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The A640G CYBA polymorphism associates with subclinical atherosclerosis in diabetes

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1. ABSTRACT

Oxidative stress is implicated in diabetes. The NADPH oxidases are the main source of superoxide in phagocytic and vascular cells, and p22phox is a key subunit. Genetic variants of *CYBA*, the human p22phox gene, associate with cardiovascular disease. We investigated the association of the A640G polymorphism with diabetes and its impact on phagocytic NADPH oxidase-dependent superoxide production and subclinical atherosclerosis. We studied 1212 subjects in which clinical parameters including carotid intima-media thickness (cIMT) were assessed. The A640G polymorphism was genotyped by TaqMan probes. In 496 subjects, the NADPH oxidase-dependent superoxide production in peripheral blood mononuclear cells was assessed by chemiluminescence. The GG genotype prevalence was significantly higher in type 2 diabetic patients than in non-diabetic subjects. Peripheral blood mononuclear cells from diabetic GG patients presented higher NADPH oxidase-dependent superoxide production than those of diabetic AA/AG patients. Within the diabetic group, GG patients presented higher cIMT levels than AA/AG patients. The A640G *CYBA* polymorphism may be a marker of oxidative stress risk and may be indicative of subclinical atherosclerosis in type 2 diabetes.

2. INTRODUCTION

Type 2 diabetes mellitus is a metabolic disease characterized by the elevation of blood glucose concentration, lipid abnormalities and vascular complications. It is a major health problem worldwide, and its prevalence is on the rise (1). Diabetes is a multifactorial disease with both genetic and environmental causes. Although the mechanisms involved in the development and progression of diabetes and its complications are complex, oxidative stress, that is, the accumulation of reactive oxygen species (ROS) due to increased production and/or decreased detoxification by antioxidants, seems to play a critical role (2).

The generation of ROS in diabetes has important vascular consequences: it reduces nitric oxide bioavailability, thus favouring endothelial dysfunction, leukocyte adhesion, proliferation, migration and apoptosis of vascular cells, platelet aggregation and thrombus formation (3). All these mechanisms contribute to atherosclerosis and cardiovascular events, which are more frequent in diabetic patients (1). In this regard, the carotid intima-media thickness (cIMT) is a marker of subclinical atherosclerosis that predicts cardiovascular risk in the general population as well as in diabetic patients (4, 5).

It is noteworthy that the family of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are key pathological pro-oxidants in diabetes, as observed both in clinical (6) and experimental studies (7, 8). Not only the vascular NADPH oxidases but also the NADPH oxidase of circulating white cells is altered in diabetes (9, 10).

The p22phox subunit is a common component of NOX1 to NOX4-dependent forms of the NADPH oxidase, and plays an essential role in NADPH oxidase activation (11). Interestingly, monocytic (10) and lymphocytic (12) p22phox levels are increased in human diabetes. One of the mechanisms that regulate p22phox levels and NADPH oxidase activity is the genetic component (13). Several allelic variants have been identified in *CYBA*, the gene encoding the human p22phox subunit, such as the A640G polymorphism, located in the 3´ untranslated region (UTR) of *CYBA* (14). Association studies of this variant with cardiovascular disease are somehow conflicting, with both positive (15) and negative (16, 17) results. In our study, we have analysed the potential association of the A640G polymorphism with diabetes. In addition, we have studied the impact of this polymorphism on superoxide release from peripheral blood mononuclear cells and its association with surrogate markers of cardiovascular risk in type 2 diabetes.

3. MATERIALS AND METHODS

3.1. Participants and clinical studies

The study population consisted of 1212 consecutive, asymptomatic subjects of Caucasian origin who attended the University Clinic of Navarra for a routine medical work-up. Subjects were confirmed as genetically unrelated through interviewing. Blood pressure was measured on three occasions using a mercury sphygmomanometer and the mean of these readings was recorded. None of the hypertensive patients presented echocardiography evidence of aortic stenosis or hypertrophic cardiomyopathy, clinical manifestations of heart failure. Type 2 diabetes was defined if the fasting glucose levels were above 125 mg/dL and/or if the patient was under hypoglycemic treatment. Obesity was defined if body mass index (BMI) was ≥ 30. Subjects were free from clinically apparent atherosclerotic disease based on: (1) absence of history of coronary disease, stroke, or peripheral artery disease; and (2) normal electrocardiogram and chest-x-ray results. Patients were excluded if they had advanced carotid atherosclerosis according to the cIMT measurements (>1.7 mm). Additional exclusion criteria were the presence of severely impaired renal function, arteritis, collagenosis, and a history of alcohol abuse. Patients with significant acute infection, according to clinical criteria by the attending physician, were also excluded.

To determine cIMT, ultrasonography of the common carotid arteries was performed with a 5- to 12-MHz linear-array transducer (ATL 500 HDI). The measurement of IMT was made 1 cm proximal to the carotid bulb of each common carotid artery at plaque-free sites. For each individual, the IMT was determined as the average of near wall and far wall measurements of each common carotid artery. Subjects were examined by the same 2 certified sonographers blinded to all clinical information. The reproducibility of IMT measurements between and within sonographers had previously been checked in individuals who returned 2 weeks later for a second examination. The intraobserver and interobserver coefficients of variation were 5% and 10%, respectively (18).

According to institutional guidelines, all subjects were aware of the research nature of the study and agreed to participate. The study was carried out in accordance with the Helsinki Declaration and the Ethics Committee of the University of Navarra approved of the protocol.

3.2. Determination of superoxide anion production

In 496 of our patients, the NADPH oxidase-dependent superoxide production was measured in peripheral blood mononuclear cells (monocytes and lymphocytes) isolated from blood samples with Lymphoprep (Axis-Shield) in response to stimulation with phorbol 12-myristate 13-acetate (PMA, 2 mg/L; Sigma) and using lucigenin (5 micromol/L; Sigma) in a chemiluminescent method that correlated well with the ferricytochrome C assay, as described previously (18, 19).

3.3. Genotyping

DNA was isolated from venous blood with the QIAamp DNA Blood Kit (Qiagen) according to the manufacturer. The A to G substitution at position 640 in the 3´ UTR (rs1049255) was genotyped by allelic discrimination, using the TaqMan probe (C_7516916_10) (Applied Biosystems) and the ABI PRISM 7000 Sequence Detector (Applied Biosystems).

3.4. Statistical analysis

Data are expressed as mean+/-SEM. Chi-square analyses were used to test for deviation of genotype distribution from Hardy-Weinberg equilibrium. Chi-square as well as binary logistic regression analyses were used to determine whether there were significant differences in genotype frequencies between cases and controls. In view of the results of the normality test (Shapiro-Wilks), variations in the clinical data were assessed either by Student's t-test or a Mann-Whitney U-test Multivariate linear regression analysis was performed to evaluate factors related to the A640G polymorphism and the possibility of interactions. Statistical analyses were performed with SPSS for Windows, version 15.0 (SPSS Inc.). $P<0.05$ was considered statistically significant.

4. RESULTS

4.1. Association of the A640G polymorphism with diabetes

We genotyped A640G polymorphism of *CYBA* in 1212 subjects. The prevalence was: AA: 366 (30.2%), AG: 604 (49.8%), GG: 242 (20.0%). The distribution followed Hardy-Weinberg equilibrium law (Chi-

Table 1. Clinical parameters of the population in study

	Non-diabetic (n=1073)	Diabetic (n=139)	*P*
Age (y)	54+/-1	60+/-1	<0.001
Gender (m/f)	827/246	112/27	0.389
BMI (kg/m^2)	28.0+/-0.1	29.9+/-0.4	<0.001
SBP (mmHg)	127+/-1	135+/-2	<0.001
DBP (mmHg)	81+/-1	81+/-1	0.190
Glucose (mg/dL)	96+/-1	154+/-4	<0.001
Insulin (pmol/L)	70.2+/-1.4	112.5+/-7.0	<0.001
HOMA index	2.56+/-0.06	6.04+/-0.40	<0.001
HDL (mg/dL)	54+/-1	50+/-1	0.002
LDL (mg/dL)	143+/-1	134+/-4	0.009
Total Cholesterol(mg/dL)	219+/-1	210+/-4	0.009
Triglycerides (mg/dL)	112+/-2	134+/-6	<0.001
cIMT (mm)	0.687+/-0.006	0.766+/-0.019	<0.001
Hypoglycemic treatment (%)	0	59	<0.001
Antihypertensive treatment (%)	29	48	<0.001
Statin treatment (%)	16	23	0.034

BMI: body mass index, DBP: diastolic blood pressure, SBP: systolic blood pressure, cIMT: carotid intima-media thickness.

Table 2. Prevalence of the A640G polymorphism in diabetes

	Non-diabetes	Diabetes	Chi-square	*P*
AA (n, %)	331, 31.0	35, 24.3	6.073	0.048 [1]
AG (n, %)	534, 50.0	70, 48.6		
GG (n, %)	203, 19.0	39, 27.1		
AA/AG (n%)	865, 81.0	105, 72.9	5.179	0.023 [2]
A allele frequency	0.5599	0.4861		
G allele frequency	0.4401	0.5139		

[1]Comparison of all three genotypes. [2]Comparison of GG versus AA/AG.

Table 3. Logistic analysis of the association of the A640G polymorphism with diabetes

	Beta	*P*	R-square of model	Chi-square of model	*P* of model
Age (years)	0.059	<0.001	0.088	56.229	<0.001
Sex (female *vs* male)	0.285	0.218			
A640G polymorphism	0.345	0.008			

square=0.064;*P*=0.801) and was in agreement with current HapMap data for Caucasian populations (20).

The general characteristics of the subjects in our study, classified according to their diabetic status is summarised in Table 1. Type 2 diabetic patients were older and with higher BMI. They presented higher systolic blood pressure levels as well as circulating glucose, insulin and HOMA levels. Besides, they had lower HDL and higher triglyceride levels, and also lower LDL and total cholesterol levels, probably due to treatment. In addition to hypoglycemic treatment, 48% of the diabetic patients were under antihypertensive treatment whereas that was the case for 29% of the patients of the non diabetic group. In addition, and in accordance with the clinical parameters above, diabetic patients presented a significantly higher cIMT, surrogate marker of atherosclerosis (4, 5).

When we studied the association of the A640G polymorphism with diabetes, we detected a significant increase in the G allele prevalence and decrease in the A allele prevalence in the diabetic group (Table 2). Importantly, a binary logistic analysis confirmed that the A640G polymorphism was associated with diabetes independently of age and sex (Table 3). In our population, the A640G polymorphism was not associated with hypertension or obesity (data not shown).

When we assessed the effect of the A640G polymorphism on clinical parameters we detected that patients with GG genotype presented higher levels of glucose, insulin, HOMA and triglycerides, and lower HDL levels (Table 4). These data, together with the data in Table 3 suggest that the GG genotype may have deleterious effects; therefore, a recessive model in which GG patients were compared with AA/AG patients was used.

4.2. Association of the A640G polymorphism with clinical phenotypes

Since diabetes is a well established risk factor for atherosclerosis, we further investigated the effect of the polymorphism on cIMT, a surrogate marker of subclinical atherosclerosis. In the diabetic group, subjects with GG genotype presented significantly (*P*=0.040) higher cIMT than patients with AA/AG genotype, whereas no differences according to genotype were detected for the non-diabetic group (Figure 1).

4.3. Association of the A640G polymorphism with superoxide production in peripheral blood mononuclear cells

In a subpopulation representative of the whole of 496 patients, we were able to perform functional studies in circulating mononuclear cells. In diabetic patients, the A640G polymorphism altered the phagocytic NADPH oxidase-dependent superoxide production in response to PMA: there was a clear trend (*P*=0.055) towards higher superoxide anion production in diabetic patients with GG genotype, whereas there were no differences according to genotype in non diabetic subjects (Figure 2). What is more,

Table 4. General characteristics of the population in study according to the genotype for the A640G polymorphism of *CYBA*

	AA (n=366)	AG (n=604)	GG (n=242)	*P* [1]	AA/AG (n=970)	*P* [2]
Age (y)	55+/-1	54+/-1	54+/-1	0.650	55+/-1	0.669
Gender (m/f)	286/80	464/140	189/53	0.863	750/220	0.795
BMI (kg/m^2)	28.2+/-0.3	28.1+/-0.2	28.4+/-0.3	0.736	28.1+/-0.1	0.484
SBP (mmHg)	129+/-1	128+/-1	129+/-1	0.365	128+/-1	0.488
DBP (mmHg)	81+/-1	81+/-1	81+/-1	0.929	81+/-1	0.759
Glucose (mg/dL)	102+/-1	101+/-1	107+/-2	0.083	101+/-1	0.034
Insulin (pmol/L)	76.4+/-2.8	77.1+/-2.8	85.4+/-3.5	0.011	77.1+/-2.1	0.003
HOMA index	2.82+/-0.12	2.95+/-0.13	3.29+/-0.17	0.006	2.90+/-0.09	0.002
HDL (mg/dL)	54+/-1	54+/-1	51+/-1	0.057	54+/-1	0.019
LDL (mg/dL)	141+/-2	142+/-2	143+/-3	0.849	142+/-1	0.610
Total Cholesterol(mg/dL)	217+/-2	218+/-2	219+/-3	0.949	218+/-1	0.752
Triglycerides (mg/dL)	114+/-4	111+/-3	123+/-5	0.035	112+/-2	0.010

BMI: body mass index, DBP: diastolic blood pressure, SBP: systolic blood pressure, cIMT: carotid intima-media thickness. [1]Comparison of all three genotypes. [2]Comparison of GG versus AA/AG.

Table 5. Multivariate analysis of the phagocytic NADPH oxidase-dependent superoxide production

	Beta	*P*	R-square of model	F of model	*P* of model
Age (years)	0.083	0.069	0.044	4.493	0.001
Sex (female *vs* male)	0.057	0.207			
Glucose (mg/dL)	0.083	0.080			
Triglycerides (mg/dL)	0.081	0.075			
A640G polymorphism (GG *vs* AA/AG)	0.093	0.038			

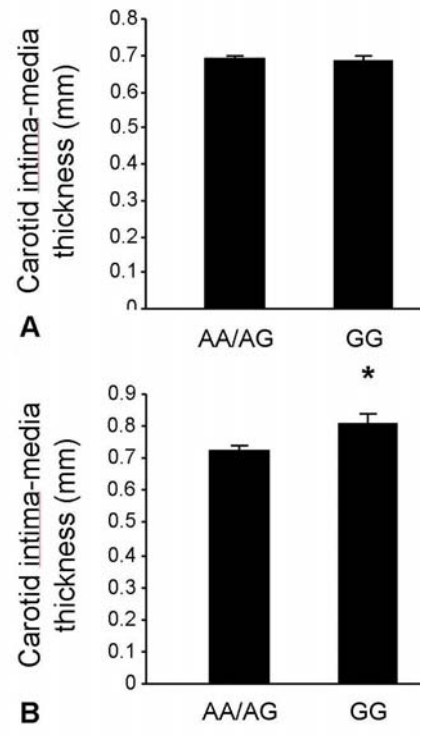

Figure 1. Carotid intima-media thickness in non-diabetic subjects (A) and in diabetic patients (B) according to genotype for the A640G polymorphism of *CYBA*. **P*<0.05.

multivariate studies showed that the A640G polymorphism was a significant determinant of the NADPH oxidase-dependent superoxide production, after adjusting for confounding factors (Table 5).

5. DISCUSSION

The first finding of our study is the association of the A640G polymorphisms of *CYBA* with type 2 diabetes, an important risk factor for the development of atherosclerosis and cardiovascular disease. The prevalence of the A640G polymorphism in our population was similar to HapMap data (20). Genotyping studies showed an increased prevalence of the G allele in diabetic patients and this association was independent from age and sex. In agreement with this, Hodgkinson *et al.* (21) detected an association of the A640G polymorphism with diabetic

complications in which a combination of the A640G and

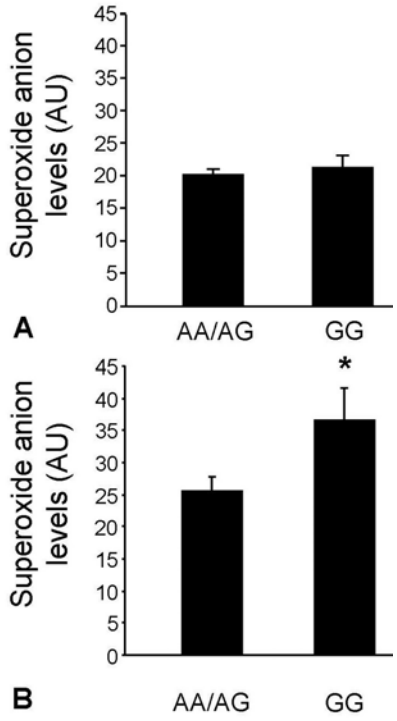

Figure 2. Phagocytic NADPH oxidase-dependent superoxide production in non-diabetic subjects (A) and in diabetic patients (B) according to the genotype for the A640G polymorphism of *CYBA*. **P*=0.055.

C242T polymorphisms of *CYBA* (T242/G640) was related to greater nephropathy risk. Association studies of this polymorphism with cardiovascular disease profiles have been conflicting: Inoue and colleagues (16) did not find an association of the variant with coronary artery disease (CAD) in a Japanese population, nor did Zafari *et al.* (17) in a white-American population. Conversely, Gardemann *et al.* (15) detected a significant reduction of the G allele in European patients with CAD. As we have detected that the A640G polymorphism is associated with diabetes but not hypertension or obesity, it can be speculated that the diabetic status of the patients in the studies mentioned above may have been a confounding factor.

The second result reported here is the association of the A640G polymorphism with the NADPH oxidase-dependent superoxide production in peripheral blood mononuclear cells. Cells from diabetic patients with GG genotype displayed higher production than those from patients with AA/AG genotypes, whereas no differences were found out in non diabetic subjects according to genotype. It is known that the NADPH oxidase activation, both in vascular (22) and circulating cells (23), is an important mechanism in atherosclerosis and is enhanced in diabetes (6). In our population, subjects with GG genotype also presented higher levels of glucose and insulin, as well as an altered lipid profile. There is evidence that glucose (24, 25) and insulin (26, 27) can activate the NADPH oxidase system and contribute to oxidative stress and a pro-inflammatory state. In fact Guzik *et al.* (6) have shown that the NADPH oxidase system is implicated in the superoxide release that takes place in vessels from diabetic patients.

The cause of the increased NADPH oxidase-dependent superoxide production of peripheral blood mononuclear cells from GG patients may be a direct genetic effect driven by the polymorphism. Although the functionality of the A640G polymorphism is currently unknown, its location in the 3´ UTR suggests it may affect mRNA processing and stability and, hence, transcriptional rate. Studies in human neutrophils (28) and lymphoblasts (29) show that the A640G polymorphism does not seem to have an impact on the NADPH oxidase-dependent superoxide production. However, the first work (28) was carried out in young, healthy volunteers as opposed to our study, in which subjects are older and may have diverse risk factors. The second study (29) was performed *in vitro* in cultured lymphoblastoids from patients with coronary artery disease, rather than being a direct *ex vivo* determination. Therefore, these two studies may lack the setting in which the effect of the A640G polymorphism is manifest. Nevertheless, further molecular studies would be necessary to assess if the A640G polymorphism leads to greater NADPH oxidase-dependent superoxide production in mononuclear cells via an increased p22phox transcription/translation.

On the other hand, the polymorphism may not be active by itself but rather a marker of risk, maybe due to other genetic causes. In this regard, preliminary linkage disequilibrium studies of the A640G polymorphism with other functional *CYBA* polymorphisms (namely the -930A/G (19) and the C242T (30)) show that linkage is low (data not shown). This suggests that, in our study, the pro-oxidant profile associated with the A640G polymorphism is not due to these other polymorphisms. We are aware that a single variant can explain only a reduced part of the phenotypic variability of a complex disease, and that the environmental factors play a role, as well. Nevertheless, a selection of markers in a certain context may help to single out patients at risk.

The third finding of our study is that in our population the A640G polymorphism shows clinical relevance, as diabetic GG subjects present higher levels of cIMT, a surrogate marker of subclinical atherosclerosis (4, 5). It is well known that one of the major complications of diabetes is atherosclerosis (31-33) and that oxidative stress is implicated (3). Our study shows that, in addition to the biochemical alterations that contribute to atherosclerosis in diabetes, the genetic component may further worsen the clinical profile (34). In agreement with it, Hayaishi-Okano *et al.* (35) showed that another *CYBA* variant, the C242T associates with cIMT. Therefore, genetic markers like the A640G polymorphism may be useful to identify patients with higher risk.

We have observed an association of the A640G polymorphism with NADPH oxidase-dependent superoxide production only in diabetic patients. Similarly, we have detected an increased cIMT in diabetic patients with GG

genotype. Our findings are similar to that of Hayaishi-Okano *et al.* (35) who detected an association of the *CYBA* C242T polymorphism with cIMT only in diabetic patients but not in controls. This observation in turn exemplifies the importance of the interaction between multiple environmental and genetic factors in complex diseases (34, 36).

Some limitations of the study should be acknowledged. First, the prevalence of diabetes in our population necessarily limits the statistical power; further studies including larger numbers of subjects should be performed to confirm the current results. Second, some of the subjects in our study were under treatment according to their cardiovascular profile (antihypertensive drugs, antiglycemic drugs and cholesterol-lowering drugs) and this may have been a confounding factor in our analysis. However, our multivariate study shows that the A640G polymorphism is a determinant of NADPH oxidase-dependent superoxide production, after correcting for glucose and triglyceride levels. Finally, no data have been presented regarding the antioxidant status. The literature suggests that, in addition to greater activity from pro-oxidant systems, diabetic patients present attenuated antioxidant defences (37, 38), which may worsen their oxidative stress status.

In summary, we have detected that the A640G polymorphisms of *CYBA* is associated with diabetes. Subjects with the GG genotype presented higher NADPH oxidase-dependent superoxide production by their peripheral blood mononuclear cells and subclinical atherosclerosis. Therefore, the A640G polymorphism may identify individuals at greater risk of developing vascular complications in the setting of type 2 diabetes mellitus.

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Key Words: Atherosclerosis, Diabetes, Intima-Media Thickness, NADPH oxidase, oxidative stress, A640G polymorphism

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