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Molecules and Cells



# Effects of the NADPH Oxidase p22phox C242T Polymorphism on Endurance Exercise Performance and Oxidative DNA Damage in Response to Aerobic Exercise Training

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**We examined the effects of the NADPH oxidase p22phox C242T polymorphism on endurance exercise performance and oxidative DNA damage in response to acute and chronic exercises. One hundred three subjects were recruited, among which 26 healthy subjects (CC: 12, TC: 12, and TT: 2) were studied during rest, exercise at 85% $VO_2$max, and recovery before and after 8 weeks of treadmill running. Lymphocyte DNA damage increased significantly in response to exercise ($p < 0.05$). There were no significant differences in plasma MDA, SOD concentrations and lymphocyte DNA damage between CC genotype and T allele group, but significant endurance training differences were observed. Endurance training increased exercise time to exhaustion in both the CC genotype and T allele groups ($p < 0.05$) but no significant difference was found between groups. The results of the current study with young, healthy, Korean men are interpreted to mean that 1) the majority had the CC genotype of the NADPH oxidase p22phox C242T polymorphism (82.5%: CC, 15.5%: TC, 1.9%: TT), 2) acute exercise increased lymphocyte DNA damage, 3) endurance training significantly increased exercise time to exhaustion, and alleviated lymphocyte DNA damage, and 4) The NADPH oxidase p22phox C242T polymorphism, however, did not alter lymphocyte DNA damage or exercise performance at rest, immediately after exercise, or during recovery.**

## INTRODUCTION

Numerous factors, which include age, gender, training levels, nutritional status, and genetic characteristics, can influence exercise performance. Among these factors, genetic characteristics are one of the most important. The reality, however, is that the genetics of exercise performance have not been extensively studied due to methodological limitations. Recently, advancements in biotechnology and molecular biological techniques have facilitated studies at the DNA level, enabling research on genetic polymorphisms and their association with various aspects of exercise to actively proceed.

Polymorphism may cause various changes in phenotype between individuals, and the difference in exercise performance may also be associated with polymorphism in specific genes. The NADPH oxidase system is an aggregate of plasma membrane-related enzymes and consists of two membrane-associated proteins (p22phox, gp91phox) and four cytosolic proteins (p40phox, p47phox, p67phox, rac1) (Cave et al., 2006). NADPH oxidase exists inside the endothelial cells and the cells of smooth muscles within blood vessels and is the main source of reactive oxygen species (ROS) (Griendling et al., 2000), which are implicated in exercise-induced oxidative damage. The p22phox C242T polymorphism results in the replacement of a histidine by a tyrosine (Dinauer et al., 1990) on the T allele associated with reduced NADPH oxidase activity (Wyche et al., 2004).

Few studies have investigated the influence of the NADPH oxidase p22phox C242T polymorphism on endurance exercise performance and/or oxidative DNA damage. Garay et al. (1974) examined the effects of genetic differences on exercise performance and reported that the distribution frequency of the allele between athletes at the Mexico Olympics in 1968 and the general public was different. Recently, Park et al. (2005) found that p22phox C242T polymorphisms in older Caucasians with relatively high cardiovascular diseases risk factors were associated with differential changes in systemic oxidative stress with aerobic exercise training.

High-intensity exercise, which involves increased oxygen consumption, has been shown to increase ROS (Alessio, 1993), which can induce the oxidation of lipids, proteins, and nucleic acids. The results of previous studies on rodents (Wierzba et al., 2006) and humans (Hartmann et al., 1994; Niess et al., 1998; Tanimura et al., 2008) indicate that a single bout of high-intensity exercise increases oxidative DNA damage. Addition-

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ally, Bejma and Ji (1999) showed that high-intensity exercise increases ROS production due to the up-regulation of NADPH oxidase activity. The results of the previous studies, therefore, suggest that NADPH oxidase could be a potential mechanism through which acute and chronic exercises may modulate systemic oxidative stress levels as well as oxidative DNA damage.

To the best of our knowledge, there are no published reports that used healthy, young subjects to examine how the NADPH oxidase p22phox C242T polymorphism affects exercise performance and lymphocyte DNA damage during rest, exercise, and recovery in humans. We therefore undertook a longitudinal study to examine the effects of the NADPH oxidase p22phox C242T polymorphism on endurance exercise performance and lymphocyte DNA damage in response to acute and chronic exercises.

## MATERIALS AND METHODS

### Subjects

One hundred three healthy, non-smoking, moderately active men between the ages of 18 and 27 years were recruited from the Yonsei University campus community by posted notices and e-mail. Following genotype analysis using a single base primer extension assay (SNP-ITTM), a total of 26 subjects were selected and 12 and 14 subjects were assigned to the CC genotype and T allele (12 TC/ 2 TT) groups, respectively. Subjects exercised 2-5 h/week with activities such as weight training, walking, cycling, and running but were not elite endurance athletes. No subjects had experienced large weight, activity or diet changes within the last 6 months. Subjects were injury- and disease-free as determined by a health history questionnaire and physical examination. All subjects provided informed consent and the study protocol was approved by an institutional ethics review board in the department of physical education at Yonsei university.

### General experimental design

Following genotype analysis using SNP-ITTM, subjects were assigned to two groups (CC genotype or T allele) and screening tests and all-out exercise trials on a treadmill at 85% $VO_2$max were conducted. Subjects began aerobic exercise training 3 days after their all-out exercise trial and continued for 8 weeks. After aerobic exercise training, one more all-out exercise trial was performed and the screening tests were repeated.

### Screening tests

$VO_2$max during running was determined during a continuous, graded exercise test on a treadmill (Q65, Quinton, USA) beginning at 1.7 mph and 10% grade and increasing 0.8-1.0 mph and 2% grade every 3 min until voluntary cessation. Respiratory gases were continuously monitored via an open circuit system (Meta Max 3B, Cortex, Germany) and recorded every minute by an on-line, real-time, personal computer-based mixing chamber system. In each test, the open-circuit system was calibrated twice before rest and exercise by using room air and a certified calibration gas (16% $O_2$ and 4% CO). $VO_2$max tests were accepted as maximal if heart rate was within 10% of the predicted heart rate and the respiratory exchange ratio (RER) values exceeded 1.1. Subjects were instructed to maintain diet and physical activity level throughout the entire experimental period. Body composition was determined by bioelectrical impedance analysis (M310, Biodynamic, USA). Three-day diet records were collected twice before and after aerobic exercise training to assess dietary habits and monitor the subjects' caloric intake and macronutrient composition. Analysis of dietary records was performed by using the Nutritionist III program (N-squared Computing, Salem, USA).

### Aerobic exercise training program

Subjects were required to exercise on the treadmill for 40 min 3 d per week with trainers who were current graduate students in our laboratory. Subjects were asked to warm-up before they started running and to cool-down after they stopped running. The intensity of training was set at 70% HRR which was calculated by Kavonen's heart rate reserve (HRR) formula (Whaley et al., 2006). The intensity was controlled by using the heart rate monitor Polar a5 (Polar, Finland), and the error margin was maintained at $\pm$ 5%. In addition to the supervised training, subjects were encouraged to exercise in any manner they desired.

### Blood sampling and analyses

Blood samples were taken at rest, after exercise to exhaustion, and after 0.5, 4, and 24 h of recovery. At each of the blood sampling time points, the levels of MDA, SOD, and lymphocyte DNA damage were determined.

*NADPH oxidase p22phox C242T genotype analysis*

To determine the NADPH oxidase p22phox C242T genotype, genomic DNA was extracted from 3 ml of whole blood using a DNA isolation kit (Gentra Genomic DNA purification kit, USA) following the protocol provided by the manufacturer. The analysis of the C242T genotype was performed using SNP-ITTM, which is a single primer extension technology (SNPstream 25 KTM System, Orchid Biosystems).

For PCR, one phosphothiolated primer and one regular polymerase chain reaction (PCR) primer were used. The sequences of these two primers were 5′-AAAGGAGTCCCGAGTGGG-3′ for the forward primer and 5′-AACATAGTAATTCCTGGTAAAGGG-3′ for the reverse primer. After 30 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min, a 7 min extension step at 72°C followed by a 1 min denaturation step at 95°C was performed. The single-stranded PCR template was placed on the flat board primer96 to which SNP-ITTM was attached in the order of 5′-AACAGCTTCACCACGGCGGTCATGT-3′. The identity of the mixed oligonucleotide was determined by a series of colorimetric responses to streptavidin-HRP and anti-FITC-AP. The yellowish-brown or blue color produced was analyzed using an ELISA reader and the final genotype was determined using the QCReviewTM program.

*Malondialdehyde (MDA) and superoxide dismutase (SOD) analyses*

Plasma MDA concentrations were determined using the BIOXYTECH LPO-586 kit (Oxis, USA). A 200 μl aliquot of plasma or standard was mixed with 640 μl of diluted N-methyl-2-phenylindele. Then, 150 μl of concentrated hydrochloric acid was added, mixed and incubated at 45°C (60 min). After cooling, the absorbance values of the standards and samples were read at 586 nm using a spectrophotometer (Hi-Tech Scientific, USA).

Plasma SOD activity was determined by using a tetrazolium-based kit (IBL, Germany). A 200 μl of the diluted radical detector and 10 μl plasma sample were added to prepared standard wells. Then, 20 μl of diluted xanthine oxidase was added and mixed for a few seconds. The reaction was incubated at room temperature. After incubation, the absorbance values of the standards and samples were read at 450 nm using a spectrophotometer (Tecan, Austria).

*Lymphocyte DNA damage analysis*

Lymphocyte DNA damage was determined by using a comet

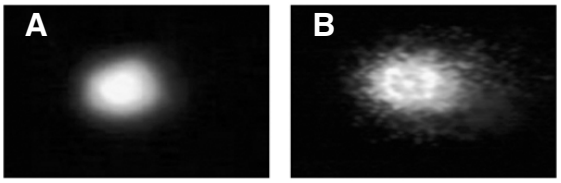


**Fig. 1.** Lymphocytes DNA image at rest (A) and immediately after exercise exhaustion (B) detected by comet assay.

assay, which shows single or double strand DNA breaks (Singh et al., 1988). For the comet assay, 130 μl whole blood was mixed with 900 μl phosphate buffered saline (PBS) and poured gently over 150 μl lymphocyte separation solution. After centrifugation at 1450 rpm (4 min), lymphocytes were pipetted out and transferred to another tube. Seventy-five microliters of low melting temperature agarose (LMA) was put into the tube and mixed with a pipette. After removing the cover glass from the slide, the mixture was poured over the slide horizontally, covered with cover glass, put on freezing plate, and refrigerated for 5 min.

The electrophoresis was conducted for 20 min at 25 V and 300 mA. When electrophoresis was finished, the nucleus was treated with fluorescence-based staining and observed under a Leica fluorescence microscope (Leica, Germany). The image of each nucleus was sent through a CCD camera (Nikon, Japan) and was analyzed through the Komet 4.0 comet image analyzing system (Kinetic Image, UK). Examples of the Comet images are as shown in Fig. 1.

### Statistical analyses

Data are presented as means ± standard deviation (SD). The significance of differences among mean values between pre- and post-training, as well as between CC genotype and T allele groups, were determined by two-way analysis of variance (ANOVA) using SPSS 12.0 for Windows. The significance of differences between the mean values among the rest, immediately after exercise and recovery were determined by one-way ANOVA. Statistical significance was set at a = 0.05.

## RESULTS

### Subject characteristics

Analysis of the NADPH oxidase p22phox C242T polymorphism in young healthy Korean men showed that 85 subjects (82.5%) had CC, 16 subjects (15.5%) had TC, and 2 subjects (1.9%) had TT genotypes. As explained above, a total of 26 subjects were assigned to CC (12) genotype and T allele (14: 12 TC/2 TT) groups. The physical characteristics of the subjects in the CC genotype and T allele groups before and after aerobic exercise training are listed in Table 1. The subjects were weight stable throughout the study period with no changes in % body fat between the CC genotype and T allele groups. There was, however, a small but significant increase in percentage body fat after 8 weeks of aerobic exercise training ($P < 0.05$).

### Exercise capacity and endurance performance

As shown in Table 1, there was a slight improvement in $VO_2$max in both the CC genotype group (2.2%), and in the T allele group (1.2%) over the eight week period, but the improvement was not significant. Additionally, there was no significant difference in $VO_2$max between the groups.

Exercise time to exhaustion increased significantly by 31.2% in the CC genotype group, and 14.7% in the T allele group throughout the training period ($P < 0.05$), but no difference was found between groups.

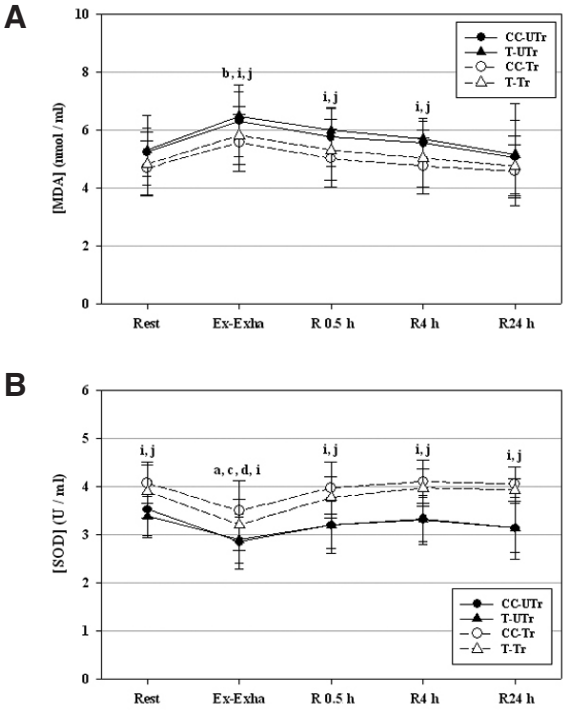


**Fig. 2.** The change in (A) MDA and (B) SOD with respect to polymorphisms pre- and post training. Values are given as means ± SD. CC-UTr: pre-training in CC genotype, T-UTr: pre-training in T allele, CC-Tr: post training in CC genotype, T-Tr: post training in T allele, Ex: exercise, Exha: exhaustion, R: recovery. Significant difference between [a]Rest and Ex-Exha in CC-UTr, [b]Rest and Ex-Exha in T-UTr, [c]Rest and Ex-Exha in CC-Tr, [d]Rest and Ex-Exha in T-Tr, [i]CC-UTr and CC-Tr, and [j]T-UTr and T-Tr.

### Plasma MDA and SOD levels

Plasma MDA levels (Fig. 2A) tended to be increased in response to exercise and returned to the resting value after four hours of recovery in both the CC genotype and the T allele groups before and after training. Plasma MDA concentrations appeared to be significantly lower at immediately after endurance training, and during the first 4 h of recovery period in both groups after training. There were no significant differences in plasma MDA levels at rest, immediately after exercise, and during recovery periods between the CC genotype and the T allele groups before and after training.

Plasma SOD levels (Fig. 2B) decreased significantly in response to exercise (except the T-UTr trial) and returned to the resting value after four hours of recovery in both the CC genotype and the T allele groups before and after training. Plasma SOD concentrations appeared to be significantly higher at rest, immediately after exercise, and during recovery periods in the CC genotype group after training; and at rest, and during the recovery period in T allele group after training. There were no significant differences in plasma SOD levels at rest, immediately after exercise, and during recovery periods between the CC genotype and the T allele groups before and after training.

### Lymphocyte DNA damage

Lymphocyte DNA damage, shown as % DNA in tail (Fig. 3A), DNA tail length (Fig. 3B), and moment (Fig. 3C) increased sig-

**Table 1.** The physical characteristics of subjects pre- and post-training

| Variable | | CC genotype (n = 12) | | T allele (n = 14) | |
|---|---|---|---|---|---|
| | | Pre-Tr | Post-Tr | Pre-Tr | Post-Tr |
| Age (yr.) | | 23.25 ± 2.90 | - | 21.57 ± 2.79 | - |
| Height (cm) | | 176.64 ± 4.62 | - | 176.39 ± 6.57 | - |
| Weight (kg) | | 72.44 ± 5.34 | 71.86 ± 5.20 | 70.46 ± 9.76 | 70.04 ± 9.14 |
| Body fat (%) | | 14.70 ± 3.65 | 13.68 ± 3.49* | 16.05 ± 4.53 | 14.18 ± 4.65* |
| Fat mass (kg) | | 10.72 ± 3.10 | 9.90 ± 3.02* | 11.53 ± 4.50 | 10.13 ± 4.16* |
| LBM (kg) | | 61.73 ± 4.23 | 61.95 ± 3.95 | 58.92 ± 6.84 | 59.91 ± 6.86* |
| $VO_2$max | (ml/kg/min) | 51.38 ± 4.13 | 52.49 ± 5.06 | 52.14 ± 6.39 | 52.78 ± 6.11 |
| | (l/min) | 3.73 ± 0.45 | 3.78 ± 0.51 | 3.64 ± 0.44 | 3.67 ± 0.43 |
| Ex. Time to Exha (min) | | 33.64 ± 4.80 | 44.12 ± 7.04* | 36.36 ± 9.05 | 41.69 ± 10.66* |

Values are given as means ± SD. Tr, training; LBM, lean body mass; $VO_2$max, maximal oxygen consumption; Ex, exercise; Exha, exhaustion; *significantly different from Pre-Tr ($p < 0.05$).

nificantly in response to exercise ($P < 0.05$) and returned to the resting value after 4 h of recovery in both the CC genotype and the T allele groups before and after training. After training, % DNA in tail appeared to be significantly lower at rest and at 4 h and 24 h of recovery period in both groups, and at 0.5 h of recovery period in the CC genotype group; DNA tail length appeared to be significantly shorter at rest and the during the recovery period in both groups; DNA tail moment appeared to be significantly lower at rest in both groups, and during the recovery period in the T allele group. There were no significant differences in lymphocyte DNA damage at rest, immediately after exercise, and during the recovery period between the CC genotype and the T allele groups before and after training.

## DISCUSSION

We investigated the effects of NADPH oxidase p22phox C242T polymorphisms on endurance exercise performance and oxidative DNA damage in response to acute and chronic exercises in humans. Key findings were that, in young healthy Korean men 1) the majority had the CC genotype of the NADPH oxidase p22phox C242T polymorphism (82.5%: CC, 15.5%: TC, 1.9%: TT), 2) acute exercise increased lymphocyte DNA damage, 3) endurance training increased running time to exhaustion, and 4) The NADPH oxidase p22phox C242T polymorphism, however, did not alter lymphocyte DNA damage or exercise performance at rest, immediately after exercise, or during recovery.

### Exercise capacity and endurance performance

The results of the present study on the effects of training on exercise capacity and endurance performance corroborate those of previous studies demonstrating that training enhances maximal oxygen consumption and endurance capacity (Jeppesen et al., 2006; Taivassalo and Haller, 2005). As shown in Table 1, the observed increase in endurance capacity in response to aerobic exercise training is probably related to the oxidative capacity of skeletal muscle (Davies et al., 1981). The reasons for the increased muscle oxidative capacity could have been due to either an increase in number, size, or alteration in the composition of mitochondria (Phillips et al., 1996). Previous studies examining the effects of endurance (Davies et al., 1981) and sprint (Davies et al., 1982a) training on exercise bioenergetics demonstrated that endurance-trained rats showed a 14% increase in $VO_2$max despite a 100% increase in muscle oxidative capacity, and that sprint-trained animals had a higher $VO_2$max in the absence of muscle oxidative capacity. These studies suggest that the mitochondrial content of muscle, or muscle oxidative capacity, is the primary determinant of endurance capacity, but that $VO_2$max is not limited by muscle oxidative capacity.

### Plasma MDA and SOD levels

MDA, which is a major thiobarbituric acid reactive substance (TBARS) and an end product of lipid peroxidation, is commonly used to determine lipid peroxidation. In this study, increased MDA levels (Fig. 2A) and decreased SOD levels (Fig. 2B) immediately after exercise to exhaustion compared to those at rest were observed. It has been reported that strenuous exercise generates ROS levels that frequently exceed the capacity of antioxidant defenses, resulting in oxidative stress (Finaud et al., 2006). The exercise-induced oxidative stress, shown as increased MDA levels, is probably related to muscle damage (Miyazaki et al., 2001). Our findings are in agreement with previous studies which demonstrated increased MDA levels and decreased antioxidant activities in response to acute, strenuous exercise (Shin et al., 2008; Toskulkao and Glinsukon, 1996).

In the present investigation, the reduced oxidative stress we observed as a result of aerobic exercise training is in accordance with previous studies that demonstrated that endurance exercise training reduces MDA levels (Miyazaki et al., 2001; Shin et al., 2008) and improves SOD levels (Shin et al., 2008). Furthermore, Urso and Clarkson (2003) reported up-regulation of blood SOD activities in trained individuals.

Recently, Bejma and Ji (1999) reported that acute exercise transiently increases ROS levels due, in part, to up-regulation of NADPH oxidase activity, which might modulate adaptations in the antioxidant system. In contrast, Adams et al. (2005) reported that regular aerobic exercise training reduces p22phox mRNA levels. These results suggest the importance of NADPH oxidase p22phox in the regulation of exercise-induced oxidative stress. In this study, we observed no significant differences in plasma MDA and SOD levels between the CC genotype and the T allele groups before and after training. These findings are, in part, in agreement with those of Stanger et al. (2001) who found no significant differences in serum MDA concentrations according to NADPH oxidase p22phox C242T genotypes.

### Lymphocyte DNA damage

Increased lymphocyte DNA damage shown as % DNA in the

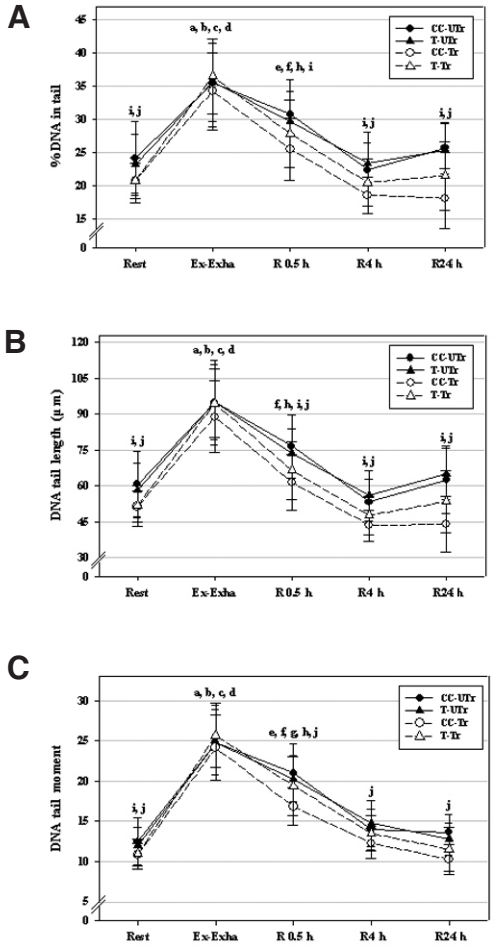


**Fig. 3.** The change in DNA damage with respect to polymorphisms pre- and post-training. (A) DNA in the tail, (B) Tail length, (C) Tail moment. Values are given as means ± SD. CC-Utr, pre-training in CC genotype; T-Utr, pre-training in T allele; CC-Tr, post-training in CC genotype, T-Tr, post-training in T allele; Ex, exercise; Exha, exhaustion; R, recovery. Significantly different between [a]Rest and Ex-Exha in CC-UTr, [b]Rest and Ex-Exha in T-UTr, [c]Rest and Ex-Exha in CC-Tr, [d]Rest and Ex-Exha in T-Tr, [e]Rest and R0.5 h in CC-UTr, [f]Rest and R0.5 h in T-UTr, [g]Rest and R0.5 h in CC-Tr, [h]Rest and R0.5 h in T-Tr, [i]CC-UTr and CC-Tr, and [j]T-UTr and T-Tr.

tail (Fig. 3A), DNA tail length (Fig. 3B), and moment (Fig. 3C), which are reliable markers of oxidative stress (Collins et al., 1996), was observed in response to treadmill running to exhaustion in both the CC genotype and the T allele groups before and after training. Our findings are in accordance with recent studies of Mastaloudis et al. (2004) and Tanimura et al. (2008) who demonstrated that high intensity exercise results in increased DNA damage. Additionally, increases in DNA damage in peripheral human white cells have been reported regardless of the exercise protocol employed, generating consensus that exercise does induce DNA damage (Hartmann et al., 2000). The mechanism of this lymphocyte DNA damage cannot be determined based on our current study. The acute increase in free radical generation during strenuous exercise might be responsible for increased lymphocyte DNA damage (Davies et al., 1982b). Furthermore, in the present study, the levels of lymphocyte DNA damage returned to the resting levels after 4-24 h of exercise to exhaustion in both the CC genotype and the T allele groups before and after training, which is in agreement with the results of recent studies by Mastaloudis et al. (2004) and Tanimura et al. (2008), who demonstrated that the level of DNA damage returned to baseline values after a single bout of strenuous exercise. Increased repair mechanisms and increased clearance of damaged cells may be responsible for this recovery after exercise.

In the present investigation, aerobic training was shown to significantly reduce lymphocyte DNA damage at rest and during the recovery period in the CC genotype and the T allele groups. There was no significant difference in lymphocyte DNA damage immediately after exercise after training when compared with untrained condition. This may be due to the fact that subjects ran longer on the treadmill after training and were tested for lymphocyte DNA damage at their exhaustion point (~100% relative exercise intensity).

As mentioned earlier, the NADPH oxidase system in endothelial and vascular smooth muscle cells plays a key role in superoxide anion ($O_2$-) production, which generates other reactive oxygen species such as hydrogen peroxide ($H_2O_2$) and the hydroxyl radical (OH-). There are four allelic variants of the gene coding for p22phox, a protein component of NADPH oxidase. One of these allelic variants is C242T that results in replacement of a histidine with a tyrosine at codon 72 of p22phox. This change involves a potential heme-binding site (Dinauer et al., 1990) and the T allele is associated with reduced NADPH oxidase activity (Wyche et al., 2004). Our findings showed that there were no significant differences in lymphocyte DNA damage between the CC genotype and the T allele groups before and after training. Our results, however, suggest that numerous factors, including NADPH oxidase, pro-inflammatory cytokines, chemo-attractants, and other proteins involved in ROS production cause lymphocyte DNA damage in humans. Further studies are required to clarify the role of the NADPH oxidase p22phox C242T polymorphism in oxidative DNA damage.

In conclusion, the results of this investigation suggest that in young healthy Korean men 1) the majority of young, healthy Korean men have the CC genotype of the NADPH oxidase p22phox C242T polymorphism (82.5%: CC, 15.5%: TC, 1.9%: TT); 2) acute exercise increases lymphocyte DNA damage; 3) endurance training significantly increased exercise time to exhaustion and alleviated lymphocyte DNA damage; and 4) the NADPH oxidase p22phox C242T polymorphism does not alter lymphocyte DNA damage or exercise performance at rest, immediately after exercise, or during recovery.

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