Apoptosis, DNA damage and ubiquitin expression in normal and *mdx* muscle fibers after exercise

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Abstract The current view indicates that after eccentric exercise myofibers are mechanically damaged and therefore an inflammatory and necrotic process occurs. In the present paper we examine the possibility that apoptosis plays a role in normal and dystrophin-deficient muscles after running. We analysed for apoptosis normal and dystrophin-deficient mouse muscles after a night of spontaneous wheel-running followed by two days of rest. Terminal deoxynucleotidyl transferase-mediated end-labeling of DNA in nuclei in tissue sections and gel electrophoresis of extracted DNA showed the presence of fragmented DNA. Furthermore, ubiquitin, a protein whose appearance is related to apoptosis, increased in muscles of both dystrophic and normal runner mice. The present findings which confirm that DNA damage is absent in muscles of sedentary mice but present in muscles of runner mice offer a new hypothesis on early events of muscle damage.

Key words: Apoptosis; Ubiquitin; Muscle damage

1. Introduction

Apoptosis is a specific form of cell death that plays an important role in development, growth regulation and diseases [1]. Apoptosis in thymus has been extensively studied and it is characterized by an early and intense double-stranded cleavage, that is normally followed by a nucleosomal ladder when the DNA is electrophoresed [2]. The nucleus morphologically appears condensed and it displays marginated chromatin. The nuclear feature (peripheral chromatin condensation, nuclear fibrillar centre, the osmiophilic nucleoplasmic granules) in an apoptotic cell, as it appears in thymus and epithelial cells, is so typical that has been considered the hallmark of apoptosis also in absence of DNA ladder [3]. Recently there have been a number of reports of cell death with the morphological features of apoptosis without the generation of a detectable ladder of nucleosomal fragments. Oberhammer et al. [4], Brown et al. [5] and Walker et al. [6] have demonstrated that DNA cleavage in apoptosis involves an initial cleavage into high molecular weight fragments (HMW) of 700–300–50 kbp, followed by endonuclease cleavage into nucleosome or oligonucleosome-sized fragments. These two steps of DNA fragmentation are catalysed by two different endonucleases and the later stage of DNA degradation (ladder) is blocked by inhibitors of serine protease and Zn [6]. Two processes must be distinguished in apoptosis, the first called 'priming' involves accumulation into the cell of the machinery which permits to apoptosis to take place, the second is the 'triggering' of primed cells into apoptosis itself [7]. Little is known about the stimuli that prime cells for apoptosis. Priming is reversible and may be part of a strategy in the regulation of cell population, since cells primed for apoptosis are all doomed to die unless rescued by specific growth factors. Apoptosis follows triggering immediately, but the time between priming and triggering may vary. Few informations are available about apoptosis in fully differentiated cells especially when cells are syncytia as the muscle fibers. Only a massive muscle cell elimination by apoptosis had been described in acute infantile spinal muscular atrophy [8]. Recently we and others showed that this could be the case for adult *mdx* mouse muscle [9,10]. In the present paper we investigate the possibility that mild exercise plays a role in DNA damage and ubiquitin expression of either normal and *mdx* muscle. Preliminary results were reported in abstract form [11].

2. Materials and methods

2.1. Animal experiment

Ten normal and ten *mdx* C57 adult mice were used at 4 weeks and were divided in runner and non-runner animals. The runners were housed in a wheel-cage and let them run spontaneously for an entire night. Two day after mice were sacrificed. The Tibialis Anterior (TA), EDL and Soleus muscles of both hind limbs were removed. Muscles from right limb were fixed in 10% formaldehyde and embedded in paraffin. The muscles were cut transversely into 6 μm thickness slices, and mounted in Canadian balsam on polylysine pre-coated slides. Muscles from left hind limbs were frozen in liquid nitrogen and stored at −80°C.

2.2. In situ DNA nicklend labeling

Paraffin embedded tissues were deparaffinized and proteins in tissue sections were digested by applying proteinase K (20 μg/ml) to specimen for 15 min at room temperature. Specimens were then washed in distilled water. Cryostat sections were fixed in 10% neutral buffered formalin for 10 min at room temperature. After washing in PBS slides were post-fixed in ethanol/acetic acid (2:1) for 5 min at room temperature. Endogenous peroxidase activity was blocked by applying 2% hydrogen peroxide in PBS on slides for 5 min at room temperature. In situ end labeling of fragmented DNA was performed using terminal deoxynucleotidyl transferase with digoxigenin-conjugated nucleotides followed by immunodetection of incorporated nucleotide using the ApopTag In Situ Apoptosis Detection Kit-Peroxidase distributed by Oncor as de-

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Abbreviations: EDL, Extensor Digitorum Longus; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ROS, reactive oxygen species.

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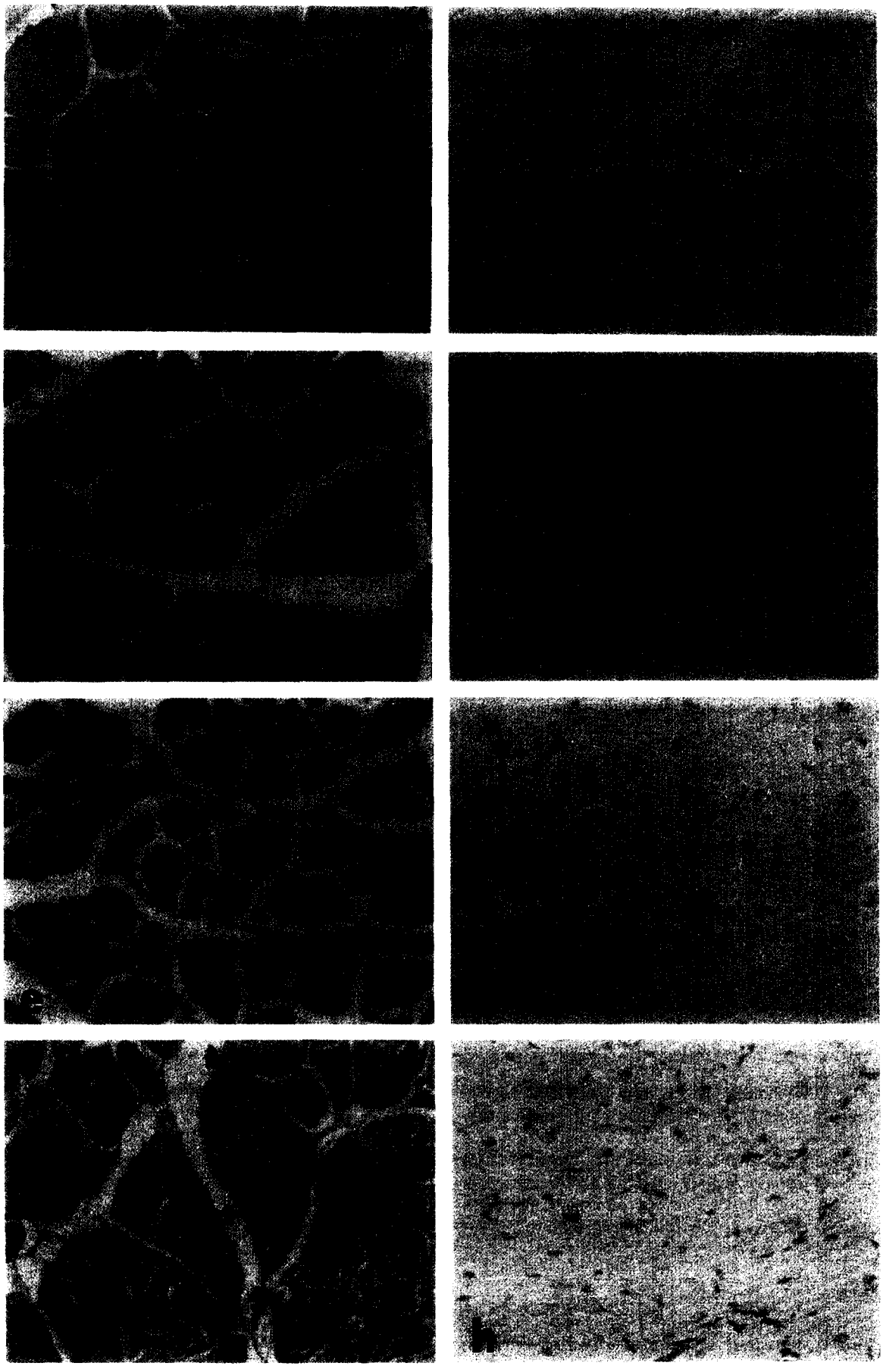

Fig. 1. In situ DNA end-labeling for detection of apoptotic myonuclei in sections of tibialis anterior muscle. Positive myonuclei appear coloured dark brown. Tibialis anterior muscle of (a) normal non-runner mouse, hematoxylin-eosin stained, (b) the same muscle with in situ nick/end labeling method, staining is undetectable. (c) normal runner mouse, hematoxylin-eosin stained, (d) runner normal mouse which presents positive myonuclei (brown) in the presence of counterstain nuclei with methyl-green (green). (e) non-runner *mdx* mouse, hematoxylin-eosin stained, (f) the same muscle with in situ nick/end labeling method, staining is undetectable. (g) runner *mdx* mouse, hematoxylin-eosin stained, (h) runner *mdx* mouse which presents positive myonuclei (brown) in the presence of counterstain with Methyl-green (green).
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scribed by the manufacturer instructions. Positive nuclei in three randomly chosen fields were counted per muscle. Results are expressed as number of positive muscles in each group on animals and percent of positive nuclei in each muscle (mean ± S.D.; statistical significance is calculated using the Student's *t*-test).

2.3. DNA isolation and analysis

For isolation and electrophoresis of DNA, one half of the five muscles of each experimental group were pooled and lysed in extraction buffer (proteinase K 1 mg/ml, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS). The samples were incubated overnight at 50°C and then treated with DNase free RNAse (100 μg/ml) for 1 hr at 50°C. DNA was phenol/chloroform extracted, ethanol-precipitated, and resuspended in 10 mM Tris-HCl, pH 8, 10 mM EDTA, pH 8 (TE). The extracts were subjected to electrophoresis either in a 2.2% agarose gel either in a 7% polyacrylamide gel and stained respectively with ethidium bromide and with silver stain.

2.4. Ubiquitin

The residual tissues of the five muscles of each experimental group were pooled and homogenized with a Polytron apparatus (PT 10 0D) in 50 mM KCl containing 10 mM EGTA, 30 μM pepstatin, 12 μM phenylmethylsulfonylfluoride (PMSF) and 1 mM benzamidine to reduce effects of endogenous proteases. An aliquot of the homogenated sample was dissolved with a volume of 10% v:v glycerol, 2.3% SDS, 5% 2-mercaptoethanol, 62.5 mM Tris-HCl pH 6.8 (Sol A). The remaining material was centrifuged at 650 × *g* for 10 min, the supernatant was collected and stored at −20°C after adding equal volume of Sol A and boiling for 5 min. Protein concentration was determined by the Coomassie brilliant blue method [12]. Aliquots of both homogenate (100 μg) and supernatant (30 μg) were analyzed by 12.5% SDS-PAGE and Western blotting. The following conditions were used for antibody binding: anti-ubiquitin from Sigma 1:300 dilution, 20°C, 2 h; anti-rabbit alkaline phosphatase linked (Sigma) 1:4000, 20°C, 1 h. Densitometric scanning of immunoblot was performed using a GS 300 Transmittance-Reflectance Scanning Densitometer (Hoefer Scientific Instruments) connected to a Macintosh Plus (Apple computer). Data were processed using the GS-370 Data System for Hoefer GS 300 Scanning Densitometer, Macintosh version according to Rossini et al. [13].

3. Results

3.1. Apoptotic myonuclei detected by in situ DNA end labeling

The running and non-running muscles of normal and *mdx* mice were tested in situ end labeling to detect DNA double strand breaks (DSB). With this method minimal digoxigenin labeling was detected in nuclei of non runner mice muscles (Fig. 1b–1f) while a large number of myonuclei became positive in both normal and *mdx* runner mice muscles (Fig. 1d–1h). In particular, the peripheral myonuclei of normal runner mice stained with hematoxylin-eosin (Fig. 1c) are positive for DSB detected with ApopTag method (Fig. 1d). The *mdx* runner mice muscles show foci of chronic inflammation with the presence of infiltrating leucocytes, and small regenerating myofibers with centrally located myonuclei (Fig. 1g). Fig. 1h shows that inflammatory cells (leucocytes with green nuclei) are negative while centrally located myonuclei are positive for apoptosis (brown). Table 1 shows that the difference in apoptotic nuclei between runner and non-runner mice muscles is statistically significant. The difference in the number of positive mice (three over five for *mdx*, vs. one over four for normal mice) though not statistically significant suggests that apoptotic events could be more frequent in the sedentary *mdx* mice.

3.2. Organization of chromosomal DNA

The DSB seen with nick/end labeling method that follow exercise coincide with alteration in the organization of chromosomal DNA in *mdx* runner mice. The fragmented chromosomal DNA can be visualized by isolating genomic DNA, subjecting it to electrophoresis size fractionation in agarose, and staining it with ethidium bromide (Fig. 2). DNA from normal runner, non runner and *mdx* non-runner muscles is high-M_r DNA and remains at the top of the gel. However, the DNA from *mdx*

Table 1
Apoptotic myonuclei detected by in situ DNA end labeling in non-running and running muscles of normal and *mdx* mice

Group	No. of animals	No. of animals with positive nuclei	Percent of positive nuclei in each animal (mean ± S.D.)	
Normal				
non-runner	4	1	0,0,3,0	1 ± 1
runner	5	5	19,30,2,18,29	20 ± 11[a]
mdx				
non-runner	5	3	4,0,6,3,0	3 ± 3
runner	4	4	28,37,34,1	25 ± 16[b]

[a]Student's *t*-test: $P = 0.014$ vs. non-runner; [b]Student's *t*-test: $P = 0.018$ vs. non-runner. The differences between normal vs. *mdx* mice are not significant.

Fig. 2. Profile of total DNA isolated from Tibialis Anterior muscle of running and non-running mouse. DNA purified by phenol/chloroform extraction. Lane 1, T cells treated with dexamethasone for 6 h; lanes 2–3, DNA extracted from *mdx* and normal non-running muscle respectively; lanes 4–5, DNA extracted from *mdx* and normal running muscle respectively.

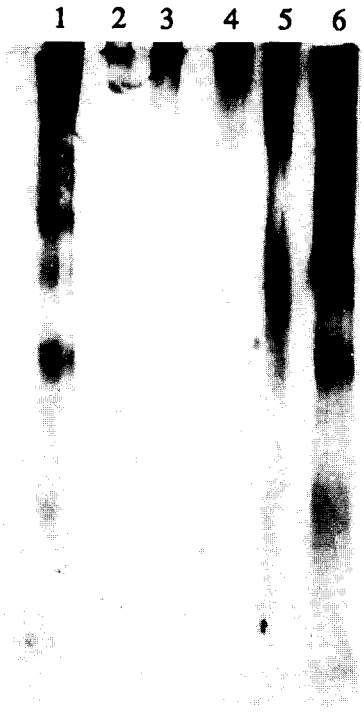

Fig. 3. Profile of total DNA purified by phenol/chloroform extraction and subjected to polyacrylamide gel electrophoresis. Lanes 1 and 6, DNA extracted from T cells treated with dexamethasone for 6 h, 100–500 ng respectively; lanes 2–3, DNA extracted from normal non-running and running muscle respectively; lanes 4–5, DNA extracted from *mdx* non-running and running muscle respectively.

runner mice presents clear signs of fragmentation. To rule out the possibility that some small percentage of the DNA was degraded into a nucleosomal ladder but was below the level of detection of ethidium bromide, the DNA was subjected to polyacrylamide gel electrophoresis followed by silver stain (Fig. 3). Although some sheared DNA was present in several samples, probably due to damage during isolation, there is no evidence of any nucleosomal ladder in normal runner, non runner and *mdx* non-runner. Instead in *mdx* runner fragmented DNA were detected particularly at nucleosomal and oligonucleosomal level.

3.3. Expression of ubiquitin

As described in literature, the programmed cell death and/or apoptosis requires de novo expression of specific sets of genes. One of these is the ubiquitin gene. We investigate the presence or absence of polyubiquitin in the supernatant of muscle homogenate after SDS-PAGE and Western blot. A polyubiquitin band (30–35 kDa) is detected in the supernatant, as previously described [14]. Fig. 4 shows that polyubiquitin amount is low in normal muscle according to the literature [15] but accumulates at higher levels when the muscles were committed to exercise.

After immunoreaction the band were scanned and quantified as described in section 2. The results confirm that polyubiquitin content is at least twice as high in runner muscles compared with non-runner mice muscles (Table 2).

4. Discussion

Muscles in adult animals are fully differentiated syncytial cells and apoptosis, which is known to be present in tissues that modulate their cellular homeostasis under the influence of growth-hormonal factors, should be, in theory, absent.

Regeneration after injury, if takes place, starts from satellite cells, substituting the loss of myofibers. It is well known that exercise in an unaccustomed muscle provokes mild injury, soreness and lactic acid accumulation. Smith [16] suggested that in adult muscle the events observed after an eccentric muscle exercise reflect the typical sequence of acute inflammation which follows the unknown primary damaging event, usually related to the mechanical overloading of the intra-inter fiber components induced by the contractile machinery. The events are divided in time lapses of 24 h and comprise accumulation of neutrophils in the first 24 h, release of lysosomal enzymes till 48 h, release of inflammation mediators and signs of repair at 72 h. The data here presented shed a light on the pathogenesis of the post-exercise muscle injury, since DSB, detectable after exercise, precedes any sign of necrosis, indicating that DNA damage is present while inflammation is in progress. We showed by in situ nick/end labeling the presence of apoptotic myonuclei in *mdx* mice [9], in line with a recent report about apoptosis in dystrophin-deficient muscles [10], and now we are able to show by DNA analysis the presence of DNA damage. In both normal and dystrophic non-runner adult muscles apoptosis is absent when analysed by electrophoresis of extracted DNA. The percent of positive nuclei (about 30%) for in situ nick/end labeling in *mdx* and normal runner mice raises the

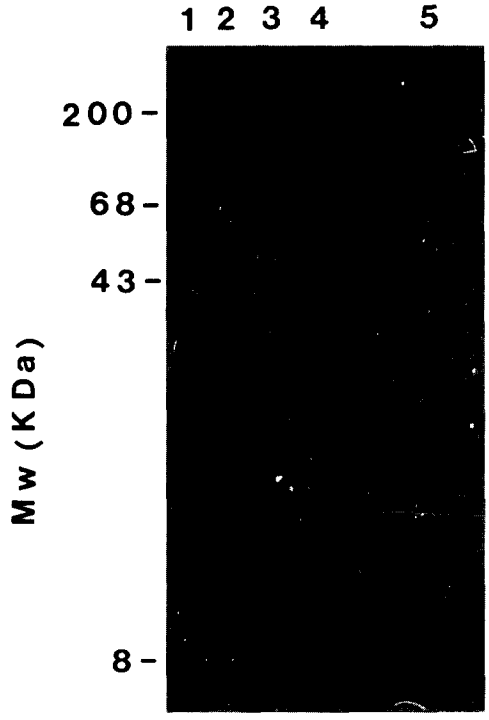

Fig. 4. Analysis of ubiquitin by Western blot of supernatant from homogenate of runner and non-runner muscles. Lane 1, normal non-runner muscle; lanes 2–3, normal and *mdx* runner muscle; lane 4, *mdx* non-runner muscle; lane 5, 1 μg of ubiquitin. Myosin heavy chain (200 kDa), bovine serum albumin (68 kDa), actin (43 kDa), and ubiquitin (8 kDa) were used to calibrate the gel electrophoresis.

Table 2
Ubiquitin content after Western blot and densitometry

Muscle	Total protein content (μg)	Polyubiquitin content (arbitrary units)
Normal		
non-runner	30	2500
runner	30	5830
mdx		
non-runner	30	3000
runner	30	5100

problem of the significance of the phenomenon, since the percent of positive myonuclei largely exceeds the percent of the slow type myofibers that are known to be prone to undergo to necrosis after mild exercise. However, the DNA analyses show the breakdown of chromosomal DNA into nucleosome-size fragments only in *mdx* runner mice muscles. The non-detected fragmentation in normal runner mice muscles could be explained by the presence of DNA fragments of so high molecular weight (50–300 kbp) that they are not detectable with normal gel electrophoresis. The observed DNA damage, probably the initiation of apoptosis process, was presumably due to an accelerated muscle metabolism during exercise or to a production of reactive oxygen species (ROS), facilitated by the increased catecholamine level induced by stress, not compensated by normal glutathione cycle and/or normal cellular ROS scavengers. Indeed the recent findings [17] that thiol compounds inhibit endonuclease and protect from apoptosis while glucocorticoid hormones, which are produced in stress conditions, facilitate the DNA fragmentation are able to explain why DSB are present in runner muscle mice. Of particular interest is the observation that a real myonecrosis follows the DNA damage observed in muscle after mild exercise only in a few percent of slow-type fibers [18]. Our data also show that chromatin fragmentations occurs with a parallel increase in polyubiquitination. At present, the genes and the proteins responsible for apoptosis are largely unknown. One candidate could be the ubiquitin [19] which can be covalently linked to cellular proteins to mark them for degradation. Increasing amount of ubiquitin are described in developing muscle *Manduca* [20] and other insects [21] which are good model for studying programmed cell death. Polyubiquitin is present in normal muscle, being related to protein turnover, and its content is increased in many muscle-wasting conditions in rodents (see for review [15]). Indeed, Schwarts et al. [22] described ubiquitin linked to actin and a preferential degradation of actin and myosin heavy chain in *Manduca* muscle. With immunoblot technique we found in runner mice muscles an increased level of an anti-ubiquitin reacting band which is present at lower level in normal mice muscles. Purification and identification are in progress, however preliminary results suggest that the protein is only polyubiquitin.

A reasonable hypothesis could be that in myofibers, with prevalent oxidative metabolism (slow type), the amount of ROS produced overwhelms natural cellular scavengers accompanied by reduction of thiol groups, and caused DNA damage. The data we have obtained are in line with the hypothesis that mild exercise is followed by inflammation but is preceded by DNA damage and apoptosis in which ubiquitin can play a pivotal role in protein degradation. Further information are needed in order to understand why differentiated cells as adult myofibers might undergo apoptosis. Gottlieb et al. [23] recently described apoptosis in rabbit cardiomyocytes after reperfusion injury, that might be the case of skeletal muscles after wheel-running. We believe that the nuclear events we here described are of the foremost importance in understanding the pathogenesis of exercise-induced damage, favouring the hypothesis of a peculiar fragility of dystrophic myofibers to normal-life stress in view of the presence of an apoptotic process in *mdx* mice after a mild exercise.

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