CHANGES OF MUSCLE-DERIVED CYTOKINES IN RELATION TO THIOL REDOX STATUS AND REACTIVE OXYGEN AND NITROGEN SPECIES

Zembron-Lacny A[1], Naczk M[2], Gajewski M[3], Ostapiuk-Karolczuk J[1], Dziewiecka H[1], Kasperska A[1], Szyszka K[1]

[1]Department of Sport Medicine and Biochemistry, Faculty of Physical Culture Gorzow Wlkp., University of Physical Education Poznan; [2]Department of Physiology, Faculty of Physical Culture Gorzow Wlkp., University of Physical Education Poznan; [3]Department of Thoracic Surgery, Medical University of Gdansk, Poland

Corresponding author:

Agnieszka Zembron-Lacny

Department of Sports Medicine and Biochemistry, Faculty of Physical Culture Gorzow Wlkp.

University of Physical Education Poznan

Estkowskiego 13, 66-400 Gorzow Wlkp., Poland

e-mail agzem@gorzow.home.pl

phone/fax 0048 95 7279158

SHORT TITLE: Integration of thiol redox with cytokine response

SUMMARY

The aim of this study was to compare the levels of the plasma muscle-derived cytokines and reactive and nitrogen species (RONS) after muscle damage triggered off by different exercises and to demonstrate the relationships between RONS, thiol redox status and myokines.

Sixteen young men participated in a 90-min run at 65% VO_2max (Ex.1) or 90-min run at 65% VO_2max finished with a 15-min eccentric phase (Ex.2, downhill running). Plasma samples were collected before and at 20 min, 24 h and 48 h after exercise.

The exercise trials significantly elevated the concentrations of plasma hydroperoxide (H_2O_2) and 8-isoprostane at 20 min rest. Myokines IL-6 and IL-10 increased at 20 min rest while IL-1β and TNFα increased at 24 h rest following both running. Ex.2 caused a significant increase in nitric oxide (NO), IL-6, IL-10 and oxidized glutathione (GSSG) levels. Thiol redox status (GSH_{total}-2GSSG/GSSG) decreased by about 30% after Ex.2 compared with Ex.1. H_2O_2 and NO directly correlated with IL-6, IL-10, IL-1β, TNFα and glutathione.

These results show that eccentric work is an important factor that enhances the production of RONS and muscle-derived cytokines, and that there is a possible participation of thiol redox status in the release of myokines to blood.

KEY WORDS: muscle damage, hydroperoxide, nitric oxide, thiol redox, interleukins

INTRODUCTION

The recent study has shown that reactive oxygen and nitrogen species (RONS) such as hydroperoxide (H_2O_2) and nitric oxide (NO) are important signalling molecules generated during muscle contraction and are involved in the regeneration and adaptation of skeletal muscle to physical work. H_2O_2 and NO are produced by enzymes superoxide dismutase (SOD) and nitric oxide synthase (NOS) which are localized to the muscle sarcolemma (isoenzymes CuZnSOD and nNOS) and mitochondria (isoenzymes MnSOD and eNOS) (Jackson *et al.* 2007). The studies in human isolated muscle and myotube culture demonstrated that H_2O_2 and NO produced within contracting skeletal muscle are key regulators of pretranslational signalling events leading to cytokines expression (Kosmidou *et al.* 2002, Steensberg *et al.* 2007).

The production of muscle-derived cytokines, called myokines by Pedersen and Febbraio (2005), is not directly induced by RONS but by thiol compounds, mainly glutathione. The ratio of reduced (GSH) to oxidized glutathione (GSSG) plays an essential role in regulating thiol-dependant signalling pathways. Changes in thiol redox status lead to conformational changes in transcriptional factors, release inhibitory subunits, or promote protein complex formations which are necessary for signal transduction or transcription to proceed (Allen and Tresini 2000). Some cytokines such as IL-1β and TNFα amplify the signal through enhancement of RONS generation and decline in thiol redox state (Allen and Tresini 2000, Ji *et al.* 2006).

The cytokine response to physical exercise has been studied by many authors in recent years (Hirose *et al.* 2004, Peake *et al.* 2005, Petersen and Pedersen 2005, Plomgaard *et al*. 2005, Steensberg *et al*. 2002, Steinberg *et al*. 2007, Zoladz *et al.* 2009). The plasma myokines increase exponentially during or after physical effort in relation to exercise intensity, duration,

mass of working muscles and type of contractions (concentric vs. eccentric). In spite of the large number of studies investigating changes in cytokines, only a few other studies have compared the effects of different types of exercise on the muscle-derived cytokines (Brenner *et al.* 1999, Peake *et al.* 2005, Carmichael *et al.* 2005). In our previous study, we reported that IL-6 dominated during training period corresponding to performance of aerobic efforts whereas TNFα reached the highest values during the start period when the anaerobic-alactate efforts and muscle damage were occurring (Zembron-Lacny *et al.* 2010). Based on these findings, the aim of the present study was to compare the effects of different types of exercise (with or without an eccentric phase) on changes in plasma muscle-derived cytokines and markers of muscle damage, and to examine the relationships between exercise-induced changes in RONS, thiol redox state and myokines in the blood.

METHODS

Sixteen physically active untrained men (age 20.7 ± 0.9 yr, height 167.7 ± 6.9 cm, body mass 74.0 ± 6.5 kg) participated in the following exercise trials: in the exercise 1 (Ex.1) which involved 90-min run at 65% VO_2max (0% gradient) and then in the exercise 2 (Ex.2) which involved 90-min run at 65% VO_2max (0% gradient) and 15-min eccentric phase (-10% gradient). The period between Ex.1 and Ex.2 was three months. Each subject was asked to avoid physical effort for 48 h before the exercise trials. During the study, subjects were also asked to avoid drugs or nutrition supplement that could interfere with immunological and pro-antioxidant evaluation. All the subjects were informed of the aim of the study and gave their written consent for participation in the project. The protocol of the study was approved by the local ethics committee in accordance with the Helsinki Declaration.

Blood samples were taken from antecubital vein to single-use containers with an anticoagulant ($EDTAK_2$) at pre-exercise and post-exercise periods (20 min, 24 h and 48 h). After collection, the samples were immediately placed in 4°C temperature. Within 10 min, they were centrifuged at 3000 g and 4°C for 10 min. Aliquots of plasma were stored at -20°C and analysed within 7 days.

Reactive oxygen and nitrogen species. Plasma hydroperoxide (H_2O_2) and nitric oxide (NO) concentrations were determined by using Oxis Research kit (USA). NO and H_2O_2 were measured immediately after plasma collection, at day of exercise study. H_2O_2 and NO detection limits were 6.25 μM and 0.5 μM respectively. The intra-assay coefficient of variation (CV) for the H_2O_2 kit and for the NO kit it were <10%. Plasma 8-isoprostanes, as a marker of RONS activity, were measured with Cayman kit (USA). 8-Isoprostane detection limit for the procedure was 2.7 $pg \cdot ml^{-1}$, and the intra-assay coefficient of variation (CV) was 6.4%.

Thiol redox status. Total (GSH_t) and oxidized glutathione (GSSG) were measured with Oxis Research kit (USA). The concentrations of GSH_t and GSSG were calculated using reduced glutathione as a standard and the results were expressed in $\mu mol \cdot l^{-1}$. Detection limits for the GSH and GSSG were 0.1 $\mu mol \cdot l^{-1}$ and 0.02 $\mu mol \cdot l^{-1}$, respectively. The intra-assay coefficients of variation (CV) for GSH and GSSG were 0.96% and 6.45%, respectively. Thiol redox status was calculated according to the following equation: $(GSH_t\text{-}2GSSG)/GSSG$. Before the measurement of glutathione, the blood samples were protected from oxidation according to the protocol of Oxis Research.

Anti- and pro-inflammatory cytokines. Plasma interleukin-6 (IL-6), interleukin-10, interleukin-1β (IL-1β) and tumour necrosis factor (TNFα) levels were determined by enzyme immunoassay methods using commercial kits (R&D Systems, USA). Detection limits for IL-

6, IL-10, IL-1β and TNFα were 0.039, 0.500, 0.023 and 0.038 pg · ml^{-1}, respectively. The average intra-assay coefficient of variation (CV) was about 8.0% for all cytokines.

Muscle damage. Plasma creatine kinase (CK) activity was used as a marker of muscle damage and was evaluated by Emapol kit (Poland). CK detection limit for the applied kit was 6 U · l^{-1}. The intra-assay coefficient of variation (CV) for the CK kit was 1.85%.

Plasma volume. Haemoglobin (Hb), haematocrit (Hct) and immune cells number were assessed using Sysmex K-4500 (Poland). The post-exercise values were corrected for changes in plasma volume according to Kraemer and Brown (1986). Relative changes in plasma volume were calculated according to the following equation:

$\%\Delta PV = 100 \times \{([Hb]1/[Hb]2) \times [100 - (Hct2 \times 0.874] / [100 - (Hct1 \times 0.874)]-1\}$, where [Hb]1 (g · dl^{-1}) and Hct1 (%) are mean initial values, [Hb]2 and Hct2 are post-exercise values. The Hct was multiplied by 0.96 and 0.91 to correct for trapped plasma and peripheral sampling respectively (Strauss *et al.* 1951).

Statistical analysis. Statistical calculations were performed using STATISTICA 8.0. Statistical significance was assessed by two-way repeated analysis of variance (ANOVA) and Tukey post-hoc test. Associations among measured parameters were analyzed using Pearson's linear regression (coefficient, r). Statistical significance was set at $P<0.05$. Results are expressed as mean and standard deviation ($x \pm SD$).

RESULTS

Muscle damage and immune cells (tab.1). CK activity, as a marker of muscle damage, was higher after trial with eccentric phase (Ex.2) than after Ex.1. Both Ex.1 and Ex.2 caused an increase in immune cells number at 20 min rest. Monocytes and neutrophils demonstrated

a tendency to higher values after Ex.2 but the differences were insignificant. The number of neutrophils highly correlated with IL-6 and IL-10 (tab.4).

Reactive oxygen and nitrogen species (tab.2). The exercise trials significantly elevated the concentrations of plasma H_2O_2 and 8-isoprostane at 20 min rest. There were no differences between Ex.1 and Ex.2. During recovery, the level of H_2O_2 and 8-isoprostane returned to initial values. NO concentration increased at 24 h after exercise only when the eccentric phase was applied (Ex.2). This can mean that eccentric contractions are more essential in the stimulation of NO than H_2O_2 synthesis.

Thiol redox status (tab.2). GSH concentration increased by 30-40% at 20 min and 24 h after both exercise trials while GSSG increased by 40% only after the running with eccentric phase (Ex.2). Finally, thiol redox status declined after Ex.2 compared to Ex.1, and it remained on the low level up to 48 h rest (fig.1). This means that eccentric exercise elevates the glutathione oxidation and delays the return to redox balance during recovery.

Anti- and pro-inflammatory cytokines (tab.3). The changes in levels of anti- and pro-inflammatory cytokines took place at different time points. IL-6 and IL-10 increased at 20 min while IL-1β and TNFα increased at 24 h after both exercise trials. However, the response of anti-inflammatory cytokine IL-6 and IL-10 was significantly higher when the eccentric phase was applied. IL-6 and IL-10 highly correlated with H_2O_2, 8-isoprostanes and also neutrophils after Ex.1 and Ex.2 (tab.4) whereas IL-1β and TNFα correlated with NO concentration after Ex.2 (IL-1β/NO: $r = 0.458$, $P<0.01$; TNFα/NO: $r = 0.660$, $P<0.001$).

DISCUSSION

Eccentric exercise has been used widely as a means of inducing muscle damage and may be a valuable tool in the study of inflammation. The muscle tissue damage promotes infiltration by inflammatory cells that, in conjunction with local muscle, endothelial, and satellite cells, produce an array of molecules to regulate the regeneration process, including TNFα, IL-1β, IL-6, IL-10 and RONS. Typical responses to muscle injury induced by eccentric exercise include loss of force-generating capacity, decreased range of motion, delayed-onset muscle soreness, swelling, and delayed increases in circulating intramuscular proteins e.g. creatine kinase (Miles *et al.* 2008, Peake *et al.* 2005, Power and Jackson 2008).

Our results show that running exercise with eccentric phase (Ex.2) caused a considerable damage in skeletal muscle expressed with the high activity of CK and the shift in immune cells, mainly monocytes and neutrophils, which can be an additional source of RONS within muscle. The presence of muscle injury particularly impacted on NO release at 24 h. Recently Lima et al. (2009) demonstrated that eccentric exercise modulates the expression of three isoenzymes of nitric synthase (NOS) in skeletal muscle through the signalling pathways controlled by nuclear factor κB (NFκB). The transcription factor NFκB is activated by H_2O_2 which also generated by contracting skeletal muscle (Ji *et al.* 2006). H_2O_2 and NO are involved in transcriptional control through thiol redox modification or nitrosation of many transcription factors which induce the expression of many molecules such as cytokines and growth factors (Hemish *et al.* 2003, Jackson *et al.* 2007, Ji *et al.* 2006, Steensberg *et al.* 2007). The relations between RONS generation and cytokine response that were observed in isolated human muscle and myotube culture, we demonstrated in blood as the correlations between H_2O_2, NO, IL-6, IL-10, IL-1β and TNFα.

The thiol redox status participates in cellular signalling pathways linking the generation of RONS with activation of transcription factors and their ability to bind with

DNA (Allen and Tresini 2000, Ji *et al.* 2006). It was confirmed that the thiol reducing agents, such as N-acetyl-L-cysteine, block transcription factors activation and cytokines synthesis whereas the oxidants, such as H_2O_2, have the opposite effects (Allen and Tresini 2000, Kosmidou *et al.* 2002, Ji *et al.* 2006). We observed that H_2O_2 and GSH increased in both exercise trials while NO and GSSG increased only when eccentric phase was applied. This may mean that NO plays more important role in oxidation of glutathione and thiol redox disturbance than H_2O_2.

The analysis of muscle-derived cytokines in blood showed an increase in IL-6 and IL-10 after both exercise trials at 20 min and the enhancement of the increase in IL-6 and IL-10 following Ex.2. The IL-6 and IL-10 remained on high level to 48 h rest after Ex.2 compared with values after Ex.1. According to Petersen and Pedersen 2005, IL-6 is essential regulator of the proliferation of satellite cells and differentiation of myoblasts. The genetic loss of IL-6 blunts the muscle regeneration and hypertrophy in vivo (Serrano *et al.* 2008). The cytokines IL-1β and TNFα increased during recovery at 24 h. The long-term presence of pro-inflammatory cytokines can also be related to muscle restoration but TNFα presence in muscle regeneration is not an absolute requirement (Cannon *et al.* 1989, Collins and Grounds 2001, Reid and Li 2001). It should be stressed that the suppression of inflammatory response by using anti-inflammatory drugs attenuates the exercise-induced increase in satellite cell number after eccentric exercise (Mackey *et al.* 2007). Based on these results, we conclude that high level of myokines, particularly IL-6 and IL-10, can be a marker of a normal reconstruction of muscle after intense exercise and adaptation to physical exercise. Recently, we observed that professional sport training significantly influences plasma cytokine levels. IL-6 was significantly higher in the preparatory period than in the play-off round in professional basketball players (Zembron-Lacny *et al.* 2010).

An important finding of our study was the integration of RONS production with cytokine response. We observed the correlations between H_2O_2 and anti-inflammatory cytokines after both exercise trials. Moreover, the high level of NO enhanced the release of IL-6 and IL-10 after Ex.2. The recent exercise study in humans demonstrated that NO is the key regulator of pretranslational signalling events leading to muscle IL-6 production. Pharmacological inhibition of NO production during exercise attenuated the increase in IL-6 mRNA levels in human skeletal muscle whereas intra-arterial infusion of an NO donor was accompanied by increase in IL-6 mRNA (Steensberg *et al.* 2007). Both NO and H_2O_2 activates transcription factor NFκB loading to IL-6 expression in skeletal muscle (Kosmidou *et al.* 2002, Lima *et al.* 2009). The combination of exercise duration with eccentric work (downhill running) increases the NO and H_2O_2 generation as well as the release of IL-6 and IL-10 from the skeletal muscle, which can improve the adaptation of organism to intense physical exercise.

These results [1)] show that eccentric work is an important factor enhancing the production of nitric oxide, oxidized glutathione and anti-inflammatory cytokines,[2)] confirm the relation of RONS generation with muscle-derived cytokines release, and [3)] indicate a possible involvement of blood thiol redox in myokines synthesis.

ACKNOWLEDGMENTS

This study was supported by a grant NN404 155534 from the Ministry of Science and Higher Education, Poland.

CONFLICT OF INTEREST

There is no conflict of interest.

REFERENCES

ALLEN GR, TRESINI M: Oxidative stress and gene regulation. *Free Radic Biol Med* **28**: 463-499, 2000.

BRENNER IK, NATALE VM, VASILIOU P, MOLDOVEANU AI, SHEK PN, SHEPARD RJ: Impact of tree different types of exercise on components of the inflammatory response. *Eur J Appl Physiol* **80**: 452-460, 1999.

CANNON JG, FIELDING RA, FIATARONE MA, ORENCOLE SF, DINARELLO CA, EVANS WJ: Increased interleukin 1β in human skeletal muscle after exercise. *Am J Physiol* **257**: 451-455, 1989.

CARMICHAEL MD, DAVIS JM, MURPHY EA, BROWN AS, CARSON JA, MAYER E, GHAFFAR A: Recovery of running performance following muscle-damaging exercise: relationship to brain IL-1beta. *Brain Behav Immun* **19**: 445-452, 2005.

COLLINS RA, GROUNDS MD: The role of tumour necrosis factor-alpha (TNF-alpha) in skeletal muscle regeneration: Studies in TNF-alpha(-/-) and TNF-alpha(-/-)/LT-alpha(-/-) mice. *J Histochem Cytochem* **49**: 989-1001, 2001.

HEMISH J, NAKAYA N, MITTAL V, ENIKOLOPOV G: Nitric oxide activates diverse signalling pathways to regulate gene expression. *J Biol Chem* **278**; 42231-42329, 2003.

HIROSE L, NOSAKA K, NEWTON M: Changes in inflammatory mediarors following eccentric exercise of the elbow flexors. *Exerc Immunol Rev* **10**: 75-90, 2004.

JACKSON MJ, PYE D, PALOMERO J: The production of reactive oxygen and nitrogen species by skeletal muscle. *J Appl Physiol* **102**: 1664-1670, 2007.

JI LL, GOMEZ-CABRERA MC, VINA J: Exercise and hormesis. Activation of cellular antioxidant signalling pathway. *Ann NY Acad Sci* **1067**: 425-435, 2006.

KOSMIDOU I, VASSILAKOPOULOUS T, XAGORARI A, ZAKYNTHINOS S, PAPAPETROPOULOS A, ROUSSOS C: Production of interleukin-6 by skeletal myotubes. Role of reactive oxygen species. *Am J Respir Cell Mol Biol* **26**: 587-593, 2002.

KRAEMER RR, BROWN BS: Alternations in plasma-volume-corrected blood components of marathon runners and concomitant relationship to performance. *Eur J Appl Physiol* **55**: 579-584, 1986.

MACKEY AL, KJAER M, DANDANELL S, MIKKELSEN KH, HOLM L, DØSSING S, KADI F, KOSKINEN SO, JENSEN CH, SCHRØDER HD, LANGBERG H: The influence of anti-inflammatory medication on exercise-induced myogenic precursor cell responses in humans. *J Appl Physiol* **103**: 425-431, 2007.

MILES MP, ANDRING JM, PEARSON SD, GORDON LK, KASPER C, DEPNER DM, KIDD JR. Diurnal variation, response to eccentric exercise, and ssociation of inflammatory mediators with muscle damage variables. *J Appl Physiol* **104**: 451-458, 2008.

PEAKE J, NOSAKA K, SUZUKI K: Charakterization of inflammatory responses to eccentric exercise in humans. *Exerc Immunol Rev* **11**: 64-85, 2005.

PEDERSEN BK, FEBBRAIO M: Muscle-derived interleukin-6 - a possible link between skeletal muscle, adipose tissue, liver, and brain. *Brain Behav Immun* **19**: 371-376, 2005.

PETERSEN AMW, PEDERSEN BK: Anti-inflammatory effect of exercise. *J Appl Physiol* **98**: 1154-1162, 2005.

PLOMGAARD P, PENKOVA M, PEDERSEN BK: Fiber type specific expression of TNF-alpha, IL-6 and IL-18 in human skeletal muscles. *Exerc Immunol Rev* **11**: 53-64, 2005.

POWER SK, JACKSON MJ: Exercise-induced oxidative stress: cellular mechanisms and

impact on muscle force production. *Physiol Rev* **88**: 1243-1276, 2008.

REID MB, LI YP: Tumour necrosis factor-alpha and muscle wasting: a cellular perspective. *Respir Res* **2**: 269-272, 2001.

STEENSBERG A, KELLER C, HILLIG T, FROSIG C, WOJTASZEWSKI JF, PEDERSEN BK, PILEGAARD H, SANDER M: Nitric oxide production is a proximal signalling event controlling exercise-induced mRNA expression in human skeletal muscle. *FASEB J* **21**: 2683-2694, 2007.

STEINBERG JG, BA A, BREGEON F, DELLIAUX S, JAMMES Y: Cytokine and oxidative responses to maximal cycling exercise in sedentary subjects. *Med Sci Sports Exerc* **39**: 964-968, 2007.

STRAUSS MB, DAVIS RK, ROSENBAUM JD, ROSSMEISL EC: Water diuresis produced during recumbency by the intravenous infusion of isotonic saline solution. *J Clin Invest* **30**: 862-868, 1951.

ZEMBRON-LACNY A, SLOWINSKA-LISOWSKA M, ZIEMBA A: Integration of the thiol redox status with cytokine response to physical training in professional basketball players *Physiol Res* **59**: (Epub ahead of print), 2010.

ZALADZ JA, MAJERCZAK J, DUDA K, CHLOPICKI S: Exercise-induced prostacyclin release positively correlates with VO_2max in young healthy men. *Physiol Res* **58**: 229-238, 2009.

Tab.1. Effect of exercise on activity of creatine kinase (CK) and number of immunological cells.

	Pre-exercise	Ex.1 vs. Ex.2	Rest 20 min	Ex.1 vs. Ex.2	Rest 24 h	Ex.1 vs. Ex.2	Rest 48 h	Ex.1 vs. Ex.2
CK U · l^{-1} Exercise 1 Exercise 2	118 ± 28 174 ± 50	*ns*	189± 46 389 ± 168*	*P*<0.05	372 ± 62** 721 ± 192***	*P*<0.001	278 ± 79 652 ± 126***	*P*<0.001
Leukocytes · 10^{-3} · μl^{-1} Exercise 1 Exercise 2	5.74 ± 0.95 6.40 ± 1.49	*ns*	10.44 ± 2.06*** 12.33 ± 3.61***	*ns*	6.14 ± 1.30 6.25 ± 1.86	*ns*	6.07 ± 0.89 5.71 ± 1.46	*ns*
Monocytes · 10^{-3} · μl^{-1} Exercise 1 Exercise 2	0.43 ± 0.12 0.41 ± 0.09	*ns*	0.64 ± 0.12 0.71 ± 0.19**	*ns*	0.61 ± 0.15 0.56 ± 0.17	*ns*	0.63 ± 0.13 0.50 ± 0.13	*ns*
Neutrophils · 10^{-3} · μl^{-1} Exercise 1 Exercise 2	3.06 ± 0.57 4.32 ± 0.87	*ns*	8.58 ± 1.57*** 9.58 ± 1.71***	*ns*	3.44 ± 0.60 4.28 ± 1.26	*ns*	3.13 ± 0.56 4.09 ± 1.82	*ns*
Lymphocytes · 10^{-3} · μl^{-1} Exercise 1 Exercise 2	1.96± 0.27 2.53 ± 0.37	*ns*	1.71 ± 0.31 1.96 ± 0.44	*ns*	2.01 ± 0.36 1.86 ± 0.38*	*ns*	1.94 ± 0.25 1.86 ± 0.56*	*ns*

Exercise 1 - 90 min run at 65% VO_2max
Exercise 2 - 90 min run at 65% VO_2max and 15 min eccentric phase
**P*<0.05, ***P*<0.01, ****P*<0.001 indicate post-exercise vs. pre-exercise values

Tab.2. Effect of exercise on concentrations of hydroperoxide (H_2O_2), nitric oxide (NO), 8-isoprostane, total (GSH_t) and oxidised glutathione (GSSG).

	Pre-exercise	Ex.1 vs. Ex.2	Rest 20 min	Ex.1 vs. Ex.2	Rest 24 h	Ex.1 vs. Ex.2	Rest 48 h	Ex.1 vs. Ex.2
H_2O_2 mmol · ml^{-1} Exercise 1 Exercise 2	3.09 ± 0.88 2.74 ± 0.86	*ns*	5.97 ± 1.06*** 6.05 ± 1.43***	*ns*	4.92 ± 0.95* 4.19 ± 0.89	*ns*	4.16 ± 0.95 5.29 ± 0.52***	*ns*
NO mmol · ml^{-1} Exercise 1 Exercise 2	26.44 ± 3.49 31.42 ± 4.45	*ns*	28.65 ± 3.21 33.55 ± 4.89	*ns*	29.87 ± 5.32 46.60 ± 5.98***	*P*<0.001	32.92 ± 4.81 35.87 ± 5.61	*ns*
8-isoprostane pg · ml^{-1} Exercise 1 Exercise 2	29.47 ± 6.30 24.38 ± 7.85	*ns*	40.08 ± 5.95* 40.92 ± 5.91***	*ns*	30.19 ± 6.39 22.00 ± 6.25	*ns*	28.22 ± 4.93 27.68 ± 7.76	*ns*
GSH_t mmol · ml^{-1} Exercise 1 Exercise 2	721 ± 118 726 ± 105	*ns*	1016 ± 161** 967 ± 67*	*ns*	978 ± 150** 1010 ± 125**	*ns*	987 ± 138** 843 ± 124	*ns*
GSSG mmol · ml^{-1} Exercise 1 Exercise 2	6.90 ± 1.58 7.84 ± 0.83	*ns*	7.69 ± 1.78 10.86 ± 1.88**	*P*<0.01	7.91 ± 1.61 11.04 ± 1.95**	*P*<0.01	7.73 ± 1.19 10.31 ± 1.77	*ns*

Exercise 1 - 90 min run at 65% VO_2max
Exercise 2 - 90 min run at 65% VO_2max and 15 min eccentric phase
**P*<0.05, ***P*<0.01, ****P*<0.001 indicate post-exercise vs. pre-exercise values

Tab.3. Effect of exercise on anti- (IL-6, IL-10) and pro-inflammatory (IL-1β, TNFα) cytokines.

	Pre-exercise	Ex.1 vs. Ex.2	Rest 20 min	Ex.1 vs. Ex.2	Rest 24 h	Ex.1 vs. Ex.2	Rest 48 h	Ex.1 vs. Ex.2
IL-6 pg · ml^{-1}								
Exercise 1	1.33 ± 0.17	*ns*	6.50 ± 1.24***	*P<0.05*	1.91 ± 0.65	*P<0.05*	1.57 ± 0.30	*P<0.05*
Exercise 2	1.90 ± 0.32		7.81 ± 1.22***		3.27 ± 0.85		2.86 ± 0.82	
IL-10 pg · ml^{-1}								
Exercise 1	7.61 ± 0.87	*ns*	16.48 ± 5.87***	*P*<0.01	8.44 ± 1.13	*P*<0.001	8.85 ± 1.70	*P*<0.001
Exercise 2	10.03 ± 1.78		19.23 ± 2.47***		13.51 ± 1.31		13.45 ± 1.76	
IL-1β pg · ml^{-1}								
Exercise 1	0.60 ± 0.13	*ns*	0.81 ± 0.17	*ns*	0.89 ± 0.10*	*ns*	0.86 ± 0.11	*ns*
Exercise 2	0.66 ± 0.14		0.73 ± 0.15		0.98 ± 0.15***		0.83 ± 0.12	
TNFα pg · ml^{-1}								
Exercise 1	2.97 ± 0.38	*ns*	3.52 ± 0.70	*ns*	4.39 ± 0.46***	*ns*	3.94 ± 0.67**	*ns*
Exercise 2	3.02 ± 0.43		3.37 ± 0.47		5.34 ± 0.67***		3.59 ± 0.61	

Exercise 1 - 90 min run at 65% VO_2max
Exercise 2 - 90 min run at 65% VO_2max and 15 min eccentric phase
**P*<0.05, ***P*<0.01, ****P*<0.001 indicate post-exercise vs. pre-exercise values

Tab.4. Relationships (correlation coefficients) between anti-inflammatory cytokines (IL-6 and IL-10) and hydroperoxide (H_2O_2), 8-isoprostanes and neutrophils in Ex.1and Ex.2 experiments.

	H_2O_2	8-isoprostanes	neutrophils
IL-6			
Exercise 1	0.575***	0.620***	0.908***
Exercise 2	0.547**	0.653***	0.847***
IL-10			
Exercise 1	0.543***	0.419***	0.678***
Exercise 2	0.572**	0.531**	0.809***

**P*<0.05, ***P*<0.01, ****P*<0.001

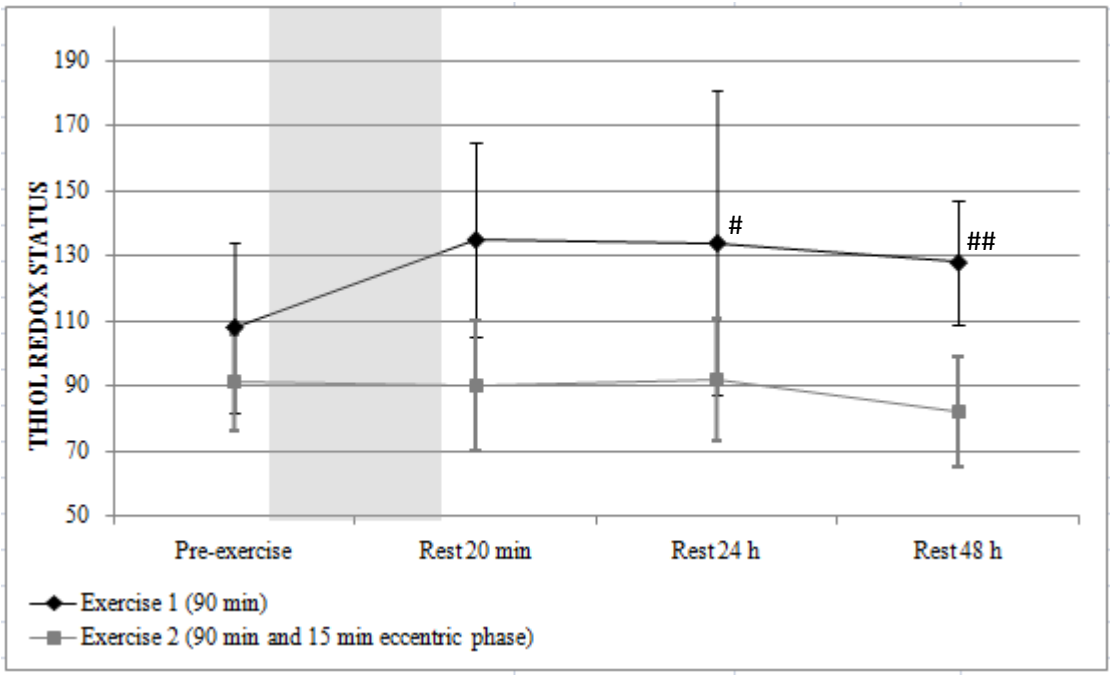

Fig.1. Changes in blood thiol redox status (GSH_t-2GSSG/GSSG); #P<0.05, ##P<0.01 indicate Exercise 1 vs. Exercise 2; grey area indicates exercise.