hand, the G894T variant in exon 7 may indeed affect eNOS protein stability, since the rare allele TT genotype had a low protein level. Our findings indicate that at least the *in vivo* local biological and hemodynamic environments are not necessary for the eNOS genotype–phenotype relationships.

However, we must note that in this Mexican American population the frequency distributions of the variants in the promoter and the intron 4 are significantly different from those in Caucasians as published early (Wang and Wang, 2000). There is also no linkage disequilibrium between the two polymorphic sites as we observed in previously (Sim *et al*., 1998; Wang and Wang, 2000). The difference in the eNOS allelic profile could be fixed after those two populations were geographically and genetically isolated. Therefore, findings from current population may be specific to the studied population. Our current study cannot rule out the

second possibility that these two sites are in functional linkage with other variant sites. However, the linkage could be in a functional *cis*-acting relationship rather than a simple genetic linkage.

We have also looked into the third possibility that the genotype–phenotype relationship is conditional on the systemic environment, such as cigarette smoking. We mimicked cigarette smoking using *in vitro* extracts (CSE) to treat endothelial cells and explore whether this will alter the genotype–phenotype relationships. Our experiments show that the genotype–phenotype relationship does not appear to be affected by the treatment of CSE as we predicted from findings at population and tissue levels. The effect of CSE on eNOS also appears to be more on the end product, that is, NO production as measured by NOx, rather than at the eNOS expression. This finding indicates that the apparent eNOS genotype–smoking interaction at population level in relation to vascular diseases would be more likely at the level of biological functional interactions rather than on eNOS expression or its enzyme activity. One possibility is that free radicals produced by cigarette smoking could interfere with biological production of NO by the eNOS.

We have further explored whether Hsp-90 and caveolin-1 alter the genotype–smoking interaction on eNOS expression. We have clearly shown that HUVECs respond to CSE challenge by increasing Hsp-90 expression. However, this Hsp-90 activation does not seem to mediate the effects on eNOS expression and NO production, or the genotype-dependent eNOS expression. While CSE has no effect on caveolin-1 expression, level of caveolin-1 appears to be associated with eNOS activity in wild-type homozygotes for intron 4 polymorphism.

In summary, our study for the first time using an *in vitro* cell culture model shows genotype-dependent eNOS expression. Despite the inherent variability with cell culture, our study has demonstrated that endothelial cells maintain the genotype-dependent expression and activity for all three genotypes even after the deprivation of *in vivo* environment. However, the smoking–genotype interaction may require such *in vivo* condition to be manifested. Neither Hsp-90 nor caveolin-1—important eNOS regulators—appears to mediate the genotype–smoking effects on eNOS expression and activity.

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Table 1

Frequency Distributions of the eNOS Polymorphisms in Promoter (T786C), Intron 4 (27 bp Repeats) and Exon 7 (G894T)

Promoter			***Intron 4***			***Exon 7***		
	N	***%***		**N**	***%***		**N**	***%***
T/T	62	65.3	4/4	8	8.4	G/G	42	44.2
T/C	28	29.5	4/5	40	42.1	G/T	29	30.5
C/C	03	3.2	5/5	41	43.2	T/T	14	14.7
T = 0.80			5 = 0.64			G = 0.59		
C = 0.20			4 = 0.36			T = 0.31		

We analyzed the linkage disequilibrium between different polymorphisms by the chi-square analysis: Promoter versus Exon 7: $\chi^2 = 7.87$, $P = 0.097$; Intron 4 versus Exon 7: $\chi^2 = 8.614$, $P = 0.072$; Promoter versus Intron 4: $\chi^2 = 3.820$, $P = 0.431$.

Table 2

Effect of Primary Smoking on eNOS and Its Regulatory Proteins

	Nonsmokers	***Primary smokers***
N	84	5
eNOS protein, pg/μg protein	14.0 ± 1.8	6.9 ± 2.4[a]
eNOS activity, cpm/mg/min	3350 ± 422	2294 ± 1761
eNOS mRNA, relative unit	0.18 ± 0.04	0.31 ± 0.31
NOx, μM/μg protein	0.311 ± 0.01	0.26 ± 0.05
Hsp90, relative intensity	0.95 ± 0.15	0.53 ± 0.34
Caveolin-1, relative intensity	0.95 ± 0.08	0.63 ± 0.15

Using analyses of variance, we tested the homogeneity in variance of the measured variables between smokers and nonsmokers. They were not significantly different for levels of eNOS protein and mRNA, NOx, Hsp90 and Caveolin-1.

[a] $P < 0.05$ by independent Student *t*-test.

Table 3

Relationships between eNOS Polymorphisms and eNOS Expression in HUVEC

Control	*eNOS mRNA*	*eNOS protein*	*eNOS activity*	*NOx*
a: Promoter, T786C				
T/T	0.18 ± 0.05	12.1 ± 1.8	32.03 ± 522	0.33 ± 0.02
T/C	0.20 ± 0.07[a]	16..6 ± 3.9	3620 ± 718	0.28 ± 0.02
C/C	0.07 ± 0.04	17.9 ± 5.9	1878 ± 996	0.30 ± 0.10
With CSE Treatment				
T/T	0.29 ± 0.07	12.7 ± 2.3	2745 ± 385	0.20 ± 0.01
T/C	0.17 ± 0.06	15.1 ± 3.2	3621 ± 776	0.17 ± 0.02
C/C	0.06 ± 0.001[a]	10.9 ± 5.7	802 ± 297	0.11 ± 0.04
b: Intron 4, 27 bp repeats				
4/4	0.43 ± 0.15[a]	6.56 ± 0.36	1383 ± 562	0.29 ± 0.06
4/5	0.25 ± 0.06	12.83 ± 2.09	4283 ± 775	0.34 ± 0.03
5/5	0.08 ± 0.04	15.66 ± 2.99	2405 ± 450	0.29 ± 0.01
With CSE Treatment				
4/4	0.36 ± 0.16	8.04 ± 11.11	1394 ± 505	0.17 ± 0.04
4/5	0.18 ± 0.04	15.68 ± 3.05	3721 ± 648	0.18 ± 0.01
5/5	0.16 ± 0.09	12.77 ± 2.57	2382 ± 418	0.19 ± 0.02
c: Exon 7, G894T				
G/G	0.16 ± 0.05	11.0 ± 1.9	2985 ± 663	0.31 ± 0.02
G/T	0.19 ± 0.07	17.8 ± 3.4	4449 ± 781	0.32 ± 0.03
T/T	0.22 ± 0.12	8.2 ± 3.8	1951 ± 593	0.26 ± 0.04
With CSE Treatment				
G/G	0.17 ± 0.06	10.9 ± 2.0	2643 ± 465	0.19 ± 0.02
G/T	0.18 ± 0.09	19.7 ± 3.5	3927 ± 781	0.18 ± 0.02
T/T	0.35 ± 0.19	5.0 ± 2.4	2009 ± 661	0.18 ± 0.02

We measured eNOS protein levels (pg/μg total protein) by ELISA, enzyme activity (cpm/mg/min) by L-arginine to L-citrulline conversion, eNOS mRNA (relative unit to the housekeeping beta-actin) and NOx by Griess reaction (μM/g proteins). These levels were compared by ANOVA.

Table 4

Effect of CSE on eNOS and Its Binding proteins in HUVEC

	Control HUVEC	*CSE- treated HUVEC*	R	P-*values for paired* T *comparisons*
Hsp90, relative intensity	1.06 ± 0.19	2.22 ± 0.68	0.470[a]	0.0640
Caveolin-1, relative intensity	0.92 ± 0.08	0.94 ± 0.09	0.888[a]	0.5570
eNOS mRNA, relative unit	0.21 ± 0.04	0.20 ± 0.05	0.702[a]	0.6840
eNOS Protein, pg/3g protein	13.40 ± 1.70	13.2 ± 1.8	0.675[a]	0.8970
eNOS activity, cpm/mg/min	3232 ± 402	2907 ± 343	0.818[a]	0.1650
NOx, μM/μg protein	0.31 ± 0.01	0.19 ± 0.01	0.226[a]	0.0001

Correlation coefficients (*R*) were estimated using Pearson bivariate correlation analyses and

[a] $P < 0.001$,

[b] $P < 0.05$.

We also compared the differences in these levels before and after CSE treatment using paired *t*-test.