OXIDATIVE STRESS-INDUCED APOPTOSIS IN RAT SKELETAL MUSCLES DURING A SINGLE RUNNING EXERCISE UP TO EXHAUSTION

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Abstract

The aim of this study was to investigate whether the oxidative stress may be the reason for apoptosis in skeletal muscles in rats. Rats were divided into two groups – controls and exposed to physical exercise. Rats were running on the treadmill at the speed of 1km/h until exhaustion. After the exercise, the concentration of lipid peroxidation markers – malonyldialdehyde and 4-hydroxyalkenes (MDA+4-HDA), and the level of reduced glutathione (GSH) was determined in the homogenates of the extensor digitorum longus (EDL) muscle and slow-twitch (ST) fibres in 2, 6, and 96 h of restitution. Aconitase activity as a marker of oxidative protein modification was determined in ST fibres and EDL muscle. Additionally, apoptosis was detected by the TUNEL technique. A significant increase in MDA+4HDA concentrations in comparison to the control group was noticed in both ST fibres and EDL muscle after 6 h. GSH concentration in 2 and 6 h after exercise was significantly decreased in ST fibres and in EDL muscle in all measurements, when compared to the control group. Aconitase activity in ST fibres and EDL muscle was also significantly decreased 2 h after the exercise when compared to the control group, but increased in 6 h of restitution. Apoptotically-changed nuclei were observed only in EDL fibres. On the basis of the results and the suggested mechanism, it can be thought that the oxidative stress triggers apoptosis in ST fibres and in EDL muscle after exercise and it starts in the mitochondria.

Key words: rats, exercise, apoptosis, oxidative stress, skeletal muscles.

Reactive oxygen species (ROS) are produced only in small amounts in the resting organism. Intense physical exercise increases their production, among other things, due to increased supply of oxygen to working muscles, which in those circumstances can exceed approximately from 100 to 200 ffold the resting values (3, 9). There are various sources of ROS in muscles during exertion but most important are increased leakage of electrons in the mitochondrial respiratory chain, where their intensified and accelerated production takes place. The damage of the cell and its death may be the consequence of increased ROS production (9). Until recently, necrosis was thought to be the only way of elimination as far as skeletal muscle fibres were concerned. Currently, it is known that they also can undergo apoptosis, which is programmed cell death. Apoptosis is a process characterised by specific changes at the molecular, biochemical, and morphological level. The process may be activated or inhibited by many factors. ROS are probably one of those factors (15). They break into macromolecule structures, block enzyme activity especially in mitochondria, and disturb energetic equilibrium of the cell, which may turn on the programmed death. Apoptosis in response to a variety of injurious stimuli has been demonstrated in several organs (13, 14, 16) but only few histological investigations confirm the appearance of apoptotically-changed nuclei in fibres of skeletal muscles (16). The mechanisms of apoptosis in skeletal muscles are not known. The knowledge concerning the mechanisms of apoptosis in mononuclear cells does not answer the question, which of those is triggered in polynuclear cells like muscular fibres? It seems quite probable that ROS itself may trigger many processes during intense contraction. Their extremely high production in mitochondria during physical exercise indicates the possibility of their contribution to the induction of the internal pathway of apoptosis, which has its origin in cell mitochondrion (10, 15).

The aim of this research was to investigate whether oxidative stress may be the reason for the apoptosis in skeletal muscle fibres of rats exposed to a physical exercise. The other purpose of the study was to investigate whether there are any differences between
susceptibility of the individual types of muscular fibres to oxidative stress and whether a single exercise may increase the oxidative stress level in individual fibres of skeletal muscles. Malonyldialdehyde + 4-hydroxyalkenes (MDA + 4-HDA), reduced glutathione (GSH), and taconitase activity were used as the markers of oxidative stress level. Aconitase activity reflects the amount of superoxide anion production during oxidative stress and it is also a marker of oxidative protein modification. This enzyme contains in its active centre a 4Fe-4S cluster susceptible to free radicals.

Material and Methods

The study was conducted on male Wistar rats, aged 3 months and weighing 200–250 g. The rats were kept according to the Local Ethical Committee on Animal Experiments in controlled environmental conditions with a 12 h day/night cycle and continuous access to standardised diet and drinking water. Twenty-four rats were divided into two groups: the control sedentary group (n=6), and a group exposed to a running exercise on a treadmill (n=18). Running speed was 1 km/h, with a 0% incline. The running was continued until the exhaustion of the animal’s strength. The mean time of the running exercise was 90 min (60–115 min). In 2 h, 6 h, and 96 h after the end of the exercise, the animals were anaesthetised by Bioketan (ketamine hydrochloride, Biowet, Poland) in the dose of 100 mg/kg b.w. and cervical spine dislocation. After that, muscles from hindlimb were quickly dissected. Slow-twitch (ST) fibres, taken from the soleus muscle and extensor digitorum longus (EDL) muscle, were prepared. Then they were cut into small pieces and frozen at -86°C until they were examined in biochemical tests. For biochemical tests, fragments of the fibres were defrosted at room temperature, weighed, and washed in 0.9% NaCl solution with EDTA. The samples were homogenised in ice-cold 20 mM TRIS-HCL buffer, pH 7.4, and centrifuged for 10 min at 4°C with the speed of 15,000 g. Supernatants were used for analysis. Markers of oxidative processes in cells were detected by means of colorimetrical method using ready-to-use reagents. Malonyldialdehyde+4-hydroxyalkenes were measured by Lipid Peroxidation Assay Kit (Calbiochem, La Jolla, USA), reduced glutathione concentration by Bioxytech GSH-400 (OXIS, USA), and aconitase activity were measured by Aconitase-340 (OXIS, USA). All the analyses were performed according to manufacturers’ instructions. For histological examination, samples of ST fibres and EDL muscles were placed for 24 h in 4% formaldehyde buffered solution and then paraffin blocks were prepared. Apoptosis was examined in paraffin sections by the TUNEL technique with use of ApopTag® Plus Peroxidase in Situ Apoptosis Detection Kit (INTERGEN, Norcross, USA).

In order to determine the significance of differences between individual groups, Friedman’s ANOVA test from Statistica 6.0 PL software (StatSoft, Poland) was used and in case of statistically-significant differences, Wilcoxon’s test was applied at the further stage. The significance of differences was set at $P \leq 0.05$ confidence level.

Results

Changes in concentrations of lipid peroxidation markers in examined muscle fibres of the control group and of those examined 2, 6, and 96 h after the exercise, are shown on Fig. 1. In the ST fibres and EDL muscle, the level of MDA+4HDA 6 h after the exercise was significantly higher in comparison to the control group and values noticed 2 and 96 h after the exercise. Fig. 2 shows the concentration of reduced glutathione. In the ST fibres, GSH concentration values were the highest in the control group.

![Fig. 1. Concentration of MDA+4HDA in ST fibres and EDL muscle.](image1)

* P<0.05 compared to the control group and values measured 2 h and 96 h after exercise

![Fig. 2. Concentration of GSH in ST fibres and EDL muscle.](image2)

* P<0.05 compared to values measured at 2 h and 6 h
** P<0.05 compared to the control group and values measured 2 h after exercise
In the 2nd and 6th h of restitution, this concentration was significantly decreasing in comparison to the control group. During 96 h, it increased slightly in comparison to the examined group in the 2nd and 6th h after the exercise. These values remained, however, lower in comparison to the control group. In the EDL muscle, the level of GSH was decreasing in the examined hours, reaching the lowest value in the 96th h of restitution. Fig. 3 shows changes in aconitase activity in ST fibres and EDL muscle. The activity of this enzyme was decreasing significantly 2 h after physical exercise in comparison to the control group in both ST fibres and EDL muscle, while in the 6th h after the exercise, its activity increased significantly in comparison to the group examined 2 h after the exercise. In comparison to control group, aconitase values in the 6th h of the examination do not show any significant differences. In the 96th h of restitution, the activity of this enzyme decreased significantly again in ST fibres, in comparison to the 6th h of restitution, remaining at the level slightly higher, but statistically insignificant, in comparison to the group examined 2 h after the exercise.

In the histological examinations, whose results are presented on Fig. 4, the number of apoptotic nuclei increased in the 96th h of the restitution. Apoptosis was absent in ST fibres.

**Discussion**

Skeletal muscle workout involves higher energy demand, which during long endurance exercise comes mainly from the oxygen process. An increase in energy demand provokes a higher oxygen supply for working muscles, which can be the source of an increased amount of reactive oxygen species (9).

In our study, we observed the results of the ROS effect on lipid structures and intracellular antioxidant concentration, and the influence of aconitase, mitochondrial enzyme activity. The effects were characterised by an increased level of MDA+4HDA in EDL muscle and ST fibres, a decrease in reduced glutathione, and a decrease in the activity of aconitase, taking part in Krebs’ cycle, observed in EDL muscle. On the basis of the results, we found that consequences of oxidative stress change during time and depend on the type of muscular fibres. The dynamics of lipid peroxidation marker change in ST fibres in 2, 6, and 96 h after the exercise. It means that in the ST fibres, for which energy for contraction is taken mainly from the oxygen process, an increase in the lipid structure damage markers was significantly higher in comparison to holding values. The fast-twitch (FT) fibres are not as susceptible to free radicals’ dangerous influences as ST fibres but in EDL muscle a high level of MDA+4HDA was observed. It is known that EDL muscle is composed of FT fibres. In a running exercise up to exhaustion, this muscle plays an important role and an eccentric type of contraction dominates, which is more destructive than the concentric type. Apoptotic nuclei were observed only in this muscle. ST fibres are more resistant to ROS and apoptosis could be observed during a higher exercise load. Changes of MDA+4HDA in ST fibres and EDL muscle were specially marked 6 h after exercise and responded to the decrease in one of the most important intracellular antioxidative agents - GSH. Its concentration measured 2 and 6 h after exercise remained significantly lower in comparison to the basic level, which clearly suggests the utilisation of this substance in order to sustain cellular redox balance and sweeping away increasing concentration of ROS.

The highest time dependent sensitivity of ST fibres to oxidative damage was noticed by other authors (5). In our earlier studies we found changing intensity of ROS generation after physical exercise (12). In this study we observed its increase 6 h after exercise as Moughan et al. (9). The result of the destructive action of ROS was noticed both in the current study and in the study by the
aforementioned authors 6 h after exercise may be related to a peak in proinflammatory synthesis of IL-6, IL-1β, or TNF-α cytokines (3, 4), increased infiltration of muscular inflammation sites by neutrophils, and an increase in the granulocyte colony-stimulating factor (G-CSF) concentration, which activates neutrophils and stimulates leukocyte degranulation, phagocytosis, leukocyte chemotaxis, and respiratory explosion (31). Increased production of ROS delayed in time may also be a consequence of hypoxia related to exercise itself and post-exercise reperfusion. The infiltration of leukocytes in the inflammation sites and their activation may also be stimulated by catecholamines, cortisol, and the growth hormone (GH), for which peak secretion takes place directly after exercise (G-CSF and cortisol) or 1 h after exercise (GH) (28). The augmentation and delayed generation of ROS may also be caused by hypoxia during exercise and post-exercise reperfusion (17). Cellular equilibrium is destroyed by a decrease in the GSH level, MnSOD activity as well as by an increase in ROS production. These mechanisms provoke a decrease in aconitase activity and cellular energy production. (6, 17). In our studies, a decrease in GSH level noticed from the 2nd h after exercise in ST fibres coincided with a decrease in aconitase activity – the enzyme necessary in the regeneration of energy needed for muscle contraction. Aconitase takes part in citrate’s conversion into isocitrate in the Krebs’ cycle (1). A decrease in its activity may also disturb cellular iron uptake (7). Aconitase high susceptibility to oxidative modification is connected with its structure. This enzyme has iron-sulphur cluster [4Fe-4S] in the active site. It has been found in vitro studies that aconitase is especially sensitive to peroxide anion – radical $[O_2^-]$ (1, 6). The elevation in the rate of superoxide generation occurring during physical exercise can lead to a corresponding enhancement in the production of $H_2O_2$ and hydroxyl radical. The latter may cause carboxylation and irreversible inactivation of aconitase through metal-dependent side specific oxidation (18). Aconitase inactivation may inhibit the free flow of the electrons in the oxidative chain, which leads to accumulation of reduced metabolites e.g. NADH. Such conditions are described as „reductive stress” and may cause an increase in ROS production as a consequence of reduced metabolite autooxidation. Then it may intensify macromolecule oxidative damage (22). In addition, ROS generation in mitochondria, cytosole inactivating aconitase, and a decreasing GSH level, may cause IRP-1 (iron regulatory protein) activation, and increase in the intracellular Fe transport as the consequence of increased expression of Tfr (transferrin receptor) (7). Ho et al. (7) established a lower non-haem iron contents in the liver, spleen, and other tissues in the exercised rats than in sedentary animals, and also that strenuous exercise could lead to a significant increase in Tfr expression and transferring-bound iron (Tf-Fe) accumulation in bone marrow erythroblasts in rats. Increased expression of Tfr could initiate apoptosis (8, 20). Intracellular changes evoked by ROS and the changes described above may initiate apoptosis in skeletal muscles, but this, of course, requires further investigation. Histological studies confirmed the appearance of apoptosis in skeletal muscles (16) but the role of such a process is not clear. Apoptosis in different types of fibres probably depends on exercise load, fibre susceptibility to free radicals, and antioxidant defence. It seems quite probable that apoptosis is one of the stages of fibre transformation in the process of skeletal muscle adaptation to physical exercise.

References