Time-Course of Exercise and Apoptosis in Dystrophin-Deficient Muscle of Mice

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Abstract

Apoptosis is a process of cell death occurring in many tissues. That apoptosis precedes necrosis in death of dystrophin-deficient muscle fibers of mdx is now accepted. Furthermore, we were the first to describe an increase of apoptotic myonuclei in mdx mice two days after spontaneous running exercise. To investigate the role of apoptosis in muscular dystrophy and to determine minimal time/characteristics of physical exercise able to induce a bout of muscle apoptosis, in the present work we studied contribution of apoptosis to exercise-induced death of muscle fiber by a time-course analysis in mdx mice. The runners were housed in a cage with an exercise wheel and allowed to run spontaneously for two hours or for an entire night (about 12 hours) and, the morning after, Tibialis Anterior of both hindlimbs removed. We checked the activity of mice by monitoring the covered distance and the time when the wheel was moving.

Apoptosis was assessed by the terminal deoxynucleotidyl transferase assay and expressed as number of apoptotic nuclei for mm^3 of muscle tissue, and by electron microscopy for morfological features.

In 12-hours running groups mdx mice present a significant minor activity (18±0.7) in comparison with 2-hours mdx runners (36±3.5 $p < 0.001$). Consequently, it seems that mdx mice have an activity peak after 2-hours exercise and then it decreases maybe because mdx muscular fibers are frailer, more fatiguable and susceptible at exercise-induced damage.

Control non-runner mdx mice present 40±13 apoptotic myonuclei/mm^3, 2-hours runner mice 84±13 ($p = 0.04$ against control) and 12-hours mice 158±32 ($p = 0.04$ against control; $p = 0.23$ against 2-hours), while in 12-hours mdx runners interstitial nuclei/mm^3(188±46) significantly decrease in comparison with 2-hours group (348±50). Electron microscopy confirm that apoptotic myonuclei increase after 2-hours running in comparison with sedentary and some more after 12-hours running. Besides it shows that apoptotic process involves satellite cells.

Besides confirming that apoptosis present in mdx mice at rest dramatically increases after exercise, results suggest that inflammation and interstitial apoptosis increase during a short-time exercise while the apoptotic process in myofibers (doubled in 2-hours runners in comparison with sedentary group) becomes more manifested after a long-term exercise, even if mdx mice activity decreases.

Key words: apoptosis, Duchenne muscular dystrophy, muscle, programmed cell death.

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Apoptosis is a process of individual cell death regulated by activation of specific genes [34]. Characteristic morphological features of apoptosis include nuclear and cytoplasmatic condensation, fragmentation of cell into apoptotic bodies which become engulfed by phagocytes, and local absence of inflammation. These changes are due to activation of nuclear endonucleases and cytoplasmatic proteases, while the death program is modulated by several regulatory genes with pro- and anti-apoptotic function [13]. During embryonic development,

apoptosis represents a mechanism of programmed cell death, designed to match the final number of a cell population with its target. In addition, apoptosis plays fundamental roles in disease, where it can be elicited by diverse stimuli such as trophic factors deprivation, oxidative stress, ischemia, excitotoxic stimuli, heat shock and toxins [38] Apoptosis, which is known to be present widely during normal development, is also the main regulator of the number and shape of muscle cells during myogenesis. In metamorphosis of tadpoles and insects the deletion of striated muscle fibers is accomplished by apoptosis and, though the morphology does not resemble classical apoptosis (cytoplasmic changes long precede changes in the nucleus), the death is clearly programmed and under physiological control [11, 17, 19]. In mammals, humans included, at the early stage of fetal muscle development, up to 40% of the cells are eliminated by apoptosis [7]. Muscle apoptosis has been described in infantile spinal muscular atrophy [8]. In neonatal rats, immature muscle cells undergo apoptosis in response to experimental injury, while mature myofibers undergo necrosis [16].

Duchenne type muscular dystrophy is caused by a lack of dystrophin. An unbalance in muscle damage and regeneration seems to be critical to the manifestation of the symptoms. However, the death of dystrophic myofiber occurs by an enigmatic process [1]. Even the late clinical onset of muscle pathology has not been explained. Recently we have shown, in line with other published results, that apoptosis occurs in skeletal muscle of adult dystrophic (mdx) mice in vivo [23, 25, 27, 28, 31, 32]. Advances in the knowledge of cell death suggest that the criteria used to characterize death of dystrophic muscle as a necrotic process are not sufficient to eliminate the possibility that apoptosis plays a role in the pathogenesis of muscle damage. Elevation of intracellular Ca^{2+} has been reported and this increase is expected to activate calcium dependent proteases (calpains) that are capable of widespread proteolysis of intracellular proteins [40], a well-known signal for induction of apoptosis. On the other hand, inflammation also occurs in well-characterized examples of apoptosis as the final step [20]. Even the suggested function of dystrophin as signal transducer [6] could explain the activation of apoptosis in dystrophin deficient muscles which lack a correct conduction of the external signals inside the cell [42]. The possibility that muscle cells undergo apoptosis has been shown by *in vitro* experiments on normal and dystrophin-deficient myoblasts [9, 21, 31, 33, 35, 43], so an unbalance of this process may disturb regulation of myoblast proliferation, differentiation, and death during regeneration of skeletal muscle. The hypothesis that apoptosis may be present during inflammation and necrotic events is sustained by the presence, around myocardium infarctual area, of apoptotic cells which showed an increased membrane permeability typical of necrotic events [10,14, 15, 36, 41]. Many experiments in different tissues, myocardium included, have shown that after ischemia and reperfusion, apoptosis is present probably due to reactive oxygen species (ROS) production [2, 10, 14, 15, 36, 41].

After the pioneering work of Kerr, in the last few years a small group of investigators has focused their attention on the process of apoptosis in adult skeletal muscles [4].

In the present work using TUNEL method we studied contribution of apoptosis to exercise-induced death of muscle fiber by a time-course analysis in *mdx* mice monitoring the real activity of mice.

Materials and Methods

Fifteen mdx adult mice were used at two months. Mice were divided into non-runner (N = 5) and runner animals (N = 10). The runners were housed in a cage with an exercise wheel and allowed to run spontaneously for 2 hours (N = 5) or an entire night (about 12 hours). 2-hours runner mice were housed in a cage without an exercise wheel for 10 hours. We checked the real activity of mice by mounting a bicycle computer on the exercise wheel. Bicycle computer measures the covered distance and the time travelled (the time only operates when the wheel is moving).

The morning after all animals were sacrificed and Tibialis Anterior of both hindlimbs were removed. Both muscles were cut at the belly. Half of muscle was prepared for electron microscopy. In brief muscles are fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2, for 2 h on ice, followed by buffer rinse and fixation for 1 h in 1% osmium tetroxide. The muscles are dehydrated in a graded series of ethanol and embedded in epoxy resin before thin sectioning for electron microscope examination. From each animal at least 50 myonuclei per section are counted. All data are expressed as mean ± standard error (SE).

Half muscle was frozen in liquid nitrogen and stored at -80°C until use.

Cryo-sections were double-labelled for the presence of lamin and for apoptotic nuclei. Slides were incubated with rabbit anti-laminin antibody (from Sigma Chemical Co., St. Louis, MO) diluted 1:10 in 1% BSA for 1 hour. The slides were then washed twice with PBS (5 min each) and incubated with rhodamine-conjugated goat anti-rabbit Ig (1:250 diluted in 1% BSA) for 1h at 37° C. Negative controls were performed by omitting the primary antibody. After the incubation with rhodamine conjugated antibody the slides were rinsed in PBS, then were labelled for fragmented DNA. In situ nick end labelling of fragmented DNA was performed using terminal deoxynucleotidyl transferase (TdT) and fluorescein-conjugated nucleotides with the *In Situ* Cell Death Detection Kit, POD (Roche) as described by the manufacturer's instructions. Negative control slides were pre-

pared by substituting distilled water for TdT Enzyme and continuing with the staining procedure as suggested by the manufacturer's instructions. Labelled nuclei were easily identified from the negative nuclei counter-stained by Hoechst 33258 and were photographed. Positive nuclei were counted and expressed as number of apoptotic nuclei for mm^3 of muscle tissue.

Results are expressed as mean±SE. Student's t-test was used for statistical analysis and data were considered statistically significant when $P < 0.05$.

Results and Discussion

Exercise-induced muscle damage can occur in daily life, and is more common in unaccustomed muscles [5, 24]. Surprisingly, the nature of pathogenetic mechanism(s) is still open to debate and so its role in progression of muscle dystrophies [6]. It is well accepted that damaged muscle fibers display signs of acute injury (disarrangement of myofibrils, mitochondrial changes, fiber necrosis and infiltration of inflammatory cells) and chronic signs of muscle injury (split fibers and central nuclei) in short-term and intermittently trained muscles, respectively. Apoptosis is a well accepted type of cell death during development of mammalian muscles, but death of adult myofibers in neuromuscular disorders and exercise-induced muscle damage is usually explained in terms of muscle necrosis [16, 44]. Recent studies have shown that adult skeletal muscles can undergo apoptosis in response to several stimuli, like dystrophinopathies, exercise, ischemia/reperfusion, denervation and atrophy (4, 12, 23, 25, 27, 28, 30, 31, 32, 37, 39]. Furthermore we showed an increase of apoptotic myonuclei and myonuclear ubiquitination in mdx mice after prolonged running [27, 28]. In previous studies we showed that in normal and mdx mice the apoptotic process has to begin during the night of wheel-running but the mice were sacrificed after 48 hours, of course this left open the question of the minimal exercise needed to induce apoptosis of muscle fibers, since the mice can run more than 5 Kms during a night [44]. To investigate the role of apoptosis in progression of muscle dystrophy and to determine minimal time of physical exercise able to induce muscle apoptosis, in the present work we studied muscle fibers after a time-course analysis in mdx mice. To determine whether and how much the mice run during 2 hours or entire night we monitored the real activity of mice by mounting a bicycle computer on the exercise wheel. Figure 1 shows that activity time was of 36%±3.5 of available time in 2-hours runners and of 18%±0.7 in 12-hours animals. The difference is significant (< 0.001). Consequently, it seems that mdx mice have an activity peak after 2-hours exercise and then it decreases maybe because mdx muscular fibers are frailer, more fatiguable and susceptible at exercise-induced damage. To determine whether apoptosis plays a role in progressive damage of dystrophic muscle we studied myofibers

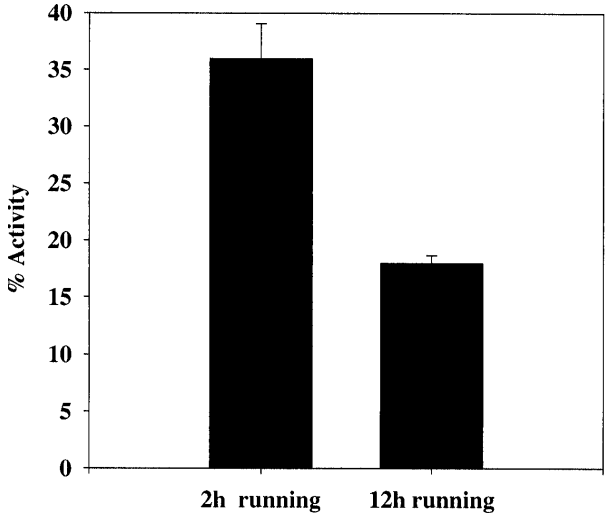

Figure 1

of mdx mice after spontaneous exercise. Tibialis Anterior muscles, both at rest and after exercise, show muscle injuries with foci of inflammation, small regenerating myofibers, round hypercontracted myofibers and fibers with centrally located myonuclei. After immunoreaction with an anti-laminin antibody, connective tissue appears to react determining if the nuclei belong to interstitial tissue, when they are surrounded by the immunoreaction, or if they are inside a myofiber, when the nuclei are outside the immunoreaction. When slides processed for laminin were subjected to in situ analysis of DNA fragmentation, nuclei positive for TUNEL were detected inside myofiber in subsarcolemmal and central position. Nevertheless the double labelling confirmed the presence of most apoptotic nuclei at interstitial level. Control non-runner mice present 40±13 apoptotic myonuclei/mm^3, and 74±6 interstitial nuclei/mm^3. Apoptotic myonuclei increase significantly in mdx muscle after 2 hours exercise (84±13) with a peak (158±32) after 12 hours of running (figure 2) and many centrally located myonuclei become positive both in small regenerating and in mature myofibers. Interstitial nuclei increase after exercise with a peak of positive nuclei after 2 hours of running and 10 hours at rest (348±50) and halve in 12-hours group respect to 2-hours runners (188±46) (figure 3).

Results suggest that inflammation and interstitial apoptosis occur during an intense short term exercise. One explanation is that phenomenon is due to an increased generation of reactive oxygen species during sustained hyperemia which accompanies and follows physical muscular activity. When there is a decreasing/interruption of physical activity hyperemia is present but the increased oxygen flow is not used in metabolic processes. An overproduction of the reactive oxygen intermediates can damage macromolecules, such as proteins, lipids and DNA of endothelial and interstitial cells [2, 22]. The free radical burst generated upon re-

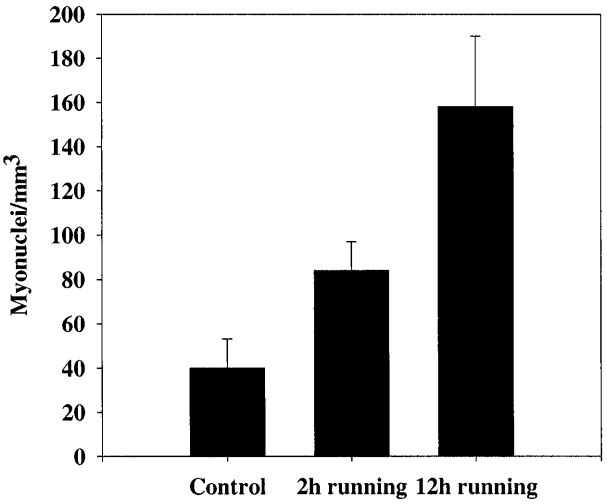

Figure 2

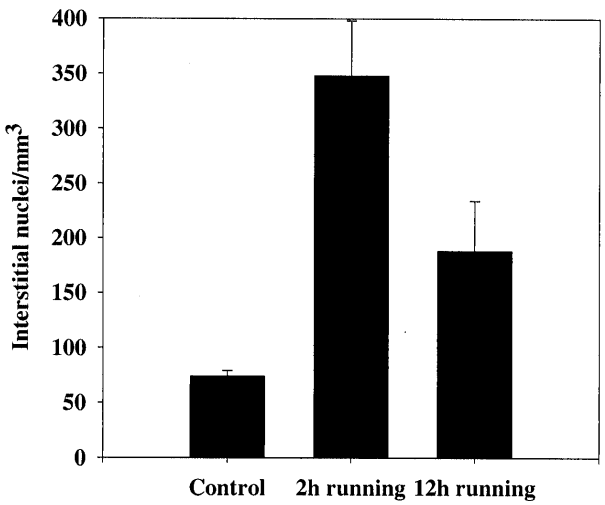

Figure 3

perfusion could be one mechanism to up-regulate pro-apoptotic and down-regulate anti-apoptotic genes. During short time exercise muscular fibers are not damaged because there is a gradient of oxygen concentration from vessels to fibers. It is well known that such events induce apoptosis in ischemia/reperfusion damage of central nervous system and of myocardium [15, 18]. It is conceivable that the same mechanisms could be present in skeletal muscles [12]. Electron microscopy confirm that apoptotic myonuclei increase after 2-hours running in comparison (2.69%±0.92) with sedentary (1.8%±0.92) and some more after 12-hours running (5.12%±1.96). Besides it show that apoptotic process involve satellite cells (data not shown).

Duchenne muscular dystrophy determine in skeletal muscle a damage similar to injury due to short time intense repeated exercise and only during long time leads to progressive muscle degeneration. A number of possible mechanisms have received attention: i) changes in plasma membrane permeability; ii) a specific defect in muscle intracellular free calcium homeostasis; iii) and a decreased mechanical stability of the sarcolemma and of the sarcomeres [1, 6, 40]. Recent studies [3, 25, 26] show the presence of apoptosis in dystrophin-deficient mouse muscle, suggesting a role in the pathogenesis of muscle disease. It is generally expected that exercise-induced damage plays a role in the myodystrophic process. Signs of apoptotic process have been detected also in DMD [29]. It is also well known that exercise in an unaccustomed muscle may provoke injury, soreness, and lactic acid accumulation. Our observations, described in the present and previous papers [27, 28], that spontaneous running in unaccustomed animals increases the number of apoptotic myonuclei in differentiated muscle fibers of adult mice strongly suggest that exercise-induced damage, or fatiguing exercise itself, activates programmed cell death in mdx muscle.

It remains to be established whether muscle apoptosis of both myofibers and endothelial cells is restricted to the repair mechanisms, as often suggested in many pathologic processes, or whether it is also part of pathogenesis of muscle damage. In our opinion observations of satellite cells apoptosis within the basement membrane of a normal appearing myofiber support the first hypothesis, at least as normal animals are concerned. It is conceivable that in this case, apoptosis of satellite cells could be part of mechanisms which eliminate unwanted “activated” satellite cells, in particular if their DNA is damaged by reactive oxygen species produced during the unbalanced perfusion phase which accompanies and/or follows several hours of running in an unaccustomed muscle. Of course, the same mechanism could be of foremost pathogenetic importance in dystrophic muscles , which are per se peculiarly exposed to damage and regeneration. If so, uncontrolled apoptosis could explain the limited ability of human dystrophic muscle to recover in the long-term by regeneration.

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Abbreviations

TUNEL, Terminal dUTP Nick End Labelling; DMD, Duchenne Muscular Dystrophy; PBS, Phosphate Buffered Solution; BSA, Bovine Serum Albumin.

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