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Functional Inducible Nitric Oxide Synthase Gene Variants Associate With Hypertension

A Case–Control Study in a Finnish Population—The TAMRISK Study

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Abstract: Increased inducible nitric oxide synthase (iNOS) activity and expression has been associated with hypertension, but less is known whether the 2 known functional polymorphic sites in the iNOS gene (g.–1026 C/A (rs2779249), g.2087 G/A (rs2297518)) affect susceptibility to hypertension. The objective of this study was to investigate the association between the genetic variants of iNOS and diagnosed hypertension in a Finnish cohort.

This study included 320 hypertensive cases and 439 healthy controls. All participants were 50-year-old men and women and the data were collected from the Tampere adult population cardiovascular risk study (TAMRISK). DNA was extracted from buccal swabs and iNOS single nucleotide polymorphisms (SNPs) were analyzed using KASP genotyping PCR. Data analysis was done by logistic regression.

At the age of 50 years, the SNP rs2779249 (C/A) associated significantly with hypertension ($P = 0.009$); specifically, subjects carrying the A-allele had higher risk of hypertension compared to those carrying the CC genotype (OR = 1.47; CI = 1.08–2.01; $P = 0.015$). In addition, a 15-year follow-up period (35, 40, and 45 years) of the same individuals showed that carriers of the A-allele had more often hypertension in all of the studied age-groups. The highest risk for developing hypertension was obtained among 35-year-old subjects (odds ratio [OR] 3.83; confidence interval [CI] = 1.20–12.27; $P = 0.024$). Those carrying variant A had also significantly higher readings of both systolic ($P = 0.047$) and diastolic ($P = 0.048$) blood pressure during the follow-up. No significant associations between rs2297518 (G/A) variants alone and hypertension were found. However, haplotype analysis of rs2779249 and rs2297518 revealed that individuals having haplotype H3 which combines both A alleles (CA–GA, 19.7% of individuals) was more commonly found in the hypertensive group than in the normotensive group (OR = 2.01; CI = 1.29–3.12; $P = 0.002$).

In conclusion, there was a significant association between iNOS genetic variant (rs2779249) and hypertension in the genetically homogenous Finnish population. Those who carried the rare A-allele of the gene had higher risk for hypertension already at the age of 35 years.

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Abbreviations: ANOVA = analysis of variance, AR = adrenergic receptor, BMI = body mass index, BP = blood pressure, eNOS = endothelial nitric oxide synthase, GWAS = genome-wide association study, iNOS = inducible nitric oxide synthase, PHE = periodic health examination, SNP = single nucleotide polymorphism, TAMRISK = Tampere adult population cardiovascular risk study.

INTRODUCTION

Nitric oxide (NO) is an important vasodilator in the cardiovascular system. The synthesis of NO is catalyzed by the enzyme family called nitric oxide synthases (NOS); neuronal (nNOS, NOS1), inducible (iNOS, NOS2), and endothelial synthases (eNOS, NOS3).[1] Of the NOS family, the most important NOS isoform in the context of basal release of vascular NO is endothelial NOS (eNOS). The endothelial release of NO is nonetheless reduced in diabetes and hypertension leading to endothelial dysfunction.[2]

Expression of iNOS can be induced in a wide range of cells and tissues especially in inflammatory conditions by cytokines and other agents, leading to production of high amounts of NO until the enzyme is degraded.[3] There have been suggestions that hypertension could have an inflammatory background[4] and there is also evidence that the amount of NO[5] and expression/activity of iNOS is increased in hypertensive patients.[6] However, the lack of hits in genome-wide association studies (GWAS) for inflammation-associated genes in hypertension,[7,8] prompted us to study whether genetic variants of the iNOS gene could be involved in hypertension at younger age.

Two common functional polymorphisms have been described for the iNOS gene and both variations lead to increased NO production. iNOS variant rs2779249 (–1026 C/A) is located in the promoter region of the gene and it has been shown that nucleotide change from C to A increases iNOS promoter transcriptional activity to fivefold leading to higher NO production. The other variant rs2297518 (2087 G/A), located in exon 6, causes an amino acid substitution from serine to leucine which increases iNOS activity (alters iNOS protein function) and conferees higher NO production based on the A-allele.[9]

The purpose of this study was therefore to investigate whether iNOS genetic variants are associated with hypertension in a Finnish population by analyzing cohorts from the Tampere adult population cardiovascular risk study (TAMRISK).[10]

METHODS

Subjects

The data for the TAMRISK study were collected from periodic health examinations (PHEs) done for 50-year-old men and women living in Tampere, Finland. TAMRISK data include information of risk factors for hypertension: blood pressure (BP), weight, parental history of cardiovascular diseases, lipid values and smoking, diabetes and exercise habits. Buccal swabs for DNA extraction and a permissions form to use PHE data were collected by mail separately of the physical examination. The DNA samples were collected during years 2006 to 2010. Informed consent was obtained from all participants. A detailed description of the design and data collection as well as protocol of baseline measurements of the study is described elsewhere.[10,11] The study protocol was approved by The Ethics Committees of the Tampere University Hospital and the City of Tampere.

Cases (n = 320) in this study were the subjects who had hypertension and/or CAD at the age of 50 years (as diagnosed by a physician by normal healthcare procedures) and for each case, at least 1 normotensive control (n = 439) with the same sex and similar smoking habits, were chosen from a PHE cohort (n = 6000). Smoking status was evaluated based on self-reporting. Of the same individuals, we also analyzed the subpopulation of men and women who had available PHE data at the age of 35, 40, 45, and 50 years. Registration of BP was made at the examination visit (mm of mercury) using calibrated mercury sphygmomanometer.

Genotyping

Genomic DNA was extracted from buccal swabs using a commercial kit (Qiagen, Inc., Valencia, CA). The samples were transferred into 96-well plates and the 2 single nucleotide polymorphisms (SNPs) for iNOS were genotyped at the LGC genomics using Competitive Allele Specific PCR (KASP) technique (LGC genomics, Hertz, UK).

Statistical Analyses

One-way ANOVA for continuous variables and Chi-square test for categorical variables were applied for the comparison of cases and controls. If the distribution was skewed, the analysis was performed using transformed values to approximately normalize the distribution. Associations of the 2 genotyped SNPs for hypertension with risk factors were analyzed by logistic regression analysis. The analysis of variance (ANOVA) for repeated measures was used to assess the differences in mean BP: s between genotypes at the age of 35, 40, 45, and 50 years. *P* values less than 0.05 were considered significant. Hardy–Weinberg equilibrium of the genotypes was calculated using OEGE (online encyclopedia calculator for genetic epidemiology studies).[12] Analyses were carried out using SPSS 20.0 for Windows (SPSS, Inc., Chicago, IL).

RESULTS

The clinical characteristics of the middle-aged (50 ± 0 years) study subjects are listed in Table 1. In addition a subgroup population (n = 417) had clinical measurements also at the age of 35, 40, and 45 years. In the whole study population the frequencies of the rs2779249 variants were 0.553 for CC (n = 444), 0.372 for CA (n = 299), and 0.075 (n = 60) for AA. Frequencies of the rs2297518 variants were 0.702 for GG (n = 567), 0.265 for GA (n = 214), and 0.032 for AA (n = 26). The measured genotype frequencies were not significantly different from the expectations of Hardy–Weinberg equilibrium ($\chi^2 = 0.96$ for rs2779249 and $\chi^2 = 1.09$ for rs2297518).

At the age of 50 years, the SNP rs2779249 (C/A) associated significantly with hypertension ($P = 0.009$); specifically, subjects carrying the A-allele had higher risk of hypertension compared to those carrying the CC genotype ($P = 0.016$, OR = 1.43; 95% CI: 1.07–1.91). Among smokers, the risk for hypertension increased to 2-fold with the A-allele carriers ($P = 0.019$, 95% CI: 1.12–3.56). In addition, we noticed a

TABLE 1. Characteristics of Study Groups at the Age of 50 Years

	Cases (n = 320)	Controls (n = 439)	*P*-Value
Age (years)	50 ± 0	50 ± 0	
BMI (kg/m^2)	28.77 ± 5.18	25.52 ± 3.62	<0.001
Hemoglobin	146.86 ± 13.50	145.37 ± 13.31	0.185
Cholesterol (mmol/l)	5.39 ± 0.98	5.37 ± 0.87	0.733
HDL cholesterol (mmol/l)	1.55 ± 0.46	1.65 ± 0.44	0.004
LDL cholesterol (mmol/l)	3.16 ± 0.87	3.17 ± 0.81	0.900
Trigycerides (mmol/l)	1.58 ± 1.27	1.23 ± 0.74	<0.001
Glucose (mmol/l)	5.18 ± 1.31	4.86 ± 0.54	<0.000
Systolic blood pressure (mm Hg)	142.65 ± 16.70	129.48 ± 14.86	<0.000
Diastolic blood pressure (mm Hg)	92.67 ± 8.80	84.47 ± 9.16	<0.000
Exercise (at least twice a week) %	64.0	71.3	0.025
Hypertension %	100	0	
Diabetes %	13.1	0	<0.000
Myocardial infarction %	2.8	0	<0.000
Family history of hypertension %	57.8	24.8	<0.000
Gender (male)%	58.4	62.8	0.255
iNOS allele distribution			
rs2779249 C/A	0.78/0.22	0.86/0.14	0.022
rs2297518 G/A	0.91/0.09	0.89/0.11	0.416

Data are presented as mean ± SD.
BMI = body mass index; HDL = high density lipoprotein; iNOS = inducible nitric oxide synthase; LDL = low density lipoprotein;

TABLE 2. Association of iNOS With Hypertension at Different Ages

iNOS Polymorphism	Genotype	Age (Years)	Hypertension, Yes/No (n)	Hypertension % of Genotype	CC vs A-Allele/GG vs A-Allele OR (95% CI)	*P*-Value (Adjusted for BMI)
rs2779249	CC	50	162/259	38.5		
	CA + AA		158/179	46.9	1.47 (1.08–2.01)	0.015
	CC	45	48/334	12.6		
	CA + AA		54/261	17.1	1.44 (0.97–2.19)	0.060
	CC	40	15/226	6.2		
	CA + AA		24/167	12.6	2.20 (1.11–4.40)	0.025
	CC	35	5/231	2.1		
	CA + AA		11/170	6.1	3.83 (1.20–12.27)	0.024
rs2297518	GG	50	225/317	41.4		
	GA + AA		96/125	43.4	1.20 (0.86–1.69)	0.270
	GG	45	74/421	14.9		
	GA + AA		27/179	13.1	0.93 (0.57–1.51)	0.767
	GG	40	30/275	9.8		
	GA + AA		11/119	8.5	0.97 (0.46–2.02)	0.928
	GG	35	11/281	3.8		
	GA + AA		6/122	4.7	1.44 (0.51–4.09)	0.495

BMI = body mass index, CI = confidence interval, iNOS = inducible nitric oxide synthase, OR = odds ratio.

significant association among the A-allele carriers with hypertension already at the age of 35 years, which continued the whole follow-up time (age groups 35, 40, 45, and 50 years) (Table 2). Also BP readings according to genetic variants confirmed these observations and showed that those who carried the A-allele had higher levels of both systolic ($P = 0.047$) and diastolic ($P = 0.048$) BP during the 15-year follow-up period (Fig. 1).

No significant associations between rs2297518 (G/A) variants alone and hypertension were found (Table 2). In order to study a possible haplotype effect on hypertension, different haplotypes from rs2779249 (C/A) and rs2297518 (G/A) were generated. Three most common haplotypes were CC–GG (n = 389, 48.8%), CA–GG (n = 153, 19.2%), and CA–GA (n = 137, 17.2%). All other haplotype combinations represented less than 6% each in the study population. In the most common haplotype group H1 (CC–GG), 145 out of 389 (37.3 %) of the carriers had hypertension at the age of 50 years, whereas 66 cases out of 137 (48.2%) in the H3-group (CA–GA) had this diagnosis. In BMI-adjusted logistic regression, the risk of hypertension was 2 times higher in the H3-haplotype group compared with the H1-group (OR 2.01, 95% CI: 1.29–3.12, $P = 0.002$) (Table 3).

DISCUSSION

In the present study, we have amplified 2 functional polymorphic sites of the iNOS gene and studied their association with hypertension in a series of Finnish men and women living in the Tampere region (TAMRISK study). We found that mutation in the promoter region (rs2779249 (C/A)) of the iNOS gene associates with increased risk of hypertension. Moreover, we also found that those having the allele A had higher risk for hypertension already at the age of 35 years. A haplotype effect on hypertension was found with the above rs2779248 and the other iNOS SNP rs2297518 (G/A), a polymorphism which leads to differences in activity of iNOS protein and further NO production.[9] Thus our results not only further suggest that iNOS gene has a functional role in hypertension, but also imply that allelic variants of this gene may affect early manifestation of the disease.

The reason for increased prevalence of hypertension among A-allele carriers of the iNOS gene is unknown. The present polymorphism rs2779249 may affect transcription of iNOS as it is in the regulatory region of the gene. In fact, Fu et al[13] have found significant differences in the promoter

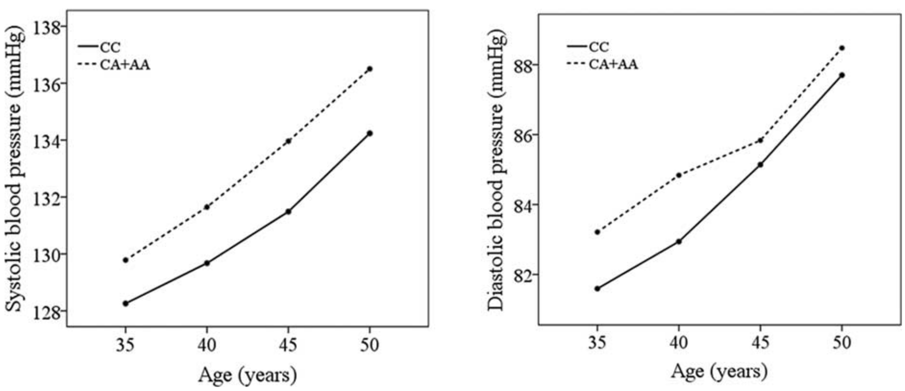

FIGURE 1. Means of SPB ($P = 0.047$) and DPB ($P = 0.048$) at different ages according to iNOS (rs2779249) genetic variants.

TABLE 3. Haplotype Frequencies for iNOS Genetic Variants and Their Estimated Association With Hypertension

Haplotype	iNOS Polymorphism		n (%)	OR (95% CI)	*P* (Adjusted for BMI)
	–1026 C > A	2087 G > A			
H1	CC	GG	389 (48.8)	Reference	—
H2	CA	GG	153 (19.2)	1.28 (0.84–1.94)	0.259
H3	CA	GA	137 (17.2)	2.01 (1.29–3.12)	0.002
H4	CC	GA	48 (6.0)	0.58 (0.27–1.24)	0.159
H5	AA	GA	27 (3.4)	0.74 (0.29–1.86)	0.515
H6	AA	GG	18 (2.3)	1.32 (0.48–3.66)	0.592
H7	AA	AA	15 (1.9)	0.86 (0.26–2.90)	0.806
H8	AA	AA	8 (1.0)	1.55 (0.35–6.88)	0.563

BMI = body mass index, CI = confidence interval, iNOS = inducible nitric oxide synthase, OR = odds ratio.

activity of the present alleles as the A-allele was associated with 5-fold increases in iNOS transcriptional activity compared with the C-allele. Lyamina et al showed that young men with high normal BP had a higher NO production than those with normal or optimum BP. In addition, the higher the BP, the more pronounced was NO overproduction. They also suggest that the inflammatory mediators may induce NO overproduction.[5] Increased concentration of NO is converted to peroxynitrite and superoxide in the prooxidant environment characteristic to essential hypertension. In addition, iNOS upregulates araginase activity, which limits NO production through eNOS. Animal model experiments provide evidence that iNOS participates in the regulation of renal function and BP[14] and it has been suggested that iNOS could be involved in the early rise in BP.[15]

The sympathetic nervous system plays a fundamental role in modulating cardiovascular functions. Catecholamine release leads to the activation of 3 β-adrenergic receptor subtypes (β1, β2, and β3-ARs), which regulate vasomotor tone. Endothelium might also control or facilitate β-AR effects of the vessels, since β-AR activation stimulates eNOS activity and could increase release of endothelial NO. In hypertension, the regulatory importance of NO is underlined by the observation that reduction of eNOS activity plays a pivotal role in BP control.[16] BP control by β-AR signaling is heavily regulated by G-protein-coupled receptor kinase (GRK2)-mediated desensitization.[17]

Permanently high catecholamine levels could lead to over activation of β-ARs, increasing eNOS activity and expression. Age-related increase in circulating catecholamines and decreased β-AR responsiveness is seen in the elderly.[18] Hyposensitivity to catecholamines is also seen during heart failure, and enhanced levels of catecholamines lead to chronic stimulation of cardiac β-ARs.[19] The third adrenergic receptor (β3-AR), which is suggested to act as a brake in sympathetic overstimulation in high catecholamine concentrations antagonizing β1- and β2-AR activity has in some conditions been shown to increase NO production through iNOS by an unknown mechanism.[20] This suggests that in aging and heart failure, there is an important relationship between adrenergic system and iNOS. In experimental heart failure in the rat, increased NO may contribute to decreased basal heart rate.[21] In fact, acute NOS inhibition led to an instantaneous recovery of the inotropic response to catecholamines in transgenic iNOS overexpressing mice.[22] Taken together, although iNOS has been previously implicated to be associated with heart failure by the adrenergic system, our findings suggest that it may have a role also in hypertension, such as that shown for eNOS.

There are only few publications concerning the role of iNOS genetic variants with hypertension and results have been conflicting apparently due to ethnic and environmental differences between populations. In opposite to our findings, Fu et al described the association of CC genotype of the iNOS rs2779249 polymorphism with hypertension in hypertensive Chinese families. They also found that the activity of a reporter gene construct containing the CC-genotype was approximately 5-fold lower than that with the A-allele.[13] Oliveira-Paula and coworkers found no significant associations between hypertension and rs2779429 polymorphism in their study. Instead, they reported that rs2297518 (G/A) polymorphism affects susceptibility to hypertension. Moreover, haplotype analysis containing pentanucleotide repeat polymorphism in the promoter region of the iNOS gene, rs2779429 (C/A) and rs2297518 (G/A) showed that S-C-A haplotype associated with hypertension and with responsiveness to antihypertensive therapy.[23] In another study, Glenn et al[24] did not find any association of 2 different repeat promoter variants of iNOS gene with hypertension in older hypertensives.

It is known that cigarette smoking results in increased levels of iNOS[25] and there are some reports showing joint effect with genotypes of the iNOS gene and smoking.[26,27] One inclusion criterion in our study population was that every case had at least one control with similar smoking habits. Markedly, when smokers were analyzed in their own group, association of A-allele of the rs2779249 polymorphic site with hypertension was even stronger.

Strengths of the study are that we have analyzed a study population with similar age and genetic background. It has been suggested that genetic determination in young individuals is much stronger than in old individuals, an observation that might be explained by increasing participation of nongenetic factors in disease etiology with aging.[28] Limitations of the study were that a possible difference in BP values at the age of 45 years and after might be difficult to establish, since practically all of the subjects who had hypertension were on BP medication by this age. In addition, each registration of BP values used in the repeated measures analysis was made at one examination visit only. Furthermore, the study subjects are from a restricted genetic pool (Finnish Caucasian), and the findings might not be extrapolated to different genetic populations.

In conclusion, our results support that A-allele of the iNOS gene (rs2779249) as well as haplotype AA (of variants rs2779249 (C/A) and rs2297518 (G/A)) contribute to increased risk of hypertension in the Finnish population. In addition, allelic variants of the gene affect the prevalence of the disease already at early middle-age.

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