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LYMPHOCYTE Cu/ZnSOD AND MnSOD GENE EXPRESSION RESPONSES TO INTENSIVE ENDURANCE SOCCER TRAINING

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ABSTRACT

The purpose of the present study was to examine the effect of physical training on the gene expression levels of Cu/Zn superoxide dismutase (Cu/Zn SOD) and Mn superoxide dismutase (Mn SOD) in young soccer players at the beginning of their professional careers. The non-trained young men underwent a professional soccer training program of 12 weeks.

Blood was obtained at two time points: before and after the 12-week endurance soccer training. Plasmatic superoxide dismutase (SOD) activity, concentration of sulfhydryl (SH) groups, superoxide anion ($O_2^{\cdot-}$), malondialdehyde (MDA), and advanced oxidation protein product (AOPP) were determined. Messenger RNA (mRNA) relative levels of SOD isoenzymes were measured in peripheral blood mononuclear cells by Real-time PCR.

After 12-week training, the soccer group experienced a significant decrease in plasmatic SOD activity and increase of $O_2^{\cdot-}$ concentration. Concentration of SH groups, MDA and AOPP were not affected by physical training, although they showed a trend to increase. Cu/Zn SOD mRNA expression levels were stable during the endurance training period but Mn SOD mRNA levels were significantly higher after the training period. The variation in Mn SOD mRNA levels was dependent on MDA concentration. Negative prediction of MDA concentration on Mn SOD mRNA expression levels remained significant after inclusion of all the other oxidative stress status parameters in a multiple regression model ($p = 0.017$). Positive prediction of Cu/Zn SOD mRNA levels on plasmatic SOD activity remained significant after inclusion of MDA, log $O_2^{\cdot-}$ and AOPP concentrations in a multiple regression model ($p = 0.025$).

Intensive endurance soccer training stimulated a beneficial increase in lymphocyte Mn SOD rather than in Cu/Zn SOD mRNA expression levels. An interaction between the oxidative stress/antioxidative defence status parameters in blood and gene expression levels of Cu/Zn SOD and Mn SOD in PBMCs was also observed.

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Introduction

Regular, non-exhausting physical exercise is a healthy practice and part of the treatment of common conditions, like obesity, diabetes mellitus or cardiovascular disease (10). Intense physical exercise causes oxidative stress (OS), which is defined as a disturbance in the prooxidant–antioxidant balance in favour of the former, because of the increased oxygen (O_2) utilization and the accelerated production of reactive oxygen species (ROS) (11). ROS, including superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$), can interact with membrane lipids, proteins and DNA and cause oxidative damage to cells and their components (3). As a result of lipid peroxidation malondialdehyde (MDA) is generated, which is a marker of oxidative lesions in body lipids (4). Apart from having potential negative effects, ROS can act as signals to directly or indirectly modify gene expression patterns involved in oxidative metabolism but the mechanisms of this modulation still need to be better understood (7, 11, 21).

Under normal physiological conditions, cells are able to balance the production of oxidants and antioxidants, resulting in redox equilibrium, because they are provided with enzymatic and non-enzymatic antioxidant defence (AOD) systems to protect themselves against or to minimize OS. Among these antioxidants, superoxide dismutases (SOD) are important enzymes in the antioxidant defence system and the first line of defence against superoxide radicals (2). They constitute an enzyme family that catalyzes the conversion of $O_2^{\cdot-}$ to H_2O_2 and O_2. Copper/zinc SOD (Cu/Zn SOD) is found predominantly in the cytoplasm, whereas manganese SOD (Mn SOD) is located in the mitochondria (27). The majority of ROS are produced in the mitochondria electron transport chain where Mn SOD is believed to play central role in protecting against oxidative damage (27), and its antioxidant effect is strongly dependent on its expression level (22).

Most of the studies engaged animal tissues to reveal the connection between exercise and gene expression of Cu/Zn SOD and Mn SOD, especially skeletal muscle, liver or heart. Few reports (7, 18) included research on humans and their lymphocytes as the easiest collectable cells vital for homeostasis. Lymphocytes are also essential mediators of inflammation and stress. It was demonstrated that leukocyte

SOD inducibility is in good correlation with susceptibility to cardiovascular and malignant diseases (20). The extent of gene expression changes, related to different exercise intensities, can be detected in lymphocytes and moderate amounts of regular exercise induce their adaptation to OS sufficiently to suppress its negative effects (2).

In the present study, we first determined the responses of plasma OS/AOD systems and lymphocyte Cu/Zn SOD and Mn SOD gene expression to intense and demanding soccer training with heavy schedule of training and competitions; and, second, explored whether there was independent prediction of OS/AOD parameters on SOD isoenzymes gene expression levels. We may speculate that Mn SOD rather than Cu/Zn SOD messenger RNA (mRNA) expression levels could be increased by chronic endurance and an intense soccer training protocol. Our results may indicate that this kind of intensive training may be more beneficial for certain groups of active sportsmen than those who start with intensive training and have other risk factors for OS.

Materials and Methods

Subjects and training

This study included twelve young members selected from the soccer club "Teleoptik", Belgrade, Serbia. Although they were previously untrained soccer players, they had been involved in different recreational physical activities. They volunteered to participate in the study from February to May 2010. The subjects had 5 to 7 training sessions per week with an average weekly training of 10 h to 15 h and participated in the National championship during the intensive training season. The trainings consisted of 15 min warming up, 60 min to 90 min working on technical and tactical elements of the game, and, at the end, stretching and cooling down for 15 min. They had intensive strength workouts twice a week. All participants were in good health, had no ongoing or previous (during the previous year) injuries, were not on any medication and were non-smokers. Participation in the study had no effect on their pre-determined training and competition schedule. Prior to enrolment on the study and at its end, all subjects were assessed for their anthropometric data (height, weight and body mass index–BMI), maximal oxygen uptake (VO_2max) test, standard blood chemistry screening and general health-screening questionnaire. VO_2max was tested during a continuous, progressive exercise test on a treadmill ergometer (Ergo XELG90 Woodway, Weil, Germany). The initial velocity was 8 km/h, increasing every 3 min by 2 km/h. Respiration parameters were analyzed using Quark b2 (Cosmed, Rome, Italy).

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the ethical committees of the Sports Medicine Association of Serbia and the Faculty of Pharmacy, University of Belgrade. The soccer players and their parents were informed about the procedures. All participants signed an informed consent before enrolment.

Blood sampling

Venous blood was collected into heparin evacuated tubes (for plasma and peripheral blood mononuclear cells – PBMCs isolation) and sample tube with serum separator gel (for serum) (Greiner Bio-one, Kremsmünster, Austria). Blood samples were taken from the same individuals at two time points, before and after a 12-week training period at rest in the morning hours in a fasting state. They were put on ice and transported to Faculty of Pharmacy's laboratory within 30 min from drawing. Plasma and serum were separated by centrifugation and multiple aliquots of each sample were stored at -80 °C until analysis. For the measurement of $O_2^{\cdot-}$, plasma from heparinised blood samples was used immediately.

Analytical methods

Measurement of lipid status and OS/AOD parameters. Total cholesterol (TC) and triglycerides (TG) were assayed by routine enzymatic methods using an ILab 300+ analyser (Instrumentation Laboratory, Milan, Italy) and Randox Laboratories (Armdore, UK) reagents. High-density lipoprotein cholesterol (HDL-C) was measured using the same method (as above) after precipitation of the plasma with phosphotungstic acid in the presence of magnesium ions. The concentration of low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula (6).

In order to evaluate the OS status in blood during soccer training, we determined the thiobarbituric acid-reacting substances (TBARs) in an assay that measures the quantity of the MDA–TBAs 1:2 adduct. The red-coloured product was detected spectrophotometrically at 535 nm as described previously by Girotti et al. (8). The rate of nitroblue tetrazolium reduction was used to measure the level of $O_2^{\cdot-}$, as described by Auclair and Voisin (1). Plasma SOD activity was measured according to a previously published method by Misra and Fridovich (17). One unit of SOD activity is defined as the activity that inhibits the auto-oxidation of adrenaline by 50 %. The concentration of sulfhydryl (SH) groups in plasma was determined using 0.2 mmol/L 5.5′-dithiobis (2-nitrobenzoic acid) (DTNB) reported by Ellman (5). The plasma advanced oxidation protein product (AOPP) concentration was determined using the method of Witko-Sarsat et al. (28). Briefly, AOPP was measured in plasma after its reaction with acetic acid. Calibration was performed with a chloramine-T standard solution in the presence of potassium iodide. The absorption was measured at 340 nm and the results are expressed as chloramine-T equivalents.

RNA extraction and Real-time PCR. Total RNA (tRNA) from PBMCs was isolated by an organic extraction method using TRIzol™ reagent (Invitrogen Life Technologies, CA, USA) previously optimised in our laboratory. Primarly, PBMCs were isolated immediately after receipt of heparinised blood in our laboratory, by density gradient centrifugation on Ficoll-Paque™ PLUS (GE Healthcare, Waukesha, Wisconsin, USA) using a slightly modified version of the manufacturer's recommendations. PBMCs' pellet was suspended in 1 mL

of TRIzol™ reagent and stored at -80 ºC. Total RNA was processed for extraction after all specimens were collected.

The concentration of isolated tRNA was determined by spectrophotometric analysis of the absorbance at 260 nm. The ratios at 260 nm and 280 nm (A260/280) as well as at 260 nm and 230 nm (A260/230) were used to assess the protein and organic purity of the tRNA solution. The integrity of the RNA samples and absence of genomic DNA contamination were demonstrated by native 1 % agarose gel electrophoresis.

Intact, high quality tRNAs from participants were subjected to complementary DNA (cDNA) synthesis in a reverse transcription (RT) process. Single-stranded cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following a three-step thermal protocol given by the manufacturer. The expression levels of the genes coding for Cu/Zn SOD and Mn SOD were measured in Real-time PCR 7500 System using the TaqMan™ Gene Expression Assays according to the manufacturer's instructions (Applied Biosystems, USA). TaqMan® Control Total Human RNA (P/N 4307281, Applied Biosystems) was used as calibrator in the relative gene expression method. Negative controls for reverse transcription (no RTase) and for real-time PCR (no cDNA) were included in the experiments.

The amount of target mRNA relative to the reference gene (β-actin) was calculated using the comparative threshold cycle (Ct) method. The relative changes in gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method, as already described (13).

Statistical methods

The distribution of variables was tested by Shapiro–Wilk's test. Statistical testing of differences in continuous variables between the groups was performed using the paired Student's *t*-test for normally distributed variables. Distributions of TG and $O_2^{\cdot-}$ were skewed, thereby logarithmic transformation of values was performed to achieve normality before statistical comparisons were made. Univariate associations were evaluated by Pearson's correlation analysis. Multiple linear regression analysis was performed to estimate the independent contribution of OS/AOD status parameters on mRNA expression levels of Cu/Zn SOD and Mn SOD. Standardized coefficients (β) of the predictor variables were calculated using the Enter method.

The results are expressed as the arithmetic mean ± SD for normally distributed variables. Log-transformed variables were expressed as the geometrical mean and the 95 % confidence interval (CI) for geometrical mean.

Statistical analyses were performed using PASW® Statistic version 18 (Chicago, Illinois, USA) and Microsoft® Office Excel 2007. Two-tailed *p*-values less than 0.05 were considered statistically significant.

Results and Discussion

Soccer players were 17.33 ± 0.49 years old with an average height of 180.8 ± 4.59 cm and average weight of 73.6 ± 8.11 kg. There were no differences in BMI (p = 0.946) after the 12 weeks of training. VO_2max was significantly higher after 12 weeks than before training, 54.76 ± 3.90 mL/min/kg and 56.54 ± 3.63 mL/min/kg (p = 0.03), respectively. An increase in VO_2max confirmed the effectiveness of high intensity and systematic endurance training and indicated an improvement in aerobic capacity (24).

Basic lipid status and OS/AOD status parameters of subjects at the beginning of the study and after 12 weeks are listed in **Table 1**. The levels of TC, TG, LDL-C, HDL-C showed no training-induced changes. The level of OS before and after 12 weeks of training was analysed in blood by determining specific markers of OS and AOD. In our group of soccer players, blood oxidants and antioxidants were in imbalance (**Table 1**), as the plasmatic SOD activity significantly decreased during the study period and at the same time the levels of $O_2^{\cdot-}$ significantly increased.

TABLE 1

Lipid status parameters, OS and AOD status parameters in 12 subjects before and after a 12-week training period

Parameters	Before Training Mean (SD)	After Training Mean (SD)	*p*
TC, mmol/L	4.42 ± 0.50	4.36 ± 0.65	0.655
TG, mmol/L*	0.79 (0.67–0.93)	0.63 (0.52–0.77)	0.070
LDL-C, mmol/L	2.88 ± 0.74	2.74 ± 0.62	0.488
HDL-C, mmol/L	1.27 ± 0.19	1.33 ± 0.17	0.120
MDA, µmol/L	1.05 ± 0.15	1.15 ± 0.16	0.212
$O_2^{\cdot-}$, µmoL NBT/min/L*	49.15 (31.28–77.22)	99.91(42.16–236.74)	0.011
AOPP, µmol/L	23.68 ± 18.60	33.61 ± 15.98	0.172
SOD, U/L	85.73 ± 52.67	32.27 ± 18.31	0.012
SH groups, mmol/L	0.50 ± 0.07	0.57 ± 0.08	0.169

Values are presented as mean ± SD.

* Mean values derived from lognormal distribution given as geometric mean values (95 % confidence interval for geometrical mean), *p* for paired Student's *t*-test.

TC: total cholesterol; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; MDA: malondialdehyde; $O_2^{\cdot-}$: superoxide anion; AOPP: advanced oxidation protein product; SOD: plasmatic superoxide dismutase activity; SH groups: sulfhydryl groups

SOD functions in the cell as one of the primary enzymatic antioxidant defences against $O_2^{\cdot-}$ (27). It is likely that continuous, exhaustive training sessions with frequent competitive matches exposed participants to increased levels of OS. The extensive production of $O_2^{\cdot-}$ overwhelmed the capacity of SOD to dismutate it. On the other hand, it is well known that physical activity increases antioxidant protection and decreases OS damage (11). The obtained low plasmatic SOD activity in our study does not need to present a risk factor for oxidative modification of cell structures. It may reflect an adaptive mechanism and possible high involvement of other

enzymatic and non-enzymatic defence systems in plasma. In our study, the marker of oxidant production was blood thiol oxidation. The content of SH groups is an indirect measure of glutathione activity in serum (25). Martinovic et al. (16) found a significant increase in the content of SH groups with the number of training years experienced by female volleyball players. However, in our case with only 12 weeks of soccer training, we observed no significant increase in the content of SH groups (**Table 1**), in agreement with Majerczak et al. (14), but did find the expected negative correlation of SH group content with AOPP levels, (r = -0.703, p = 0.011).

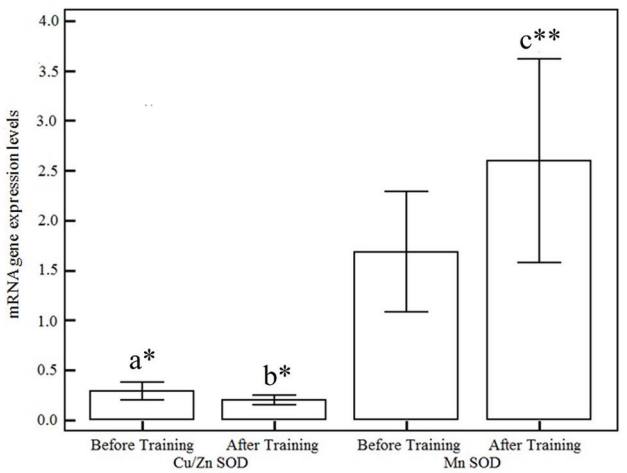

Fig. 1. Effects of 12-week soccer training on Cu/Zn SOD and Mn SOD mRNA expression levels and the difference between them: (**a**) significantly different from Mn SOD before training; (**b**) significantly different from Mn SOD after training; (**c**) significantly different from Mn SOD before training; *p < 0.001, ** p < 0.05 (Student's paired t-test). Values are presented as mean ± SD.

Changes in the levels of $O_2^{\cdot-}$ can induce alteration in the AOD system leading to the activation or silencing of genes encoding defensive enzymes and transcriptional factors. Its excessive generation during exercise is responsible for nuclear factor κB (NFκB) and activator protein-1 (AP-1) activation which up-regulate the expression of the antioxidant enzymes, catalase, glutathione peroxidases, SODs, especially Mn SOD (26). Based on our results, Cu/Zn SOD mRNA expression levels showed no significant differences between occasions (p = 0.106) but Mn SOD mRNA expression was significantly greater (p = 0.031) after training than before. Differences between the isoenzyme gene expression levels were highly significant in favour of Mn SOD for both, before and after training (p = 0.0004 and p = 0.0002, respectively) (**Fig. 1**).

Both SODs are involved in a similar biochemical pathway but they differ in location and transcriptional regulation. Cu/Zn SOD has a stable expression and its product regulates the reactive species turnover in the cytoplasm, maintaining cells in homeostasis. In contrast, Mn SOD expression is unsteady and sensitive to many intracellular and environmental factors, provides the main defence against OS within mitochondria (27). Significant increase in Mn SOD mRNA expression and the observed non-significant down-regulation of Cu/Zn SOD expression after training might indicate an enhancement of AOD capacity in mitochondria of mononuclear cells and its preference as a training-induced OS site (15). Most $O_2^{\cdot-}$ is produced in mitochondria and metabolized by the Mn SOD residing in them and the rest is metabolized by Cu/Zn SOD (27). Further, we may speculate high inducibility of the Mn SOD gene can indicate recruitment of AOD by long-term exercise but the preservation of inducibility of the Cu/Zn SOD gene in mononuclear cells of young soccer players. Such results are consistent with the findings of Gomez-Cabrera et al. (9) and Suzuki et al. (23), where ROS-induced up-regulation of Mn SOD but had no effects on the regulation of Cu/Zn SOD. Regulation of the Cu/Zn SOD gene that differs from regulation of Mn SOD was independent from oxidative metabolism in PBMC. This seems to be a result of adaptation to endurance training. Garcia-Lopez et al. (7) investigated the effects of a 21-week period of progressive strength or endurance training on PBMC antioxidant enzyme gene expression and activity in healthy middle-aged untrained men and found that endurance-training induced significant changes only in Mn SOD mRNA, while Cu/Zn mRNA expression levels remained unchanged. The program of strength or endurance exercise training had no significant effects on the activity of either Cu/Zn SOD or Mn SOD in lymphocytes. Moreover, they found discrepancies between the activities of isoenzymes and mRNA expression levels, similar to us (data not presented). It was expected that changes in mRNA expression would correlate well with their activities or protein levels (19), though changes in the activity of SOD isoenzymes were not associated with corresponding changes in mRNA expression. Adaptation of the organism to OS is achieved by increasing activity of antioxidants and gene expression, especially through stimulation of Mn SOD gene, mostly because mitochondria are the biggest production cite of ROS when intensive energy consumption occurs. This is a very important part of the defence against intracellular OS where enormous production of $O_2^{\cdot-}$ could initiate activation of a transcriptional regulation factor, NF-κB whose incorrect regulation could have been implicated in progression of cancer, inflammation, improper immune responses and other diseases.

The correlations between gene expression and OS/AOD status parameters both before and after the training period were tested with univariate analysis. No significant associations between them were found before training, but after 12 weeks of intensive training the MDA concentration was highly significantly negatively correlated with Mn SOD mRNA expression levels (r = -0.714, p = 0.009), although its concentration did not change significantly after intensive training. It was known that higher plasma MDA levels were determined to be a training-induced OS marker (15). Plasmatic SOD activity was highly significantly positively correlated with Cu/Zn SOD mRNA expression levels (r = 0.778, p = 0.005). Plasmatic SOD activity represents the sum of activities of all isoenzymes, but Cu/Zn SOD is a much more abundant isoenzyme in the plasma than Mn SOD (12).

Multiple linear regression analysis was performed to identify the determinants of Mn SOD and Cu/Zn SOD mRNA

expression levels and SOD activity in subjects after 12 weeks of training. An independent predictor of higher Mn SOD mRNA expression level was lower MDA concentration (β = -0.675, p = 0.017) after inclusion of OS status parameters (log $O_2^{\cdot-}$ and AOPP concentrations) in the Enter linear regression model. However, after inclusion of all OS/AOD parameters in the model, MDA concentration lost its independent effect on Mn SOD mRNA expression level (β = -0.402, p = 0.063). Low levels of MDA, as a late-stage OS biomarker of injured cells and subcellular structures, could in turn up-regulate the gene coding for Mn SOD. Involvement of enzymatic and dietary antioxidants which can inhibit lipid peroxidation, would have been beneficial for Mn SOD expression.

In contrast to plasmatic SOD activity, no determinants were found for Cu/Zn SOD mRNA expression levels. However the same analysis showed that Cu/Zn SOD mRNA expression level was an independent determinant factor affecting plasmatic SOD activity (β = 0.624; p = 0.025) after inclusion of MDA, log $O_2^{\cdot-}$, AOPP concentrations in a multiple regression model. On the other hand, when the independent variables were all OS/AOD parameters, the Cu/Zn SOD mRNA expression level was not a significant predictor of SOD activity (β = 0.73, p = 0.069). Intensive trainings did not change the citosolic Cu/Zn SOD expression levels, but caused radical fall in SOD activity. This fall would be more prompt in a certain group of examinees who were exposed to higher level of OS status parameters like obese people. Together with elevated MDA concentration, which down-regulated Mn SOD expression, they would be exposed to even higher OS. To the best of our knowledge, this is the first time that prediction of MDA as an OS parameter for Mn SOD mRNA expression and prediction for Cu/Zn SOD mRNA expression levels on plasmatic SOD activity were observed. Nevertheless, these findings need to be confirmed in a larger group of examinees.

Up-regulation of Mn SOD and its higher variability in lymphocytes was expected when subjects were exposed to a high level of OS. Cytoplasmtic Cu/Zn SOD gene expression levels in soccer players after 12 weeks of exhaustive training did not differ significantly and its expression became more stable, as demonstrated by a 13 % smaller inter-individual coefficient of variation after training (data not presented).

Conclusions

In conclusion, intense and endurance soccer training induced up-regulation of Mn SOD mRNA expression levels while Cu/Zn mRNA levels remained unchanged. Our results also revealed an interaction between the OS/AOD status parameters in blood and gene expression levels of Cu/Zn SOD and Mn SOD in PBMCs. Lower MDA concentration was found to be a highly predictive parameter for higher gene expression levels of Mn SOD and higher Cu/Zn SOD mRNA levels were found to be an independent predictor for higher plasmatic SOD activity in PBMC. Moderate, rather than intensive physical exercises may be more beneficial for people exposed to multiple OS status parameters.

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