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Rapid Letter

The A640G and C242T p22*phox* Polymorphisms in Patients with Coronary Artery Disease

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ABSTRACT

Oxidative stress plays a significant role in the pathogenesis of coronary artery disease (CAD). A p22*phox*-based NAD(P)H oxidase acts as a potent superoxide-generating system in the vasculature. We studied the association of the A640G and the C242T polymorphisms with clinical risk factors, endothelial function, and severity of CAD in a cohort of 216 patients referred for coronary angiography. The frequency of p22*phox* genotypes for AA, AG, and GG was 22.5, 52.3, and 25.2%, and for CC, CT, and TT 35.5, 51.3, and 13.2%, respectively. The A640G and the C242T polymorphisms were not associated with severity of CAD and endothelial function. The frequency distribution of the genotypes among patients with or without angiographically significant CAD did not reach statistical significance. Our study does not support a functional role for the A640G or C242T polymorphisms either in the severity of CAD or in determining endothelial function in older men. *Antioxid. Redox Signal.* 4, 675–680.

INTRODUCTION

OXIDATIVE STRESS plays a significant role in the pathogenesis of coronary artery disease (CAD) by contributing to altered control of vasomotor tone, atherosclerosis, and hypertension (1). The vascular tissue is a rich source for reactive oxygen species, including superoxide, hydrogen peroxide, and nitric oxide. Currently, attention is focused on p22*phox*-based NAD(P)H oxidases as critical determinants of the redox state of blood vessels, with effects on vascular tone via alterations in endothelium-dependent relaxations and on vascular smooth muscle growth (9). The NADPH oxidase is a multicomponent oxidase: a plasma membrane-associated cytochrome b558, composed of two subunits, p22*phox* and gp91*phox* or nox, two cytosolic components, p47*phox* and p67*phox*, and a small molecular weight G protein, either rac-1 or rac-2 (9, 15). Functionally, p22*phox* appears to be critical for the activity of the oxidase, because antisense inhibition of p22*phox* expression in vascular smooth muscle cells decreases superoxide and hydrogen peroxide production by these cells (19).

Although the association of the C242T genotype with CAD has been extensively studied and results have been conflicting, most studies find no association of this genotype with CAD (5, 8, 12, 13, 16). With regard to the A640G polymorphism, no association of the A640G polymorphism with the presence of CAD was found in a Japanese population, but the AA genotype of the A640G polymorphism was suggested to be independently associated with the presence and extent of CAD in a predominantly Caucasian population (8,12). There is a lack of functional studies investigating the A640G genotypes in relation to NAD(P)H oxidase activity and endothelial function. We hypothesized that the A640G polymorphism might affect extent and severity of CAD as measured by coronary angiography. We studied endothelium-dependent and -independent vascular reactivity as an indicator of the functional consequences of p22*phox* polymorphisms (2).

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MATERIALS AND METHODS

Patient population

The study sample comprised 216 patients who underwent coronary angiography between March 1999 and August 2000. All subjects provided informed consent, and the study was approved by the Institutional Review Board. Patients were referred for coronary angiography to the Atlanta Veterans Affairs Medical Center. All patients who agreed to participate in the study provided information about coronary risk factors and medications. Coronary risk factors were assessed as followed: smokers were defined as both current and past smokers. They were considered to have hypertension if they met the criteria of the World Health Organization or had been treated with antihypertensive drugs. They were hypercholesterolemic if their fasting total serum cholesterol was >220 mg/dl or they were taking lipid-lowering agents, and had diabetes mellitus if they met the diagnostic criteria of the World Health Organization or had been treated for diabetes.

Coronary angiography

Coronary vessels with at least 50% stenosis were defined as diseased. By means of coronary angiography, the study population was divided into three groups: (a) subjects without any angiographically detectable CAD or with coronary arterial stenosis of <50%, (b) subjects with single-vessel disease, and (c) subjects with multi-vessel disease.

Blood sampling, lymphocyte immortalization, and genotyping

Blood samples were collected during cardiac catheterization. Lymphocytes were isolated and immortalized by an Epstein–Barr virus transformation procedure via a modification of Neitzel's method, and genomic DNA was isolated (14).

The DNA fragment containing the A640G polymorphic site was amplified from genomic DNA by polymerase chain reaction (PCR) (12). *Dra III* restriction fragment length polymorphism (RFLP) analysis was used to analyze the A640G genotypes.

The DNA fragment containing the C242T polymorphic site of the $p22^{phox}$ gene was amplified from genomic DNA by PCR. *Rsa I* RFLP analysis was used to test for the presence of C242T polymorphism. To avoid possible mistyping of the genotypes, due to incomplete digestion, a second *Rsa I* restriction site was included in the PCR amplification product as an internal control.

Brachial ultrasonography

Assessment of endothelial function was performed in the brachial artery using high-resolution vascular ultrasonography (Toshiba SSH-140A) as per the guidelines for the ultrasound assessment of endothelium-dependent (flow-mediated) and endothelium-independent (nitroglycerin-induced) vasodilation of the brachial artery (6).

Statistical analysis

Age differences for the three categories of vessel disease were analyzed by analysis of variance. We used logistic regression to contrast the prevalence of dichotomous risk factors across the three categories of vessel disease. The prevalence of the G and T alleles of the $p22^{phox}$ polymorphisms were contrasted across the three categories of vessel disease by the chi-squared statistic. Adherence to Hardy–Weinberg equilibrium was assessed by a standard goodness-of-fit model using the chi-squared distribution.

RESULTS

The characteristics of the study patients are displayed in Table 1. There was a statistically significant difference in age among the categories of vessel disease with the highest mean age observed among subjects with multi-vessel CAD. As expected, smoking and hypercholesterolemia were significantly related to extent of vessel disease, and a history of diabetes was more common among subjects with multi-vessel disease, but the difference was not statistically significant. A history of hypertension was not consistently related to extent of ves-

TABLE 1. DEMOGRAPHIC CHARACTERISTICS AND RISK PROFILE OF STUDY SUBJECTS

Variable	*Nonsignificant CAD* (n = *52*)	*Single-vessel CAD* (n = *37*)	*Multi-vessel CAD* (n = *127*)	p*
Age	57.1 ± 9.3	56.4 ± 17.4	63.2 ± 9.9	<0.001
Male	49 (94.2)	35 (94.6)	127 (100)	0.03
Smoking status	33 (63.5)	26 (70.3)	105 (82.7)	0.02
Hypercholesterolemia	29 (55.8)	25 (67.6)	105 (82.7)	<0.001
Diabetes	15 (28.8)	10 (27.0)	54 (42.5)	0.09
Hypertension	33 (63.5)	24 (64.9)	91 (71.6)	>0.20
Family history of CAD	18 (34.6)	11 (29.7)	46 (36.2)	>0.20
History of PVD†	2 (3.9)	4 (10.8)	21 (16.5)	0.04

Age is presented as mean ± SD. The other variables are presented as number (or percentage in parentheses) of subjects with the given characteristics.

**p* value contrasting the three categories.

†PVD = peripheral vascular disease.

sel disease. A family history of CAD was unrelated to extent of vessel disease, whereas a personal history of peripheral vascular disease was significantly related to extent of vessel disease.

A640G and C242T polymorphisms and severity of CAD

The distribution of genotypes and the allele frequencies of the two polymorphisms of the p22^{phox} gene are summarized in Table 2. The allele frequencies among all subjects for both polymorphisms were in Hardy–Weinberg equilibrium. The G allele frequency of the A640G polymorphism was 0.51, 0.58, and 0.48, for no or <50%, single, and multi-vessel disease, respectively ($p > 0.20$). The frequency of the T allele for the C242T polymorphism was 0.38, 0.40, and 0.37 for the corresponding three categories of vessel disease, and this difference, too, is not statistically significant ($p > 0.20$).

A640G and C242T polymorphisms and endothelial function

It is possible that despite the absence of an effect on clinical expression of CAD, the polymorphism will manifest its effects on more subtle determinants of vascular oxidative stress, such as endothelial function. For these studies, we compared the effects of the p22^{phox} genotypes on flow-mediated and nitroglycerin-induced vasodilation.

The change in flow-mediated brachial artery diameter was somewhat lower among subjects with the homozygous A640/A640 (AA) genotype (median = 2.62, $n = 6$) compared with subjects with either the heterozygous A640/G640 (AG) or homozygous G640/G640 (GG) genotypes (median = 4.46, $n = 21$; Fig. 1A), but the mean difference (−1.59) did not reach statistical significance ($p = 0.09$) after adjustment for extent and severity of CAD. Furthermore, the change in brachial artery diameter with sublingual nitroglycerin was somewhat higher among subjects with the AA genotype (median = 15.6) compared with subjects with the AG/GG genotypes (median = 13.7), but the mean difference was not statistically significantly different ($p > 0.20$) after adjustment for extent and severity of CAD (Fig. 1B). Thus, presence of the

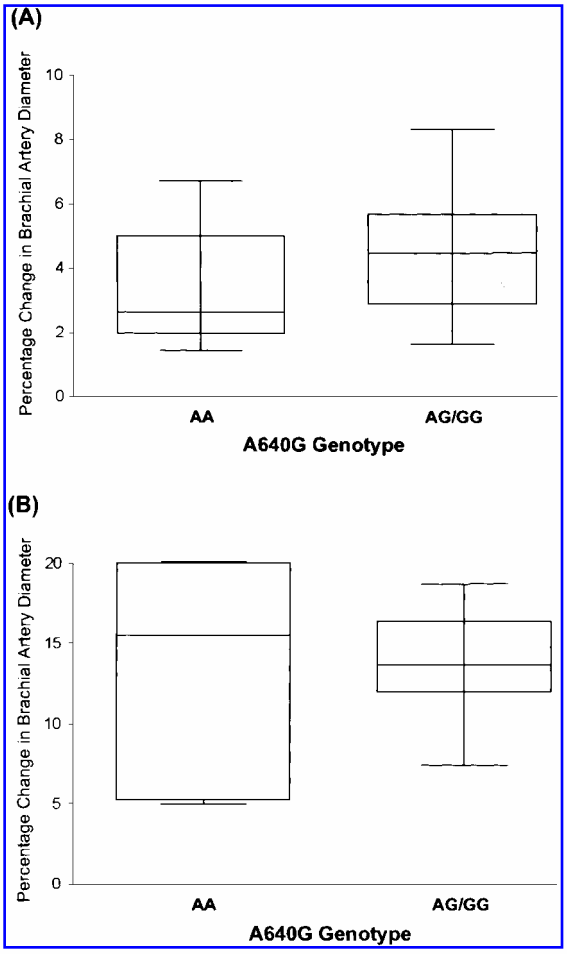

FIG. 1. Box-and-whiskers plot of effects of reactive hyperemia (A) and nitroglycerin (B) on the percent change in brachial artery diameter in patients with and without the AA genotype. Differences between groups were not statistically significant for flow-dependent ($p = 0.09$) and nitroglycerin-induced vasodilation ($p > 0.20$).

TABLE 2. GENOTYPE DISTRIBUTION OF A640G AND C242T P22^{PHOX} POLYMORPHISMS AND SEVERITY OF CAD BY CORONARY ANGIOGRAPHY

Genotype	*Nonsignificant CAD* (n = *50*)	*Single-vessel CAD* (n = *31*)	*Multi-vessel CAD* (n = *118*)
A640G polymorphism			
AA	12 (24.0)	5 (16.1)	32 (27.1)
AG	25 (50.0)	16 (51.6)	59 (50.0)
GG	13 (26.0)	10 (32.3)	27 (22.9)
G allele frequency	0.51	0.58	0.48
C242T polymorphism			
CC	18 (36.0)	12 (38.7)	46 (39.0)
CT	26 (52.0)	13 (41.9)	57 (48.3)
TT	6 (12.0)	6 (19.4)	15 (12.7)
T allele frequency	0.38	0.40	0.37

Data are presented as number (or percentage in parentheses) of subjects with the given genotype.

AA genotype did not affect endothelium-dependent or -independent brachial vascular function in patients with CAD. Because of the small sample size, multivariate analysis could not be performed to test whether this lack of relationship between AA genotype and vascular response to flow was due to the confounding influence of other factors that alter endothelial function.

The change in flow-mediated brachial artery diameter was similar for subjects with the homozygous C242/C242 (CC) genotype (median = 3.75, n = 11) and those with either the heterozygous C242/T242 (CT) or homozygous T242/T242 (TT) genotypes (median = 4.24, n = 16, Fig. 2). However, after adjustment for degree of vessel involvement, the mean difference was not statistically significant ($p > 0.20$). Finally, the change in brachial artery diameter with sublingual nitroglycerin was somewhat higher among subjects with the CC genotype (median = 15.6) compared with subjects with the CT/TT genotypes (median = 12.9), but the mean difference was not statistically significantly different ($p = 0.097$) after adjustment for extent and severity of CAD (Fig. 2B).

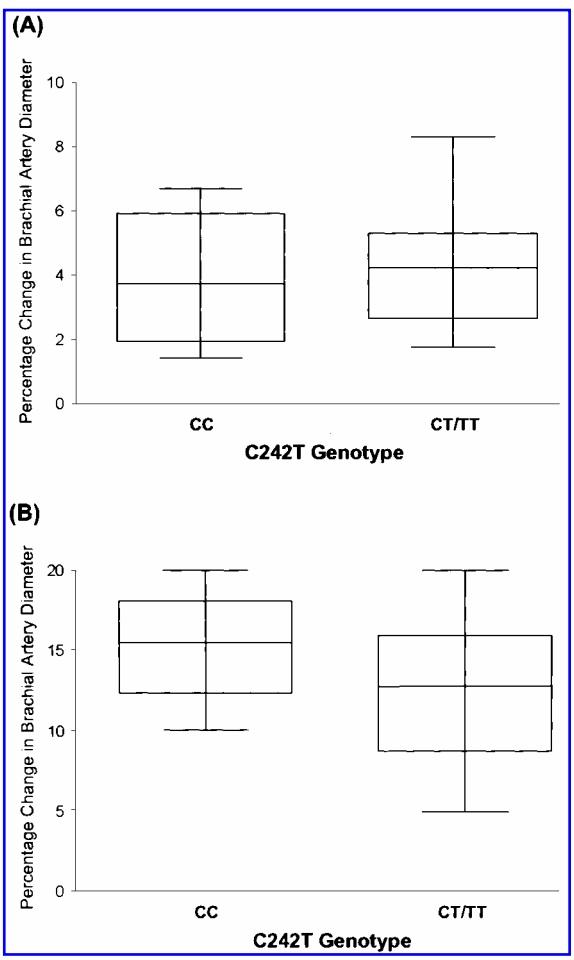

FIG. 2. Box-and-whiskers plot of effects of reactive hyperemia (A) and nitroglycerin (B) on the percent change in brachial artery diameter in patients with and without the T allele. Differences between groups were not statistically significant for flow-dependent ($p > 0.20$) and nitroglycerin-induced vasodilation ($p = 0.097$).

DISCUSSION

The results of this study show, by means of coronary angiography in a sample of predominantly older men, that the A640G and C242T p22^{phox} genotypes of the NAD(P)H oxidase are not associated with extent and severity of CAD. Although the change in flow-mediated brachial artery diameter was somewhat lower among subjects with the AA genotype compared with the other genotypes, this difference did not reach statistical significance after adjustment for extent and severity of CAD.

In a recent study, NAD(P)H oxidase activity was studied in saphenous veins from patients undergoing routine coronary artery bypass surgery, and it was demonstrated that the 242T allele is associated with significantly reduced vascular NAD(P)H oxidase activity, independent of other clinical risk factors for arthrosclerosis (10).

The C242T polymorphism of the p22^{phox} gene has been suggested to affect the heme-binding site, which is predicted to alter electron flow and superoxide production (15). The C242T polymorphism was found to be more prevalent in healthy Japanese patients compared with patients with established CAD (12). Subsequently, five studies with different ethnic backgrounds found conflicting results regarding the association between the p22^{phox} C242T polymorphism and prevalence or severity of CAD (Table 3) (4, 5, 8, 13, 16).

The AA genotype of the A640G polymorphism, which is located in the 3′untranslated region of p22^{phox}, has been suggested to be independently associated with the presence and extent of CAD (8). In contrast, our data demonstrate no association between the A640G polymorphism of the p22^{phox} gene and severity of CAD. This lack of relationship persisted even after correction for differences in other risk factors that may predispose to CAD. Our findings concur with the results of the study of Inoue *et al.,* who did not find an association between the A640G genotype and the presence of CAD (12). The design of our study is comparable to the investigation of Gardemann *et al.,* assessing extent and severity of CAD by means of coronary angiography (8). These divergent observations may be caused by the smaller size of our study sample, differences in genetic background, and the criteria used for phenotypic definitions.

Impaired endothelium-dependent vasodilation has been demonstrated previously in subjects with clinical atherosclerosis, as well as those with risk factors associated with the future development of CAD (6). There are two published studies reporting contrasting results regarding association between coronary endothelial function and C242T p22^{phox} genotypes (13, 17). Our study is the first investigation studying the effect of A640G genotype on brachial endothelial function. We found no statistically significant difference in either endothelium-dependent or -independent brachial vascular responses between patients with or without the AA genotype. Furthermore, there was no association between the C242T genotypes and brachial vasoreactivity, supporting the lack of

TABLE 3. CLINICAL ASSOCIATION STUDIES OF p22^{PHOX} POLYMORPHISMS IN PATIENTS WITH AND WITHOUT CAD

Study (year)	*Population**	*Age*	*T allele frequency*	*G allele frequency*	*Clinical association*
Ref. 12 (1998)	Japan CAD: 201 Control: 201	 54.9 ± 10.0 59.8 ± 7.6	 0.08 0.13	 0.61 0.59	T allele carries less risk for CAD.
Ref. 5 (1999)	Australia CAD: 550 Control: 139	 56.9 ± 0.3 54.5 ± 0.7	 0.33–0.35 0.31–0.32	 Not performed	T allele is not associated with CAD.
Ref. 13 (1999)	U.S.A. CAD: 149 Control: 103	 60 ± 0.8 57 ± 1.1	 0.42 0.34	 Not performed	T allele is not associated with CAD.
Ref. 8 (1999)	Germany CAD: 1,706 Control: 499	 62.7 ± 9.3 58.5 ± 10.5	 0.33–0.35 0.32–0.34	 0.49–0.51 0.52–0.53	T allele is not associated with CAD; AA genotype is associated with multi-vessel CAD.
Ref. 16 (1999)	Singapore CAD (I): 126 Control (I): 154 CAD (C): 151 Control (C): 167	 55.2 ± 0.7 53.9 ± 1 55.3 ± 0.8 54.5 ± 0.9	 0.4 0.38 0.1 0.09	 Not performed	T allele is not associated with CAD.
Ref. 4 (2000)	U.S.A. CAD + F: 156 CAD + P: 152	 58.4 ± 8.0 59.1 ± 7.9	 0.30 0.35	 Not performed	T allele is associated with CAD progression.

*C = Chinese, F = fluvastatin, I = Asian-Indian, P = placebo.

association between the C242T polymorphism and coronary endothelial function reported previously (13).

As the average age of our study population was 60 years and clinical coronary risk factors were present in the majority of our patients, we believe that older age, the latency of the atherosclerotic disease, and the masking effects of other risk factors that also impair endothelial function may have confounded the possible effect of the polymorphisms, when the severity and extent of CAD were assessed. Observations of our laboratory suggest that neutrophil-generated NADPH oxidase-dependent superoxide production is affected by p22^{phox} genotypes in younger subjects without CAD or coronary risk factors (18).

In conclusion, our study does not support a functional role for the A640G or the C242T polymorphisms either in the extent and severity of CAD or in altering endothelial function in older men with CAD. Further studies are clearly needed to demonstrate whether the p22^{phox} polymorphisms cause a relevant alteration in the function or level of the protein encoded by the gene, because it is likely that polymorphisms causing even minor changes in the function of p22^{phox}-containing oxidases could influence early atherogenesis and the progression of CAD.

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ABBREVIATIONS

CAD, coronary artery disease; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

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