Signs of Necrosis and Inflammation Do Not Support the Concept of Apoptosis as the Predominant Mechanism During Early Atrophy in Immobilized Muscle

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Abstract

The contributions of potential autophagic or inflammatory reactions to the induction of skeletal muscle atrophy during immobilization are not yet completely understood. The aim of this study was to comparably investigate them over a short period of immobilization, with special emphasis on biochemical data and their probable morphological correlates. Twenty-four Charles River mice were divided into four experimental groups (each n=6) with one hindlimb immobilized for different periods of time (24h, 48h, 72h, and 7days, respectively). After cervical dislocation, both soleus muscles were completely removed and their wet weight was determined. The β-glycuronidase activity (βGlu), for muscle fiber autophagic response, and myeloperoxidase activity (MPO), for lysosomic activity of leukocytes were determined, always related to muscle protein content. All data were compared to the contralateral control muscles (=100%). A part of each muscle was examined morphologically in LM and EM. Muscle wet weight decreased progressively, most pronounced at 24h (15%), but slowing down already after 48h. βGlu activity increased significantly at 24h, returning to the basal levels at 48h group. At 48h and 72h, MPO showed a significant increase. The morphologic examination revealed the presence of lysosomes, mitochondrial swelling, and edema especially at 24h and 48h. Leucocytes infiltration was largely observed after 48 hours of immobilization. These data suggest that during immobilization atrophy, muscle wasting is initiated by an autophagic reaction followed by an inflammatory response. It therefore appears that necrosis is more important than apoptosis in immobilization atrophy.

Key words: muscle atrophy, immobilization, β-glucuronidase, myeloperoxidase, necrosis, inflammation.

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Although several studies have described alterations in functional, structural, and biochemical properties of skeletal muscles after immobilization (for review see [3, 7]), little is known about the underlying cellular mechanisms leading to wasting of muscle tissue. However, the most important processes appear to be triggered during the first days of immobilization [4, 16].

Several intrinsic tools of fiber wasting could be responsible for muscle atrophy induced by immobilization, contributing to an increased degradation of proteins [18] and a marked reduction in protein synthesis [19]. Such results in eventual structural disturbances and degeneration [16] probably triggered by a loss of calcium homeostasis [23]. The autophagic reaction could also play an import instrument in this destructive concert since an increase in lysosomic activity has been described in immobilized muscles, particularly during the first days [5, 26]. On the other hand, experiments with animals flown on a Shuttle [9] have suggested that the loss in muscle mass due to hypogravity resulted from apoptosis as the main mechanism of atrophy. However, different models of experimental atrophy (e.g. tenotomy, immobilization, denervation, weightlessness) cannot easily be compared

with regard to the results and to the underlying mechanisms [3].

It had been shown in overloaded muscles that inflammatory cells invaded into muscle acting as scavengers to remove fiber debris and allowing for consecutive muscle repair [6]. Leukocytes could also, in the sense of an extrinsic aggressive mechanism to the fibers, induce or at least aggravate the development of structural abnormalities observed after muscle immobilization [8] considering the cytotoxic effects of their components liberated to the interstitial space.

The aim of this study was to investigate the contribution of intrinsic autophagic factors to muscle atrophy and to search for signs of an inflammatory response over a short period of immobilization, with special emphasis on biochemical data and their probable morphological correlates. It is expected from these experiments to gather more data explaining the mechanisms of muscle atrophy either towards predominant necrosis or towards apoptosis.

Material and Methods

The experiments were performed after approval of the local ethic committee for animal experimentation on 24 Charles River mice (males, aged 12 weeks with a body weight of 36-40g) maintained under standardized conditions with regard to housing environment (four animals per cage at 22±2°C) and feeding following the international guidelines for the care and use of laboratory animals.

The right hindlimb of all animals was immobilized under light pentobarbital sodium anesthesia with a plaster cast for different periods of time, and the animals were assigned to four groups (6 mice/group) according to the time of immobilization: 24h (group 1d), 48h (group 2d), 72h (group 3d) and 1 week (group 7d). All casts were adjusted to neutral positions of the ankle and knee joints. The animals of each group were sacrificed by cervical dislocation and the plaster cast was removed after the respective experimental periods. Both soleus muscles were completely dissected and their wet weight was determined with an accuracy of $1x10^{-5}$ g using a Kern® 870 balance. The left soleus muscles were taken as control in all groups.

Each muscle was immediately divided into two pieces. One was frozen at -70ºC for further biochemical analysis. The other part, for qualitative LM and EM evaluation, was prefixed with 2.5% glutaraldehyde and was transferred to 2.5% glutaraldehyde for 2 hours in portions of $1mm^3$ each. The specimen were postfixed with 1% osmiumtetroxide, dehydrated in graded alcohol, and embedded in Epon. Semithin sections for light microscopy were stained with toluidine blue, ultrathin sections for electron microscopy were contrasted with uranylacetate and lead citrate.

The frozen portion of the muscle was thawed and mechanically homogenized in 1 ml of tris-buffer at pH 7.4 for further determination of the following biochemical parameters: (i) myeloperoxidase (MPO) activity as a principal marker for lysosomic activity of leukocytes determined by spectrophotometry using a commercial kit (MPO-EIA, Nº 21013D, Bioxytech®) and (ii) β-glycuronidase (βGlu) activity as a principal marker for muscle fiber autophagic activity, determined by spectrophotometry using a commercial kit (325A, Sigma Diagnostics). The enzyme activities were normalized to the muscle protein content measured by spectrophotometry using a commercial diagnostic kit (690A, Sigma Diagnostics).

All data were expressed as percentages of the contralateral control muscles (=100%) using means with standard deviations. For statistical analysis between the different experimental groups, the one-way ANOVA for repeated measurements was used with post hoc comparison using the Scheffe F-test. The level of significance was set at α of 5%.

Results

Muscle wet weight decreased significantly over the period of immobilization, (Fig. 1) obviously most pronounced (15%) at 24h of immobilization. After 48h, the decrease in muscle weight started to slow down,

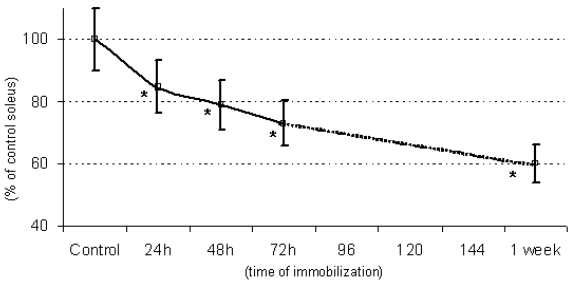

*Figure 1. Mean values and standard deviations of soleus muscle wet weight (expressed as % of the contralateral muscles) after different periods of immobilization. * Significant differences (p<0.05) from controls.*

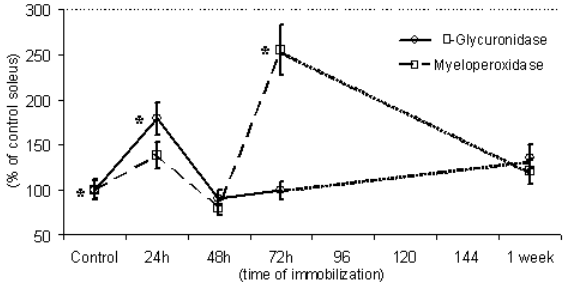

*Figure 2. Mean values and standard deviations of β-Glycuronidase activity and myeloperoxidase activity in soleus muscle (expressed as % of the contralateral muscle) after different periods of immobilization. * Significant differences (p<0.05) from controls.*

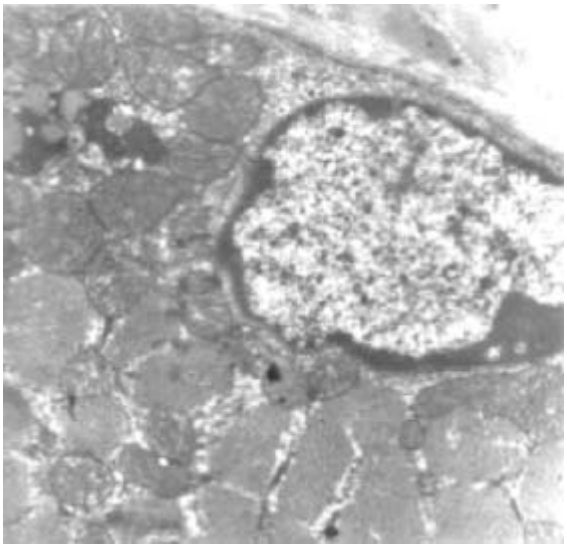

Figure 3. Secondary lysosomes (upper left corner) and some mitochondrial alterations in a muscle fiber immobilized for 1 day; note the normal appearance of the nucleus; original magnification x12,000.

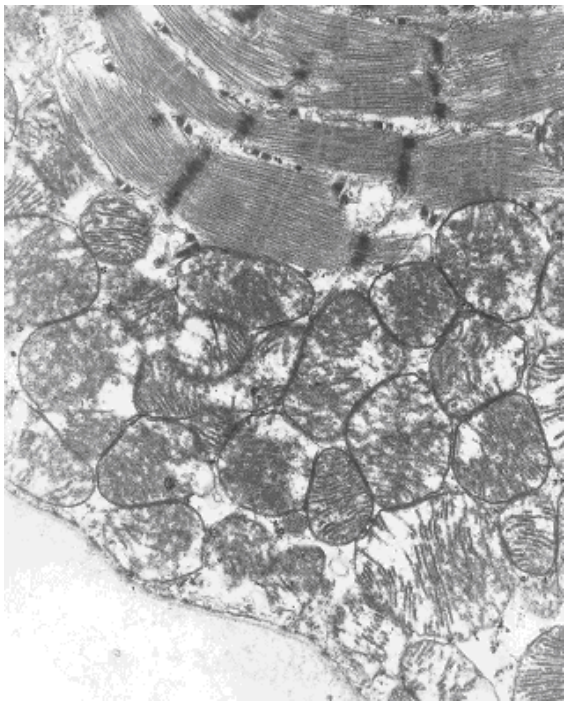

Figure 4. Pronounced mitochondrial swelling with cristolysis after 1 day; original magnification x24,000.

until the muscle had experienced a weight loss of about 40% after 1 week (Fig. 1). The βGlu activity increased significantly at 24h, returning to the basal levels at 48h (Fig. 2). At 48h and 72h the MPO showed a significant increase (Fig. 2) indicating the presence of activated leukocytes.

The control muscles did not show significant alterations of the above parameters over the experimental period, nor did they show remarkable signs of pathomorphology.

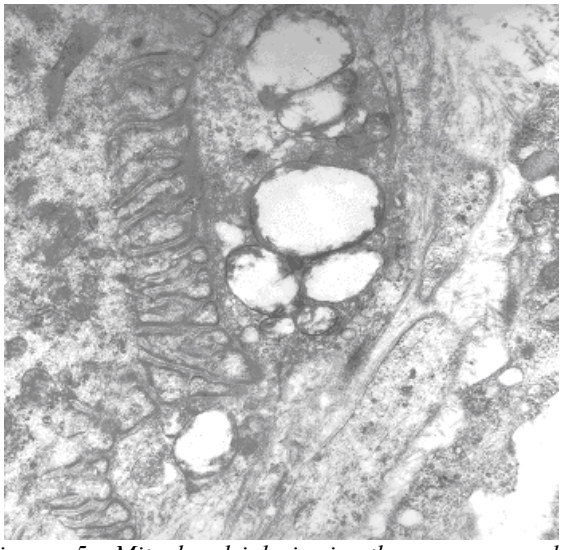

Figure 5. Mitochondriolysis in the neuromuscular junction after 2 days; original magnification x24,000.

The encountered morphological alterations in immobilized muscles appeared to be time dependent, eventually being more severe with longer immobilization periods. At 24 hours of immobilization, vast lysosomes, mitochondrial swelling also at the neuromuscular junctions, and edema were observed in the muscle fibers (Fig. 3, 4, 5). Already after 48 hours of immobilization, but more pronounced after 72 hours, several invading leukocytes were encountered, particularly eosinophils (Fig. 6) and phagocyting cells as well as natural killer cells. Moreover, some interstitial edema was observed. Activated satellite cells (Fig. 7) and central nuclei with numerous nucleoli were also present, suggesting an increase in protein synthesis. The fiber sarcolemma often appeared scalloped with pseudopodia-like processes protruding into the interstitium (Fig. 8). This typical alteration in fibers undergoing atrophy tended to increase until 1 week of immobilization. The cellular edema was also more pronounced at 1 week of immobilization (Fig. 9). The interstitial space was enlarged at the same time, filled with collagen fibers and activated fibroblasts, and many fibers presented residual bodies but not many secondary lysosomes. No signs were found suggestive for apoptosis at any of the time points studied.

Discussion

Taking contralateral muscles as controls in immobilization studies, one has to be aware that the control legs had to sustain some mechanic stress (though the immobilized animals moved less and were intentively housed in small cages) because of the hindimb monopedal movement pattern and also because of the additional weight of the plaster cast. It therefore cannot be excluded that the control muscles experienced

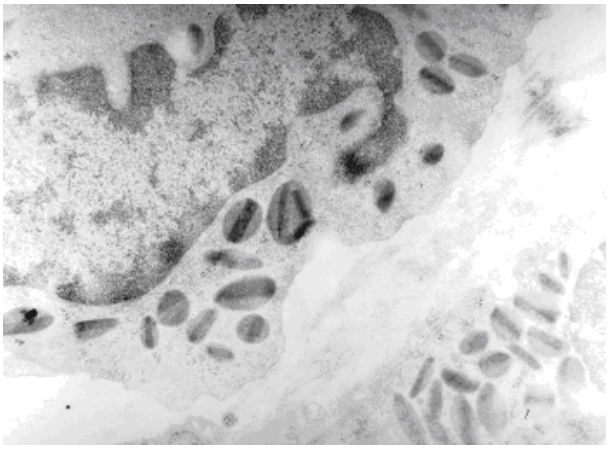

Figure 6. Eosinophilic granulocyte in the muscle after 3 days; original magnification x24,000.

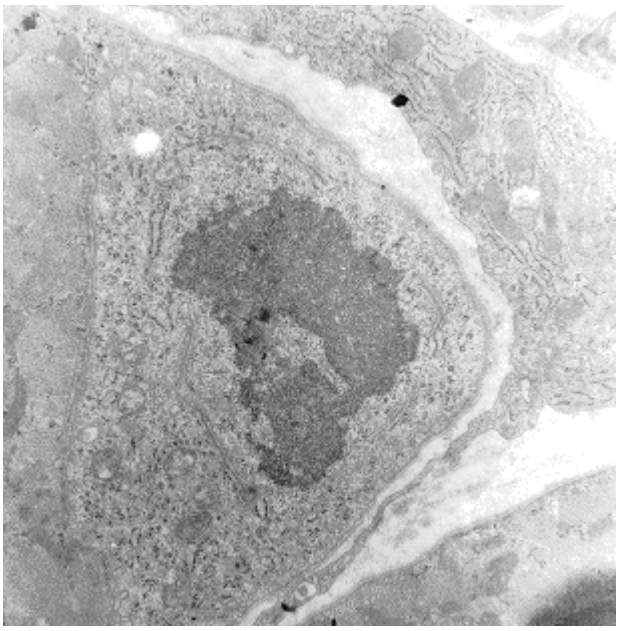

Figure 7. Activated satellite cell after 3 days; original magnification x24,000.

some kind of overload. The parameters studied, however, of all control mucles had not changed singnificantly over the experimental period, which should justify taking them as valid controls.

The loss in muscle wet weight during one week of immobilization was in the same range like described for muscle fiber area of immobilized mice and followed the same pattern with more initial atrophy fading towards the end of a week [2]. The morphological alterations found in the immobilized muscles were typical for atrophy and in accordance with those described by other authors using similar experimental setups [22]. Such has recently also been described in disused muscles of old subjects [21]. The structural impairments even extended to the neuromuscular junction, which have to be remodelled during remobilisation [28].

It appears therefore that any mechanism had been present that have to be held responsible for inducing

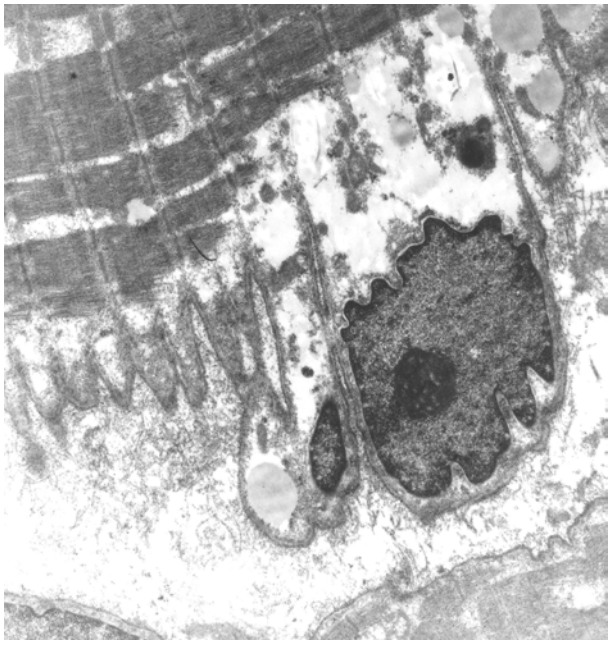

Figure 8. Edematous fiber with scalloped sarcolemma after 7 days; original magnification x24,000.

muscle atrophy during the first seven days of immoblization. The first functional alterations observed in this study was a marked increase in βGlu activity with the structural correlate of many lysosomes found in the muscle fibers. The lysosomal system is probably activated by a loss of calcium homeostasis in the sense of intracellular calcium accumulation, which has been described to occur during the early phase of immobilization [11]. The administration of a calcium channel blocker has been demonstrated to be beneficial in reducing muscle atrophy [23, 27]; this suggests that this mechanism (and most probably also the early autophagic process) may play, like in the suspension model [11], a pivotal role in initiating muscle atrophy during immobilization.

Although the present study did not determine the rate of protein synthesis or degradation, it is suggested that degradation is an important factor in immobilization atrophy opposed to the suspension model with a predominantly reduced protein synthesis rate as the cause of atrophy [12, 19]. Beyond the lysosomal autophagic activity that has been documented at both, the morphological and biochemical level in this study, another molecular mechanism could explain the protein breakdown in immobilized muscle: The ubiquitin-proteasome pathway leads to accelerated proteolysis especially in myofibrillar proteins [17], and polyubiquitin expression has been reported to be increased fivefold in immobilized muscle [24]. With regard to the activation of those two mechanisms, oxidative stress imposed on immobilized muscle, especially during the early phase, has to be considered [14, 15]. It can not only severely impair membrane functions and is assumed to be strongly linked to the

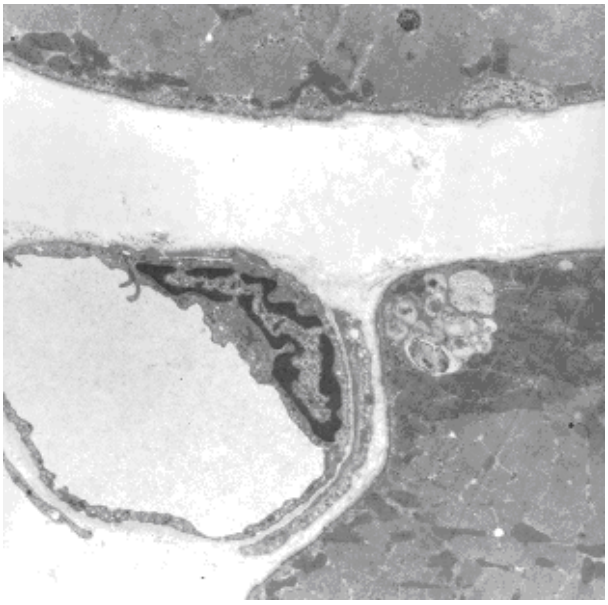

Figure 9. Interstitial edema after 7 days, note residual bodies in the lower right muscle fiber; original magnification x14,400.

loss of calcium homeostasis, but also exerts direct protein oxidation contributing to a higher protein degradation rate. Pharmacological protection against oxidative stress during the early phase of immobilization atrophy appeared to be a promising approach to minimize atrophy [4]. However, the contribution or timing of either of the above described mechanism to the net atrophy still remains to be studied.

Considering many recent studies reporting apoptosis as an important mechanism of reduction of muscle mass during atrophy, it appeared somewhat strange that we did not find any signs typically described for apoptosis at the morphological level [20]. It is widely accepted that apoptosis occurs at an early stage in muscle atrophy induced by hindlimb suspension or spaceflight [1, 9], and if apoptosis was also important in immobilization atrophy, we should have been able to find any morphological signs in this short-term study. However, histochemical markers suggestive for apoptosis have been found in immobilized muscles, but it had been emphasized that also fibroblasts and endothelial cells contributed to the population of apoptotic nuclei [22].

If apoptosis was a determinant factor in the early phase of immobilization atrophy, a concomitant inflammatory response would have to be excluded [20], since these two events are not associated to each other. In contrast, the present findings elicited a distinct secondary inflammatory reaction documented by an increase in MPO activity, which is a lysosomic marker of neutrophils and macrophages [10, 25] and by the frequent occurrence of various leukocytic cells whithin the muscle. Like shown in exercise-overloaded muscles [6] the inflammatory cells invaded into muscle might also contribute to mechanisms of degradation and following necrosis considering the cytotoxic effects of their components liberated to the interstitial space. Moreover, they play an important role as scavengers to remove debris of necrotic fiber segments.

It is concluded that for the experimental model of immobilization atrophy, maybe different from other forms to induce muscle atrophy, necrosis rather than apoptosis seems to play the major role in muscle tissue wasting, and this necrotic process is triggered by autophagic mechanisms followed by an inflammatory reaction.

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