Barbara Morawin[*]
Karol Kalina[**]
Mateusz Rynkiewicz[***]
Agnieszka Zembroń-Łacny[****]

NITRIC OXIDE GENERATION IN RESPONSE TO INTENSE PHYSICAL EXERCISE

INTRODUCTION

Essential processes in the regeneration of an injured muscle are the proliferation of satellite cells and vascularization. Myogenesis and angiogenesis are a prerequisite for the subsequent morphological and functional healing of the injured muscle. It leads to rebuilding of the damaged myocytes and vessels, restoration of the blood flow and restoration of the oxygen supply to the tissue. NO plays a key role because it can act as a signal molecule and vasodilator, and can promote activation of several growth factors which are extracellular signals regulating functions of muscular, vascular and nervous systems (Fillipin et al. 2009, Kuang et al. 2008).

NO mediates expression of cytoskeletal proteins in response to mechanical stimuli and is essential for the addition of sarcomeres when working length is chronically increased. NO, as a cellular mediator in signal transmission, can use several signalling pathways such as activation of guanylyl cyclase, inhibition of cytochrome c oxidase in the mitochondrial electron transport chain or S-nitrosylation of transcription factors, including AP-1 (*activator protein-1* controls about 80 genes), NF-κB (*nuclear factor* κB controls about 300 genes) and HIF-1 (*hypoxia-inducible factor-1* controls 200 genes) (Lima-Cabello et al. 2010, Lira et al. 2010, Fillipin et al. 2009, Kuang et al. 2008).

NO is generated continuously by skeletal muscle through the conversion of L-arginine to L-citrulline by the nitric oxide synthase (NOS), a production that is increased by contractions. A skeletal muscle normally expresses

[*]**Barbara Morawin** – Akademia Wychowania Fizycznego w Poznaniu; doktorant.
[**]**Karol Kalina** – Gimnazjum w Czaplinku; doktorant.
[***]**Mateusz Rynkiewicz** – Sportowe Centrum Diagnostyczne Fit-Test.
[****]**Agnieszka Zembroń-Łacny** – Katedra Wychowania Fizycznego, Uniwersytet Zielonogórski.

the neuronal (type I or nNOS), the inducible (type II or iNOS) and the endothelial (type III or eNOS) isoforms of NOS. nNOS is strongly expressed in fast-twitch muscle fibres and localized to the muscle sarcolemma where it as associated with the dystrophin complex. eNOS is localized to the muscle mitochondria. Abnormalities in specific isoforms, such as nNOS and eNOS, have been reported in muscle diseases with mitochondrial deficiencies, indicating that specific NOS activities and expression may be involved in the pathogenesis of these diseases. Increased nNOS activity and expression were observed in muscle fibbers with mitochondrial proliferation, suggesting that it is related to mitochondrial biogenesis. However, the exact mechanisms involved in these abnormalities are not clear (Tengan et al. 2012). nNOS content in human skeletal muscle is 60% higher in athletes than non-athletes while studies investigating eNOS provided conflicting results (McConell et al. 2007).

The study was designed to explain whether a single exercise trial increases NO generation and whether changes in NO level are related with skeletal muscle damage and body composition in non-athletes.

MATERIAL AND METHODS

Subjects. Eighteen healthy untrained women and men participated in the study (tab.1). 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 bioethics committee in accordance with the Helsinki Declaration.

Body composition. Body mass (BM) and body composition (fat-free mass FFM and fat mass FM) were estimated with the application of Bioelectrical Impedance Method (BIA) by using Tanita Body Composition Analyser BC-418MA (Japan) calibrated prior to each test session in accordance to the manufacturer's guidelines. Duplicate measures were taken with the participant in a standing position; the average value was used for the final analysis. The recurrence of measurement was 98%. The measurements were taken between 7.00 and 8.00 a.m. before blood sampling. Additionally, FFM and FM indexes were calculated according to the definition of Van Itallie et al. (1990): FFMI = FFM(kg)/height(m^2) and FMI = FM(kg)/height(m^2). Note that, mathematically, BMI (kg/m^2) = FFMI + FMI. Thus, measured FFMI, FMI and %FM values falling below the values for a BMI of 18.5 kg/m^2 were defined as low; measured FFMI, FMI and %FM values falling in the range for BMI between 18.5 and 25.0 kg/m^2 were considered normal, and values above that range were considered high and very high (Bahadori et al. 2006).

Exercise trial. The incremental and progressive exercise test was performed on a treadmill Trackmaster TM310 (USA) at the temperature of 22°C between 8 a.m. and 2 p.m. All subjects began at 4.5 mph running speed and it was increased by 0.5 mph every 2 min until the maximal level of recorded parameters was achieved. Breath-by-breath oxygen uptake was continuously recorded using Oxycon Mobile ergospirometry system (Viasys Healthcare Inc., USA). Heart rate was continuously recorded during the test using a portable heart rate telemetry device Polar Sport Tester T61 (Finland).

Blood sampling. Blood samples were taken from the elbow vein at rest, 1 min, 30 min, 24 h and 48 h after exercise. Within 20 min, they were centrifuged at 1000 x g and +4°C for 10 min. Aliquots of serum were stored at -70°C.

Table 1

Anthropometric and body composition data in subjects

	Women n=6	**Men n=12**	**ANOVA HSD Tukey**
Age years	21.3 ± 2.2	21.3 ± 2.0	$p > 0.05$
Height cm	165.0 ± 3.6	184.0 ± 10.2	$p < 0.01$
Weight kg	64.2 ± 7.4	81.9 ± 17.7	$p < 0.01$
BMI kg/m^2	23.6 ± 3.1	24.0 ± 3.8	$p > 0.05$
FFM kg	48.1 ± 4.3	70.2 ± 11.3	$p < 0.001$
FFMI kg/m^2	17.7 ± 1.6	20.4 ± 2.8	$p < 0.01$
FM kg	16.2 ± 4.0	11.7 ± 8.1	$p > 0.05$
FMI kg/m^2	6.0 ± 1.7	3.3 ± 2.2	$p < 0.001$
VO_2max mL/kg/min	48.9 ± 1.7	58.4 ± 7.9	$p < 0.05$

Biochemical analysis. Lactate concentration in the capillary blood was assessed before and at 1 min after exercise trial using LKM 140 Dr Lange Kit (Germany). Serum myoglobin (Mb) was used as a marker of muscle damage, and was evaluated by Oxis Research ELISA kit (USA). Mb detection limit was 5 ng/mL, and the intra-assay coefficient of variation (CV) for the Mb kit was <6%. Serum nitric oxide (NO) concentration was determined by using the Oxis Research kit (USA). NO detection limit was 0.5 nmol/mL, and the intra-assay coefficient of variation (CV) for the NO kit was <10%.

Statistical analysis. Statistical calculations were performed using STATISTICA 10. Statistical significance was assessed by one-way analysis of variances (ANOVA) and a post-hoc test (HSD Tukey). Associations among

measured parameters were analyzed using Pearson's linear regression (coefficient, r). Statistical significance was set at $p<0.05$. The results are expressed as mean and standard deviation (mean ± SD).

RESULTS

Body composition (Tab. 1). There were significant differences in body composition between women and men and thus significant differences in BMI. 83% women and men demonstrated normal BMI ranged from 18.5 to 24.9. Figures 1 and 2 show a significantly positive relation between BMI and FFMI, and the same between BMI and FMI. Normal FFMIs ranged from 14.8 to 18.5 kg/m^2 in women, and from 13.6 to 22.4 kg/m^2 in men which corresponded very well with studies done by van Itallie et al. (1990), Schutz et al. (2002) and Bahadori et al. (2006). The FMI values were 3.8 to 7.3 kg/m^2 in women, and from 1.2 to 6.2 kg/m^2 in men for the normal BMI ranges. BMI highly correlated with FFMI and FMI (fig.1-2). Furthermore, FMI significantly correlated with VO2max ($r=-0.521$, $p<0.001$). BMI and FFMI did not correlate with maximal oxygen uptake. This means that fat mass index composition can be related with VO_2max value.

Myoglobin and nitric oxide (Tab. 2). Mb and NO concentrations significantly increased at 1 min after exercise and remained on high level at 24 h post-exercise. Mb concentration was higher in men than women at 24h and 48h after exercise while NO concentration was similar. The positive correlation was observed between Mb and NO concentrations in both groups (Fig.3-4). This means that muscle injury following exercise is necessary to increase in NO production.

Table 2

Effect of exercise trial on myoglobin (Mb) and nitric oxide (NO) concentrations

	Before exercise	**After exercise 1 min**	**After exercise 30 min**	**After exercise 24 h**	**After exercise 48 h**
Mb ng/mL					
Women	58.47 ± 14.61	233.61 ± 55.47*	120.77 ± 28.46*	44.81 ± 20.68*♯	54.10 ± 11.55♯
Men	76.91 ± 26.19	275.82 ± 102.63	181.83 ± 80.32	198.77 ± 74.88	117.08 ± 53.56
NO nmol/mL					
Women	11.83 ± 1.81	18.91 ± 1.06*	17.38 ± 1.83	15.24 ± 2.22	14.19 ± 1.35
Men	12.19 ± 3,57	18.82 ± 2.95	15.04 ± 2.36	16.02 ± 3.92	15.10 ± 3.42

* significant differences ($p<0.05$) compared to initial level (before exercise)
♯ significant differences ($p<0.05$) between women and men

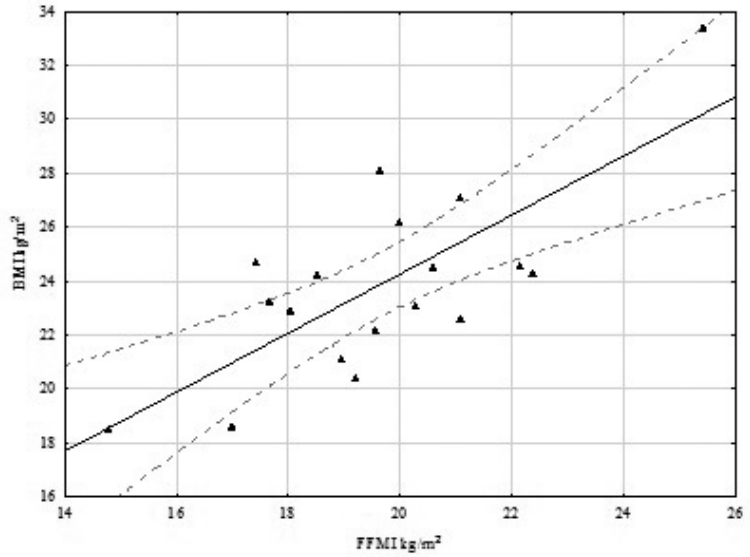

Figure 1. The relationship between a body mass index (BMI) and fat-free mass index (FFMI); r=0.745, p<0.001.

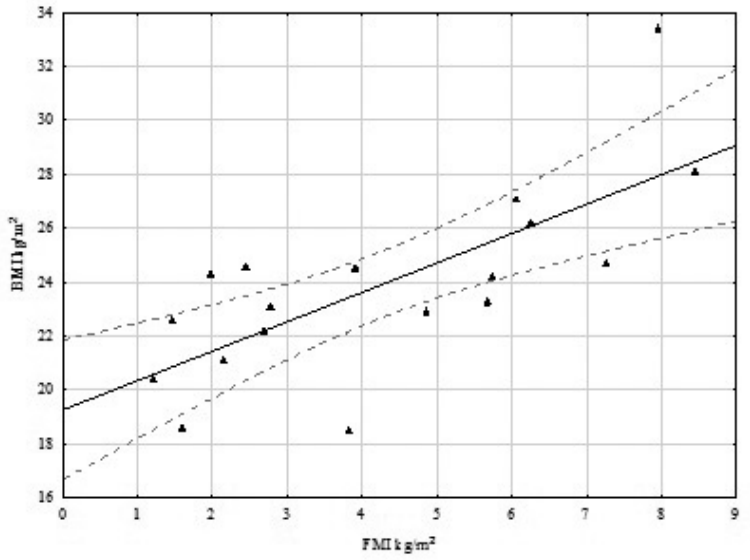

Figure 2. The relationship between a body mass index (BMI) and fat mass index (FM); r=0.731, p<0.001.

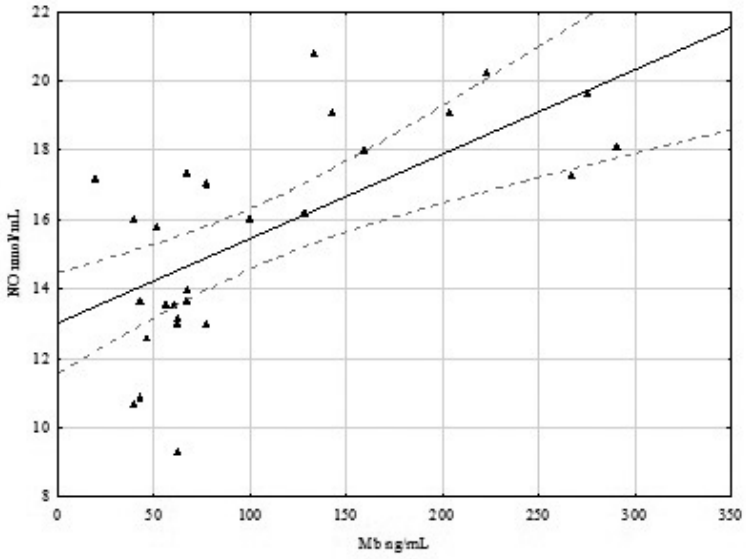

Figure 3. The relationship between myoglobin (Mb) and nitric oxide (NO) in women; r=0.636, p<0.001.

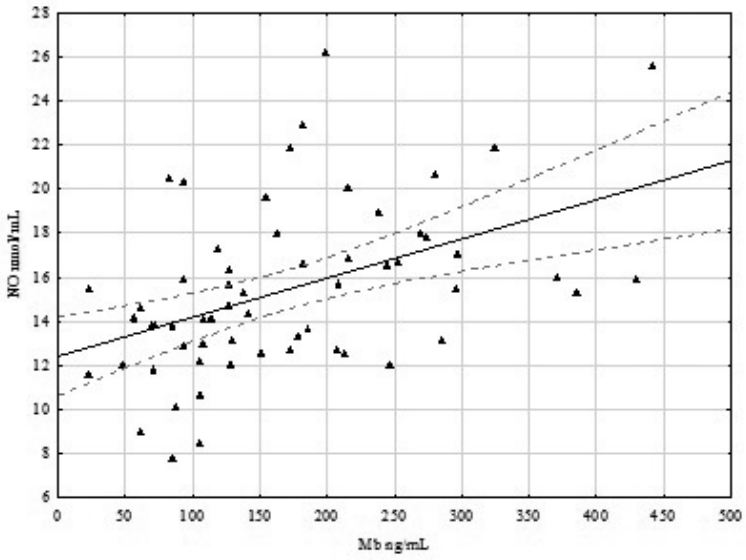

Figure 4. The relationship between myoglobin (Mb) and nitric oxide (NO) in men; r=0.455, p<0.001.

DISCUSSION

Many studies have demonstrated that intracellular NO production is required for the exercise adaptation that occurs in cardiovascular system and skeletal muscles in response to exercises (Anderson 2000, Filippin et al. 2009). Our results demonstrated that incremental exercise elevates NO level. Contrary to our study, Yamamoto et al. (2007) showed no acute effects of aerobic exercise on either their serum NO concentration. This result agrees with the previous study by Gonzales et al. (2007) indicating a lack of any significant pre- to post-exercise changes in NO production. Nevertheless, the authors have stressed that post-exercise NO release requires the further study. According to Lima-Cabello et al. (2010) muscle NO production occurs in signalling pathways controlled by H2O2. Previously, we observed the simultaneous generation of H_2O_2 and NO after running eccentric exercise (Zembroń-Łacny et al. 2010).

Our study demonstrated that elevated NO level was related with muscle damage induced by incremental exercise. One of the first investigations on the role of NO in skeletal muscle damage was reported by Anderson (2000) who mechanically crushed muscle of wild, NO knock-out or NO inhibited mice and observed very different repair processes to reveal the importance of NO in damage repair. It followed that NO facilitates the activation of satellite cells, which are located in the basal lamina of skeletal muscle, and this is one of the first steps in the repair process. Moreover, it appears that the beneficial function of NO in damage repair is not just restricted to satellite cell proliferation and differentiation but also to fusion. Hence, NO and the signalling agent of NO, cGMP, the antagonist of myostatin, activate follistatin, which is a negative regulator of myogenesis. Despite the well described role of NO in the repair of muscle injury, it is possible that the mechanism controlling the repair after unaccustomed exercise-induced damage might be different from those after crush injury (Radak et al. 2012). Interestingly, treadmill running related overuse of tendons results in increased NO production, which suggests a role in the repair process (Szomor et al. 2006). The induction of mechanical damage to a gastrocnemius muscle has been shown to result in increased NO formation, which is believed to initiate a signalling process for damage repair (Valko et al. 2007). In addition, the importance of NO to muscle function has been demonstrated as NO inhibition resulted in severe reduction in walking speed of rats (Wang et al. 2001).

Recently, it was suggested that NO may affect body composition, and NO precursor-arginine may offer great promise in preventing and treating obesity in humans (Wu et al. 2012). Unfortunately, we did not observe any

relationships between the initial NO concentration and components of body composition as well as FFMI and FMI. However, the concept of FFMI and FMI was created in analogy to the BMI and appears to be of interest in the classification of overweight and overfat patients (respectively underweight and underlean). The partitioning of BMI into FFMI and FMI is obviously not possible without associated measurements of body composition. Note that the original idea of calculating the FFM and FM indexes, in analogy to the BMI, was proposed several years ago. The potential advantage is that only one component of body weight, i.e. FFM or FM, is related to the height squared (Schutz et al. 2002). Surprisingly, these indexes have not found a wide application yet, probably because appropriate reference standards have yet to be defined. By determining these indexes, quantification of the amount of excess (or deficit) of FFM, respectively FM, can be calculated for each individual. Considering that BMI is the sum of FFMI and FMI, an increase or a decrease in BMI could be accounted for by a rise or a drop in one component, in the other or in both components. Therefore, the advantage of the combined use of these indices is that one can judge whether the deficit or excess of body weight is selectively due to a change in FFM vs. FM or both combined (Bahadori et al. 2006, Schutz et al. 2002, Van Itallie et al. 1990). For example, an individual of 1.95m and 103 kg has a BMI of 27.1 kg/m^2 and would be judged as overweight. This would be true if his FMI is higher than the reference values and conversely if his FFMI is not simultaneously elevated. FMI for this individual was 6.0 and corresponded from 1.2 to 6.2 kg/m^2 in men for the normal BMI ranges in our study.

In conclusion, exercise-induced skeletal muscle damage is necessary to generate NO which can play an important role in muscle regeneration and adaptation to intense exercise. However, our study did not confirm the relationship between NO generation and body composition.

Literatura

Anderson J. E. (2000), A role for nitric oxide in muscle repair: nitric oxide-mediated activation of muscle satellite cells, Mol Biol Cell, 11, pp. 1859-1874.

Bahadori B., Uitz E., Tonninger-Bahadori K., Pestemer-Lach I., Trummer M., Thonhofer R., Brath H., Schaflinger E. (2006), Body composition: the fat-free mass index (FFMI) and the body fat mass index (BFMI) distribution among the adult Austrian population – results of a cross-sectional pilot study. Int J Body Comp Res, 4, pp. 123-128.

FILIPPIN L. I., MOREIRA A. J., MARRONI N. P., XAVIER R. M. (2009), Nitric oxide and repair of skeletal muscle injury. Nitric Oxide, 21, pp. 157-163.

GONZALEZ D., MARQUINA R., RONDON N., RODRIGUEZ-MALAVER A., REYES R. (2007), Effects of aerobic exercise on nitric oxide, uric acid, antioxidant activity and oxidative stress in human saliva. Med Sci Sports Exerc, 39, pp. 354.

KUANG S., GILLESPIE M. A., RUDNICKI M. A. (2008), Niche regulation of muscle satellite cell self-renewal and differentiation. Cell Stem Cell, 2, pp. 22-31.

LIMA-CABELLO E., CUEAVAS M. J., GARATACHEA N., BALDINI M., ALMAR M., GONZÁLEZ-GALLEGO J. (2010), Eccentric exercise induces nitric oxide synthase expression through nuclear factor-κB modulation in rat skeletal muscle. J Appl Physiol., 108, pp. 575-583.

LIRA V. A., BROWN D. L., LIRA A. K., KAVAZIS A. N., SOLTOW Q. A., ZEANAH E. H., CRISWELL D. S. (2010), Nitric oxide and AMPK cooperatively regulate PGC-1? in skeletal muscle cells. J Physiol., 588, pp. 3551-3566.

MCCONELL G. K., BRADLEY S. J., STEPHENS T. J., CANNY B. J., KINGWELL B. A., LEE-YOUNG R. S. (2007), Skeletal muscle nNOS protein content is increased by exercise training in humans. Am J Physiol Regul Integr Comp Physiol., 293: pp. 821-828.

RADAK Z., NAITO H., TAYLOR A. W., GOTO S. (2012), Nitric oxide: Is it the cause of muscle soreness? Nitric Oxide., 26, pp. 89-94.

SZOMOR Z. L., APPLEYARD R. C., MURRELL G. A. (2006), Over-expression of nitric oxide synthases in tendon overuse. J Orthop Res., 24, pp. 80-86.

SCHUTZ Y., KYLE U. U. G., PICHARD C. (2002), Fat-free mass index and fat mass index percentiles in Caucasians aged 18-98 y. Int J Obesity, 26, pp. 953-960.

TENGAN C. H., RODRIGUES G. S., GODINHO R. O. (2012), Nitric oxide in skeletal muscle: role on mitochondrial biogenesis and function. Int J Mol Sci., 13, pp. 17160-17184.

VALKO M., LEIBFRITZ D., MONCOL J., CRONIN M. T., MAZUR M., TELSER J. (2007), Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol., 39, pp. 44-84.

VAN ITALLIE T. B., YANG M-U, HEYMSFIELD S. B., FUNK R. C., BOILEAU R. (1990), Height-normalized indices of the body's fat-free mass and fat mass: potentially useful indicators of nutritional status. Am J Clin Nutr, 52, pp. 953-459.

Wang M. X., Murrell D. F., Szabo C., Warren R. F., Sarris M., Murrell G. A. (2001), Nitric oxide in skeletal muscle: inhibition of nitric oxide synthase inhibits walking speed in rats. Nitric Oxide., 5, pp. 219-232.

Wu Z., Satterfield M. C., Bazer F. W., Wu G. (2012), Regulation of brown adipose tissue development and white fat reduction by L-arginine. Curr Opin Clin Nutr Metab Care., 15(6), pp. 529-538.

Yamamoto K., Kondo T., Kimata A., Ueyama J., Shirotori A., Okada Y., Sakui D., Nakashima M., Yamada S. (2007), Lack of effect of aerobic physical exercise on endothelium-derived nitric oxide concentrations in healthy young subjects. Nagoya J Med Sci, 69, pp. 167-172.

Zembroń-Łacny A., Naczk M., Gajewski M., Ostapiuk-Karolczuk J., Dziewiecka H., Kasperska A., Szyszka K. (2010), Changes of muscle-derived cytokines in relation to thiol redox status and reactive oxygen and nitrogen species. Physiol Res, 59, pp. 945-951.

Barbara Morawin
Karol Kalina
Mateusz Rynkiewicz
Agnieszka Zembroń-Łacny

Generacja tlenku azotu w odpowiedzi na intensywny wysiłek fizyczny

Słowa kluczowe: tlenek azotu, mioglobina, skład ciała, uszkodzenie mięśni, wysiłek fizyczny.

Tlenek azotu (NO), jako cząsteczka sygnałowa, odgrywa kluczową rolę w odbudowie włókien mięśniowych szczególnie we wczesnej fazie po uszkodzeniu, inicjując proliferację komórek macierzystych mięśni (ang. *satellite cells*), angiogenezę i synaptogenezę w aksonach motoneuronu, i w efekcie wzrost siły skurczu mięśnia. Celem badań była ocena zmian stężenia NO pod wpływem intensywnego wysiłku fizycznego, zależności między generacją NO a uszkodzeniem mięśni szkieletowych i składem ciała.

Badania przeprowadzono z udziałem 18-osobowej grupy kobiet i mężczyzn w wieku 21,3 ± 2,0 lat. Badani zostali poddani testowi wysiłkowemu o wzrastającej intensywności do odmowy. Krew pobierano przed wysiłkiem, w 1 min, 30 min, 24 godz i 48 godz po wysiłku. W surowicy krwi oznaczono stężenie mioglobiny (Mb; wskaźnik uszkodzenia mięśni) i tlenku azotu (NO) metodami immunoenzymatycznymi. Analizę składu ciała dokonano metodą impedancji bioelektrycznej (ang. *bioelectrical impedance*, BIA).

Stężenie Mb i NO wzrosło statystycznie istotnie w 1 min po zakończeniu wysiłku, i utrzymywało się na wysokim poziomie do 24 godz po wysiłku. Między stężeniem Mb a NO zaobserwowano dodatnią korelację ($r=0{,}446$, $P<0{,}001$). Ponadto wykazano zależność pomiędzy komponentami składu ciała a maksymalnym pochłanianiem tlenu. Wskaźnik masy tkanki tłuszczowej (ang. *fat mass index*) korelował z wartością VO_2max ($r=-0{,}521$, $P<0{,}001$), ale nie korelował ze stężeniem NO.

Na podstawie przeprowadzonych badań można stwierdzić, że uszkodzenie mięśni wywołane intensywnym wysiłkiem fizycznym zwiększa wytwarzanie NO, który może odgrywać istotną rolę w adaptacji organizmu do wysiłku fizycznego.