Sudden Spontaneous Exercise Increases Myonuclear Ubiquitination and Apoptosis of Dystrophin Deficient Muscle

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Abstract

In multicellular organism, homoeostasis is maintained through a balance between cell proliferation, cell differentiation and cell death. Physiological cell death occurs primary through an evolutionary conserved form of cell suicide called apoptosis. Apoptosis or programmed cell death is an active multi-step process characterized by morphological, biochemical and molecular events which requires coordinated regulation of specific genes (Steller, 1995. Science 267, 1445). This program of cell suicide plays a major role in development, in tissues with high cellular turnover and contributes to the pathogenesis of several human deseases. In vitro experiments on normal and dystrophin deficient myoblasts added informations on regulation of myoblast proliferation, differentiation and death during regeneration of skeletal muscle, but few informations are available about the role of apoptosis in adult muscles. First observations came from studies on myocardium that displayed apoptosis in infarction (Tanaka et al. 1994. Circulation Res. 75, 426; Gottlieb et al. 1994. J. Clin. Invest. 94, 1621; Umansky et al. 1995. Cell Death Differ. 2, 235; Itoh et al. 1995. Am. J. Pathol. 146, 1325). Recently we and others showed apoptosis in skeletal muscle of adult mdx mice in vivo (Podhorska-Okolow et al. 1995. Basic Appl. Myol. 5, 87; Sandri et al. 1995. FEBS letters 373, 291; Tidball et al. 1995. J. Cell Sci. 108, 2197; Smith, et al. 1995. Cell Death Differ. 2, 243; U. Carraro, 1995. Basic Appl. Myol. 5, 371). One of the first genes shown to be up-regulated during programmed cell death was ubiquitin. Ubiquitin appears to play an essential role in irradiation-induced death of human peripheral lymphocytes, in some neurodegenerative and autoimmune disorders, and more recently in programmed cell death of Manduca sexta muscles. In mammalians, different conditions of muscle wasting reveal an increased expression of ubiquitin (Wing, et al. 1995. Biochem. J. 307, 639). Apoptosis plays a major role in several diseases including viral infections, auto immune diseases, cancer, cardiac infarct, and neurological disorders (Thompson 1995. Science 267, 1456). In this view dystrophin deficient (mdx) mice were subjected to mild exercise to study role of apoptosis in pathogenesis of dystrophinopathies (Anderson et al., 1992. Trends Biochem. Sci. 17, 289; Best et al. 1994. Basic Appl. Myol. 4, 77; V. Toescu et al. 1994. Basic Appl. Myol. 4, 217; Ohlendieck, 1994. Basic Appl. Myol. 4, 277; Carraro, 1995 Basic Appl. Myol. 5, 371) and skeletal muscles were analysed for apoptosis and ubiquitin. After exercise apoptotic myonuclei increased from 2-3% to 30% in mdx mice, while muscles from sedentary congenit control mice (BALB c) were negative for apoptosis. Expression of ubiquitin correlated with the exercise and marked the myonuclei positive for apoptosis. Thus exercise results in a massive increase of ubiquitin expression tightly related with programmed cell death in mdx mice. In vitro myoblasts committed to apoptosis showed an increased expression of ubiquitin early in death process. These findings confirm that DNA breaking, absent in muscles of sedentary normal mice but present in mdx mice at rest, dramatically increases after exercise, and shed new light on exercise-induced muscle damage and on its progression in dystrophinopathies.

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Key words: apoptosis, ubiquitin, Duchenne muscular dystrophy, exercise-induce muscle damage, programmed cell death.

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Apoptosis or programmed cell death is an active multi-step process characterized by morphological, biochemical and molecular events which requires coordinated regulation of specific genes [17]. This program of cell suicide plays a major role in development, in tissues with high cellular turnover and contributes to the pathogenesis of several human diseases [21]. *In vitro* experiments on normal and dystrophin deficient myoblasts [15, 16] add information on regulation of myoblast proliferation, differentiation and death during regeneration of skeletal muscle, but few information is available on the role of apoptosis in adult muscles. Some observations come from studies on myocardium, since it can display apoptosis after ischemia and reperfusion [5, 7, 20, 24]. Recently we shown in line with other published results apoptosis in skeletal muscle of adult *mdx* mice *in vivo* [3, 12, 15, 16, 22].

One of the first genes up-regulated during programmed cell death is ubiquitin gene [18]. Ubiquitin appears to play an essential role in irradiation-induced death of human peripheral lymphocytes, in some neurodegenerative and autoimmune disorders [4], and in programmed cell death of *Manduca sexta* muscles [6]. In mammalians, different conditions of muscle wasting reveal an increased expression of ubiquitin [25].

To determine whether ubiquitin plays a role in progressive damage of dystrophic muscle we studied myofibers of *mdx* mice after a mild spontaneous exercise. Sedentary *mdx* mice and congenit BALB c mice were used as controls.

Material and Methods

Ten *mdx* and ten congenit control BALB c adult mice were used. *Mdx* mice were divided in runner (N = 5) and non-runner animals (N = 5). The runners were housed in a wheel-cage, let them run spontaneously for an entire night. We stress that the wheel is the one counselled to give the mice a more enjoyable environment; so no distress is caused per se by the wheel, which is moved only if the mouse run in it. The day after the mice were housed in a standard cage, and then sacrified after additional 24 hrs. Tibialis Anterior of both hind limbs were removed. Both muscles were cut at the middle bell, half of the right limb muscle was prepared for electron microscopy, the other half was fixed in 10% formaldehyde and embedded in paraffin. Half of the muscle from left hind limbs was used to isolate myonuclei, the other half was frozen in liquid nitrogen and stored at -80°C until use.

Ubiquitin policlonal antibody developed in rabbit (SIGMA) was used to label ubiquitin and initiated proteins. At least 400 nuclei per section were counted in each muscle. Positive myonuclei are given as percentage of counted nuclei.

DNA fragmentation in myonuclei was visualized by the Apoptag kit (Oncor, Gaithersburg, MD), under the specific conditions established for skeletal muscle specimens by Podhorska et al. [12]. Positive nuclei were counted after Methyl-green counterstain and expressed as percentage of counted nuclei. At least 400 nuclei per section were counted.

Results and Discussion

Light mycroscopy of muscles of dystrophic mice, both at rest and exercised, shows foci of muscle injury with inflammatory cells, small regenerating myofibers and myofibers with centrally located myonuclei, while muscles of sedentary BALB c mice present homogeneous well-defined fibers with peripheral myonuclei. After immunoreaction with an anti-ubiquitin antibody BALB c myofibers appeared poorly reacting due to low level of ubiquitin expression in physiological conditions, while some cytoplasmic stain distinguishes slow and fast fibers [25]. Myofibers of sedentary *mdx* mice present a positive reaction in some peripherally-located nuclei, and in small regenerated myofibers (submitted manuscript).

On the other hand, in *mdx* muscle after exercise many centrally located myonuclei are positive both in small regenerating and in mature myofibers, while foci of inflammation are negative (Fig 1a). The high turnover of ubiquitin and the 24 hr of rest after exercise exclude that ubiquitin is induced in parallel with Heat Shock Proteins by the stress due to exercise *per se* [9]. On the other hand, it is well documented that ubiquitin is tightly bound to histones or to some other proteins of the nuclear matrix after DNA damage.

When the slides were processed for *in situ* analysis of DNA fragmentation, numerous myonuclei in exercised muscles of *mdx* mice were positive for apoptosis (Fig. 1b). As we recently described, muscles of sedentary *mdx* mice show 2-3% of apoptotic myonuclei, while BALB c muscles are negative [15]. The increase of the percentage of positive myonuclei for ubiquitin in *mdx* muscles after exercise correlates with the increased number of apoptotic myonuclei (Table 1).

When DNA analysis by pulsed field gel electrophoresis is performed on isolated myonuclei the results reveal that: i) no DNA fragments are detectable in BALB c muscles; ii) some fragments at 200 kb and at 50 kb are present in muscles of sedentary *mdx* mice in good correlation with the 2-3% of apoptotic myonuclei found with Apo-Tag kit; and iii) an increased amount of DNA fragments are detected in muscles of *mdx* mice after exercise together with a smeared pattern of DNA, which suggests a complete digestion of DNA typical of the necrotic process. The possibility that inflammatory cells contributed to DNA fragmentation is not excluded, but a myonuclear origin of the DNA fragments is suggested by the presence in myonuclei of apoptotic features detected by *in situ* nick-end labelling and by electron microscopy. Normal myofibrillar fields around apoptotic nuclei distinguish myonuclei from nuclei of satellite cells, endothelia, fibroblasts and eventual invading macrophages. In 15% of nuclei of *mdx* muscles after exercise electron microscopy documents typical features of apoptosis with condensed chromatin around myonuclear membrane (submitted manuscript).

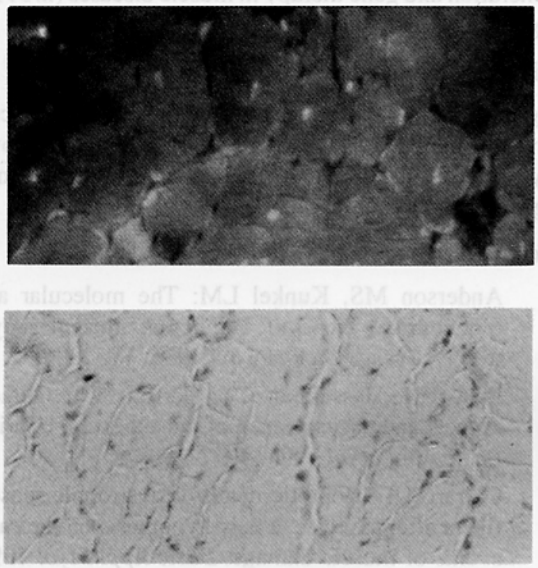

Figure 1. Exercise-induced ubiquitination of myonuclear proteins and DNA fragmentation in Tibialis anterior muscle of dystrophic (mdx) mouse. a, ubiquitin immunolocalization in muscle fiber: numerous cells presents a positive reaction in centrally located myonuclei in running mdx mouse; b, in situ analysis of DNA fragmentation detected by digoxigenin nucleotide labelling of 3'-OH DNA ends (Apoptag, Oncor) shows that a high proportion of centrally-located myonuclei are positive after exercise in mdx mice.

Massive activation of proteases is one candidate in triggering cell apoptosis and it is implicated in nuclear proteins catabolism and in lamin-DNA fragmentation [8, 10]. Which is the protease system associated is still unknown, one candidate could be ubiquitin. Ubiquitin, binding proteins for successive degradation, influences the life of several important proteins for apoptosis such as p53, c-myc, BAG-1 [9, 19], and a relationship between ubiquitin and DNA fragmentation is clearly shown in the present paper.

When the distribution of ubiquitin and ubiquitin-conjugated proteins is investigated by SDS-PAGE and Western blot [13, 14] in supernatants and myofibrills of muscle homogenates, low level of free ubiquitin is constantly shown in all studied muscles, in good agreement with published data [4, 6, 25]. This observation could be related to the ceased expression of stress proteins two days after exercise, since shock and other stress cause only transient increase in free ubiquitin [9]. In the soluble fraction of exercised *mdx* muscle we detect an increased content of ubiquitin-conjugated proteins compared with muscles of both *mdx* and BALB c mice at rest: the exercised *mdx* muscles contain at least ten times of the amount present in the muscles of sedentary mice. Similar results are obtained in the myofibrillar fractions. The highest level of ubiquitination is detected in *mdx* mice after exercise. Densitometry of ubiquitin-reacting bands shows that ubiquitin linked to contractile proteins increases two-three times in comparison with the ubiquitin amount of the *mdx* and BALB c sedentary mice muscles (submitted manuscript). On the other hand, *in situ* analysis suggests that exercise-induced ubiquitin is preferentially linked with nuclear proteins. This has been related with DNA damage and could be important for fragmentation of histones or nuclear matrix proteins, as lamin, or for changes of nuclear structure during the apoptotic process [8, 10]. Also, some myoplasm proteins were labelled indicating that proteinase activity is generalized. The widespread expression of ubiquitin and its capacity to link with multiple nuclear and cytoplasmic

Table 1. Apoptosis and ubiquitination of myonuclei in muscles of sedentary and exercised mdx mouse.

Group	Per cent of positive nuclei			
	Apoptotic nuclei		Ubiquitinated nuclei	
	Individual mice	mean ± SEM	Individual mice	mean ± SEM
BALB c				
non runner	0, 0, 3, 0	1 ± 1	0, 0, 5, 0, 0	1 ± 2
mdx				
non runner	4, 0, 6, 3, 0	3 ± 3	6, 4, 8, 7, 8	6 ± 2
runner	28, 22, 34, 20, 25	25 ± 5	32, 30, 28, 38, 37	33 ± 4

proteins suggests a major role in regulating apoptosis and other mechanisms of muscle damage. Recent *in vitro* studies underlay the role of cell death in regulating myoblast proliferation at fusion [15, 16], and this could be relevant in regenerating myofibers of *mdx* mice, in particular after exercise. On the other hand, *in vivo* apoptotic myonuclei were found in mature myofibers indicating a pathogenetic role of the mechanisms of programmed cell death in exercise-induced muscle damage in dystrophynopathies. The secondary pathogenetic processes by which a lack of dystrophin/dystrophin associated glycoproteins leads to progressive muscle degeneration in muscular dystrophies is an open issue. A number of possible mechanisms have received attention: changes in plasma membrane permeability, a specific defect in muscle intracellular free calcium homeostasis, and a decreased mechanical stability of the muscle plasma membrane and of the sarcomer [1-3, 11, 23]. It is a general expectation that exercise-induced damage plays a role in the myodystrophic process and that modifications of the training programs of muscles may have some importance in influencing muscle degeneration in patients with muscular dystrophies. It is well known that exercise in an unaccustomed muscle provokes mild injury, soreness and lactic acid accumulation. Our observations that a spontaneous running in unaccustomed animals increases the number of apoptotic myonuclei in differentiated muscle fibers of adult *mdx* mice shed a light on the pathogenesis of the post-exercise muscle injury. We suggest that exercise-induced damage or fatiguing exercise itself activates the program of cell suicide in *mdx* muscle possibly because of unbalanced calcium homeostasis or because of an increased generation of reactive oxygen species during reperfusion. Muscle cells initiate the apoptotic process activating the process of DNA fragmentation and the protease system. Only some myofibers reach the final steps of apoptosis, i.e., chromatin condensation and apoptotic body formation. In spite of the clear difference between sedentary and exercised *mdx* mice observed, myonuclei showing apoptotic features by electron microscopy were one/half of positive myonuclei for both ubiquitin and *in situ* DNA end-labeling. In conclusion, exercise-induced muscle damage in *mdx* mice suggests new roles of ubiquitin related to nuclear events, and it provides evidence for a new and provoking pathogenesis in dystrophinopathies, which could open new pharmacologic strategies in managements of exercise-induced muscle damage and muscle dystrophies.

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