Effects of regular exercise on neutrophil functions, oxidative stress parameters and antibody responses against 4-hydroxy-2-nonenal adducts in middle aged humans

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Abstract

Regular exercise has recognized health benefits, partly because it reportedly lowers the levels of the oxidation products of proteins and DNA at rest, in contrast with the effect of acute exercise. However, when we compared oxidative response markers in active middle-aged subjects with those in sedentary ones, the level of urinary 8-OHdG was higher in active subjects. Because neutrophils are the first line of defense against a variety of infectious diseases, we then compared the cell density, functions and apoptosis of neutrophils in active subjects with those in sedentary ones. The cell density of neutrophils and phagocytosis of opsonized zymosan by neutrophils were higher in active subjects, being similar with the reported effects of acute exercise. To determine any beneficial effects of oxidative stress in active subjects, we then compared the levels of antibodies against 4-hydroxy-2-nonenal adducts in active subjects with those in sedentary ones, because 4-hydroxy-2-nonenal is one of the most common bioactive aldehyde products of oxidative stress, and because the IgM class of antibodies against oxidized low-density lipoprotein is associated with atheroprotective properties. The level of the IgM but not the IgG class of antibodies against 4-hydroxy-2-nonenal adducts was higher in active subjects. Overall, this study revealed that our active

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middle-aged subjects showed both oxidative responses and a higher IgM response to reactive carbonyl derivatives, possibly providing a basis for a health benefit by exercise in our active subjects.

Key words: regular exercise, oxidative response, anti-oxidative response, antibodies against 4-hydroxy-2-nonenal adducts.

Introduction

Regular exercise is beneficial for health. Although the underlying mechanisms for the positive effects of regular exercise have not been fully elucidated, there is the widely accepted hypothesis that each bout of exercise causes oxidative stress that eventually induces an anti-oxidative response, thereby augmenting the capacity of our body to exert the anti-oxidative response (16). If this mechanism holds true, then repeated bouts of exercise, i.e. regular exercise, may decrease the oxidation products of proteins and DNA at rest (16). This is perhaps why regular exercise or a physically active lifestyle reduces the risk of the development of coronary artery disease and ameliorates the symptoms in patients with established cardiovascular disease (21). It may also partly account for the association between regular exercise and reduced risk of other diseases, including type 2 diabetes, cancer and senile dementia (6, 21). It should be noted that a major cause of these diseases is oxidative stress.

The oxidation products can be measured non-invasively based on the levels of reactive carbonyl derivatives (RCD) of serum proteins (22) and urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) (14). Serum proteins are secreted from cells and react with reactive oxygen species (ROS) outside the cells, whereas 8-OHdG is derived from the intracellular oxidation products of DNA. Therefore, the levels of carbonylated serum proteins and 8-OHdG are affected by extracellular and intracellular ROS, respectively, although they are not mutually exclusive.

Regular exercise may also be beneficial in reducing common cold symptom duration and severity, possibly by means of the products of white blood cells including ROS derived from neutrophils. Neutrophils are a major constituent of white blood cells, and are one of the main producers of ROS in the body. Consequently there is the possibility that the cell density of neutrophils and/or the capability to produce ROS may be related to the beneficial effects of regular exercise. In addition, if regular exercise induces an anti-oxidative response in our body, neutrophil apoptosis may decrease, thereby possibly increasing our capacity to combat against pathogens.

Recently, RCD, such as formaldehyde-generated RCD, were implicated as the active moieties that drive adaptive immune responses (12). Therefore, there is the possibility that regular exercise causes an adaptive immune response through RCD. Clinical and experimental studies have suggested that the IgG class of antibodies against oxidized low-density lipoprotein (OxLDL) is associated with pro-atherogenic properties, whereas the IgM class of antibodies against OxLDL is associated with atheroprotective properties (10). This raises the possibility that regular exercise may cause a higher IgM response to RCD, thereby decreasing the incidence of atherosclerosis.

In this study, we recruited middle-aged male games players (soccer) and sedentary males of similar age, and examined the effects of regular exercise on the oxidation products of proteins and DNA at rest and on apoptosis of neutrophils, the cell density and ROS production of neutrophils. We also examined the possibility that regular exercise may cause a higher IgM response to RCD such as 4-hydroxy-2-nonenal (HNE) adducts.

Materials and methods

Experimental design

This experiment was designed to examine the relationship between regular exercise and health, and was approved by the bioethical committee of Toho University School of Medicine. The experiments were carried out in three different periods, (1) from 28 May 2005 to 2 July 2005, (2) from 30 October 2005 to 4 December 2005, and (3) from 30 September 2006 to 18 November 2006. We recruited 37 male volunteers involved in regular exercise (soccer) (55.3±7.7 years-old; 41-67 years-old) and 15 male volunteers who were not regularly active (53.4±6.9 years-old; 44-65 years-old) for this experiment. All the active volunteers had played soccer for 2 to 3 hours every Sunday for more than 10 years. Three to four of the volunteers joined the experiment at 10 am on each Saturday when they had not exercised on that day. We obtained informed consent from all the volunteers under the condition that personal information would be number coded and stored anonymously. Then, 25 ml of peripheral blood was taken from each volunteer by a nurse to obtain serum and a neutrophil-rich population. Urinary samples (5 ml each) were also obtained from each volunteer in the morning. Serum and urine samples were aliquoted and stored at -80°C until analysis. Each volunteer then performed a supervised VO_2max test based on the Astrand-Rhyming submaximal cycle ergometer test, on an Aerobike 75XLII (Combi Wellness Co. Ltd., Tokyo). VO_2max in active subjects was higher than that in sedentary ones ($p<0.01$) (Fig. 1), as expected.

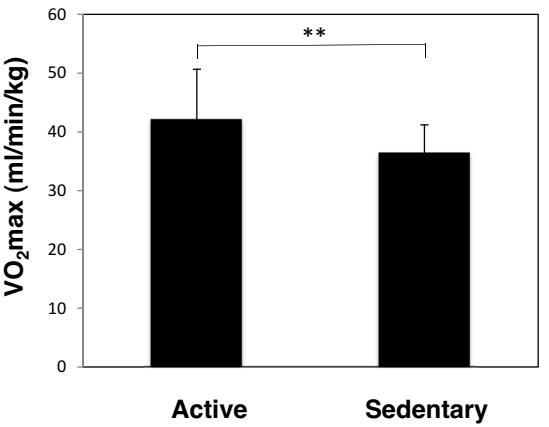

Fig. 1. VO_2max.
The VO_2max data are expressed as means ± standard error. The difference is statistically significant ($p<0.01$, **).

Measurement of urinary 8-OHdG

Urinary 8-OHdG was determined using a high sensitivity ELISA kit (NOF, Tokyo). The obtained values were corrected for urinary creatinine concentration.

Measurement of carbonylated serum albumin

Carbonylated serum albumin was measured by derivatization of carbonyls

with dinitrophenylation, followed by Western blot analysis according to the method previously described (13). The amount of carbonylated serum albumin was normalized to serum albumin concentration, which was determined by Western blot analysis with anti-human serum albumin antibodies.

Preparation of a neutrophil-rich population
Ten ml of peripheral blood was mixed with an equal volume of 3% dextran in saline, followed by incubation for 30 min at room temperature. The supernatant was then centrifuged, and the red cells lysed with distilled water for 30 to 60 s. Neutrophils were then separated from lymphocytes by Ficoll density gradient centrifugation, the purity being 54±17% (n=45) based on FSC vs. SSC profiles on flow cytometric analysis using a FACS Calibur (Becton Dickinson, CA).

Phagocytosis of zymosan by a neutrophil-rich population
Zymosan (*Saccharomyces cerevisiae;* Sigma) was labeled with FITC and then washed several times with PBS. The zymosan was then opsonized with fetal calf serum (3). A neutrophil-rich population was incubated with either opsonized or unopsonized FITC-zymosan (15 mg/ml) at the cell density of 1.25 x 10^6 cells/ml for 30 min at 37°C, followed by flow cytometric analysis. Neutrophils were identified by means of FSC vs. SSC profiles.

Superoxide anion production by a neutrophil-rich population
Two hundred μl of a neutrophil-rich population at the density of 1.25 x 10^6 cells/ml was left in a tube for 3 min at room temperature. Three min after the addition of 25 μM lucigenin, the cells were stimulated with PMA (0.81 μM), followed by measurement of chemiluminescence with a luminometer.

Induction and analysis of apoptosis (9)
A neutrophil-rich population was incubated for 24 h at the cell density of 5 x 10^5 cell/ml in RPMI1640 medium containing 7% fetal calf serum. The cells were then washed and stained with FITC-Annexin V (Bender MedSystems, Vienna, Austria) and/or propidium iodide (PI) for 10 min at room temperature. Cells were then analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, CA). For flow cytometric analysis of apoptosis, neutrophils were identified by means of FSC vs. SSC profiles. Annexin V single-positive cells are defined as early apoptotic ones, and Annexin V and PI double-positive cells as late apoptotic ones.

Measurement of antibodies against HNE adducts in serum
Antibodies against HNE adducts were measured according to the method published previously (8). Briefly, 50 μl of 5 μg/ml bovine serum albumin in 0.05 M carbonate buffer (pH 9.6) was added to each well of a 96-well U-bottomed vinyl plate (BD Falcon), followed by incubation for 2 h at room temperature. After washing with PBS, 50 μl of 200 μM HNE in PBS or a vehicle control was added to each well, followed by incubation for 4 h at room temperature. After washing with PBS containing 0.05% Tween 20 (wash buffer), PBS containing 3% skim milk was added, followed by incubation for 2 h at room temperature. After washing with wash buffer, samples or rabbit anti-HNE adducts antibodies (Academy Biomedicals) as a positive control were added, followed by incubation for 2 h at

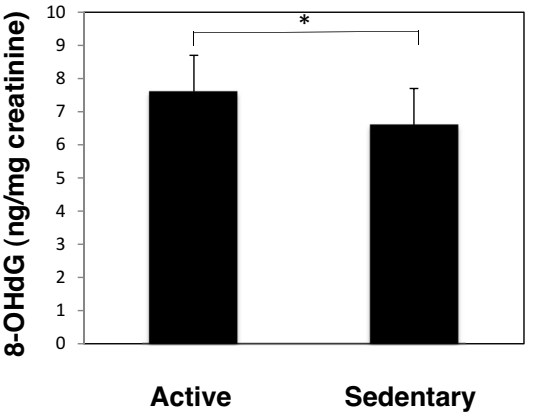

Fig. 2. Urinary 8-OHdG.
Urine samples were obtained from each subject, and the levels of urinary 8-OHdG were measured by means of a specific ELISA. The data were subjected to correction as to creatinine concentration, and expressed as means ± standard error. The difference is statistically significant ($p<0.05$, *).

room temperature. After washing, each well was incubated with biotinylated secondary antibodies (anti-rabbit IgG antibodies (American Qualex), anti-human IgM antibodies (Southern Biotech), anti-human IgG antibodies (Southern Biotech), or anti-human IgA antibodies (VECTOR)), streptavidin-HRP (R&D), and the substrate, ABTS (Sigma).

Statistics
The data were analyzed by means of Student's *t* test. *p* values of less than 0.05 were considered significant.

Results

Effects of regular exercise on urinary 8-OHdG and carbonylated serum albumin.
Urinary 8-OHdG and carbonylated serum albumin were chosen as markers of oxidative responses. As shown in Fig. 2, the level of urinary 8-OHdG in active subjects was higher than that in sedentary ones ($p<0.05$). Carbonylated serum albumin was then detected by means of derivatization of carbonyls through dinitrophenylation (13) (Fig. 3A, B, and C). The levels of carbonylated serum albumin, however, were not significantly different between active and sedentary subjects (Fig. 3D).

Effects of regular exercise on the cell density and functions of neutrophils
We then determined the neutrophil cell density in the peripheral blood of each subject, because acute exercise is known to release neutrophils from bone marrow into the periphery (19), and because regular exercise lowers the neutrophil density (1). The neutrophil cell density in active subjects was higher than that in sedentary ones ($p<0.05$) (Fig. 4).

We then determined the functions of neutrophils, namely phagocytosis and superoxide anion production. The superoxide anion can be quantitated by means of luminol- or lucigenin-enhanced chemiluminescence, the latter being superior to the former due to the high selectivity as to the superoxide anion produced outside of cells (2). We therefore employed lucigenin-enhanced chemiluminescence for determination of superoxide anion. Although zymosan was phagocytosed by neutrophils, opsonization with fetal calf serum enhanced phagocytosis (6.3% vs. 28%) (Fig. 5A, B, and C). Phagocytosis of opsonized zymosan by neutrophils of active subjects was higher than that by neutrophils of sedentary ones ($p<0.05$)

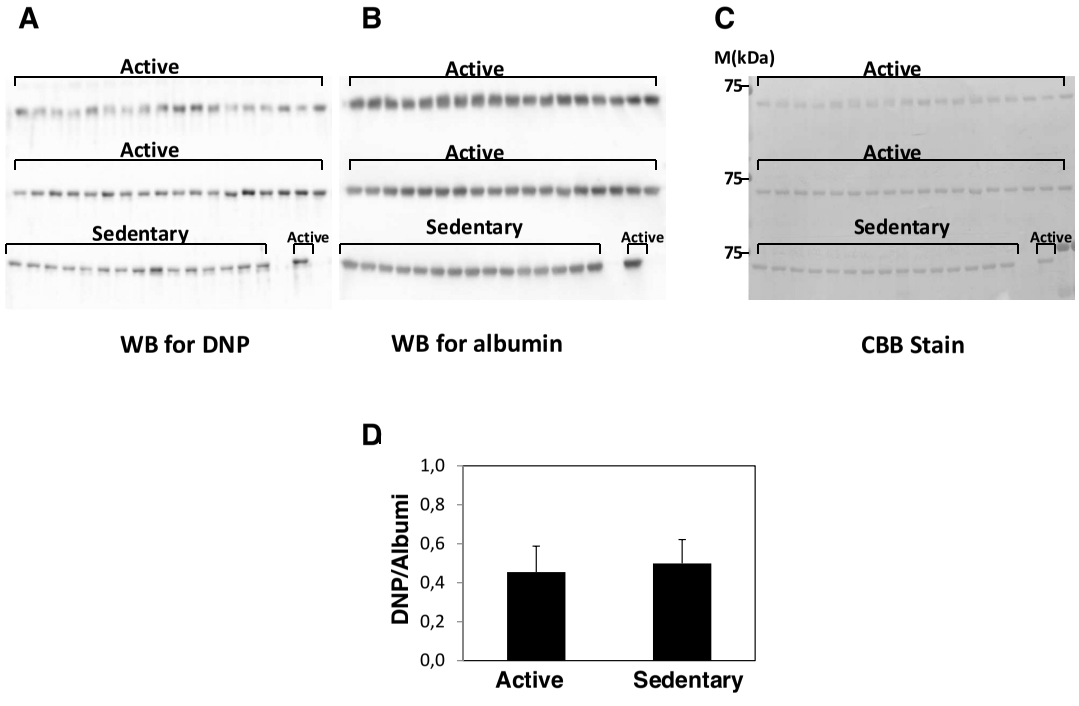

Fig. 3. Carbonylated serum albumin.
(A, B, C) Serum was obtained from the venous blood of each subject, and the level of carbonylated serum albumin was measured by derivatization of carbonyls with dinitrophenylation, followed by Western blot analysis according to the method previously described (A). The amount of carbonylated serum albumin was normalized with regards to the amount of serum albumin (B). The results of CBB staining are also shown in (C). (D) The data are expressed as means ± standard error. The difference is not statistically significant.

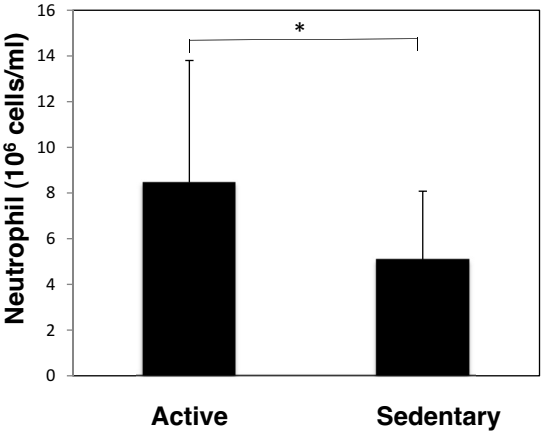

Fig. 4. Neutrophil cell density.
The cell density of a neutrophil-rich population was determined and corrected to the percentage of neutrophils, which was determined by FSC vs. SSC profiles on flow cytometric analysis. The data are expressed as means ± standard error. The difference is statistically significant ($p<0.05$, *).

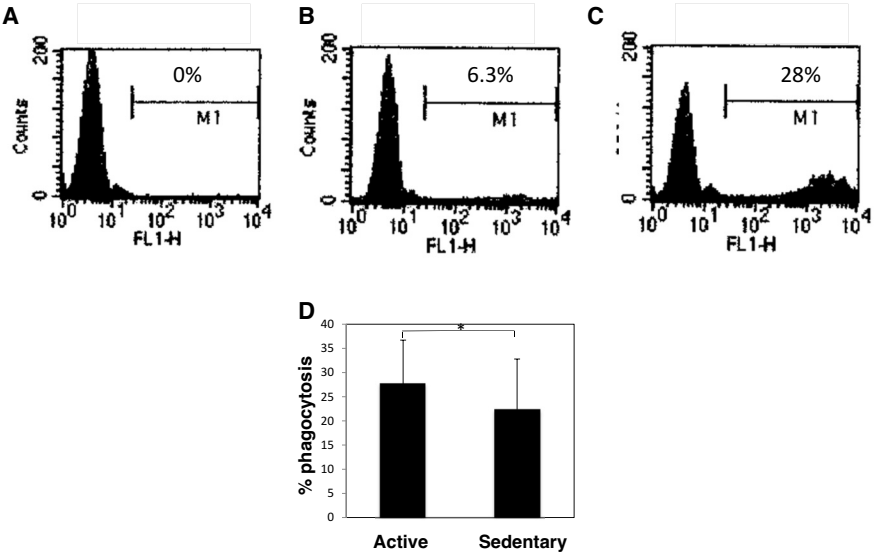

Fig. 5. Phagocytosis of opsonized zymosan by neutrophils.
(A, B, C) A neutrophil-rich population was obtained, and incubated alone (A), or with FITC-labeled unopsonized (B) or opsonized zymosan (C). (D) The data are expressed as means ± standard error. The difference is statistically significant ($p<0.05$, *).

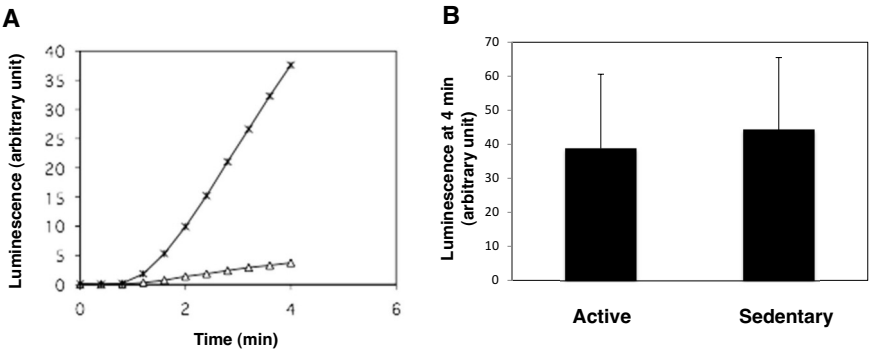

Fig. 6. PMA-induced production of the superoxide anion by neutrophils. (A) A neutrophil-rich population was obtained and lucigenin was added to it. They were then incubated alone (triangles) or with PMA (crosses), followed by measurement of chemiluminescence with a luminometer. (B) The data are expressed as means ± standard error. The difference is not statistically significant.

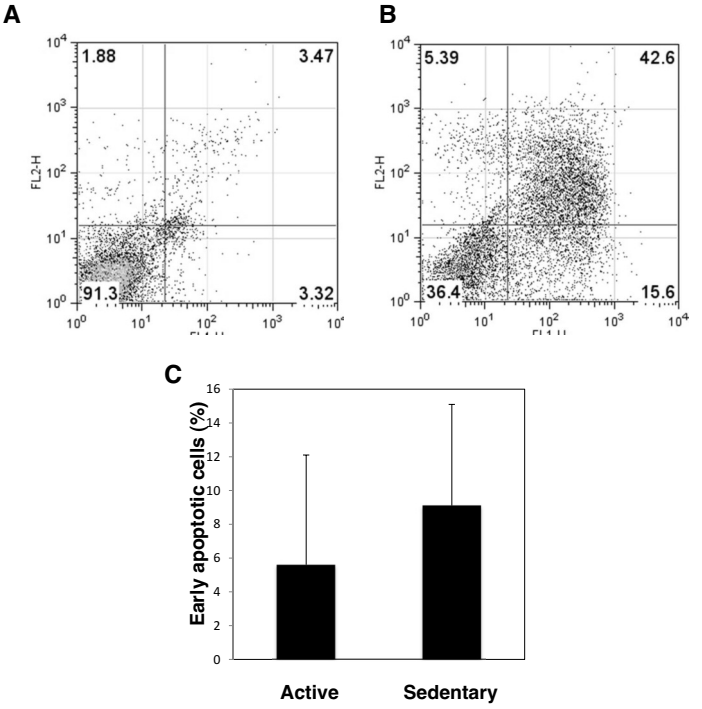

Fig. 7. Apoptosis of neutrophils after culturing.
(A, B) A neutrophil-rich population was obtained, and apoptosis was immediately analyzed (A) or analyzed after 24-h culture (B). FITC fluorescence is shown on the abscissa, and PI fluorescence on the ordinate. (C) The data are expressed as means ± standard error. The difference is not statistically significant ($p<0.1$).

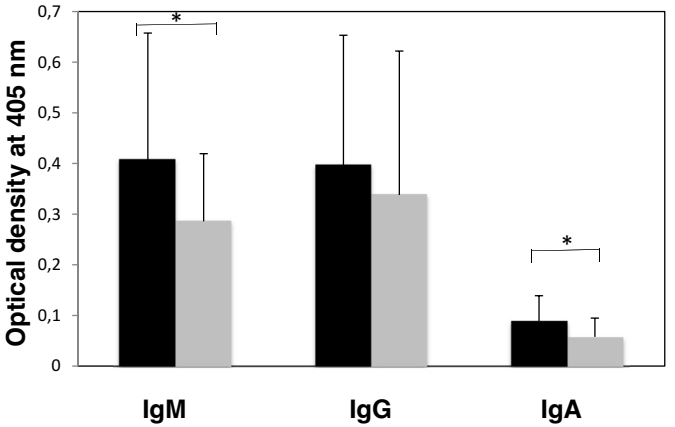

Fig. 8. The IgM, IgG, and IgA classes of anti-HNE adduct antibodies in serum. The data are expressed as means ± standard error (black for active subjects, gray for sedentary subjects). The differences in IgM and IgA are statistically significant ($p<0.05$, *), whereas that in IgG is not statistically significant.

(Fig. 5D). When neutrophils are stimulated with PMA, they produce the superoxide anion after a short time lag (Fig. 6A). However, superoxide anion production was not significantly different between active and sedentary subjects (Fig. 6B).

Neutrophils undergo apoptosis upon culturing, and the process can be quantitated with a flow cytometer. Before culture, neutrophils showed marginal levels of apoptosis, those of early and late apoptosis being 3.3 and 3.5%, respectively (Fig. 7A), whereas, after 24h-culture, they showed significant levels of apoptosis, those of early and late apoptosis being 15.6 and 42.6%, respectively (Fig. 7B). Early apoptosis in neutrophils of active subjects was lower than that in neutrophils of sedentary ones, although the difference was statistically not significant ($p<0.1$) (Fig. 7C). Late apoptosis was also not different significantly between active and sedentary subjects (data not shown, $p>0.1$).

Effects of regular exercise on the levels of antibodies against HNE adducts

HNE is one of the most common bioactive aldehyde products of oxidative stress (5), and it was reported that model antigens modified by HNE or other aldehydes resulted in antigen-specific Th2-type antibody responses under adjuvant-free conditions (12). We therefore determined the levels of antibodies against HNE adducts (IgM, IgG, and IgA) in serum after the serum had been diluted appropriately. Fig. 8 shows the results for one-hundredth diluted samples. The levels of IgM and IgA in active subjects were higher than those in sedentary ones ($p<0.05$). Although the levels of IgM, IgG and IgA should not be compared with each other due to the lack of individual standards, the IgA response would be weaker than the IgM and IgG ones, judging from the results of one-tenth and one-thousandth diluted samples (data not shown).

Discussion

In this study, we found significant differences in the level of urinary 8-OHdG, the cell density of circulating neutrophils and the phagocytosis of opsonized zymosan by neutrophils, which were all higher in active subjects. Neutrophil apoptosis after culturing, on the other hand, was lower in active subjects, although statistically not significant. The level of the IgM but not the IgG class of antibodies against HNE adducts was significantly higher in active subjects.

Since the urinary volume affects the concentration of urinary 8-OHdG, the urinary 8-OHdG data were corrected for creatinine concentration. Although we expected that regular exercise would decrease the oxidation products of protein and DNA at rest, the corrected urinary 8-OHdG levels were higher in active subjects compared with those in the sedentary males. In contrast, the level of carbonylated serum albumin did not differ between active and sedentary subjects. One of the plausible reasons for the different responses to oxidative stress between DNA and the serum protein would be the location of ROS production that causes the modifications, i.e. extracellular vs. intracellular; this is an area worthy of further study.

Neutrophil cell density was higher in active subjects, contrary to our expectation. In one study involving highly trained cyclists who were cycling distances of 120 km per week, the neutrophil cell density at rest was significantly lower than that in sedentary subjects, although in these cyclists acute exercise caused an increase in the neutrophil cell density to a similar extent as sedentary subjects (1). One of the plausible reasons for the discrepancy between our study and that of Blannin et al. is that the participants in the latter study were much younger and were also more highly trained than our active subjects, as judged from the VO_2max values. The other possible reason is that the carry-over effect from the last bout of exercise would persist in the active subjects even after a week. On the contrary, acute exercise results in first, rapid and profound neutrophilia, followed by a second, delayed increase in the blood neutrophil count a few hours later, the magnitude of which is related to both the intensity and duration of the exercise (15, 17). The initial increase is likely due to demargination caused by shear stress and catecholamines, whereas the later increase may be due to cortisol-induced release of neutrophils from the bone marrow (11).

Neutrophil phagocytosis of opsonized zymosan was higher in active subjects, contrary to our expectation. In contrast, PMA-induced superoxide anion production did not differ between active and sedentary subjects. Neutrophil phagocytosis and oxidative burst activity are increased by an acute bout of exercise (15, 17, 19) or by moderate exercise (18), whereas they are decreased in highly trained subjects (1). Of note, Blannin et al (1) used formazan deposits as an indicator of phagocytosis, which are actually an indicator of intracellular ROS production, whereas we employed FITC-labeled opsonized zymozan for analysis of phagocytic activity.

Neutrophil apoptosis was delayed in active subjects, although statistically not significant. This was in good agreement with a recent publication (20), in which the authors suggested that regular exercise induces an anti-oxidative response of neutrophils.

All the above data except for neutrophil apoptosis raise the possibility that an increase in ROS that can increase HNE adduct generation may induce an anti-HNE adduct antibody response of the Th2 type. This possibility may be supported by an increase in the IgA class of anti-HNE adduct antibodies, because Th2 cells promote preferential B cell production of IgA in mice (4) and presumably in humans (7). On the other hand, an increase in the IgM class of anti-HNE adduct antibodies may suggest one beneficial outcome of an increase in ROS in the active subjects in this study. This is because the IgG class of antibodies against oxidized low-density lipoprotein (OxLDL) is associated with pro-atherogenic properties, whereas the IgM class of antibodies against OxLDL is associated with atheroprotective properties (10). The level of urinary 8-OHdG appeared to be more closely related to the IgM class of anti-HNE adduct antibodies than the other classes of anti-HNE adduct antibodies, although the difference was statistically not significant. The mechanism underlying the increases in the IgM and IgA classes of anti-HNE adduct antibodies in active subjects should be explored in the future by means of experimental animals.

To summarize, this study demonstrated that active middle-aged males showed both oxidative responses and a higher IgM response to HNE adducts, possibly through increases in the levels of ROS, compared with sedentary males of similar age. Thus this study provides a basis for a health benefit by exercise in active individuals.

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