NADPH oxidase p22phox gene variants are associated with systemic oxidative stress biomarker responses to exercise training

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OXIDATIVE STRESS PLAYS an important role in the pathogenesis of atherosclerosis. Recently, two large trials in humans showed that systemic oxidative stress level is correlated with cardiovascular disease (CVD) and its various risk factors (19, 34).

Regular physical activity has been reported to be the most effective nonpharmacological intervention to enhance endogenous antioxidant capacity and alleviate oxidative stress-induced tissue damage (25, 28, 32). Several studies indicate that genetics may contribute to the systemic redox state and the susceptibility to oxidative damage, and mild to moderate genetic heritabilities of the redox state have been observed as well (21, 22, 41). However, none of the previous exercise intervention studies focused on a genetic association with the systemic oxidative stress level response to exercise training.

The NADPH oxidase system plays a key role in generating reactive oxygen species (ROS), including superoxide and hydrogen peroxide in phagocytic cells, fibroblasts, vascular smooth muscle cells, and endothelial cells (2, 10, 29). The NADPH oxidase system is regulated systemically in veins and arteries, which strengthens the importance of the molecular regulation of the enzyme in CVD (11).

The NADPH oxidase system is composed of multiple subunits including membrane-associated gp91phox, p22phox, and three regulatory components: p40phox, p47phox, and p67phox (24). The p22phox forms the α-subunit of cytochrome b558 and is the final electron transporter from NADPH to molecular oxygen in both phagocytic and nonphagocytic NADPH oxidase (39).

The gene encoding p22phox is located on chromosome 16q24 and several allelic polymorphisms in the p22phox gene have been reported (20). The C242T polymorphism results in the replacement of histidine by tyrosine located in the putative heme-binding site. A study on the functional aspects of the C242T polymorphism suggests that the T allele is associated with significantly reduced NADPH oxidase activity (12). The A640G polymorphism is located in the 3′ untranslated region and may affect transcriptional rate. Furthermore, both polymorphisms have been found to be independently associated with CVD prevalence, although the results have been inconsistent (7, 14).

An acute bout of exercise increases the production of ROS through mechanisms involving the NADPH oxidase system (3). As an adaptation to acute exercise-induced oxidative stress, the increased level of ROS induces antioxidant enzyme gene expression via an intracellular signaling pathway, which involves the redox regulated transcription factor, NF-κB (17). Furthermore, using p22phox knockout mice, it has been demonstrated that the NADPH oxidase system plays a critical role in NF-κB activation (4). Therefore, the NADPH oxidase system is a potential mechanism through which exercise training may modulate systemic oxidative stress levels.

The purpose of the present study was to investigate whether p22phox C242T and A640G gene polymorphisms were associated with the systemic oxidative stress biomarker response to aerobic exercise training. We hypothesized that there would be an interactive effect between exercise training and p22phox genotypes on systemic oxidative stress levels.

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MATERIALS AND METHODS

Fifty-nine apparently healthy sedentary men (n = 26) and women (n = 33) volunteered to participate in this study. Subjects responded to media advertisements and underwent a telephone interview to assess their initial eligibility. The study was approved by the Institutional Review Board of the University of Maryland, College Park. All subjects provided their written, informed consent during their first laboratory visit. Subjects for this study had either at least one lipid abnormality (see Screening) or high or high-normal blood pressure, or both. Hypertensive subjects using antihypertensive medications were tapered off the medications before participating in this study. In addition, subjects were required to be sedentary, 50–75 yr of age, nondiabetic, nonsmoking, free of CVD, and have body mass index <37 kg/m². All women were more than 2 yr postmenopausal and maintained their hormone replacement therapy regimen, either on or not on hormone replacement, for the duration of the study.

Screening. Subjects’ medical histories were reviewed on their first laboratory visit to ensure they met the study inclusion and exclusion criteria listed above. A 12-h overnight fasting blood sample was drawn for plasma lipoprotein lipid profile analysis. Subjects had to have at least one National Cholesterol Education Program lipid abnormality (cholesterol >200 mg/dl, HDL cholesterol <35 mg/dl, triglycerides >200 but <400 mg/dl) without any lipid-lowering medication. Each subject also had to have total cholesterol and LDL cholesterol <90th and HDL cholesterol >20th percentile for their age and gender group to ensure they did not have familial hypercholesterolemia.

Each subject had their fasting plasma glucose level determined and underwent a 2-h 75-g oral glucose tolerance test. Those with fasting plasma glucose >126 mg/dl or a 2-h glucose level >200 mg/dl were excluded from the study. Subjects qualified on the basis of these tests underwent a physical examination and a physician-supervised maximal treadmill exercise test to screen for cardiovascular, pulmonary, or other chronic diseases that would preclude them from exercise training.

Dietary stabilization. All qualified subjects then underwent 6 wk of dietary instruction (2 days/wk, 1 h/session) with a registered dietician instructing them in how to maintain the American Heart Association Step I Diet. Subjects had to follow this prescribed diet and be weight stable for >3 wk before undergoing baseline testing.

Baseline testing. At the completion of the 6-wk dietary stabilization period and before beginning exercise training, all qualified subjects completed baseline testing that consisted of measurement of plasma lipoprotein lipids and maximal oxygen consumption (V\textsubscript{O\textsubscript{2}} max). V\textsubscript{O\textsubscript{2}} max was determined by indirect calorimetry during a graded exercise test to exhaustion as described previously (42). V\textsubscript{O\textsubscript{2}} max was then used to derive valid exercise prescriptions for the exercise training intervention. Blood samples were obtained on two separate occasions after an overnight 12-h fast to measure plasma lipoprotein lipid profiles.

Thiobarbituric acid assay. Plasma thiobarbituric acid (TBA) reactive substances (TBARS) were measured by a modification of the method of Buege and Aust (5) and expressed in nanomoles per milliliter malondialdehyde equivalents (MDAeq). Blood samples were drawn after an overnight fast. For measures after exercise training, blood samples were drawn between 24 and 36 h after the subjects’ most recent exercise session. Subjects were not allowed to take anti-inflammatory drugs for the 24 h before blood sample collection. In addition, a questionnaire regarding recent (<1 mo) infection and inflammation was administered before blood sample collection. If subjects had a recent infection and/or inflammation, then the blood sample collection was rescheduled. The plasma from the blood samples was stored in a −80°C freezer until the assay was performed. In the modified method, butylated hydroxytoluene (Sigma) and sodium dodecyl sulfate (Life-Technologies, GIBCO) were added to the reaction mixture to prevent ex vivo lipid peroxidation and to inhibit sialic acid reactivity, respectively (16, 23). After the TBA–MDA reaction procedure, the chromogen was extracted with n-butanol (Sigma). Two hundred microliters of the upper butanol phase were then placed into a flat-bottom 96-well plate, and the absorbance was read at 532 nm. To correct for background absorption of other non-TBA-reacted pigments present in plasma, absorbance values at 572 nm were subtracted from those at 532 nm.

On the basis of previous methodological considerations (15), we performed a quality-control trial and optimized the assay protocol for sampling condition and combined current knowledge about the assay to improve its reliability. To estimate the reliability of our assay, we tested plasma levels of MDAeq in seven control subjects, comprised of four men and three women, over 3 consecutive months. Subjects visited the laboratory three times on the same calendar day of the month (men) or on the same day of their menstrual cycle (women). Physical exercise and anti-inflammatory drugs were not allowed for 24 h before blood samples were obtained. All blood samples were obtained between 7:00 and 9:00 AM and after a 12-h fast. All samples were assayed in duplicate, and standards were assayed in triplicate. The statistical analysis for the reliability test was conducted to determine total error and the retest correlation (13). On the basis of the separately conducted reliability test, the coefficient of variation was 7.9% across the 3 consecutive months, and the intraassay correlation coefficient was 0.91 (95% confidence interval = 0.70–0.98) and the \( r^2 = 0.896 \) on the basis of the linear regression analysis (Fig. 1).

NADPH p22phox C242T and A640G genotyping. Genomic DNA was extracted from peripheral blood by standard methods. The Rsa I restriction site gain created by the C242T substitution and the Dra III site loss created by the A640G substitution were genotyped by standard restriction fragment length polymorphism techniques as described by Inoue et al. (14).

Exercise training. The 6 mo of supervised endurance exercise training consisted of 3 sessions/wk. All training sessions began and concluded with appropriate stretching/warm-up and cool-down exercises. Heart rate monitors were used to monitor exercise intensity and to ensure that subjects trained at a heart rate corresponding to the prescribed intensity. The training program was initially 20 min of exercise at 50% V\textsubscript{O\textsubscript{2}} max and gradually progressed to 70% V\textsubscript{O\textsubscript{2}} max for 3 consecutive months of our reliability study participants. MDA, malondialdehyde; ICC, intraassay correlation coefficient; 95%CI, 95% confidence interval.

Fig. 1. Regression analysis of individual thiobarbituric acid-reactive substances (TBARS) values during 3 consecutive months of our reliability study participants. MDA, malondialdehyde; ICC, intraassay correlation coefficient; 95%CI, 95% confidence interval.
Table 1. Subject characteristics and polymorphic frequencies for the genotype, allele, and haplotype groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>C242T Genotype</th>
<th>A640G Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC (n = 23)</td>
<td>CT (n = 28)</td>
</tr>
<tr>
<td>Age, yr</td>
<td>58.4 ± 1.0</td>
<td>60.0 ± 1.2</td>
</tr>
<tr>
<td>Gender, male/female</td>
<td>8/15</td>
<td>14/14</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28.5 ± 0.7</td>
<td>27.5 ± 0.8</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>203.9 ± 6.8</td>
<td>203.7 ± 7.5</td>
</tr>
<tr>
<td>HDL-C</td>
<td>46.3 ± 4.0</td>
<td>48.3 ± 2.6</td>
</tr>
<tr>
<td>LDL-C</td>
<td>125.9 ± 6.3</td>
<td>128.0 ± 6.2</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>170.2 ± 18.3</td>
<td>143.7 ± 10.8</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>134.0 ± 2.9</td>
<td>157.5 ± 3.2</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>84.7 ± 1.5</td>
<td>86.3 ± 2.0</td>
</tr>
<tr>
<td>V̇O₂ max, ml/kg ′min ′¹</td>
<td>24.5 ± 1.0</td>
<td>25.4 ± 1.0</td>
</tr>
<tr>
<td>Genotype frequency</td>
<td>0.39</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Values are means ± SE except for frequency data. Because of the absence of lipid profile data in 9 subjects, sample sizes for total cholesterol, HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), and triglyceride analysis were reduced in each genotype group. The sample sizes for lipid profiles were CC(20), CT(23), TT(7), AA(15), AG(18), and GG(17). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; V̇O₂ max, maximal oxygen consumption.

RESULTS

TBARS decreased by 16% (4.00 vs. 3.30 nM/ml) after exercise training in the entire group (P < 0.001). The range of the percent changes in TBARS was between −50 and +65% (median: −14.9%). Women had higher initial and final TBARS than men, but the TBARS changes with exercise training between men and women were not significantly different. Thus, the data from men and women were combined for the further comparisons in the testing of a gene association.

Demographic and initial levels of CVD risk factors were comparable among genotype groups for both polymorphisms (Table 1). Allele and genotype frequencies of the C242T and A640G genotypes were in Hardy-Weinberg equilibrium (χ² = 0.05 and χ² = 2.39, respectively; both P > 0.05) and were similar to previous studies (8, 35, 37) (Table 1).

There was a significant decrease in TBARS with training in the CC and CT genotype groups (both P < 0.01), but not in TT homozygotes for the C242T polymorphism. TBARS was significantly decreased in all A640G genotype groups (P < 0.01 for both AA and AG; P < 0.05 for GG). In both C242T and A640G variants, there were no statistical differences among genotype groups in the amount of TBARS changes with exercise training; however, in the recessive model, A allele carriers had a significantly greater decrease in TBARS after exercise training compared with the A allele noncarrier group for A640G polymorphism (Table 2). Also, there was a signif-

Table 2. Changes in TBARS in p22phox C242T and A640G genotype groups

<table>
<thead>
<tr>
<th>C242T</th>
<th>Recessive Model</th>
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<tbody>
<tr>
<td></td>
<td>Dominant Model</td>
</tr>
<tr>
<td></td>
<td>CC</td>
</tr>
<tr>
<td>Baseline MDAeq, nM/ml</td>
<td>4.02 ± 0.3</td>
</tr>
<tr>
<td>Changes with ExTr</td>
<td>−0.71 ± 0.2</td>
</tr>
<tr>
<td>A640G</td>
<td>AA</td>
</tr>
<tr>
<td>Baseline MDAeq, nM/ml</td>
<td>3.92 ± 0.3</td>
</tr>
<tr>
<td>Changes with ExTr</td>
<td>−0.74 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. P values represent comparison in change values among genotype groups in each model. MDAeq, malondialdehyde equivalent; ExTr, exercise training.
significant interactive effect between exercise training and the A640G polymorphism on changes in TBARS ($P = 0.05$) (Fig. 2).

The diplotype analysis showed that TBARS decreased significantly in the CCAG, CTAA, and CTAG diplotype groups, whereas no significant decrease was observed in the other groups (Fig. 3A). There was a significant difference ($P < 0.05$) in TBARS changes among C-A haplotype carrier groups (Fig. 3B).

**DISCUSSION**

Increased systemic oxidative stress plays an essential role in the progression of cardiovascular complications related to CVD risk factors including atherosclerosis, hypertension, diabetes, nephropathy, and obesity. Modulation of oxidative stress and the antioxidant system is now considered a valuable treatment to reduce CVD risk and prevent CVD (18). Although many studies have suggested that exercise training enhances endogenous antioxidant capacity, the mechanisms underlying this effect are not fully understood. It is believed that acute exercise is an oxidative stressor that transiently induces an additional oxidative stress that is not sufficiently counterbalanced by the body’s antioxidant defense system. The acute exercise-induced oxidative stress stimulates antioxidant enzyme gene expression and ultimately enhances total antioxidant capacity (17).

Although the belief that aerobic exercise training decreases systemic oxidative stress levels is widely accepted, there is a paucity of long-term aerobic exercise intervention studies in which changes in systemic oxidative stress levels were determined. In the present study, there was a highly significant decrease in systemic oxidative stress levels with long-term aerobic exercise training, measured by TBARS, in apparently healthy middle-aged to older previously sedentary individuals. However, the range of individual changes in TBARS levels after exercise training was highly variable (range: −50 to +65%), which suggested that there may have been a genetic contribution to the changes in systemic oxidative stress levels. Also, the notion of a genetic contribution is supported by previous studies in which a mild to moderate genetic heritability of the redox state was found (21, 22, 41).

The most important finding of the present study was the association of A640G p22phox gene polymorphism with changes in plasma TBARS levels with exercise training. In other words, subjects with one or two copies of A allele at the A640G locus showed greater reduction in TBARS level after exercise training than those without an A allele. This finding suggests a recessive model of a genetic contribution. In addition, there was also a significant interactive effect observed between A640G genotype and exercise training on TBARS level. In contrast, for the C242T polymorphism, although there was a trend for a greater reduction in the plasma TBARS with training in CC and CT genotype groups compared with TT homozygotes, the difference was not statistically significant.

The variance in TBARS change includes nine nonresponders (15%). We found that none of these nine nonresponders had two copies of C-A haplotype, which was assumed by diplotype analysis. This finding was confirmed in six additional nonresponders from 28 African-American subjects whose data are not reported here. Therefore, it is likely that the effects of the C242T and A640G p22phox gene polymorphisms are additive.

In the present study, the haplotype analysis based on diplotype data showed that the C-A haplotype carriers reduced TBARS levels with exercise training. Using a diplotype analysis to assess interactions between two polymorphisms is more reasonable when the two sequence variations are not in linkage disequilibrium; in other words, one gene polymorphism behaves independently of the other within a chromosome. To the best of our knowledge, no evidence can be found showing that there is a significant linkage disequilibrium between the two p22phox polymorphisms. Hodgkinson et al. (12a) reported a strong association of T242/G640 diplotype with the incidence of nephropathy, inferring that the combination of the two polymorphisms may be more informative when determining the genetic contribution to certain biological phenotypes.

There was no significant difference between the genotype groups in baseline TBARS levels. These results remained after adjusting for age and gender. Nakano and colleagues (30) found no difference in MDAeq levels between NADPH oxidase C242T genotype groups in diabetic patients. Stanger et al. (38) also reported that the C242T polymorphism was not associated with lipid peroxidation as measured by MDAeq levels. Our baseline results agree with these two previous studies.

The ability to detect interactions between genes and environmental factors is determined by the precision with which the environmental exposure and phenotype variables are mea-
sured as well as the magnitude of effect and sample size (43). In our study, rigorously screened older subjects underwent diet stabilization and a highly standardized exercise training program for 6 mo. The inherent problem of nonspecificity of the TBA method remains even after modification of the assay protocol. However, the assay method was quality controlled by a separate trial.

The importance of p22phox in the NADPH oxidase family has been studied intensely by investigating its molecular structure, biochemical properties, and intracellular location (9, 40). Downregulation of p22phox gene expression in vascular smooth muscle cells by antisense RNA leads to decreased superoxide production, indicating that p22phox is critical for superoxide production in the vasculature (6). As mentioned earlier, acute exercise transiently increases ROS levels owing, in part, to upregulation of NADPH oxidase activity, which may modulate adaptations in the antioxidant system (3). On the other hand, a recent study showed that regular aerobic exercise training reduced p22phox mRNA levels along with other NADPH oxidase subunits including gp91phox and Nox 4 (1). This would be another mechanism by which exercise training promotes adaptations to the systemic oxidative stress level. Therefore, NADPH oxidase activity is implicated in the aerobic exercise-training-induced adaptation in redox state.

Sequence variations in the p22phox gene, specifically a C to T missense polymorphism at exon 4 and an A to G 3′-UTR polymorphism, have been independently associated with NADPH oxidase activity, vascular endothelial function, and the incidence of CVD (7, 12, 14). Generally, both mutations appear to be beneficial, meaning that the T242 and G640 alleles are associated with lower NADPH oxidase activity and, therefore, reduced susceptibility to oxidative damage.

There is no direct evidence to link NADPH oxidase activity level to the rate of antioxidant enzyme gene expression induced by exercise. However, NADPH oxidase activity is clearly important in superoxide production, which leads to a nuclear translocation and DNA binding of NF-E2 related factor 2 (Nrf2). Nrf2 is a transcription factor that is an activator of antioxidant response element (ARE)-mediated transcription through its nuclear translocation and binding to AREs (27, 31). Furthermore, gene expression for several important antioxidant enzymes, including catalase, glutathione peroxidase, glutathione S-transferases, and Mn-superoxide dismutase, may depend on Nrf2 binding to ARE sequences in their promoter regions (26, 44). Papaiahgari et al. (33) have proposed that inhibition of NADPH oxidase retards both Nrf2 translocation and ARE-mediated transcription. Considering this, the present relation of C242 and A640 allele, which link to higher NADPH oxidase activities, with exercise training-induced decreases in TBARS may be attributed to the greater ROS-mediated stimulation on antioxidant enzyme system during exercise in these genotype groups. Future studies are required to investigate the role of the

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**Fig. 3.** A: TBARS changes among NADPH p22phox diplotype groups. The figure illustrates changes of TBARS levels after exercise training in each diplotype group. The first 2 letters (C or T) indicate diplotype in C242T polymorphism, and the following 2 letters (A or G) indicate diplotype in A640 G polymorphism. TTGG diplotype was excluded because only 1 sample was available. *P < 0.05, **P < 0.01 for the difference within genotype group. B: comparison of changes in TBARS level after exercise training between C-A haplotype carriers and noncarriers. The hetero-hetero genotype group, CTAG (n = 11), was excluded in this analysis because the linkage phase could not be established with certainty.
NADPH oxidase system in exercise training-induced antioxidant capacity enhancement through redox-regulated transcription factors.

Finally, fluid shear stress, one of the key features of exercise-induced stress in the vessel, activates vascular NADPH oxidase and p22phox expression (36). It has been speculated that p22phox genotype may affect the NADPH oxidase activity response to exercise-induced flow-mediated shear stress and that this may determine the amount of antioxidant enzyme gene expression levels in the vasculature.

A previous study reported that, on average, women exhibited higher oxidative stress levels compared with men (19). However, because we found no significant interaction on the basis of sex, in the present study, the data for men and women were combined in the analysis of genetic effects on exercise training-induced changes in systemic oxidative stress.

In the present study, systemic oxidative stress level was measured by the TBA assay, the most widely used method. The intraclass correlation coefficient of our methods was 91%. However, there are several limitations with the colorimetric assay method. These limitations include nonspecific formation of TBA-derived adducts with interfering substances (e.g., aldehydes other than MDA), ex vivo oxidation, existence of other pigments in plasma, and the influence of diet. Considering some of these assay limitations, we used a modified assay method and standardized sampling conditions to minimize biological and/or experimental error and performed a separate trial for quality control. Even though the latter three limitations were addressed by the assay modification, sampling conditions, and the separate quality control study; the production of non-MDA-derived TBARS could not be addressed. Future studies should include HPLC methods to measure MDA or the measurement of urinary 8-isoprostane PGF2α.

In conclusion, we found that p22phox C242 and A640G polymorphisms were associated with differential changes in systemic oxidative stress with aerobic exercise training. To our knowledge, this is first report of a genetic contribution to the adaptive response of systemic oxidative stress to exercise training.

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