Effects of Exercise Training on Superoxide Dismutase Gene Expression in Human Lymphocytes

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This research was conducted for the purpose of studying the influence of exercise training on the level of SOD mRNA expression in human lymphocytes. 10 first-year high school boys (age 15-16), members of the high-school football club, were subjected to exercise training for a period of 3 months; using a bicycle ergometer before and after the exercise training, maximal exercise test was conducted. The subjects trained 2 hours a day, 6 days a week over the span of 3 months, as a regular part of their club activities. Blood samples were taken before and immediately after maximal exercise test before and after training, at rest and after training at the end of month 1, 2 months and the end of the training period; expression of manganese-SOD (Mn-SOD) mRNA and copper-zinc SOD (Cu/Zn SOD) mRNA in their lymphocytes was measured. Results showed significant increases in both maximal oxygen uptake (VO2max) and maximal voluntary ventilation (MVV), (p<0.001 and p<0.01, respectively), and the physiological effects of exercise training were verified. No significant changes were seen in expression of Mn-SOD and Cu/Zn-SOD mRNA at rest as a result of training. In maximal exercise tests, Cu/Zn-SOD mRNA expression levels showed no significant changes either before or after exercise training. Although Mn-SOD mRNA expression levels showed significant increases before exercise training in comparison to before maximal exercise test (p<0.05), significant changes after exercise training were not observed. From these results, the effects of exercise training on lymphocyte SOD mRNA were demonstrated to exert beneficial effects on Mn-SOD functions for an acute bout of exercise rather than on SOD mRNA expression at rest.

Keywords: Mn-SOD, Cu/Zn-SOD, mRNA, lymphocyte, exercise training

1. Introduction

It is known that exercise elevates oxygen uptake up to 20 times over rest (Astrand and Rodahl, 1986), and that production of free radicals also increases in reactive oxygen species with increases in oxygen uptake (Jenkins, 1988). The reactive oxygen species thus produced combines with lipids, protein and DNA, giving rise to oxidation damage (Davies et al., 1982; Jenkins, 1993; Aruoma, 1994). As a defensive strategy, we have antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase and catalase, against reactive oxygen species (Sen, 1995). Among these antioxidant functions, SOD, which specifically metabolizes the superoxide radical (O2·-) into oxygen and hydrogen peroxide (H2O2), stands out as a leading antioxidant. It is a vital enzyme in the metabolism of reactive oxygen species (Chance et al., 1979; Fridovich, 1995). Two types of SOD are primarily known among mammals, the quadrimer manganese-SOD (Mn-SOD), which includes manganese, and the dimer copper/zinc-SOD (Cu/Zn-SOD), with copper and zinc (Taniguchi, 1992).
Among previous researches studying the relationship between exercise and SOD, studies have been made on SOD in blood, skeletal muscle, liver, etc.; there have been reports of rises in Mn-SOD and Cu/Zn-SOD activity or concentration through exercise training (Jenkins et al., 1984; Higuchi et al., 1985; Ohno et al., 1992, 1993a; Gore et al., 1998b; Miyazaki et al., 2001). However, in the case of taking blood SOD levels as an index, there are many unknowns concerning the origin of the SOD. For Cu/Zn-SOD in the blood, in particular, it is known that it is excreted in the urine (Ohno et al., 1993b). Thus we must be careful to note that relative concentrations in the blood do not necessarily reflect the state of reactive oxygen species metabolism in the system (Ookawara et al., 1999). Moreover, in regard to changes in SOD concentration in cellular tissues, it is hard to specify whether the level of concentration is the result of production in the target cellular tissues or a rise in metabolism. In order to solve this problem, recent researches have studied the index of mRNA expression in relation to skeletal muscle, heart and liver, and there have been reports of increased expression of Mn-SOD and Cu/Zn-SOD mRNA through exercise training (Oh-ishi et al., 1997a; Rush et al., 2000; Ennezat et al., 2001). However, most of the reports on the relation between exercise and expression of SOD mRNA have been researches on skeletal muscle or liver and heart in rats and pigs, as conducting experiments on these resources in humans is extremely difficult. In addition, there have been few reports of studies of the relation between exercise and human SOD mRNA.

Therefore, in this research, in order to obtain data concerning human SOD mRNA and exercise, we took lymphocytes as our research material. Lymphocytes are cells vital for maintenance of homeostasis. Moreover, SOD in lymphocytes is known to be involved in various illnesses that are treatable with exercise (Feher et al., 1987, 1988; Muzes et al., 1990; Uchimura et al., 1999). In addition, lymphocytes are easier to collect than skeletal muscle or liver and heart cells, making repetitive study simpler.

There are many research issues remaining in studies pertaining to exercise and reactive oxygen species. Research on SOD mRNA, in particular, is lacking, and conclusions still include many assumptions. Furthermore, there is very little clarity about the antioxidant capability of SOD increased through exercise training, not only during exercise, but also concerning issues such as whether it is effective in preventing or curing illnesses. In this regard, it is believed that studying the effect of exercise on lymphocyte SOD, which is involved in many illnesses, is more effective in solving the above issues than studying skeletal muscle SOD.

Therefore, in this research, with reference to the report confirming the training effect of human exercise training (Ookawara et al., 1999), we conducted exercise training on first-year high school students who had not exercised regularly in the previous three months, with the purpose of studying the influence of exercise training on the expression of Mn-SOD mRNA and Cu/Zn-SOD mRNA in lymphocytes.

2. Materials and Methods

2.1. Subjects

Subjects were 10 healthy male high school students, members of the football club (mean ± SD age = 15.4 ± 0.5 years; height 164.6 ± 4.8 cm; weight 52.1 ± 6.4 kg). Participation in the experiment was obtained after prior sufficient explanation to the subjects and their parents of the contents of the study, its purpose, experimental methods and the dangers involved in the maximal exercise test and blood sampling.

2.2. Training and maximal exercise testing

Training took place over a period of 3 months, 6 days per week, 2 hours per day, as a part of regular football club activity (15 minutes of warm up, 20 minutes of review of basic techniques, 60 minutes of scrimmage, 15 minutes of cooling down). This was taken as the period of exercise training.

Maximal exercise test was conducted before and after this exercise training and maximal oxygen uptake (VO2max) was measured. Maximal exercise test was conducted on a bicycle ergometer (Combi 232C, Combi Co., Japan); after 3 minutes rest, pedaling at 50 rpm was continued until fatigue was reached, the load gradually increased by 50 watts every three minutes. At the same time, a graded exercise system (ML-4500, Fukuda Sangyo Co., Japan) and a breath analysis system (Oxycon Sigma, Fukuda Sangyo Co., Japan) were used to measure heart rate and exhalation volume. Final post-exercise training maximal exercise test was conducted 3 days after the final exercise training.
2.3. Blood sampling and lymphocyte isolation

Heparin-added blood sampling from an antecubital vein was conducted before exercise training, 1 and 2 months after the beginning of exercise training and before and after the maximal exercise tests before and after exercise training. However, during the period of exercise training, sampling was conducted at rest before practice, and post-maximal exercise test sampling was conducted immediately after exercise. We drew the obtained blood samples into test tubes (5 ml), diluted it with the same amount of phosphate-buffered saline (PBS), poured 5 ml of Lymphoprep™ (Nycomed Pharma, AS, Norway) on top and centrifuged it at 800×g for 20 minutes at 20°C to separate the lymphocytes. The separated lymphocytes were washed 3 times in PBS and, after adjusting the number of cells to 1×10⁶/ml, they were stored at –80°C until the mRNA could be extracted.

2.4. SOD mRNA analysis

For extracting mRNA, we used TRIZOL® Reagent (Invitrogen Life Technologies, New Zealand), following the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). We washed the extracted mRNA 2 times in a 75% ethanol water solution and stored them at –80°C until reverse transcription (RT) could be made.

We dissolved the extracted mRNA in 20μl of TE buffer (Cosmo Bio Co., Japan) and subsequently drew 1μl into microtubes, added RNase Free H₂O (Toyobo Co., Japan) 9μl, 5×RTase buffer (Toyobo Co., Japan) 4μl, random primer (25 pmol/μl, Toyobo Co., Japan) 1μl, dNTPs mixture (10 mM each, Toyobo Co., Japan) 2μl, RNase inhibitor (10 u/μl, Toyobo Co., Japan) 1μl, and M-MLV RTase (RNase H-) (10 u/μl, Toyobo Co., Japan) 2μl. Using a thermal cycler (PCR System 9700, Applied Biosystems, Japan), we reacted it for 10 minutes at 30°C, 20 minutes at 42°C, 5 minutes at 99°C and 5 minutes a 4°C to synthesize cDNA. The synthesized cDNA was stored at -80°C for polymerase chain reaction (PCR).

We used the real-time PCR method for measurement of Mn-SOD and Cu/Zn-SOD mRNA. Real-time PCR mixtures consisting of Universal PCR Master Mix (Applied Biosystems, Japan), forward primer (Sawady Technology Co., Japan), reverse primer (Sawady Technology Co., Japan), and TaqMan Probe (Sawady Technology Co., Japan) were prepared on ice, and 20μl of this mixture were poured into each well of a 96-well reaction plate (Applied Biosystems, Japan). Into this we added 5μl of 1:10 cDNA-distilled water solution, and, using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Japan), after reacting for 2 minutes at 50°C and 10 minutes at 95°C, we conducted 40 cycles, 1 cycle consisting of 15 seconds at 95°C and 60 seconds at 60°C, detecting the curve of the increase in PCR production in real time. Taking β-actin as the inner control for Mn-SOD and Cu/Zn-SOD, we conducted real-time PCR by the same method. The degree of Mn-SOD and Cu/Zn-SOD mRNA was calculated after normalization to levels of β-actin mRNA.

For Mn-SOD, Cu/Zn-SOD and β-actin sequences, we accessed the National Center for Biotechnology Information (NCBI) Home Page and looked them up, using Primer Express® software (Applied Biosystems) to determine the forward primer, reverse primer and TaqMan Probe sequence for Mn-SOD, Cu/Zn-SOD and β-actin, respectively (Table 1).

2.5. Statistical analysis

All results were shown in mean ± standard deviation. VO₂max, MVV and Mn-SOD and Cu/Zn-SOD mRNA expression in lymphocytes before

| Tab. 1. Mn-SOD, Cu/Zn-SOD and β-actin sequences used for real-time PCR |
|-----------------------------|-----------------------------|
| **Gene** | **Sequence** |
| Mn-SOD | Forward primer 5'-TGCTGCGTTGCTCCAAATCAGG-3' |
| Cu/Zn-SOD | Forward primer 5'-GGTGTGGCCGATGTGTCTATT-3' |
| β-actin | Forward primer 5'-CAGGTTCATCACTTGGCAAT-3' |

and after maximal exercise test were determined by the Wilcoxon signed rank test. For comparison of Mn-SOD and Cu/Zn-SOD mRNA expression in lymphocytes during exercise training in lymphocytes examined, we employed one-way ANOVA, conducted with Fisher’s PLSD post hoc test. In addition, \( p<0.05 \) was regarded as significant.

3. Results

3.1. Physical Characteristics

\( \dot{V}O_{2}\text{max} \) and MVV both increased significantly compared to before exercise training (16.3%, \( p<0.001 \); 12.4%, \( p<0.01 \), respectively) (Figure 1 and Figure 2). Exercise time and exertion both showed significantly high values after exercise training, from 13.9 minutes (SD 1.8) and 219 watts (SD 30) to 15.2 minutes (SD 1.3) and 253 watts (SD 27), \( p<0.05 \), respectively). We could find no significant difference in weight or height in the subjects before and after exercise training.

3.2. Expression of SOD mRNA during exercise training

We were unable to confirm significant changes in Mn-SOD mRNA expression in lymphocytes at rest during the exercise training period (Figure 3). Nor could we confirm significant changes in Cu/Zn-SOD mRNA expression in lymphocytes during the exercise training period (Figure 4).
3.3. Expression of SOD mRNA during exhausting exercise test

Before exercise training, Mn-SOD mRNA expression in lymphocytes after maximal exercise test showed significantly high values compared to before maximal exercise test ($p<0.05$), but after exercise training, we could not confirm significant changes (Figure 5).

Both before and after exercise training, significant changes in Cu/Zn-SOD mRNA expression in lymphocytes could not be confirmed (Figure 6).

4. Discussion

The 2 hours per day, six days per week exercise training over 3 months of this study were confirmed to have physiological exercise results, with increases in $\dot{V}O_{2\text{max}}$, MVV and time of exercise of 16.3%, 12.4% and 10.9%, respectively, as in the report of Ookawara et al. (1999). However, we could not confirm significant changes in Mn-SOD and Cu/Zn-SOD mRNA expression in lymphocytes at rest during the exercise training period. This result is similar to reports that could not confirm Mn-SOD and Cu/Zn-SOD mRNA expression in skeletal muscle at rest after exercise training in rats made to undergo exercise training (Hollander et al., 1999; Oh-ishi et al., 1997a, b). As for the reason, Hollander et al. (1999) hypothesized that mRNAs have much shorter half-lives than enzyme proteins and can be degraded rapidly after transient upregulation (Gorecki et al., 1991), the Mn-SOD and Cu/Zn-SOD mRNA expression in skeletal muscle harvested 48 hours after exercise training might have already returned to rest level. In fact, a research group that included Hollander has confirmed a significant increase in Cu/Zn-SOD mRNA expression in harvested skeletal muscle 24 hours after exercise training compared to before exercise training (Gore et al., 1998a). In addition, Hollander et al. (2001) confirmed significant increases in Mn-SOD mRNA expression in the skeletal muscle of rats immediately after a single bout of exercise, 1 hour later and 2 hours later, but they reported that they could not confirm significant change after 48 hours. Post-exercise training blood sampling in this research occurred 72 hours after the last club activity. In addition, the samplings taken after 1 month and 2 months were made 48 hours after the 6-day activity finished. It cannot be confirmed that skeletal muscle and lymphocytes show the same changes, but we do believe that the time elapsed until sampling affected this research and led to the inability to confirm significant changes in Mn-SOD and Cu/Zn-SOD mRNA expression in lymphocytes at rest during exercise training. As a result, it was demonstrated that Mn-SOD and Cu/Zn-SOD mRNA expression in lymphocytes at rest is not easily influenced by exercise training.

There is no report studying the influence of a single bout of exercise on lymphocyte SOD mRNA expression, but in the report of Tauler et al. (2003), who studied lymphocyte SOD activity before and after dual marathons, significant increases in
lymphocyte SOD activity were demonstrated after dual marathons, and it was shown that lymphocyte SOD is affected by oxidation stress from a single bout of exercise. In our own study, we could not confirm significant changes in Cu/Zn-SOD mRNA expression in lymphocytes either before and after training as a result of the maximal exercise test. In contrast, Mn-SOD mRNA expression in lymphocytes after the maximal exercise test before exercise training showed significant increases compared to before the maximal exercise test. There have been several reports similar to ours confirming significant changes in Mn-SOD mRNA expression only. Hollander et al. (2001) demonstrated significant increases in Mn-SOD mRNA expression in skeletal muscle in rats after exercise. They hypothesized that Mn-SOD mRNA expression was increased in response to increased oxidation stress resulting from the exercise. In addition, Oh-ishi et al. (1997a) divided rats into exercise training groups and non-exercise training groups. Each group was subjected to a single bout of exercise, and Mn-SOD mRNA expression in skeletal muscle among the non-exercise training group showed significant decreases after a single bout of exercise. However, no significant changes were confirmed for the exercise training group. The reason given for this was that the non-exercise training group received muscle damage from oxidation stress and that Mn-SOD mRNA expression in skeletal muscle decreased, but that the exercise training group were equipped to withstand oxidation stress through exercise training, and they reported it as a result of the effects of exercise on Mn-SOD mRNA in skeletal muscle. It is believed that the difference in influence of transient exercise on the two types of SOD is due to the difference in regions, Mn-SOD primarily in the mitochondria and Cu/Zn-SOD primarily in the cytoplasm. That is, during exercise, oxygen uptake increases, and in conjunction the production of reactive oxygen species increases, and most of the reactive oxygen species in the mitochondrial electron transport. It is known that 80% of the O$_2^-$ produced in the mitochondria is metabolized by the Mn-SOD residing there, while the other 20% is eliminated by the Cu/Zn-SOD residing in the cytoplasm (Nohl and Hegner, 1978). Therefore, we can consider lymphocyte SOD mRNA to be similar to skeltal muscle, and that Mn-SOD is more easily influenced by exercise than Cu/Zn-SOD. In addition, Pajovic et al. (2000) exposed lymphocytes to radiation, and the results of the study of the influence on lymphocyte Mn-SOD and Cu/Zn-SOD revealed that Mn-SOD only increased after exposure. They reported the result as lymphocyte Mn-SOD rising to cope with oxidation stress on the lymphocytes from the exposure to radiation, and that lymphocyte Mn-SOD plays a vital role against oxidation stress to the lymphocytes. As a result, it could be thought that one factor in the significant change in lymphocyte Mn-SOD only might involve the fact that the lymphocytes themselves were subjected to oxidation stress by exercise, and to cope with it Mn-SOD mRNA expression was enhanced.

There are several reports studying the effects of exercise training not only SOD mRNA expression but also at the same time on SOD concentration and SOD activity. None of these reports confirm significant changes in SOD mRNA expression through exercise training, but they show significant increases in SOD concentration or SOD activity (Gore et al., 1998b; Hollander et al., 1999; Oh-ishi et al., 1997a, b). The reason for this is hypothesized to be that as a result of improvements in transcription efficiency or RNA stability, even without significant changes in mRNA expression, SOD concentration and SOD activity bring about significant changes (Clerch et al., 1996; Fazzone et al., 1993). In this research, we studied only SOD mRNA expression, so we cannot venture into the territory of conjecture, but we can speculate that before exercise training, an increase in lymphocyte Mn-SOD mRNA expression was needed against oxidation stress from exhaustion exercise, but after exercise training lymphocyte Mn-SOD mRNA transcription efficiency or RNA stability was improved, so that the body was able to cope with the oxidation stress of the same exhaustion exercise without increasing Mn-SOD mRNA expression in the lymphocytes, showing the possibilities for improving Mn-SOD mRNA function through exercise training. However, in order to clarify the details of this, it will be necessary to measure SOD concentration and activity in the same samples.

Through this research, we have shown that exercise training also has benefits for Mn-SOD mRNA in lymphocytes. In addition, we have also demonstrated that, in lymphocytes, exercise training influences Mn-SOD functions in response to a single bout of exercise more than SOD mRNA expression at rest.

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