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The association of endothelial constitutive Nitric Oxide Synthase polymorphisms with family history of coronary heart disease in men

Research Article

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Abbreviations: acute myocardial infarction, (AMI); coronary heart disease, (CHD); endothelial nitric oxide synthase, (ecNOS); myocardial infarction, (MI); polymerase chain reaction, (PCR)

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Summary

It has been reported that endothelial nitric oxide synthase (ecNOS) gene polymorphism is associated with the risk of CHD, acute myocardial infarction (AMI) and atherosclerosis but hitherto no subjects with a family history of CHD have been examined. 292 native Saudi males of matching ages were drawn from normal, healthy male volunteers attending the blood bank at Alshmasee and the King Khalid University Hospital in Riyadh, Saudi Arabia. Blood samples were collected for the determination of lipids profiles using routine laboratory methods and Genotype was determined by polymerase chain reaction and restriction fragment length polymorphism analysis. The genotype frequencies for bb, ab and aa were 31.5, 53 and 5.5% respectively and the calculated allele frequencies for the ecNOS4b (0.65) and ecNOS4a (0.35) were not statistically different. The subjects were divided according to the family history of CHD, with an excess of individuals homozygous for bb and aa among the subjects who have a history of CHD standing at 61% and 12%, compared with those who do not have a history of CHD (59% and 4% respectively, p= 0.04). The ecNOS gene was found to be associated with family history of Coronary heart disease in Saudis male subjects more attention to these patients should be considered.

I. Introduction

Coronary Heart Disease (CHD) is a major public health problem which has been associated with various risk factors, including hypertension, hyperlipidaemia, diabetes mellitus and smoking (Simons, 1986; Jorde, 1988) However, in some individuals, CHD is not associated with conventional risk factors, suggesting that other genetic factors are involved in the predisposition to coronary atherosclerosis and its thrombotic complications (Nora et al, 1980; Marenberg et al, 1994).

Several studies show a clustering of CHD risk factors in the people of Saudi Arabia (Al-Nuaim, 1997; Khattab et al, 1999; Musaiger, 2002; Al-Nozha et al, 2002; Hakim et al, 2003; Al-Rukban, 2003; Al-Shehri et al, 2004). The prevalence of diabetes mellitus and CHD in Saudis is 24% and 6% respectively (Al-Nozha et al, 2004a, b). Osman, (2000) and Al-Nuaim, (1997) have shown a high prevalence of metabolic risk factors for CHD in Saudi subjects and a regional variation in the prevalence of the disease. Changes in lifestyle are clearly important to the current epidemic of obesity, diabetes and CHD in Saudis, but genetic factors may also contribute to the risk of CHD in this population. Moreover, the prevalence of smoking in Saudi Arabia is very high and has become a significant public health problem there (Al-Nuaim, 1997; Osman, 2000). In another study in Saudi Arabia, it was found that 19% of stroke patients registered from 1989-1993 were smokers (Al-Rajeh et al, 1998).

These essential roles of NO in vascular regulation suggest that a derangement in endothelial NO synthesis might lead to the development of atherosclerosis (Cooke et al, 1992). It has been reported that the ecNOS gene a/b polymorphism caused by four (allele ecNOSa) or five (allele ecNOSb) repeats of a 27-base pair sequence in intron 4 of the ecNOS gene is associated with the risk of

CHD, AMI (Thomas et al, 2002) and atherosclerosis (Cooke et al, 1992).

The human ecNOS gene is located on chromosome 7q 35-36 and comprises 26 exons spanning 21 kb: A number of variable tandem repeats and dinucleotide repeats [(CA)n] have been identified in the ecNOS gene (Janssens et al, 1992; Marsden et al, 1992; Sessa et al, 1992; Miyahara et al, 1994; Nadaud et al, 1994). Among the reported polymorphisms in the endothelial ecNOS gene, a close association has been shown to exist between the allele (four repeats) in intron 4 and the onset of CHD in an Australian population (Khattab et al, 1999). The effects of conventional risk factors such as smoking, hypertension, diabetes and HDL on the association between the ecNOS gene and ischaemic stroke have been determined in other populations (Bonnardeaux et al, 1995; Wang et al, 1996; Asanuma et al, 2001; Basset et al, 2002).

Nitric oxide has recently been implicated in a number of diverse physiological processes, including smooth muscle relaxation, inhibition of platelet aggregation, neurotransmission, immune regulation and penile erection (Furchgott, 1989; Dudzinski et al, 2006). Nitric oxide is produced from L-arginine by nitric-oxide synthase with a concomitant production of L-citrulline. There appear to be at least three distinct isoforms of nitric-oxide synthase (Furchgott, 1989; Yui et al, 1991a,b; Iyanagi, 2005). All three isoforms contain consensus sequences for the binding of FMN, FAD, and NADPH cofactors, and the structures of the isoforms have close homology to cytochrome p-450 reductase. The NOSs N-terminus bind tetrahydrobipoterine and heme, and the N- and C-terminal domains are linked by a short sequence that binds calmodulin (Bredt et al, 1991).

Hence, we investigated whether the polymorphism in intron 4 of the ecNOS gene is an independent risk factor for CHD in Saudi population.

II. Patients and methods

A. Patients

The subject population was drawn from normal, healthy male volunteers attending the blood bank at Alshmasee and King Khalid University Hospital in Riyadh, Saudi Arabia. Ethical approval was obtained from the local institutions, and written informed consent was obtained from each participant in the study. Information on sociodemographic characteristics, personal and family medical history and health-relevant behaviors, including smoking, exercise and diet was obtained by a standardized interview at the time of venesection. Height and weight were measured and blood pressure was measured once with a standard mercury sphygmomanometer.

Two 5 mL non fasting blood samples were obtained in EDTA coated vacuum tubes. After centrifugation for 10 minutes at 1000 rpm, plasma was stored at -20 ºC in 1.5 mL aliquots; the remaining red blood cells were stored at -20 ºC in 4 mL tubes for DNA extraction. Plasma total cholesterol, HDL-cholesterol and triglycerides were determined by routine enzymatic methods with a Roche modular analyzer. Apolipoproteins A I (apo A I) and Apolipoproteins A II (apo A II) were measured by a commercial immunoturbidmetric assay using a Roche modular analyzer.

B. Genotyping

Genomic DNA was extracted from buffy coats as described previously (Hayden et al, 1987). The Taq I polymorphism was originally described by Drayna and Lawan (Drayna, 1987). Ec NOS genotypes were determined with minor modifications by a polymerase chain reaction (PCR) using oligonucleotide primers (sense: 5'-AGGCCCTATGGTAGTGCCTTT-3'; antisense, 5'-TCTCTTAGTGCTGTGGTCAT-3' Prizma Laboratory Products Industry and Trade Co. LTD., Istanbul, TR) which flank the region of the 27bp direct repeat in intron 4 as described previously. Reactions were performed in a total volume of 24µL containing 500ng genomic DNA, 10 pmol of each primer, 0.2mm dNTP, 0.5U Taq DNA polymerase (MBI Fermentas Inc., New York, NY). The thermocycling procedure (Perkin Elmer Cetus, DNA Thermal Cycler 480, USA and Eppendorf Mastercycler Personal 5332, Germany) consisted of initial denaturation at 95°C for 5 min, 35 cycles of denaturation for 94°C for 1 min., annealing at 55°C for 1 min and extension at 72°C for 1 min. The PCR products were analyzed using 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The large allele, ecNOS4b, contains 5 tandem 27bp repeats and the smaller allele, ecNOS4a, contains 4 repeats. The sizes of the PCR products were 393bp and 420bp respectively for the ecNOS4a and ecNOS4b alleles.

C. Statistical analysis

Statistical manipulations and sample difference testing were carried out using SPSS version 10 for Windows (SPSS, Evanston, IL, USA). Data were tested for normality using normal probability plots and, if appropriate, transformed to produce a normal distribution. Differences among genotypes were tested on transformed data using one-way ANOVA. Due to multiple group comparisons, the Bonferroni correction was used to ascertain the statistically significant differences between the group means. Correlations were investigated using Pearson's correlation coefficient. The frequencies of discrete variables such as genotypes were compared by a chi-square test and of continuous variables by *t*-test or analysis of variance. We used logistic regression analysis for the association between presence of CHD and polymorphism. The presence of CHD was regarded as the dependent variable and the ecNOS4 polymorphism, gender, hypertension, diabetes, age, lifetime smoking, BMI, lipids parameters and lipoprotein levels were regarded as independent variables.

This statistical test was also performed to examine whether the genotype frequencies were in Hardy-Weinberg equilibrium.

III. Results

The characteristics of the Saudi male subjects (N = 262) are shown in **Table 1**, for both the whole group and for the three ecNOS genotypes. Comparison of age, BMI and the incidence of several conventional risk factors for CHD, including systolic and diastolic blood pressure, lipids, glucose and lipoproteins between ecNOS genotypes (aa + ab and bb) revealed no significant difference (**Table 1**).

The calculated allele frequencies for ecNOS4b and ecNOS4a were 0.65 and 0.35 respectively. The genotype frequencies for bb (0.35), ab (0.59) and aa (0.6) were significantly different from their expected values (R2 = 11.6, p <0.01). The distribution of the three investigated polymorphisms significantly deviated from the Hardy-Weinberg equilibrium exhibiting 50% decrease in aa, 17% decrease in bb, and 29.5% increase in ab genotypes as compared to their respective expected frequencies.

Table 1. Demographic, biochemical and life-style characteristic of the populations and according to eNOS genotype. Data shown are the mean (SD).

CHARACTERISTICS	TOTAL	BB	AB	AA
Numbers	262	92	154	16
Age (years)	29.4(8.4)	29.2(8.2)	30.7(9.6)	28.9(7.9)
BMI (Kg)	28.9(7.1)	28.5(8.6)	30.3(5.9)	28.9(6.7)
Current smoker (Numbers)	114	62	44	8
Family history of CHD (Numbers)	49	27	15	7
F. Glucose (mmol/L)	5.4(1.1)	5.8(0.8)	5.2(1.1)	5.5(1.2)
Cholesterol (mmol/L)	4.6(0.9)	4.7(1.0)	4.5(0.6)	4.5(0.9)
HDL (mmol/L)	0.97(0.3)	.9(.2)	0.9(0.2)	0.9(0.3)
Triglyceride (mmol/L)	2.2(1.4)	2.3(1.6)	2.1(1.3)	2.1(1.2)
SPB	118.7(12.7)	120.9(12.8)	117.9(12.3)	117.6(13.0)
DPB	75.8(10.4)	74.7(9.8)	77.1(7.8)	75.9(11.4)
Hips (cm)	106.6(17.2)	106.9(27.2)	98.7(9.9)	106.6(9.2)
Waist (cm)	94.6(12.7)	98.7(13.9)	360.0(484.9)[a]	92.7(12.2)[b]
APOA1{mg/dl)	117.4(17.1)	115.8 (16.3)	119.2(16.9)	118.5(18.02)
APOA11{mg/dl)	43.7(7.2)	42.8(7.8)	45.2(10.9)	44.2(6.8)

[a]p= 0.001 (ANOVA) between groups 1 and 3.
[b]p=0.01 between groups 2 and 3

Table 2. Clinical characteristics and metabolic parameters of Subjects without history of CHD (No CHD) and subjects with history of CHD.

Characteristics	N0 CHD	CHD	P value
Numbers	206	56	
Age (years)	30.9(9.4)	28.7(7.8)	0.12
BMI (Kg)	27.9(9.02)	28.9(7.02)	0.51
Family history of CHD (Numbers)	0	56	
F. Glucose (mmol/L)	5.4(1.2)	5.6(1.6)	0.576
Cholesterol (mmol/L)	4.6(0.9)	4.6(0.9)	0.68
HDL (mmol/L)	0.9(0.3)	0.9(0.2)	0.14
Triglyceride (mmol/L)	2.2(1.2)	2.2(1.4)	0.66
SPB	120.9(11.9)	118.7(12.6)	0.30
DPB	75.8(7.03)	75.6(11.2)	0.66
Hips (cm)	106.5(19.1)	105.3(9.04)	0.83
Waist (cm)	158.2(229.3)	94.2(13.2)	0.038
APOA1{mg/dl)	118.3(14.6)	118.6(17.4)	0.78
APOA11{mg/dl)	43.9(7.4)	44.2(7.1)	0.7

Data are presented as mean (SD)

Categorical distribution subjects according to HDL level (**Table 3**) smoking habit (**Table 4**) and family history of diabetes (**Table 5**) failed to show any significant difference with respect to distribution of the ecNOS4a genotype and allele prevalence. The subjects with the history of CHD showed 61/ ba and 12/ aa as expand to 59% bb and 4% aa in the individual without the history of CHD (P<0.04), clinical characteristic (**Table 2**). Where as family history of CHD was significantly associated with the homozygous presentation of alleles (**Table 6**). In analyzing the dominant effect of the ecNOS4a allele, the prevalence of the non-bb genotype (AA+BB) was found to be significantly higher in the group which had a history of CHD than in the control group (54%versus 64%, p< 0.05).

Stepwise regression for all the groups showed a significant association (P<0.05) between the ecNOS genotype and physical activity (in a group which took 20 minutes' exercise daily), with R^2 0.2.

IV. Discussion

Several studies have investigated the relation between ecNOS gene polymorphism and CHD, myocardial infarction (MI) and atherosclerosis and have produced varied or contradictory results (Tsukada et al, 1998; Thomas et al, 2002). It was found that the 4a allele was

associated with CHD but not with previous MI (Wang et al, 1997; Tsukada et al, 1998). Whereas, positive associations of 4a compared with 4b were reported in African-Americans with MI (Hooper et al, 1999), Caucasians with MI and CHD (Fowkes et al, 2000), Japanese patients with MI (Ichihara et al, 1998) and Australians with severely stenosed arteries and a history of MI (Wang et al, 1996). Other studies on German (Sigusch et al, 2000) or Japanese (Elbaz et al, 2000) patients fails to observe such an association. Since this polymorphism is in a non-coding region, it could merely be a genetic marker which is associated with the functional mutation. This is the first study to have found a relationship between the ecNOS gene and people with a history of CHD. The main finding of the present study is that sequence polymorphisms of the ecNOS gene locus are associated with a history of CHD, suggesting the pathophysiological of ecNOS 4a and 4b in the development of CHD in the Saudi population. The functional significance of ecNOS gene polymorphisms has been reported by several investigators (Wang et al, 1996; Ichihara et al, 1998; Elbaz et al, 2000; Sigusch et al, 2000; Yoshimura et al, 2000).

Table 3. Distribution of individuals between high and low HDL group for ecNOS polymorphism, the number of Individuals is given for High HDL (HDL cholesterol >1.2 mmol/L) or low HDL (HDL cholesterol<1.1). This is also expressed as a percentage of the total number of individuals in that group.

	Low HDL group		***High HDL group***	
Polymorphism	**Numbers**	%	**Numbers**	%
BB	116	57	40	67
AB	74	37	17	28
AA	11	6	5	5

Chi-Sq=2.1 p-value=0.35 Df=2

Table 4. Distribution of individuals between subjects without history of CHD (No CHD) and subjects with history of CHD (H.CHD). This is also expressed as a percentage of the total number of individuals in that group.

	No CHD		***H. CHD***	
Polymorphism	**Numbers**	%	**Numbers**	%
BB	125	59	34	61
AB	77	37	15	27
AA	9	4	7	12

Chi-Sq=6.2 p-value=0.044 Df=2

Table 5. Distribution of individuals between smoking and non smoking subject. This is also expressed as a percentage of the total number of individuals in that group.

	Non-smokers		***Smoking***	
Polymorphism	**Numbers**	%	**Numbers**	%
BB	91	62	62	56
AB	48	33	44	38
AA	8	6	8	6

Chi-sq= 1.53 p-value= 0.47 DF=2

Table 6. Distribution of individuals between subjects without history of diabetes (No H.DM) and subjects with history of diabetes (H.DM). This is also expressed as a percentage of the total number of individuals in that group.

	No.H.DM		***H.DM***	
Polymorphism	**Numbers**	%	**Numbers**	%
BB	100	64	58	54
AB	48	31	42	39
AA	8	5	8	7

Chi-sq=2.9 p-value = 0.23 DF=2

The genotype distribution of our subjects is within the Hardy-Weinberg equilibrium. Our result also showed significant differences in both genotype distribution and allele prevalence between the two groups, with or without a history of CHD. Elbaz and colleagues (2000) observed a significant difference in the distribution of genotypes when analysis were restricted to pairs of cases and matched controls, both free of previous cardiovascular and cerebrovascular history (cases: 50.0% *GG*, 40.1% *GT*, 9.9% *TT*; controls: 36.0% *GG*, 50.8% *GT*, 13.2% *TT*). Moreover, Wang et al found that ecNOS genotype was associated with a history of myocardial infarction (Wang et al, 1996). Previous studies have also shown that ecNOS gene polymorphism is responsible for variations in the genetic control of the plasma concentration of nitric oxide metabolites (Nava et al, 1995; Tsukada et al, 1998). Moreover, nitric oxide can inhibit vascular smooth cell proliferation (Sakar et al, 1995), which is responsible for the synthesis and assembly of the macromolecules which strengthen the fibrous cap. Therefore, there is a possibility that the inhibition of smooth muscle cell proliferation with changing ecNOS activity determined by ecNOS genotype contributes to the formation of a friable fibrous cap (Libby, 1991). Finally, brief exercise training may alter the gene expression for the enzyme, the constitutive endothelial NO synthase, which forms NO and may be part of the vascular adaptation seen after aerobic exercise training. Furthermore, if there is a genetic predisposition to produce NO, as in world class athletes or animals bred to race, NO may contribute to spectacular exercise performance (Shen et al, 1995). One potential mechanism which may contribute to the enhanced production of nitrite in vessels from exercised dogs may be the induction of the calcium-dependent ecNOS gene (Sessa et al, 1995). In our study we found an association between the ecNOS gene and physical activity. The high prevalence of Obesity, diabetes and CHD in Saudi (Al-Nuaim, 1997; Osman et al, 2000; Al-Nozha et al, 2002, 2004a, b; Al-Rukban, 2003) can explained the effect of the history of CHD on the association between gene polymorphism and CHD development so this polymorphism seems most useful for future research in CHD patients.

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