

C242T *CYBA* Polymorphism of the NADPH Oxidase Is Associated With Reduced Respiratory Burst in Human Neutrophils

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Abstract—Oxidative stress contributes to the pathogenesis of atherosclerosis. p22^{phox}-based NAD(P)H oxidases exist in the vessel wall, acting as important superoxide-generating systems in the vasculature. Some studies have identified reduced atherosclerosis in the presence of the C242T *CYBA* polymorphism, whereas others have not. Because vascular p22^{phox} is identical to neutrophil p22^{phox}, we studied the association between the C242T, A640G, and -930^{A/G} *CYBA* polymorphisms and the quantity of superoxide produced from neutrophils isolated from healthy adults to determine if these polymorphisms had any functional impact on NADPH oxidase function. Neutrophils were isolated from 90 subjects by Percoll density gradient centrifugation. Genotypes were determined by polymerase chain reaction (PCR) and restriction mapping, as well as real-time PCR. The oxidative burst was stimulated with phorbol 12-myristate 13-acetate. Superoxide was quantified using the superoxide dismutase inhibitable oxidation of the spin probe hydroxylamine 1-hydroxy-3-carboxy-pyrrolidine, detected by electron paramagnetic resonance. Superoxide production was significantly affected by the C242T polymorphism, being 8.7 ± 0.7 , 7.9 ± 0.6 , and 5.9 ± 1.2 $\mu\text{mol/L}$ per minute per 10^6 neutrophils for the C242T CC, CT, and TT genotypes, respectively ($P < 0.05$). In contrast, the A640G and the -930^{A/G} polymorphisms did not alter the neutrophil respiratory burst. Phagocytic respiratory burst activity in homozygous individuals with the T allele of the C242T *CYBA* polymorphism is significantly lower than of wild-type carriers and heterozygous individuals. Because p22^{phox} exists in both the neutrophil and vessel wall, vascular oxidative stress is likely diminished in individuals with this polymorphism. (*Hypertension*. 2004;43:1-6.)

Key Words: atherosclerosis ■ neutrophils ■ oxidative stress ■ polymorphism ■ risk factors

Oxidative stress plays a significant role in the pathogenesis of coronary artery disease (CAD) by altering vasomotor tone, enhancing atherosclerosis and contributing to hypertension.¹ It is clear that vascular cells can produce reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and nitric oxide (NO). Currently, attention is focused on p22^{phox}-based NAD(P)H oxidases as major sources of ROS in blood vessels.^{2,3} The molecular structure of vascular NA(D)PH oxidases is similar to the phagocytic NADPH oxidase that is responsible for the respiratory burst. The neutrophil oxidase is a multicomponent enzyme system composed of membrane and cytosolic components. The membrane components, known as the cytochrome b558, comprise 2 subunits, p22^{phox} and gp91^{phox}. The cytoplasmic components include p47^{phox} and p67^{phox}, p40^{phox}, and rac-2.² The p22^{phox} subunit is central to the normal functioning of the oxidase because it stabilizes the large subunit and serves a docking function for the cytosolic factors. Functionally,

p22^{phox} is critical for the activity, because antisense inhibition of p22^{phox} expression in vascular smooth muscle cells decreases superoxide and hydrogen peroxide production in response to angiotensin II by these cells.^{4,5}

In human coronary arteries, p22^{phox} is expressed in atherosclerotic areas more intensely than in nonatherosclerotic areas.⁶ To date, several polymorphisms of *CYBA*, the gene encoding p22^{phox}, have been identified. The C242T polymorphism is located on chromosome 16q24, exon 4, at position 242 of *CYBA*.⁷ The C242T polymorphism encodes a CAC-TAC replacement, which predicts the nonconservative substitution of histidine-72 with a tyrosine residue. The A640G gene polymorphism is located in the 3' untranslated region of *CYBA* with no frank amino acid substitution.^{8,9} The -930^{A/G} gene polymorphism is located in the p22^{phox} promoter, and is associated with higher promoter activity.¹⁰ Given the critical role of the p22^{phox}-based vascular NAD(P)H oxidases in ROS generation in the vasculature, it is conceiv-

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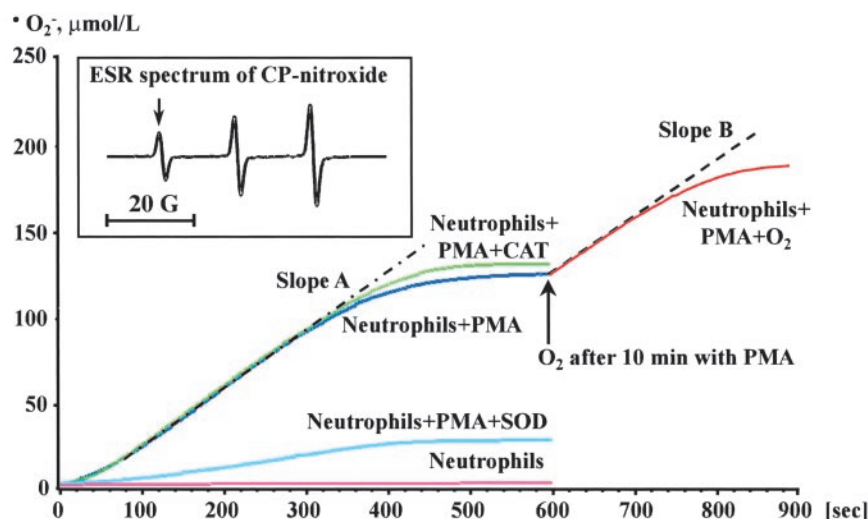


Figure 1. Representative ESR tracing of neutrophil superoxide production. Inset depicts the ESR signature scan of CP-nitroxide. All kinetic scans were performed by measuring the increasing amplitude of the low magnetic field peak (solid arrowhead). Resting neutrophils are represented in purple, neutrophils (2.0×10^6 cells) stimulated with 200 nmol/L PMA are in blue, neutrophils preincubated with 100 U SOD before PMA stimulation are in light blue, and neutrophils preincubated with SOD and 50 μ g/mL catalase (CAT) before PMA stimulation are in green. The red line represents neutrophils stimulated by PMA, which have been reoxygenated after reaching the plateau.

able that altered activity of $p22^{phox}$ may modify risk for atherosclerosis. Indeed, the association of the *CYBA* genotypes with CAD was reported previously in clinical association studies, and the results were contradictory. Inoue et al reported a significant protective effect of the C242T polymorphism on the presence of CAD in Japanese subjects.¹¹ In contrast, Cahilly et al found the C242T polymorphism to be a marker of progression of CAD.¹² We and others found no association between C242T *CYBA* polymorphism and the severity of CAD detected by coronary angiography, suggesting no functional role of the polymorphism in prevalence and severity of CAD.^{13–17} The conflicting data on whether this polymorphism contributes to CAD is likely because multiple long-standing risk factors and atherosclerotic burden confound the possible effect of this polymorphism on a polygenic disease such as atherosclerosis.

Importantly, to our knowledge to date, there has not been a precise assessment of the impact of these polymorphisms on NADPH oxidase function. This is noteworthy, because in the absence of an alteration in NADPH oxidase activity, it is highly unlikely that a polymorphism in $p22^{phox}$ would alter vascular function or predispose to CAD. In view of this, we examined the functional consequences of *CYBA* polymorphisms using a cell type in which the NADPH oxidase subunit composition is well defined in a population of healthy adults. This approach was chosen to eliminate the contribution of other sources of superoxide present in the vasculature, confounding risk factors, and atherosclerotic burden. Because polymorphisms are shared across tissues and $p22^{phox}$ is common to all known NAD(P)H oxidases, measurement of neutrophil respiratory burst activity permits assessment of the effect of a given polymorphism on enzyme function in multiple tissues. We therefore examined the effect of the C242T, A640G, and $-930^{A/G}$ polymorphisms on neutrophil NADPH oxidase activity.

Methods

Subjects

The study group consisted of 90 healthy adults who gave informed consent for participation. Coronary risk factors in this study were defined as described previously.¹⁷ Subjects were excluded if there

was a history for CAD, if >2 risk factors for CAD were present, if there was clinical evidence for an acute or chronic infection, and if they were older than 45 years. This study was approved by the Emory University Institutional Review Board and the procedures followed institutional guidelines.

Blood Sampling and Genotyping

DNA was extracted from venous blood samples, and the DNA fragment containing the C242T and A640G polymorphic sites of *CYBA* was amplified from genomic DNA by polymerase chain reaction (PCR) as previously described.¹⁷

The $-930^{A/G}$ polymorphism in the promoter of the *CYBA* gene was determined using the TaqMan (Applied Biosystems) PCR method and the ABI PRISM 7900HT Sequence Detector (Applied Biosystems). Clear identification of each genotype was independently validated with sequencing by Emory DNA sequencing core facility as well as by DNA high-performance liquid chromatography using Transgenomic WAVE DNA fragment analysis.

Neutrophil Isolation

Soon after venous blood sampling, neutrophils were isolated by Percoll density gradient centrifugation as previously described.¹⁸

Superoxide Measurement

Superoxide production by human neutrophils was measured using electron spin resonance spectroscopy (ESR) with the superoxide dismutase (SOD) inhibitable oxidation of the spin probe CPH (Figure 1), which after reaction with superoxide yields a stable nitroxide radical with a half-life of several hours.^{19,20} Neutrophils (1×10^6) were incubated with 5 mmol/L CPH and stimulated by the addition of 200 nmol/L of phorbol 12-myristate 13-acetate (PMA). All ESR samples were prepared using chelated phosphate-buffered saline, pH 7.4. ESR spectra and kinetics were recorded in a 50- μ L glass capillary tube at room temperature using a Bruker EMX spectrometer. ESR spectra were obtained using the following instrument settings: field sweep, 60 G; microwave frequency, 9.822 GHz; microwave power, 20 mW; modulation amplitude, 1 G; conversion time, 163.840 ms; time constant 327.68 ms; sweep time, 167.772 s; receiver gain, 1×10^4 . ESR kinetic scans were obtained over 10 minutes in unstimulated neutrophils and after PMA stimulation in the presence or absence of 100 units of SOD (Figure 1). The rate of superoxide formation was measured by monitoring the amplitude of the low-field component of the ESR spectrum as previously described.²⁰ The concentration of CP-nitroxide was determined from a calibration curve for intensity of the ESR signal at various known concentrations of CP-nitroxide. The rate of superoxide production was calculated from the accumulation of the CP-nitroxide, obtained from the time scan of CPH oxidation (Figure 1). For this purpose, the

Clinical and Demographic Characteristics of Study Subjects

Characteristic	Study Subjects
Age, y	33 (20–45)
Gender, n (%)	
Male	55 (61)
Female	35 (39)
Risk factors, n (%)	
Family history of CAD	18 (20)
Hypercholesterolemia	2 (2)
Hypertension	1 (1)
Smoking	2 (2)
Ethnicity, n (%)	
White	78 (86)
Black	6 (7)
Other	6 (7)

Values are mean or n (%).

kinetics of CP-nitroxide were integrated during the first 300 sec using WinEPR software (Bruker Biospin Corp) and results were expressed as $\mu\text{mol/L } \text{O}_2^-/\text{min}$ per 10^6 cells. These results were confirmed in a subset of subjects in whom we demonstrated that this ESR-based method provided results that correlated well with the cytochrome C assay ($r=0.8$) and measurement of oxygen consumption (data not shown).

Measurement of p22^{phox} Protein Levels

Western blotting was performed as previously described.⁵ Briefly, equal amount of protein from neutrophils were applied on 12% SDS-polyacrylamide gels. Polyclonal anti-p22^{phox} antibody and horseradish peroxidase-labeled donkey anti-rabbit immunoglobulin (Santa Cruz and Amersham) were used as primary and secondary antibodies. The signals were detected by the enhanced chemiluminescence method and quantified by densitometry.

Statistical Analysis

Exact procedures for the binomial distribution were used to obtain 95% confidence intervals for the prevalences of the T and G alleles of the *CYBA* polymorphisms. The observed distribution of the genotypes compared with that expected under Hardy-Weinberg equilibrium were evaluated by a 1-degree-of-freedom χ^2 test.²¹ Differences in the means of neutrophil-generated respiratory burst activity according to the genotypes of the 3 *CYBA* polymorphisms were evaluated by standard ANOVA.²² Finally, we also used the Wilcoxon rank-sum test, a nonparametric procedure when data were not normally distributed.²³ All statistical analysis was implemented using Stata.²⁴

Results**Subject Characteristics and Genotype Frequencies**

Demographic and clinical characteristics are summarized in the Table. Among whites, the distribution of the C242T genotypes were 34 CC, 33 CT, and 11 TT; the distribution of the A640G genotypes were 23 AA, 34 AG, and 21 GG; and the distribution of the $-930^{\text{A/G}}$ genotypes were 14 AA, 38 AG, and 24 GG. The genotypes for each polymorphism were in Hardy Weinberg equilibrium. Among whites, the prevalence of the T allele of the C242T polymorphism was 0.35 (0.28, 0.43)_{95%}, the prevalence of the G allele of the A640G polymorphism was 0.49 (0.41, 0.57)_{95%}, and the prevalence of the G allele of the $-930^{\text{A/G}}$ polymorphism was 0.56 (0.48, 0.65)_{95%}. The corresponding allele prevalences for blacks

were 0.17 (0.02, 0.48)_{95%}, 0.58 (0.28, 0.85)_{95%}, and 0.58 (0.28, 0.85)_{95%}. However, these prevalences were based on only 6 persons and hence were imprecise.

Relationship Between Superoxide Generation and Genotype

The mean levels of superoxide generation in our entire cohort ($n=72$) was $0.08 \pm 0.005 \mu\text{mol/L } \text{O}_2^-/\text{min}$ per 10^6 cells in the basal state, and $8.3 \pm 0.4 \mu\text{mol/L } \text{O}_2^-/\text{min}$ per 10^6 cells after PMA stimulation ($n=90$). Neutrophils from subjects with the CC and CT genotypes of the C242T *CYBA* polymorphism produced significantly more superoxide than those from subjects with the TT genotype ($P=0.038$; Figure 2A). After correcting for basal production, the TT genotype neutrophils generated only 32% as much superoxide as CC genotype neutrophils. Superoxide production from unstimulated neutrophils of subjects with the CC, CT, and TT genotypes was not statistically significantly different ($P>0.20$).

Neutrophils from subjects with AA, AG, and GG genotypes of the A640G *CYBA* polymorphism produced $8.1 \pm 1 \mu\text{mol/L } \text{O}_2^-/\text{min}$ per 10^6 cells, $7.8 \pm 0.7 \mu\text{mol/L } \text{O}_2^-/\text{min}$ per 10^6 cells, and $7.9 \pm 0.8 \mu\text{mol/L } \text{O}_2^-/\text{min}$ per 10^6 cells, respectively.

Superoxide production from stimulated neutrophils of subjects with the AA, AG, and GG genotypes were not statistically significantly different ($P>0.20$, Figure 2B). There was lower superoxide production in unstimulated neutrophils of subjects with the GG genotype ($0.08 \pm 0.01 \mu\text{mol/L } \text{O}_2^-/\text{min}$ per 10^6 cells) compared with AG and AA genotypes ($0.12 \pm 0.01 \mu\text{mol/L } \text{O}_2^-/\text{min}$ per 10^6 cells and $0.12 \pm 0.02 \mu\text{mol/L } \text{O}_2^-/\text{min}$ per 10^6 cells) ($P<0.02$).

Neutrophils from subjects with AA, AG, and GG genotypes of the $-930^{\text{A/G}}$ polymorphism produced $8.3 \pm 1.1 \mu\text{mol/L } \text{O}_2^-/\text{min}$ per 10^6 cells, $8 \pm 0.5 \mu\text{mol/L } \text{O}_2^-/\text{min}$ per 10^6 cells and $8.4 \pm 1.1 \mu\text{mol/L } \text{O}_2^-/\text{min}$ per 10^6 cells, respectively. Superoxide production by either stimulated or unstimulated neutrophils of subjects with the AA, AG, and GG genotypes were not statistically significantly different ($P>0.20$, Figure 2C).

Effect of C242T Polymorphism on p22^{phox} Expression

One possible explanation for the observed decrease in NADPH oxidase activity is a decreased expression of p22^{phox}. However, p22^{phox} protein levels were equivalent in the CC, CT, and TT genotypes (Figure 3).

Discussion

Our study demonstrates the effect of 3 *CYBA* polymorphisms on phagocytic superoxide production. Healthy adults who carry the TT genotype of the C242T *CYBA* polymorphism have a significant reduction in respiratory burst compared with wild-type carriers, suggesting decreased activity of the NADPH oxidase in these individuals. In contrast, neither the A640G nor the $-930^{\text{A/G}}$ *CYBA* polymorphism affects superoxide production.

During the past decade, p22^{phox}-based NAD(P)H oxidases were identified as major sources of vascular oxidative stress.² Azumi et al demonstrated that p22^{phox} was expressed in human coronary arteries, and its expression was more intense in atherosclerotic than nonatherosclerotic arteries.⁶ In athero-

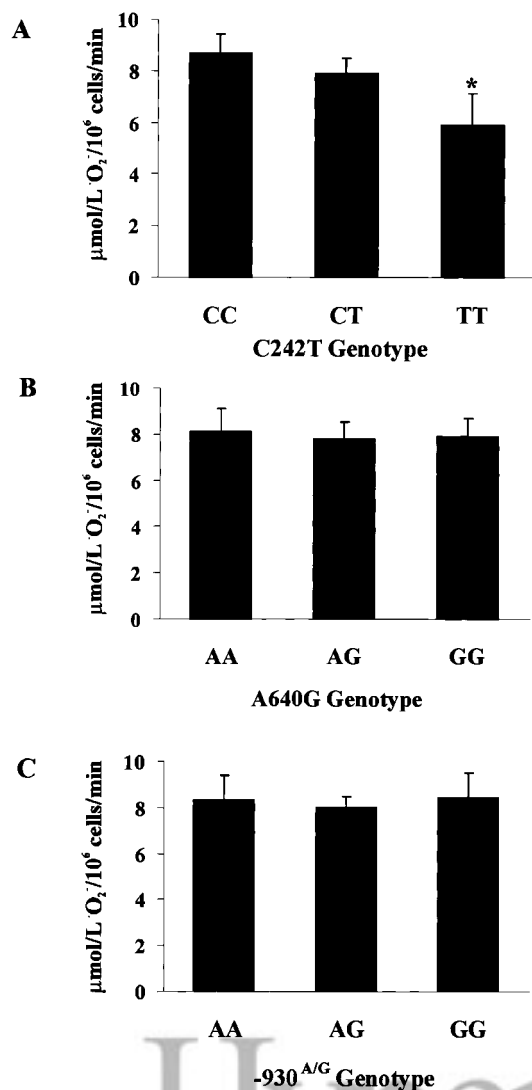


Figure 2. Neutrophil-generated respiratory burst activity and *CYBA* genotypes. A, Neutrophil-generated respiratory burst activity in relationship to the C242T *CYBA* genotypes. Neutrophils with the CC (n=44) and CT (n=34) genotypes produced significantly more superoxide than TT (n=12) genotype neutrophils stimulated by 200 nmol/L PMA. Data are given as mean±SEM. **P*=0.038. B, Neutrophil-generated respiratory burst activity in relationship to the A640G *CYBA* genotypes. Differences between AA (n=25), AG (n=37), and GG (n=27) genotypes were not statistically significant for neutrophils stimulated by 200 nmol/L PMA. Data are given as mean±SEM. C, Neutrophil-generated respiratory burst activity in relationship to the -930^{A/G} genotypes. Differences between AA (n=17), AG (n=43), and GG (n=27) genotypes were not statistically significant for neutrophils stimulated by 200 nmol/L PMA. Data are given as mean±SEM.

sclerotic lesions, p22^{phox} was present around lipid core and shoulder regions and was localized in T-lymphocytes, endothelial and smooth muscle cells, as well as fibroblasts.²⁵ More recently, a significant association between superoxide generation, p22^{phox} expression, and oxidized low-density lipoprotein cholesterol was shown.²⁶

We and others showed the critical role of p22^{phox} in NAD(P)H oxidase activity in different cell lines and tissues.^{4,27} The p22^{phox} subunit binds to gp91^{phox} and nox1, and

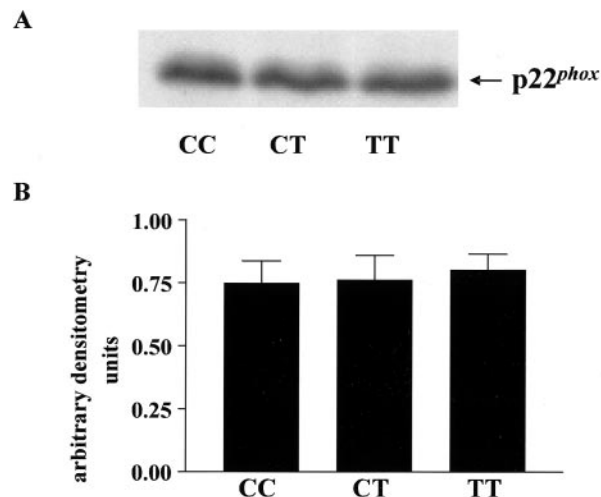


Figure 3. Western blot analysis of p22^{phox} expression in human neutrophils. A, Protein was extracted from neutrophils with the C242T *CYBA* genotypes (n=9) and blotted against p22^{phox}; a representative immunoblot of each genotype is presented. B, Quantification of p22^{phox} protein expression levels by densitometry are expressed as arbitrary units.

appears to be necessary for mutual stability (unpublished observations).²⁸ Previous observations suggested that gp91^{phox} is the sole heme binding subunit of the NADPH oxidase; however, functional assembly of the active NADPH oxidase to mediate electron transfer for superoxide generation requires both subunits of flavo cytochrome b558.^{29,30} Because the gp91^{phox}, p22^{phox} subunit stoichiometry is 1:1, and because the presence of p22^{phox} is necessary to stabilize the native heme environment of the cytochrome b558, it is conceivable that polymorphisms in the *CYBA* gene reduce the ability of p22^{phox} to anchor gp91^{phox} and act as a scaffold, and subsequently alter NADPH oxidase activity.³¹

Our observations regarding decreased respiratory burst activity in subjects with the TT genotype confirm a functional role for this polymorphism and support and extend the observations made by Guzik et al.³² They demonstrated that the 242T allele is associated with reduced vascular NAD(P)H oxidase activity in saphenous veins of CAD patients, independent of other clinical risk factors.³³ In contrast to these observations, in our study population, the C242T *CYBA* polymorphism does not affect basal activity of the phagocytic enzyme, but leads to ≈30% reduced activity on stimulation. Because vascular tissue contains multiple enzymes with NAD(P)H oxidase activity, it is difficult to make an absolute correlation between p22^{phox}-based oxidase activity and genotype in this system.³⁴ For this reason we isolated neutrophils that express significant amounts of a well-defined NADPH oxidase of single molecular composition, this system permits us to directly investigate the relationship between *CYBA* polymorphisms and enzymatic activity. The close agreement between our study and Guzik's indicates that the correlation found between the C242T *CYBA* polymorphism, p22^{phox}-based oxidase activity and endothelial function might be a relevant clinical marker for CAD.

There are 2 limitations of studies of this type. First, CAD patients have multiple risk factors and significant atheroscle-

rotic burden, making it difficult to investigate the effect of a polymorphism free of other factors known to increase superoxide. This was not an issue here, because our study subjects were young, had no history of CAD, and very few had coronary risk factors. Second, methods used to measure superoxide are subject to many potential problems, such as redox cycling observed with higher doses of lucigenin, signals caused by direct reduction of the substrate, as with cytochrome C, and poor quantification.³⁵ In this study, we used ESR to measure NADPH oxidase activity, which is extremely specific and not subject to many of the difficulties encountered with other methods. The use of the cyclic hydroxylamine is also an advantage, because it is not subject to reduction to a spin-inactive state by reductants in cells, which is a problem encountered with commonly used nitron spin traps.

A reduced level of oxidative stress would either prevent or delay the development of or protect the vasculature against oxidant-mediated conditions such as atherosclerosis and endothelial dysfunction by maintaining a higher bioavailability of NO.³⁶ Furthermore, atherosclerotic plaque rupture in areas of the shoulder region where NADPH oxidase-containing monocytes/macrophages and neutrophils are recruited is known to be promoted by superoxide.^{37–39} In addition, neutrophils from patients with acute myocardial infarction and peripheral obstructive atherosclerotic disease produce more superoxide anion compared with neutrophils of healthy controls.^{40,41} The potential for identifying subjects with genetically altered susceptibilities to oxidative stress and confirmation of the presence of the C242T CYBA polymorphism thus could provide a novel genetic marker for cardiovascular risk assessment. Nevertheless, we cannot exclude the possibility that the C242T CYBA polymorphism could be in linkage equilibrium with another functional polymorphism.

In conclusion, our study supports a functional role for the C242T CYBA polymorphism in altering respiratory burst in healthy adults. Clearly, further long-term, prospective studies among larger populations are needed to elucidate the role of specific genotypes in relation to the development of vascular disease, because it is conceivable that polymorphisms causing even minor changes in the function of p22^{phox}-containing oxidases influence atherogenesis and the progression of CAD.

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References

- Alexander RW. Hypertension and the pathogenesis of atherosclerosis: oxidative stress and the mediation of arterial inflammatory response. *Hypertension*. 1995;7:447–451.
- Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res*. 2000;86:494–501.
- Harrison DG. Endothelial function and oxidant stress. *Clin Cardiol*. 1997;20:11–17.
- Ushio-Fukai M, Zafari AM, Fukui T, Ishizaka N, Griendling KK. p22^{phox} is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulates angiotensin II-induced hypertrophy in vascular smooth muscle cells. *J Biol Chem*. 1996;271:23317–23221.
- Zafari AM, Ushio-Fukai M, Akers M, Yin Q, Shah A, Harrison DG, Taylor WR, Griendling KK. Role of NADH/NADPH oxidase-derived H₂O₂ in angiotensin II-induced vascular hypertrophy. *Hypertension*. 1998;32:488–495.
- Azumi H, Inoue N, Takeshita S, Rikitake Y, Kawashima S, Hayashi Y, Itoh H, Yokoyama M. Expression of NADH/NADPH oxidase p22^{phox} in human coronary arteries. *Circulation*. 1999;100:1494–1498.
- Dinauer MC, Pierce EA, Bruns GA, Curnutte JT, Orkin SH. Human neutrophil cytochrome b light chain (p22^{phox}): gene structure, chromosomal localization and mutations in cytochrome-negative autosomal recessive chronic granulomatous disease. *J Clin Invest*. 1990;86:1729–1737.
- Rae J, Noack D, Heyworth PG, Ellis BA, Curnutte JT, Cross AR. Molecular analysis of 9 new families with chronic granulomatous disease caused by mutations in CYBA, the gene encoding p22^{phox}. *Blood*. 2000;96:1106–1112.
- De Boer M, de Klein A, Hossle JP, Seger R, Corbeel L, Weening RS, Roos D. Cytochrome b558-negative, autosomal recessive chronic granulomatous disease: two new mutations in the cytochrome b558 light chain of the NADPH oxidase (p22-phox). *Am J Hum Genet*. 1992;51:1127–1135.
- Moreno MU, San Jose G, Orbe J, Paramo JA, Belouqui O, Diez J, Zalba G. Preliminary characterisation of the promoter of the human p22^{phox} gene: identification of a new polymorphism associated with hypertension. *FEBS Letters*. 2003;542:27–31.
- Inoue N, Kawashima S, Kanazawa K, Yamada S, Akita H, Yokoyama M. Polymorphism of the NADH/NADPH oxidase p22^{phox} gene in patients with coronary artery disease. *Circulation*. 1998;97:135–137.
- Cahilly C, Ballantyne CM, Lim DS, Gotto A, Marian AJ. A variant of p22^{phox}, involved in generation of reactive oxygen species in the vessel wall, is associated with progression of coronary atherosclerosis. *Circ Res*. 2000;86:391–395.
- Li A, Prasad A, Mincemoyer R, Satorius C, Epstein N, Finkel T, Quyyumi AA. Relationship of the C242T p22^{phox} gene polymorphism to angiographic coronary artery disease and endothelial function. *Am J Hum Genet*. 1999;86:57–61.
- Cai H, Duarte N, Wilcken DE, Wang XL. NADH/NADPH oxidase p22^{phox} C242T polymorphism and coronary artery disease in the Australian population. *Eur J Clin Invest*. 1999;29:744–748.
- Gardemann A, Mages P, Katz N, Tillmanns H, Haberbosch W. The p22^{phox} A640G gene polymorphism but not the C242T gene variation is associated with coronary heart disease in younger individuals. *Atherosclerosis*. 1999;145:315–323.
- Saha N, Sanghera DH, Kambh MI. The p22^{phox} polymorphism C242T is not associated with CHD risk in Asian Indians and Chinese. *Eur J Clin Invest*. 1999;29:999–1002.
- Zafari AM, Davidoff MN, Austin H, Valppu L, Cotsonis G, Lassegue B, Griendling KK. The A640G and C242T p22^{phox} polymorphisms in patients with coronary artery disease. *Antioxid Redox Signal*. 2002;4:675–680.
- Fraticeilli A, Serrano CV Jr., Bochner BS, Capogrossi MC, Zweier JL. Hydrogen peroxide and superoxide modulate leukocyte adhesion molecule expression and leukocyte endothelial adhesion. *Biochim Biophys Acta*. 1996;1310:251–259.
- Roubaud V, Sankarapandi S, Kuppusamy P, Tordo P, Zweier JL. Quantitative measurement of superoxide generation and oxygen consumption from leukocytes using electron paramagnetic resonance spectroscopy. *Anal Biochem*. 1998;257:210–217.
- Dikalov S, Skatchkov M, Bassenge E. Spin trapping of superoxide radicals and peroxynitrite by 1-hydroxy-3-carboxy-pyrrolidine and 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine and the stability of corresponding nitroxyl radicals towards biological reductants. *Biochem Biophys Res Commun*. 1997;231:701–704.
- Sham P. *Statistics in Human Genetics*. New York: John Wiley & Sons; 1998.
- Snedecor GW, Cochran WG. *Statistical Methods*. 8th Edition. Ames, IA: Iowa State University Press; 1989.
- Wilcoxon F. Individual comparisons by ranking methods. *Biometrics*. 1945;1:80–83.
- StataCorp. *Stata Statistical Software: Release 7.0*. College Station, TX: Stata Corporation; 2001.

25. Yokoyama M, Inoue N, Kawashima S. Role of the vascular NADH/NADPH oxidase system in atherosclerosis. *Ann N Y Acad Sci*. 2000;902:241–247.
26. Azumi H, Inoue N, Ohashi Y, Terashima M, Mori T, Fujita H, Awano K, Kobayashi K, Maeda K, Hata K, Shinke T, Kobayashi S, Hirata K-I, Kawashima S, Itabe H, Hayashi Y, Imajoh-Ohmi S, Itoh H, Yokoyama M. Superoxide generation in directional coronary atherectomy specimens of patients with angina pectoris. Important role of NAD(P)H oxidase. *Circulation*. 2002;22:1838–1844.
27. Foubert TR, Bleazard JB, Burritt JB, Gripenroeg JM, Baniulis D, Taylor RM, Jesaitis AJ. Identification of a spectrally stable proteolytic fragment of human neutrophil flavocytochrome b composed of the NH₂-terminal regions of gp91^{phox} and p22^{phox}. *J Biol Chem*. 2001;276:38852–38861.
28. DeLeo FR, Burritt JB, Yu L, Jesaitis AJ, Dinauer MC, Nauseef WM. Processing and maturation of flavocytochrome b558 include incorporation of heme as a prerequisite for heterodimer assembly. *J Biol Chem*. 2000;275:13986–13993.
29. Yu L, Quinn MT, Cross AR, Dinauer MC. Gp91^{phox} is the heme binding subunit of the superoxide-generating NADPH oxidase. *Proc Natl Acad Sci U S A*. 1998;95:7993–7998.
30. Biberstine-Kinkade KJ, Yu L, Stull N, LeRoy B, Bennett S, Cross A, Dinauer MC. Mutagenesis of p22^{phox} histidine. *J Biol Chem*. 2002;277:30368–30374.
31. Huang J, Hitt ND, Kleinberg ME. Stoichiometry of p22-phox and gp91-phox in phagocyte cytochrome b558. *Biochemistry*. 1995;34:16753–16757.
32. Guzik TJ, West NE, Black E, McDonald D, Ratnatunga C, Pillai R, Channon KM. Functional effect of the C242T polymorphism in the NAD(P)H oxidase p22^{phox} gene on vascular superoxide production in atherosclerosis. *Circulation*. 2000;102:1744–1747.
33. Guzik TJ, West NEJ, Black E, McDonald D, Ratnatunga C, Pillai R, Channon KM. Vascular superoxide production by NA(D)PH oxidase: association with endothelial dysfunction and clinical risk factors. *Circ Res*. 2000;86:e85–e90.
34. Lassegue B, Clemens RE. Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol*. 2003;285:R277–R297.
35. Tarpey MM, Fridovich I. Methods of detection of vascular reactive species: nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite. *Circ Res*. 2001;89:224–236.
36. Zafari AM, Harrison D, Griendling KK. Vascular oxidant stress and nitric oxide bioactivity. *Endothelium, Nitric Oxide, and Atherosclerosis*. Armonk, NY: Futura Publishing Co Inc; 1999.
37. Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. *J Clin Invest*. 1996;98:2572–2579.
38. Galis ZS, Asanuma K, Godin D, Meng X. N-acetylcysteine decreases the matrix-degrading capacity of macrophage-derived foam cells: new target for antioxidant therapy? *Circulation*. 1998;97:2445–2453.
39. Naruko T, Ueda M, Haze K, van der Wal AC, van der Loos CM, Itoh A, Komatsu R, Ikura Y, Ogami M, Shimada Y, Ehara S, Yoshiyama M, Takeuchi K, Yoshikawa J, Becker AE. Neutrophil infiltration of culprit lesions in acute coronary syndromes. *Circulation*. 2002;106:2894–2900.
40. Sanchez de Miguel L, Arriero MM, Farre J, Jimenez P, Garcia-Mendez A, de Frutos T, Jimenez A, Garcia R, Cabestrero F, Gomez J, de Andres R, Monton M, Martin E, De la Calle-Lombana LM, Rico L, Romero J, Lopez-Farre A. Nitric oxide production by neutrophils obtained from patients during acute coronary syndromes: expression of the nitric oxide synthase isoforms. *J Am Coll Cardiol*. 2002;39:818–825.
41. Mohacs A, Kozlovsky B, Kiss I, Seres I, Fulop T Jr. Neutrophils obtained from obliterative atherosclerotic patients exhibit enhanced resting respiratory burst and increased degranulation in response to various stimuli. *Biochim Biophys Acta*. 1996;1316:210–216.



Hypertension

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