Functional Effect of the C242T Polymorphism in the NAD(P)H Oxidase p22phox Gene on Vascular Superoxide Production in Atherosclerosis

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Background—Increased superoxide anion production increases oxidative stress and reduces nitric oxide bioactivity in vascular disease states. NAD(P)H oxidase is an important source of superoxide in human blood vessels, and some studies suggest a possible association between polymorphisms in the NAD(P)H oxidase CYBA gene and atherosclerosis; however, no functional data address this hypothesis. We examined the relationships between the CYBA C242T polymorphism and direct measurements of superoxide production in human blood vessels.

Methods and Results—Vascular NAD(P)H oxidase activity was determined in human saphenous veins obtained from 110 patients with coronary artery disease and identified risk factors. Immunoblotting, reverse-transcription polymerase chain reaction, and DNA sequencing showed that p22phox protein, mRNA, and 242C/T allelic variants are expressed in human blood vessels. Vascular superoxide production, both basal and NADH-stimulated, was highly variable between patients, but the presence of the CYBA 242T allele was associated with significantly reduced vascular NAD(P)H oxidase activity, independent of other clinical risk factors for atherosclerosis.

Conclusions—Association of the CYBA 242T allele with reduced NAD(P)H oxidase activity in human blood vessels suggests that genetic variation in NAD(P)H oxidase components may play a significant role in modulating superoxide production in human atherosclerosis. (Circulation. 2000;102:1744-1747.)

Key Words: atherosclerosis ■ endothelium ■ superoxide ■ genetics

Oxidative stress plays critical roles in cardiovascular physiology and in the pathogenesis of vascular disease.1 Superoxide anion and its derivatives activate redox-sensitive signaling pathways, stimulate vascular smooth muscle cell mitogenesis, and regulate the signaling of hypoxia and apoptosis. In addition, nitric oxide scavenging by superoxide reduces the bioactivity of nitric oxide and produces peroxynitrite, a strong oxidant that nitrosylates cellular proteins and lipids. Increased superoxide production is a feature of vascular disease states, including atherosclerosis, hypertension and diabetes.2

NAD(P)H oxidase is present in vascular smooth muscle cells and endothelial cells, and it seems to be a major source of superoxide production in animal models of vascular disease3 and in human atherosclerosis.4 NAD(P)H oxidase is a multisubunit enzyme complex comprising p47phox, p67phox, gp91phox, p22phox, and Rac-2 proteins. The p22phox subunit is required for oxidase activity in smooth muscle cells, and it is expressed in human coronary arteries.5

Recent interest has focused on the possible association of polymorphisms in the CYBA gene, which encodes p22phox, with atherosclerosis.6,7 The CYBA C242T polymorphism results in a substitution of Tyr for His at residue 72 of p22phox, leading to speculation that this polymorphism may modulate enzyme activity by affecting heme binding.6 Recent data suggest that the CYBA 242T allele is associated with the increased progression of coronary disease.7 However, the relationship between the CYBA C242T polymorphism and vascular NAD(P)H oxidase activity in human blood vessels remains unknown.

Accordingly, we sought to investigate the functional effect of the CYBA C242T polymorphism on vascular superoxide production in human blood vessels. We found that the CYBA 242C/T alleles are expressed in p22phox mRNA in blood vessels and that the 242T allele is associated with significantly reduced vascular NAD(P)H oxidase activity in human blood vessels independent of other clinical risk factors for atherosclerosis.

Methods

Patients

Segments of human saphenous veins and mammary arteries were obtained from patients undergoing routine coronary artery bypass...
surgery at the John Radcliffe Hospital, Oxford, UK. Collection of tissue specimens was approved by the local Research Ethics Committee, and informed consent was obtained. Clinical risk factors (hypercholesterolemia, smoking, diabetes, and hypertension) were categorized as described previously.3

Genotyping and p22phox Expression
The C to T substitution at position 242 in the CYBA coding sequence was typed by RsaI digestion of specific polymerase chain reaction (PCR) products amplified from genomic DNA, as described previously.6 Reverse transcription (RT)-PCR of cDNA synthesized from mRNA extracted from vessel segments was performed using specific intron-spanning primers (forward: 5’CGCTGGCGTCCGGCT-GATCTCTCA3’; reverse: 5’ACGCACACGCCAGTAGTGA-GATT3’). RT-PCR products (341 bp) were digested with RsaI or were sequenced using automated dye sequencing in both directions. Western immunoblotting of vessel homogenates for p22phox protein was performed using polyclonal antibodies provided by Dr Imajoh-Ohmi (Tokyo, Japan).3

Superoxide Determination
Basal and NADH-stimulated superoxide production were measured from vascular segments using lucigenin-enhanced chemiluminescence (5 μmol/L lucigenin), as previously described.3

Statistical Analysis
Data are expressed as mean±SEM. Statistical significance of differences between superoxide production was assessed by Student’s t tests. Relationships between superoxide production and risk factors or CYBA genotype were analyzed using ANOVA (type III sums of squares). P<0.05 was considered statistically significant.

Results
Patient Characteristics
Saphenous veins were obtained from 110 patients (89 men and 21 women) undergoing coronary artery bypass grafting. Demographic and clinical characteristics are shown in Table 1.

Frequencies of p22phox C242T Genotypes
The frequency of the C allele at CYBA position 242 was 67% and that of the T allele was 33%. Genotype frequencies were as follows: CC, 45.4%; CT, 43.6%; and TT, 10.9%. These frequencies were in accordance with the Hardy-Weinberg distribution (χ²=0.02, P=0.99).

Relationship Between Vascular Superoxide Generation and CYBA Genotype
To investigate the relationship between vascular NAD(P)H oxidase activity and CYBA genotype, we compared both basal and NADH-stimulated superoxide production in vessel segments from patients with or without the CYBA 242 T allele (Figure 1). The addition of NADH stimulated superoxide release >10-fold (19.9±2 at baseline versus 285±13 after NADH; P<0.001). Both basal and maximal NAD(P)H oxidase activity were significantly lower, by ≈30%, in vessels from patients with the T allele (CT/TT genotypes) compared with those from patients without the T allele (CC genotype). No differences in superoxide production existed between the CT and TT genotypes (CT: n=48, 251±16; TT: n=12, 247±24; P=NS).

An analysis of major clinical risk factors for atherosclerosis that could affect vascular superoxide production revealed that diabetes and hypercholesterolemia were strongly associated with increased NAD(P)H oxidase activity (Table 2), but the presence of the CYBA 242T allele remained independently associated with reduced vascular NAD(P)H oxidase activity (P<0.001). In particular, the CC and CT/TT groups were well-matched for the proportion of diabetics (CC, 11 of 50; CC/TT, 15 of 60; P=0.2) and for drug therapies, including hydroxymethylglutaryl–coenzyme A reductase inhibitors and angiotensin-converting enzyme inhibitors. We also measured NADH-dependent superoxide production from paired internal mammary artery segments from 30 patients and found a close correlation with saphenous vein superoxide production in individual patients (r=0.6, P<0.01). In accordance with our findings in saphenous veins, NADH-dependent superoxide production in mammary arteries was significantly lower in those with the CT/TT genotype (n=17) than in those with the CC genotype (n=13; 150±17 versus 210±21; P=0.04).

Expression and Molecular Identification of p22phox in Human Blood Vessels
We evaluated p22phox expression in saphenous veins using Western immunoblotting of vascular homogenates and RT-
PCR of saphenous vein mRNA with restriction analysis and DNA sequencing. Immunoblotting revealed p22phox protein in saphenous vein homogenates from patients with different CYBA genotypes (Figure 2). Similarly, RT-PCR of saphenous vein mRNA generated a specific product in all genotypes; DNA sequencing showed an identity with neutrophil p22phox. Furthermore, restriction analysis and sequencing of RT-PCR products from patients with CC, CT, or TT genotypes confirmed the expression of the alleles in each case, in accordance with the genotype determined directly from genomic DNA. We also found p22phox protein, mRNA, and C/T allelic variants in samples from the internal mammary artery.

These data confirm that p22phox is expressed in saphenous veins (both mRNA and protein), that the molecular identity is the same as that expressed in neutrophils and mammary arteries, and that the expression of C and/or T alleles in blood vessels corresponds with genotype.

**Discussion**

We studied the relationship between vascular superoxide production and the CYBA C242T polymorphism in the gene encoding the NAD(P)H oxidase p22phox subunit. We found that the 242T allele is associated with significantly lower basal and NADH-stimulated vascular superoxide production in human blood vessels from patients with atherosclerosis. We also confirmed the molecular identity and allele-specific expression of p22phox in human vessels. These findings provide direct functional data relating vascular superoxide production in human atherosclerosis to genetic polymorphisms in the CYBA gene. Previous studies have examined the incidence of CYBA polymorphisms in association with coronary artery disease. Inoue et al. found that the 242T allele was associated with a reduced risk of coronary artery disease, whereas other case-control studies had conflicting findings. However, the genotyping of patients in the prospective Lipoprotein and Coronary Artery Study (LCAS) suggested a significant association between the CYBA 242T allele and the progression of coronary atherosclerosis.

By analyzing a quantitative trait, vascular NAD(P)H oxidase activity, we found a significant functional effect of this polymorphism, thus supporting a potential role for CYBA polymorphisms in vascular disease pathogenesis. Our functional data agree with the previous observation that the presence of the CYBA 242T allele seems to exert a dominant effect, because we found an equal reduction in vascular NAD(P)H oxidase activity in patients with CT or TT genotypes compared with the CC genotype.

Our findings suggest that the CYBA 242T allele is associated with reduced NAD(P)H oxidase activity under both

| TABLE 2. Risk Factors Associated With Vascular NADH-Dependent Superoxide Release |
|------------------|------------------|------------------|------------------|------------------|
| Risk Factor      | Patients, n      | Superoxide Production, RLU/s per mg vessel |
|                  | With RF | Without RF | With RF | Without RF | P      |
| CYBA 242T allele | 60      | 50        | 248 ± 19 | 336 ± 15 | 0.001  |
| Hypertension     | 81      | 29        | 294 ± 17 | 269 ± 20 | 0.1    |
| Hypercholesterolemia | 71      | 39        | 323 ± 19 | 225 ± 16 | 0.02   |
| Smoking          | 42      | 68        | 336 ± 20 | 256 ± 15 | 0.5    |
| Diabetes mellitus| 26      | 84        | 369 ± 21 | 262 ± 14 | 0.001  |

Values are no. of patients or mean ± SEM. RF indicates risk factor; RLU, relative light units.

Type III sums of squares ANOVA was performed using clinical risk factors as categorical covariates and NADH-dependent superoxide production as the dependent variable.
basal and maximal (NADH-stimulated) conditions, although the mechanisms underlying these effects and their implications for the pathogenesis of atherosclerosis remain unclear. One plausible mechanism is the resulting His to Tyr substitution at position 72 of p22phox that could possibly disrupt heme binding at the active site.6,10 Because most studies suggest that vascular superoxide production by NAD(P)H oxidase is increased rather than decreased in atherosclerosis, the association of the T allele with reduced oxidase activity may seem counterintuitive.10 However, the balance between antioxidant stress and up-regulation of antioxidant defenses remains poorly defined; it is possible that increased superoxide production could augment protective mechanisms against disease progression. For example, nitric oxide–mediated vasorelaxations do not seem to be affected by the CYBA C242T polymorphism.11 Furthermore, NAD(P)H oxidase may play important roles in regulating other cellular processes, such as oxygen sensing, smooth muscle cell mitogenesis, redox-sensitive gene expression, and apoptosis, which could all provide targets for the differential effects of p22phox variants in atherosclerosis.4,10 Alternatively, the CYBA 242T allele may be an indirect marker for other genetic variants that regulate p22phox protein activity, or affect atherosclerotic progression. Finally, our in vitro assays using saphenous veins may not reflect superoxide production in coronary arteries in vivo.

In conclusion, the CYBA C242T polymorphism significantly affects vascular superoxide production in patients with coronary artery disease. The presence of the 242T allele is independently associated with reduced basal and NADH-stimulated superoxide production, suggesting a potentially important role for genetic variation in NAD(P)H oxidase in human atherosclerosis.

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