Genotype dependent and cigarette specific effects on endothelial nitric oxide synthase gene expression and enzyme activity

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Abstract **We explored the interactive effects of endothelial nitric oxide synthase (eNOS) genotypes and cigarette smoking on protein levels and enzyme activity in 33 postpartum placentas. Whilst the eNOS protein levels were lower in the rare allele (0.48 ± 0.11, $n = 9$ vs. 1.05 ± 0.10, $n = 24$, $P < 0.01$), the eNOS enzyme activity was about 7-fold higher in the rare allele (4556.2 ± 255.4 vs. 621.8 ± 180.5 cpm/mg/min, $P < 0.01$). Smokers had lower eNOS protein levels (1.07 ± 0.09 vs. 0.50 ± 0.19, $P < 0.05$) in both alleles. It reduced the eNOS activities only in the rare allele (non-smokers: 6143.8 ± 251.2, $n = 5$, smokers: 2968.5 ± 259.4, $n = 4$, 52% reduction, $P < 0.01$). We conclude that associations between eNOS polymorphism and protein levels and enzyme activities are modifiable by smoking, the effects of smoking are dependent on the eNOS genotypes.**

Key words: Atherosclerosis; Endothelial nitric oxide synthase polymorphism; Endothelial nitric oxide synthase enzyme activity; Cigarette smoking; Gene–environment interaction

1. Introduction

Nitric oxide (NO) plays a key role in regulating physiological functions in nearly all systems and has important implications for atherogenesis [1]. Both high and low NO can be pathological while the physiological range of the NO levels is determined by effector and defense systems in the recipient tissues. Biological NO is mostly produced enzymatically from L-arginine by a family of three NO synthases (NOSs) [2,3], neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3). The eNOS (7q35–36), confined mainly to endothelium and modestly to platelets, is essential in maintaining basal vascular NO production which regulates blood flow, particularly coronary artery blood flow. Reduction in basal NO release may predispose to hypertension, thrombosis, vasospasm and atherosclerosis [4]; and restoration of NO activity can induce regression of pre-existing intimal lesions [5].

Among many reported polymorphic markers in eNOS gene, associations between these polymorphisms and vascular diseases have not been consistent [6–10]. We reported a significant interactive effect between cigarette smoking and the eNOS polymorphism on coronary artery disease (CAD) [11]. In smokers, but not in non-smokers, there was a significant excess of homozygotes for the rare four repeats of 27 bp allele (eNOS4/4) in patients with severely stenosed arteries, compared with those with no or mild stenosis. However, eNOS4/4 individuals also had significantly higher NO levels than those with one or two common five repeat alleles (eNOS5/5+eNOS5/4) in healthy non-smokers [12]. We also reported a $T_{-781} \rightarrow C$ base substitution at the promoter region and the $G_{894} \rightarrow T$ ($Glu_{298} \rightarrow Asp$) mutation at exon 7 of the eNOS gene not associated with CAD nor NO levels [13,14].

Smokers have reduced endothelial dependent vessel relaxation, accelerated atherosclerotic plaque development and premature CAD [15,16]. Cigarette smoking is also a rich source of exogenous NO which can affect endogenous NO production [17]. A recent study showed that cigarette smoke extract can irreversibly inhibit pulmonary artery eNOS [18]. But the exact molecular mechanisms of how cigarette smoke affects endogenous NO production and how endogenous NO attenuates the cigarette effects are not clear. Cigarettes could either up- or down-regulate eNOS and/or iNOS or their necessary cofactors. It could also directly block NO-initiated signal transduction pathways for its biological effects or directly reduce bioavailable NO by reacting with cigarette-generated O_2^- to produce more peroxynitrite.

In light of the inconsistent findings for the associations between the reported eNOS polymorphisms and vascular diseases, and the potential influence of cigarette smoking on the associations, we hypothesized that eNOS expression is eNOS genotype dependent and modifiable by cigarette smoking. The manifestations of certain eNOS genotype related phenotypic traits are conditional on the presence or absence of a specific environmental factor, e.g. cigarette smoking. We wished to explore the interrelations among eNOS polymorphisms, eNOS gene expression, eNOS enzyme activity and cigarette smoking. While it is practically difficult to measure eNOS expression in human coronary artery directly, placental tissue, which is a rich source of eNOS and virtually devoid of nNOS [19,20], can be used for such a purpose. We investigated our hypothesis by measuring the genotypes and phenotypes of eNOS in human placental tissues.

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; CAD, coronary artery disease

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2. Materials and methods

2.1. Placenta collection

Human placental tissues were collected from 33 pregnancies delivered by Caesarean sections in the third trimester. There were 21 full term normal pregnancies and 12 pregnancies complicated with preeclampsia and/or fetal intrauterine growth restriction. Within 30 min after the delivery of the placenta, a section of placental tissue (~2 cm×2 cm×2 cm) was excised from mid-region of the placenta excluding the infarct tissues. The tissue was further divided into 10 small aliquots before being snap frozen in liquid nitrogen. They were stored at −80°C until analysis (1–12 months). Placentas were collected from the Department of Obstetrics and Gynaecology at Westmead Hospital. They were transported in dry ice to the Cardiovascular Genetics Laboratory at Prince of Wales Hospital for determinations of eNOS mRNA and protein levels and eNOS genotypes. The eNOS activity was measured in the Orthopaedic Research Institute at St. George Hospital. Samples were identified only by laboratory codes during all experimental procedures. Participating researchers in each laboratory were cross-blinded for the results and identity of the samples until the final statistical analysis. The study was approved by the Ethics Committees of University of New South Wales and of Western Sydney Area Health Service. Signed consent was obtained from every mother before Caesarean section.

2.2. DNA extraction and eNOS genotyping

Tissues (100 mg) were minced and ground with a pre-chilled mortar and pestle in 1.2 ml digestive buffer (100 mM NaCl, 10 mM Tris, 25 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), 1 μg/ml proteinase K, pH = 8.0). The mixtures were incubated in tightly capped tubes at 50°C for 16 h with shaking. This was followed by an addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuging at 1700×*g* for 10 min. DNA was precipitated with 1/2 volume 7.5 M ammonium acetate and two volumes of chilled 100% ethanol and centrifuged at 1700×*g* for 2 min. DNA genotypes for $T_{-781} \rightarrow C$ base change at the promoter region, 27 bp repeat polymorphism at intron 4 and $G_{894} \rightarrow T$ mutation at exon 7 were determined by PCR methods described previously [11,13,14].

2.3. RNA extraction and eNOS mRNA quantitation

Tissues (weight 100 mg) were cut into tiny pieces and homogenized in 1 ml of Trizol Reagent (Life Technologies, Cat. No. 15596). RNA was precipitated by isopropanol. The extracted RNA was then DNase-treated before being dissolved in RNA storage solution (1 mM sodium citrate, pH 6.4, Ambion, Bresatec Cat. No. AM-7001). We used the Superscript™ Preamplification System (Gibco BRL, Cat. No. 18089-011) for the first strand cDNA synthesis primed with oligo dT. The same amount of total RNA in duplicate was used for first strand cDNA synthesis. This was followed by PCR using eNOS specific primers and 3 μl target cDNA in a 20 μl reaction volume. The completed PCR reaction solution was mixed with exactly 7 μl of loading dye, and exactly 10 μl of which was loaded in duplicate onto an 8% polyacrylamide gel electrophoresis (PAGE) gel in a Bio-Rad Mini-Protein electrophoresis apparatus. Electrophoresis was conducted for 30 min at 110 V. Gels were then stained with 250 ml ethidium bromide solution (10 μl 10 mg/ml ethidium bromide in 250 ml double-distilled H_2O) for 10 min. The images were captured with a Kodak Digital Science DC40 camera on a UV box with a 2 s exposure time. Net intensity was read with a constant band area using the Kodak Digital Science Electrophoresis Documentation and Analysis System. β-Actin was also amplified as a house-keeping gene and was subjected to reverse transcription (RT)-PCR simultaneously with the target eNOS cDNA amplification. The eNOS mRNA levels were expressed as the ratio of the net eNOS band intensity to the net β-actin band intensity. Given the intrinsic limitations that quantitative RT-PCR has, our measurements can only be regarded as semi-quantitative. However, since we were comparing the differences between eNOS genotypes, the nature of semi-quantitation was satisfactory.

2.4. eNOS protein quantitation by Western blotting

An aliquot of tissue (weight 150 mg) was minced finely with a sterile razor blade. It was then transferred into a pre-chilled tissue grinder to be homogenized on a setting of 12 strokes in 5 ml lysis buffer (5 mM Tris–HCl, pH = 7.5, 1% Triton X-100 and protease inhibitor cocktail tablet (Roche, Cat. No. 1873580)). The homogenized tissue was then transferred into a pre-chilled Beckman centrifuge tube and centrifuged at 20 000×*g* for 30 min at 4°C. The supernatant was recovered and aliquoted into Eppendorf tubes. The total soluble protein concentration in the supernatant was determined by the Bradford method (Bio-Rad, Cat. No. 500-0006). The extracted proteins were then diluted to the same concentration for all samples in the following Western blotting procedures. Protein samples were denatured by heating at 90°C for 3 min in denaturing buffer (0.5 M Tris pH 6.8, 28% glycerol, 6 mM EGTA, 6% SDS, bromophenol blue 14% β-mercaptoethanol). Exactly 10 μl of denatured sample was loaded in duplicate to the denaturing SDS–PAGE gel. Proteins were separated by a 4.4% stacking gel and 10% separation gel (120 V for 90 min). The gel to nitrocellulose transfer was conducted using a Bio-Rad transblot at 100 V for 3 h. The transferred membrane was blocked overnight in blocking buffer (3% BSA in TBS) with constant shaking. This was followed by incubating with rabbit anti-human eNOS primary antibody (rabbit polyclonal anti-synthetic peptide corresponding to the C-terminal sequence of human eNOS, Cayman, Cat. No. 160880) diluted 1:1000 in 0.5% polyvinyl-pyrrolidone (PVP) in TBS. Goat anti-rabbit IgG conjugated with HRPO (Sigma, Cat. No. A-6154) was used as a secondary antibody diluted 1:40 000 in PVP/TBS. We used Supersignal Ultra chemiluminescent substrate to detect the signal (Pierce, Cat. No. 34075) and the image was captured on an X-ray film with an exposure time of 1 min (Kodak Scientific Imaging Film, BIOMAX ML, Cat. No. 178 8207). The membrane was re-probed for the detection of the actin band (42 kDa) using rabbit anti-human actin antibody (Sigma, Cat. No. A5060) as a house-keeping control. The net intensity of the eNOS band (~135 kDa) was analyzed by Kodak Digital Science Electrophoresis Documentation and Analysis System. The ratio of the net intensity of the eNOS band to actin band of the same sample was used as an estimation of the eNOS protein concentration.

2.5. NOS activity measurements

Samples were ground into a fine powder in liquid nitrogen using a mortar and pestle, and sonicated in five volumes of homogenization buffer (50 mM Tris–HCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 2 μg/ml leupeptin, 5 μg/ml pepstatin A, 500 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride and 1 μg/ml E-64). Conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the homogenates as previously described [21]. In brief, 30 μl of tissue homogenates was incubated in a reaction mixture containing 20 μl of [^{3}H]-L-arginine (10 μCi/ml), 1 mM NADPH, 300 IU/ml calmodulin, 5 μM tetrahydrobiopterin, with or without 2 mM $CaCl_2$, with or without 2.5 mM EGTA, with or without 2.5 mM N^G-monomethyl-L-arginine for 30 min at 37°C. Reactions were stopped by adding 0.5 ml of ice cold Tris buffer (pH 5.5) containing 1 mM EGTA and 1 mM EDTA. Reaction mixtures were applied to Dowex 50W (Na^+ form) packed in chromatography spin columns for ion-exchange chromatography and centrifuged at 1000×*g* for 4 min. The radioactivity of the eluent (recovered [^{3}H]-L-citrulline) was measured by liquid scintillation. The data represented the mean of samples from each placenta, each being assayed in quadruplicate. Since there are no nerve endings in the placenta, the calcium dependent NOS activity in the placental tissue is likely to represent eNOS. Two different adjustments were used in presenting the NOS activities. The eNOS activities were adjusted by the amount of total proteins of the homogenates (cpm/mg/min) to represent the total eNOS activity capacity of the tissue. The activities were adjusted by the specific eNOS protein levels to reflect the specific eNOS enzyme activity per unit of eNOS protein.

2.6. Statistical analysis

Results are presented as mean ± S.E.M. and compared by ANOVA for more than two groups and Student's *t* test for between groups. A factorial model of the univariate analysis of variance was employed to assess the main effects and interactive effects of independent factors to the phenotypic variances. The two-tailed *P* values are reported.

3. Results

3.1. Effects of cigarette smoking on eNOS expression and enzyme activity

Among 33 samples, there were 21 full term normal pregnancies. The rest of the placentas ($n = 12$, 36%) were obtained from pregnancies complicated with preeclampsia and/or intra-

Table 1
Effects of cigarette smoking and pregnancy complications on levels of eNOS mRNA and protein, and enzyme activities

Status (n)		eNOS			
		mRNA	Protein	Activity[a]	Activity[b]
Smoking	No (24)	1.15 ± 0.11	1.07 ± 0.09	473.7 ± 72.2	997.5 ± 467.1
	Yes (9)	1.14 ± 0.33	0.50 ± 0.19*	544.5 ± 145.3	1297.5 ± 460.7
Pregnancy	Normal (21)	1.35 ± 0.15	0.95 ± 0.11	401.1 ± 53.2	697.0 ± 197.4
	Complicated (12)	0.75 ± 0.09*	0.79 ± 0.16	676.9 ± 152.7*	1843.7 ± 993.8

Complicated pregnancies including preeclampsia and/or intrauterine growth retardation. The levels of eNOS protein and mRNA were presented as the ratios of eNOS specific band intensity to the house-keeping β-actin band intensity in quantitative Western blotting and RT-PCR. ANOVA was used to compare the differences and two-tailed P values are reported.
[a]The eNOS activity was adjusted by the levels of total protein of the tissue homogenates (cpm/mg protein/min).
[b]The eNOS activity was adjusted by the levels of specific eNOS protein determined in Western blotting.
*$P < 0.05$.

uterine growth retardation. Among complicated pregnancies, four (33%) were smokers, but five of the 21 (23.8%) with normal pregnancies were smokers. However, there was no significant association between cigarette smoking and abnormalities during pregnancy. For all samples, eNOS protein levels and enzyme activities were not correlated ($r = 0.28$, $n = 33$, $P = 0.116$). There were also no statistically significant correlations between the eNOS mRNA and eNOS protein levels ($r = 0.12$, $P = 0.34$), and enzyme activities ($r = 0.22$, $P = 0.25$). However, when the correlation analysis was conducted among samples of non-smoking with normal pregnancies, the correlation was significant between eNOS protein levels and enzyme activities ($r = 0.56$, $n = 16$, $P = 0.012$).

Cigarette smoking appeared to down-regulate the eNOS expression at the translational level since eNOS protein concentrations but not the mRNA concentrations were decreased in smokers (Table 1). However, the eNOS activities also tended to be higher in placentas with low eNOS protein concentration in smokers. This change was more dramatic when the enzyme activity was adjusted by the eNOS protein concentration rather than the total protein concentration (Table 1). Cigarette smoking did not appear to have any effect on the iNOS activity (Table 2).

The eNOS gene expression in complicated pregnancies was reduced at the transcriptional level. Both eNOS mRNA and protein concentrations in placenta of complicated pregnancies were low (Table 1). In contrast, the eNOS activity (adjusted by the total protein amount used in the assay) was increased among the placentas of complicated pregnancies. The increase in eNOS enzyme activities was even more considerable after they were adjusted by the amount of specific eNOS protein. Although calcium independent (iNOS) activity was also detectable in the placental tissue, it was much lower than the calcium dependent (eNOS) activity. There was no consistent change in iNOS activity between placentas of normal and complicated pregnancies (Table 2).

3.2. Effects of eNOS polymorphisms on eNOS expression and enzyme activity

The 27 bp repeat polymorphism in intron 4 of the eNOS gene was in linkage disequilibrium with the $T_{-781} \rightarrow C$ substitution in the promoter region ($\chi^2 = 10.969$, $P = 0.004$). It should be noted that two rare CC homozygotes for the $T_{-781} \rightarrow C$ substitution were also heterozygotes for the rare four repeat allele at intron 4. However, neither the 27 bp repeat polymorphism in intron 4 nor the $T_{-781} \rightarrow C$ substitution in the promoter was in linkage with the $G_{894} \rightarrow T$ mutation at exon 7 ($\chi^2 = 0.869$, $P = 0.648$, and $\chi^2 = 6.574$, $P = 0.160$, respectively).

The levels of eNOS mRNA and protein, and enzyme activities were assessed in relation to $T_{-781} \rightarrow C$ substitution in the promoter, the 27 bp repeat polymorphism at intron 4, and the $G_{894} \rightarrow T$ mutation at exon 7 (Table 3). The eNOS protein concentrations in the CC genotype for the $T_{-781} \rightarrow C$ substitution were only 16% of the level in common TT homozygotes. The eNOS mRNA concentrations also tended to be lower in the CC homozygotes, but the difference was not statistically significant. Both eNOS mRNA and protein levels in TC heterozygotes were similar to those in TT homozygotes. Whilst there was no significant difference in the eNOS enzyme activities adjusted by total protein concentration between different genotypes, the eNOS activities adjusted by the eNOS protein levels were about 4-fold higher in the CC homozygotes than that in TT homozygotes (Table 3).

Since the frequency of the 27 bp repeat polymorphism in Caucasian population is very low [11], we were unable to obtain placental samples homozygous for the rare four repeats allele. However, the eNOS mRNA and protein levels

Table 2
Effects of cigarette smoking and pregnancy complications on total NOS, iNOS and eNOS activities

Status (n)		NOS activities		
		Total NOS	iNOS	eNOS
Smoking	No (24)	567.5 ± 88.8	93.8 ± 23.6	473.7 ± 72.2
	Yes (9)	658.7 ± 176.3	114.3 ± 35.0	544.5 ± 145.3
Pregnancy	Normal (21)	490.4 ± 60.7	89.3 ± 17.5	401.1 ± 53.2
	Complicated (12)	796.5 ± 196.7	119.6 ± 47.6	524.6 ± 193.6

The total NOS activities include both calcium dependent and calcium independent NOS activities. They were measured by inclusion of calcium in the reaction buffer. The iNOS activity was measured by the absence of calcium and the eNOS activity was deduced by subtracting the iNOS from the total NOS activity. Since there is no nerve ending in placenta, the majority calcium dependent NOS is eNOS.

Table 3
Effects of eNOS polymorphisms on levels of eNOS mRNA and protein, and enzyme activities

Genotypes (*n*)		eNOS			
		mRNA	Protein	Activity[a]	Activity[b]
$T_{-781} \rightarrow C$	TT (17)	1.24 ± 0.12	0.95 ± 0.12	556.6 ± 110.4	709.8 ± 143.8
	TC (14)	1.12 ± 0.22	0.94 ± 0.14	423.1 ± 66.4	1280.0 ± 798.6
	CC (2)	0.49 ± 0.12	0.15 ± 0.01*	441.8 ± 286.5	2815.3 ± 1682.6
27 bp repeat at intron 4	5/5 (24)	1.32 ± 0.13	1.05 ± 0.10	446.5 ± 65.8	519.2 ± 73.2
	5/4 (9)	0.69 ± 0.14*	0.48 ± 0.11**	617.0 ± 160.1	2572.8 ± 711.7**
$G_{894} \rightarrow T$	GG (17)	1.02 ± 0.10	0.84 ± 0.11	577.7 ± 115.0	1349.1 ± 656.1
	GT (13)	1.28 ± 0.24	0.98 ± 0.16	437.5 ± 55.0	846.9 ± 319.6
	TT (2)	1.76 ± 0.54	1.16 ± 0.13	302.7 ± 63.8	269.6 ± 82.9

The levels of eNOS protein and mRNA were presented as the ratios of eNOS specific band intensity to the house-keeping β-actin band intensity in quantitative Western blotting and RT-PCR.
[a]The eNOS activity was adjusted by the levels of total protein of the tissue homogenates (cpm/mg protein/min).
[b]The eNOS activity was adjusted by the levels of specific eNOS protein determined in Western blotting.
*$P < 0.05$.
**$P < 0.01$.

in eNOS5/4 were already 45% of the levels in eNOS5/5 (Table 3). It is consistent with an allele dosage effect and the effect was at the transcriptional level. The eNOS activities adjusted by the eNOS protein levels, on the other hand, increased nearly 5-fold in eNOS5/4 (Table 3).

For the $G_{894} \rightarrow T$ mutation at exon 7, we observed a slight but linear increase in the eNOS mRNA and protein levels with the presence of one or two of the rare 'T' alleles suggesting an allele dosage effect (Table 3). However, the eNOS enzyme activities decreased with the presence of the rare 'T' allele. This decrease was more prominent for the eNOS activities adjusted by eNOS protein levels. The eNOS activities in TT homozygotes and GT heterozygotes were only about 20% and 63% of the activities in GG homozygotes (Table 3). However, none of the differences was statistically significant.

3.3. Interactive effects of cigarette smoking and eNOS polymorphisms on eNOS expression and enzyme activity

We assessed the effect of cigarette smoking on eNOS protein concentrations in eNOS5/4 and eNOS5/5 genotypes separately. Cigarette smoking decreased the eNOS protein levels from 1.09 ± 0.12 to 0.90 ± 0.27 (18% reduction) for eNOS5/5, and from 0.42 ± 0.27 to 0.27 ± 0.28 (36% reduction) for eNOS5/4. The difference in eNOS levels between the eNOS5/5 and eNOS5/4 genotypes was unaltered by cigarette smoking. However, the eNOS activities were significantly depressed by cigarette smoking only in the eNOS5/4 genotype (non-smokers: 6143.8 ± 251.2, $n = 5$, smokers: 2968.5 ± 259.4, $n = 4$, 52% reduction). Interestingly, enzyme activity was even slightly elevated in the eNOS5/5 homozygotes (non-smokers: 521.3 ± 110.8, $n = 19$, smokers: 722.2 ± 251.1, $n = 5$, 38% increase). Using a factorial model of the univariate analysis of variance, we identified only the 27 bp repeat polymorphism at intron 4 interacting with cigarette smoking and pregnancy complications on eNOS enzyme activities whilst the main effects of the polymorphism remained significant (Table 4). Although the levels of eNOS mRNA and proteins also changed consistently indicating interactive effects, they were not statistically significant (Table 4). Neither the $T_{-781} \rightarrow C$ substitution nor the $G_{894} \rightarrow T$ mutation interacted with cigarette smoking or complicated pregnancies. Furthermore, in the analytical model controlling for the effects of these two confounding factors, neither of these two base changes had statistically significant main effects on the eNOS activities.

4. Discussion

It is widely known that not every smoker develops athero-

Table 4
Interactions between cigarette smoking, pregnancy complications and eNOS intron 4 polymorphism on levels of eNOS mRNA and protein, and activities (estimated mean ± S.E.M. by the model)

Smoking	Pregnancy (*n*)	Genotype (*n*)	eNOS			
			mRNA	Protein	Activity[a]	Activity[b]
No	Normal (16)	eNOS5/5 (13)	1.37 ± 0.16	1.05 ± 0.13	384.1 ± 102.6	477.8 ± 124.6
		eNOS5/4 (3)	0.91 ± 0.29	0.73 ± 0.24	412.4 ± 185.0	684.1 ± 224.7
	Abnormal (8)	eNOS5/5 (6)	0.87 ± 0.24	1.13 ± 0.20	587.3 ± 151.1	564.7 ± 183.4
		eNOS5/4 (2)	0.89 ± 0.58	0.10 ± 0.48	1 203.8 ± 369.9	11 603.5 ± 449.4
Yes	Normal (5)	eNOS5/5 (4)	1.89 ± 0.29	1.04 ± 0.24	363.4 ± 184.9	472.6 ± 224.7
		eNOS5/4 (1)	0.61 ± 0.58	0.16 ± 0.48	728.3 ± 369.9	4 497.9 ± 449.4
	Abnormal (4)	eNOS5/5 (1)	1.04 ± 0.58	0.77 ± 0.48	746.5 ± 369.9	971.7 ± 449.4
		eNOS5/4 (3)	0.36 ± 0.33	0.37 ± 0.28	657.2 ± 213.6	1 439.1 ± 259.4

After the other factors were controlled for in an univariate ANOVA of factorial model, the main effects of the polymorphism, smoking and complicated pregnancy on eNOS levels were $F = 7.119$, $P = 0.013$; $F = 0.480$, $P = 0.495$ and $F = 0.392$, $P = 0.537$. There was no significant interactive effect among three factors on eNOS levels. The main effects of the polymorphism, smoking and complicated pregnancy on eNOS enzyme activities adjusted by eNOS protein level were $F = 300.865$, $P = 0.001$; $F = 42.987$, $P = 0.001$ and $F = 86.667$, $P = 0.001$. The interactive effects of smoking vs. the polymorphism, complicated pregnancy vs. the polymorphism, and smoking vs. polymorphism vs. abnormal pregnancy on eNOS activities were $F = 55.387$, $P = 0.0001$; $F = 64.284$, $P = 0.0001$ and $F = 251.558$, $P = 0.0001$, respectively.
[a]The eNOS activity adjusted by the levels of total protein of the tissue homogenates.
[b]The eNOS activity adjusted by the levels of specific eNOS protein.

sclerosis and not every genetically susceptible individual, e.g. familial hypercholesterolaemia, develops myocardial infarction. It is the coexistence of adverse environmental factors on the background of genetic susceptibility which determines the initiation and progression of atherosclerosis. Our previous study has illustrated a gene–environment interaction model at population levels by demonstrating that the four repeat allele in intron 4 of the eNOS gene is associated with an increased CAD risk [11]. However, we have also reported that the rare four repeat allele is associated with higher basal NO production in healthy non-smokers [12]. Since a reduced basal NO production is often associated with an increased CAD risk, the results of the two studies appear to be paradoxical. The results of the present study have provided some explanations for the apparent paradoxical findings in our patient and health populations that the rare allele was associated with CAD risk in patient smokers but higher NO levels in control non-smokers [11,12].

We found that the eNOS mRNA and protein levels were significantly lower in the eNOS5/4 than the eNOS5/5 genotypes. In contrast, the eNOS enzyme activity was about 5-fold higher in the eNOS5/4 than that in the eNOS5/5, which is consistent with the rare allele being associated with a higher NO production [12]. However, whilst cigarette smoking decreased the eNOS protein levels in the common and the rare allele, it reduced the eNOS enzyme activities only in the eNOS5/4 genotype to less than half of the non-smokers. The enzyme activities for the eNOS5/5 were even modestly elevated in cigarette smokers. Therefore, although the rare four repeat allele may produce more NO in non-smokers, the NO generating capacity by the eNOS is seriously compromised in smokers in the rare four repeat allele but not the common five repeat allele. The findings provide mechanisms at the tissue level for the genotype dependent and environment specific model in regulating eNOS expression and enzyme activity. Although no homozygote for the rare four repeat allele was available for the present study, the effect of the heterozygote was so large that an allele dosage effect can be expected.

Whilst the polymorphism-related regulation in eNOS gene expression is most likely to be at the transcriptional level, mechanisms for the association between the low eNOS protein levels and high enzyme activities remain to be explained. It is difficult to speculate that the changes in eNOS level could directly cause protein conformational changes which result in a more active form of eNOS. It is known that the eNOS normally functions as dimers conditional on the presence of many cofactors [22–25]. The level changes in eNOS protein could indirectly influence the bioavailability of cofactors or the effective formation of eNOS dimers, and therefore the eNOS enzyme activity. Furthermore, it is intriguing that cigarette smoking regulates eNOS enzyme activity also genotype dependent on the intron 4 polymorphism. The cigarette smoking only down-regulated the eNOS enzyme activities in the rare four repeat allele carriers. Clearly more studies are needed to understand the mechanisms.

We further reported that the $T_{-781} \rightarrow C$ substitution at the promoter region was also associated with reduced eNOS protein levels. As we observed for the intron 4 polymorphism, the eNOS enzyme activities tended to be elevated in the rare allele homozygotes. It adds yet another example that a low eNOS level was coincidental with an elevated eNOS enzyme activity. Since these two polymorphisms are in linkage disequilibrium and both CC homozygotes are also four repeat heterozygotes, it is not clear which of these two sequence variants is functional in gene regulation. Lacking of an allele dosage effect and no interaction between cigarette smoking and the $T_{-781} \rightarrow C$ substitution would indicate that the $T_{-781} \rightarrow C$ substitution is not functional. On the other hand, the $G_{894} \rightarrow T$ ($Glu_{298} \rightarrow Asp$) mutation at exon 7 had a slightly elevated eNOS protein level. But the eNOS specific enzyme activity was lower in the mutant, which also appears to be allele dose dependent. There is no interactive effect between the mutation and cigarette smoking on either eNOS levels or eNOS enzyme activities. Whilst no study has revealed that this amino acid change can cause any conformational change in eNOS protein, our results suggest that it could reduce enzyme activity.

In conclusion, we have demonstrated a genotype dependent and environmental specific model in regulating eNOS expression and enzyme activity at the tissue level. This model has wider application to other phenotypic variables. It indicates that recognition of a specific genotype–environment pair, e.g. eNOS intron 4 polymorphism–cigarette smoking, would have significant impacts on understanding the aetiology of atherogenesis and implementing effective measures in prevention and intervention. Further studies at the DNA level are needed to understand the molecular mechanisms responsible for the genotype dependent and environment specific changes observed in populations and tissues.

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