

Apoptosis induced by proteasome inhibition in human myoblast cultures

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Dysfunction of the ubiquitin-proteasome system has recently been implicated in the pathogenesis of some untreatable myodegenerative diseases characterized by the formation of ubiquitinated inclusions in skeletal muscles. We have developed an *in vitro* model of proteasomal dysfunction by applying inhibitors of the proteasome to primary adult human skeletal muscle cultures. Our data show that proteasome inhibition causes both cytoplasmic accumulation of ubiquitinated inclusions and apoptotic death, the latter through accumulation of active caspase-3.

Key words: proteasome, myoblasts, apoptosis, aggregates.

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Paper accepted on May 9, 2006

European Journal of Histochemistry
2006; vol. 50 issue 2 (Apr-Jun):109-118

The ubiquitin-proteasome system (UPS) is the major pathway responsible for intracellular protein degradation in eucaryotic cells (Glickman, 2002).

Increasing evidence suggests that impaired UPS activity could be involved in the pathogenesis of some untreatable myodegenerative diseases such as oculopharyngeal muscular dystrophy (OPMD), myofibrillar myopathy (MM), and inclusion body myositis (IBM) (Abu-Baker, 2003; Ferrer, 2004; Fratta, 2004; Fratta, 2005). These diseases are characterized by the presence of proteinaceous aggregates in muscle cells (Calado, 2000; De Bleecker, 1996; Askanas, 1991), most likely formed by an inefficient degradation of misfolded proteins by UPS. In addition, in sporadic IBM the presence of misfolded protein correlates with a reduced activity of the proteasome proteolytic activity (Fratta, 2005). Despite this evidence, however, the molecular mechanisms by which proteasome impairment and/or aggregate formation could lead to skeletal muscle fibre loss in these myodegenerative diseases are still unclear. Likewise recent data have also demonstrated significant changes of proteasome functions in aged skeletal muscle, suggesting that sarcopenia in aging may partially result from accumulation of oxidized and ubiquitinated proteins, insufficiently degraded by UPS (Ferrington, 2005; Husom, 2004; Martinez-Vicente, 2005).

Although several studies have analyzed the effect of proteasomal impairment in various cell lines (Shinohara, 1996; Lopes, 1997; Bellas, 1997), the effects of proteasome dysfunction in human skeletal muscle cells have been poorly investigated. In the present study we have examined the formation of ubiquitinated aggregates and the mechanism of cell death in cultured human skeletal myoblasts after treatment aimed at the proteasome inhibition.

Materials and Methods

Subjects and muscle biopsies

Skeletal muscle biopsies were obtained from 7 healthy subjects undergoing orthopedic surgery for no malignant condition (mean age 35 ± 6 years). Biopsies were performed in the upper limb muscles. The study was approved by the Ethics Committee of IRCCS Istituto Auxologico Italiano, and the informed consent for muscular biopsy was obtained.

Myoblasts cultures

Myoblasts were derived according to the method of Blau and Webster (Blau, 1981) and grown in HAM's F10 medium (Sigma, St Louis, MO) supplemented with 15% Fetal Bovine Serum (Gibco / Invitrogen, San Diego, CA), Albumine from Bovine Serum 0.5 mg/mL, Epidermal Growth Factor 10 ng/mL, Insulin 4 ng/mL, Dexamethasone 0.39 μ g/mL, Penicillin 100 units/mL, and Streptomycin 0.1 mg/mL. All cultures were incubated at 37°C in a humid atmosphere containing 5% CO₂. Cultures were characterized by immunocytochemistry using antibodies to desmin (Chemicon, Temecula, CA; Calbiochem/Oncogene Research Products, Cambridge, MA). To confirm further myogenicity of the cultured cells, we observed the fusion process which forms multinucleate myotubes after seven days in differentiating medium containing Dulbecco's modified Eagle's medium supplemented with 10 μ g/mL insulin and 5% fetal bovine serum (Meola, 1991).

Immunofluorescence studies

Cell monolayers were washed twice with Phosphate Buffered Saline (PBS), fixed in 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, thoroughly washed, and then blocked with 10% Normal Goat Serum (NGS). Primary antibody incubation was carried out overnight at 4°C. Immunoreaction controls involved the omission of primary antibody. After several washings with 10% PBS/NGS, cells were incubated with the appropriate secondary antibody for 1 hour, washed several times and mounted in a fluorsave solution (Calbiochem). Primary antibodies included anti-ubiquitin (Chemicon) and anti-20S proteasome antibodies (Biomol International). Antimouse and antirabbit Cy3- and Cy2- conjugated antibodies were supplied by

Jackson ImmunoResearch (West Grove, PA). Samples were observed under a Leica DMIR2 microscope, and digital images were assembled using Imaging Software LeicaFW4000.

Cell viability

We investigated the effects of two different proteasome inhibitors (PIs), reversible inhibitor MG132 and irreversible inhibitor lactacystin, at concentration previously reported to block the proteolytic activity of the proteasome (Wagenknecht, 2000; Canu, 2000), on the survival of adult human myoblasts. We measured cell viability by Coulter Counter and MTS assays. For MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assays cells were seeded at 10^4 cells per well in 96-well plates, adhered for 24 hours, incubated with PI, and then analyzed by Cell Titer 96 Aqueous Cell Proliferation assay (Promega) after 24, 48 and 72 hours, according to manufacturer's protocol. For Coulter Counter assays, myoblasts were seeded in 6-well dishes at the density of 3×10^5 /well. After 24 hours, PI or vehicle was added to the culture medium in triplicate wells. After 24, 48, 72 hours cells were collected in isotonic solution and counted in triplicates with Z2 Coulter Counter (Beckman-Coulter).

Western blot analysis

Muscle cells were rinsed with cold PBS and harvested by scraping and centrifugation, then lysed on ice for 15 minutes in a buffer containing 20 mM TRIS pH 7.5, 150 mM NaCl, 1mM EDTA, 1 mM EGTA, 1% Triton X-100, and complete protease inhibitors (Roche). Lysates were briefly sonicated and protein concentration measured using the Bio-Rad Protein-Assay-Reagent (Bio-Rad Laboratories) and BSA as a standard. The homogenates were centrifuged for 10 minutes at 10,000 x g and the supernatants were then diluted in 3 x SDS sample buffer (180 mM Tris-HCl pH 6.8, 6% SDS, 30% glycerol, 300 mM DTT and 0.02% bromophenol blue). Equal amounts of proteins were separated on polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech, Little Chalfont, UK), incubated in blocking buffer for 1 hour (5% low fat milk in TBST, 50 mM TRIS pH 7.6, 0.15 M NaCl, 0.05% Tween 20), and probed overnight with the primary antibody in TBST.

Antibodies for active fragment of caspase-3 and for poly(ADP-ribose) polymerase (PARP) were obtained from Cell Signaling Technology (Beverly, MA). Antibody for p32 fragment of caspase-3 was obtained from Upstate. Recombinant human caspase-3 was obtained from Sigma. Protein bands were visualized with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham).

Immunoprecipitation

Equal amounts of precleared lysates (3000 μ g) were rotated overnight at 4°C with 1:100 rabbit anti-active caspase-3 (p17-p20). Antigen-antibody complexes were immobilized by rotation for 2 hours at 4°C with 1:10 protein A/G Sepharose (Amersham). The samples were separated on polyacrylamide gel electrophoresis and the lanes were cut in two pieces which were separately incubated with anti-active caspase-3 (p17-p20) or anti-ubiquitin antibodies.

In situ TUNEL assay

Myoblast monolayers were fixed with 4% paraformaldehyde and assayed with the Cell Death Detection Kit TMR Red (Roche Diagnostics, Grenzach-Wyhlen, Germany) according to the manufacturer's instructions. The apoptotic index was expressed as a percentage of TUNEL-positive nuclei on all the nuclei, stained with 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI).

DEVDase, LEHDase, and IETDase activity

Caspase activities were monitored by colorimetric kits (Chemicon). Protein concentration was measured by the Bio-Rad Protein-Assay-Reagent (Bio-Rad Laboratories, München, Germany) and BSA as a standard. Samples of $2-3 \times 10^6$ cells, maintained in identical culture conditions, were rinsed in cold PBS and lysed in Cell Lysis buffer. Cell lysates were centrifuged for 5 minutes at $10,000 \times g$ and the supernatants stored at -80°C. Lysates were incubated at 37°C for 4 hours in Caspase Assay buffer supplemented with specific colorimetric substrate (DEVD-pNA, IETD-pNA, LEHD-pNA respectively for caspase-3, -8 and -9). Samples were measured spectrophotometrically at 405 nm and the results were calibrated with known concentrations of p-nitroanilide. The caspase activities were calculated as pmol of p-nitroaniline liberated/hours \times μ g protein.

Statistical analysis

Unless otherwise stated all data are expressed as mean \pm SEM. Data were subjected to a test for normality. Because data showed a normal distribution, we used a parametric analysis of variance (ANOVA), followed by Tukey test to detect significant differences among groups. Statistical significance was set at $p < 0.05$.

Results

Muscle cells characterization

Myoblast cultures were characterized by immunocytochemistry using antibodies to desmin, a protein expressed only in myogenic cells (Kaufman, 1988; Furling, 2001). Myogenic purity, calculated as proportion of cells expressing desmin, was estimated as the ratio between stained and unstained cells. At 7 day in culture (DIV 7) selected areas of each well, comprising together approximately 5% of the well surface, were scanned in triplicate for the presence of desmin positive cells out of the total cells counted. Desmin positive myoblast percentage resulted higher than 98% in all cultures (Figure 1A). To confirm further myogenicity of the cultured cells, we observed the fusion process which forms multinucleate myotubes after seven days in differentiating medium (Figure 1B).

Proteasome inhibition induces formation of ubiquitinated inclusions

Impairment of UPS has been shown to increase cellular levels of ubiquitinated proteins (Figueiredo-Pereira, 1994). As shown in figure 2, MG132 or lactacystin (LC) treatment induced a time dependent increase of ubiquitinated proteins in human myoblasts (Figure 2A-B). Such accumulation started within 6 hours of exposure to 2 μ M MG132 or 30 μ M LC. Lower drug concentrations (1 μ M MG132 or 15 μ M LC) induced slower accumulation, detectable only after 12 hours. In some cell types (Canu, 2000) accumulation of ubiquitinated proteins is a normal occurrence during apoptosis. Conversely, in myoblasts, well-known apoptotic stimuli, such as staurosporine (Columbaro, 2001) or mitochondrial toxin Azide, themselves did not lead to the increase of ubiquitinated proteins (Figure 2C).

We next assessed, by immunofluorescence analyses, whether such an intracellular increase of ubiquitinated proteins would also be accompanied by

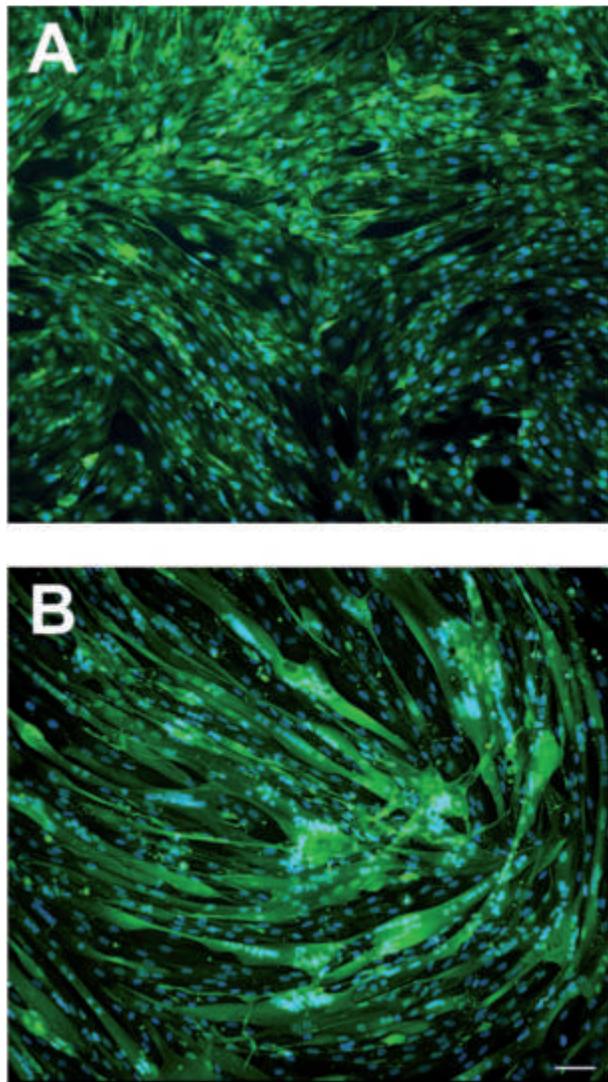


Figure 1. Desmin immunostaining in human skeletal. A) myoblasts at DIV 7 and B) myotubes. 98% of total cells stained for desmin. Scale bar represents 50 μm .

modified intracellular distribution. There was a generalized increase in ubiquitin immunofluorescence after adding PIs, compared to the low-level diffuse staining observed in control cultures (Figure 2D-H, green fluorescence). Moreover, following the treatment with PIs (Figure 2E-H), a proportion of myoblasts appeared containing discrete cytoplasmic regions of intense ubiquitin (green fluorescence) and 20S-proteasome (red fluorescence) immunoreactivity. Merged images (Figure 2E-H, right panel) were consistent with the formation of protein aggregates positive for ubiquitin and 20S-proteasome. Kinetic analysis showed that aggregate formation peaked ~ 48 hours after PIs application and were not detectable earlier than 24 hours (*data not shown*).

Proteasome inhibition induces apoptosis in human myoblasts

Proteasome inhibitors MG132 (1-10 μM) and lactacystin (15-45 μM) induced a significant cytotoxicity that became evident already at 24 hours after myoblast culture treatment (Figure 3A-C). Concomitantly with the marked decreased viability, optical microscopy showed altered morphology and cell detachment from tissue culture plates (Figure 3D).

To examine whether the reduction in the cell number was caused by apoptotic cell death, we performed in situ tdt-UTP-nick-end-labeling (TUNEL) assay. In normal growth conditions, apoptotic myoblasts were $2\pm 1\%$, whereas a time and dose dependent apoptotic index increase occurred after PIs treatment (Figure 4A). When the myoblasts were treated with the PIs simultaneously with agents known to prevent apoptosis, such as the general caspase inhibitor ZVAD-FMK or irreversible caspase-3 inhibitor DEVD-FMK, cell death was significantly reduced (figure 4B). The apoptotic index in myoblasts treated with the PIs plus DEVD-FMK was $5\pm 1\%$ and $6\pm 2\%$ after 24 and 48 hours respectively (*data not shown*).

Caspases activation is a crucial event in apoptotic processes and caspase-3 activation is the key event leading to muscle-fiber apoptosis (Tews, 2005). To investigate the mechanism by which proteasome inhibition acts to induce apoptosis in primary myoblasts, we at first measured caspase-3 activity by colorimetric assay, at different times after PIs treatment. As shown in Figure 5A, caspase-3 activity, in control myoblast cultures, was not significantly different during the entire period of cultivation, whereas it was dramatically increased by PIs, in proportion to the duration of the treatment. Specifically, 12 hours corresponded to the early point at which caspase-3 activation was detected (Figure 5A). Myoblast cultures co-treated with caspase-3 inhibitor DEVD-FMK did not show significant caspase-3 activation. In line with these observations, cleavage of poly (ADP-ribose) polymerase, a known substrate of caspase-3 (Lazebnik YA Nature 1994), was only detected when caspase-3 was activated by treatment with MG132 or LC (Figure 5B). Furthermore, PARP cleavage in myoblasts was prevented by co-incubation with DEVD-FMK.

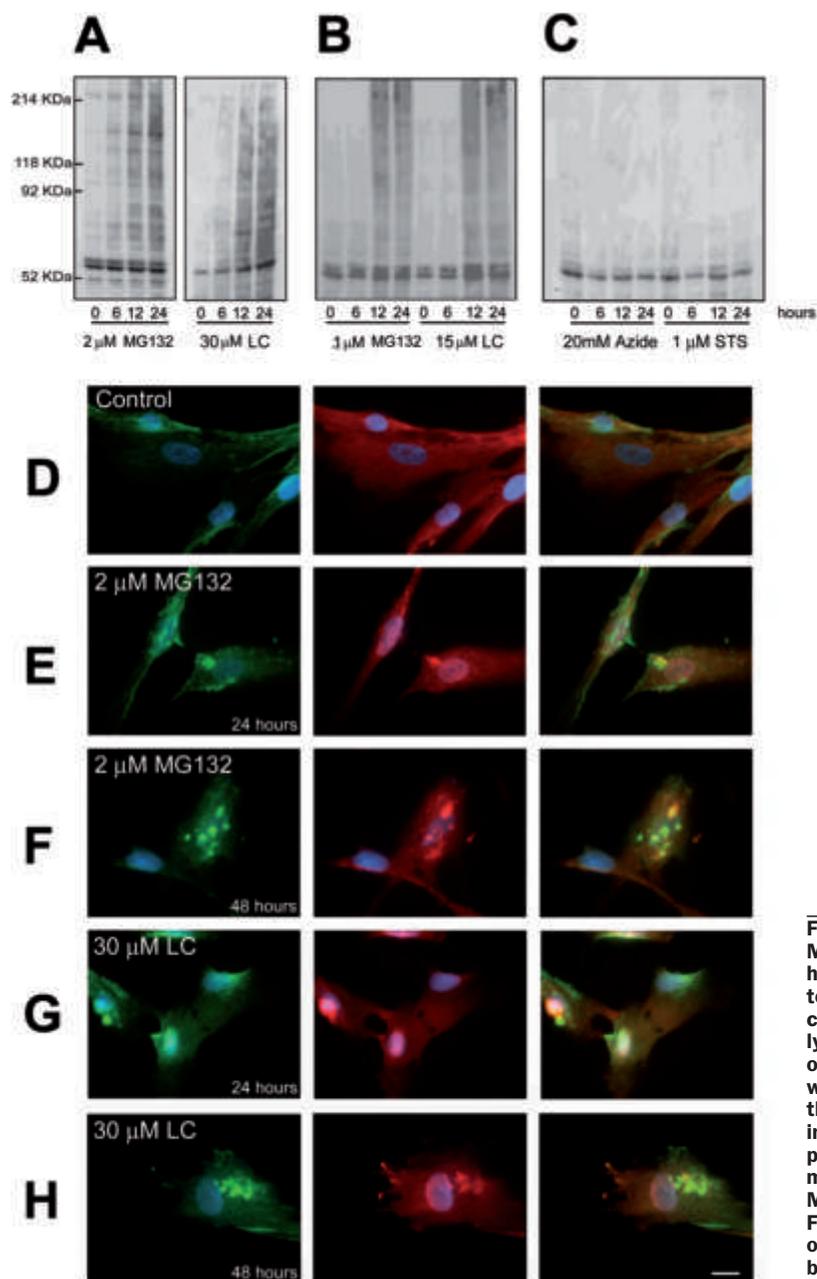


Figure 2. A-B) Myoblasts were treated with MG132 (1-2 μM) or LC (15-30 μM) for 6-12-24 hours. Equal amounts of proteins were subjected to SDS-PAGE and immunoblotted with the polyclonal anti-ubiquitin antibody. C) Total cell lysates from myoblasts exposed to 20 mM Azide or 1 μM staurosporine (STS) for 6-12-24 hours were separated by SDS-PAGE and probed with the anti-ubiquitin antibody. Double immunostaining for ubiquitin (green fluorescence) and 20S proteasome (red fluorescence) in control myoblasts. D) and in myoblasts treated with MG132 or LC for 24 hours. E, G) or 48 hours. F, H) Co-localization is shown in merged images on the right panel (yellow fluorescence). Scale bar represents 10 μm.

Caspase-3 activation mechanisms in PIs treated myoblasts

To be active, caspase-3 needs to be cleaved from an inactive larger proform (p32) into small fragments (the large p20 and the short p12 subunits) by initiator caspases-8 and -9. Subunit p20 is subsequently separated from its prodomain to form the active subunit p17 by auto-processing (Thornberry, 1998). Western blot analysis with an antibody specific for large active caspase-3 subunits (p20 and p17) showed, already after 12 hours, formation of active 17 KDa fragment in myoblasts under treatment with MG132 or lactacystin, in agreement

with previous colorimetric assay results (figure 6A). Western blot analysis also showed the accumulation of p20 subunit and higher molecular weight fragments immunoreactive for caspase-3. Conversely, western blot analysis with an antibody specific for caspase-3 proform (p32) showed no changes in p32 level after treating myoblasts with PIs (Figure 6B).

To investigate the mechanism of p17 and p20 accumulation in human myoblasts after proteasome blockade, we measured the caspase-8 and -9 activities by enzymatic assay. As positive control we assessed the caspase-9 activation after treating

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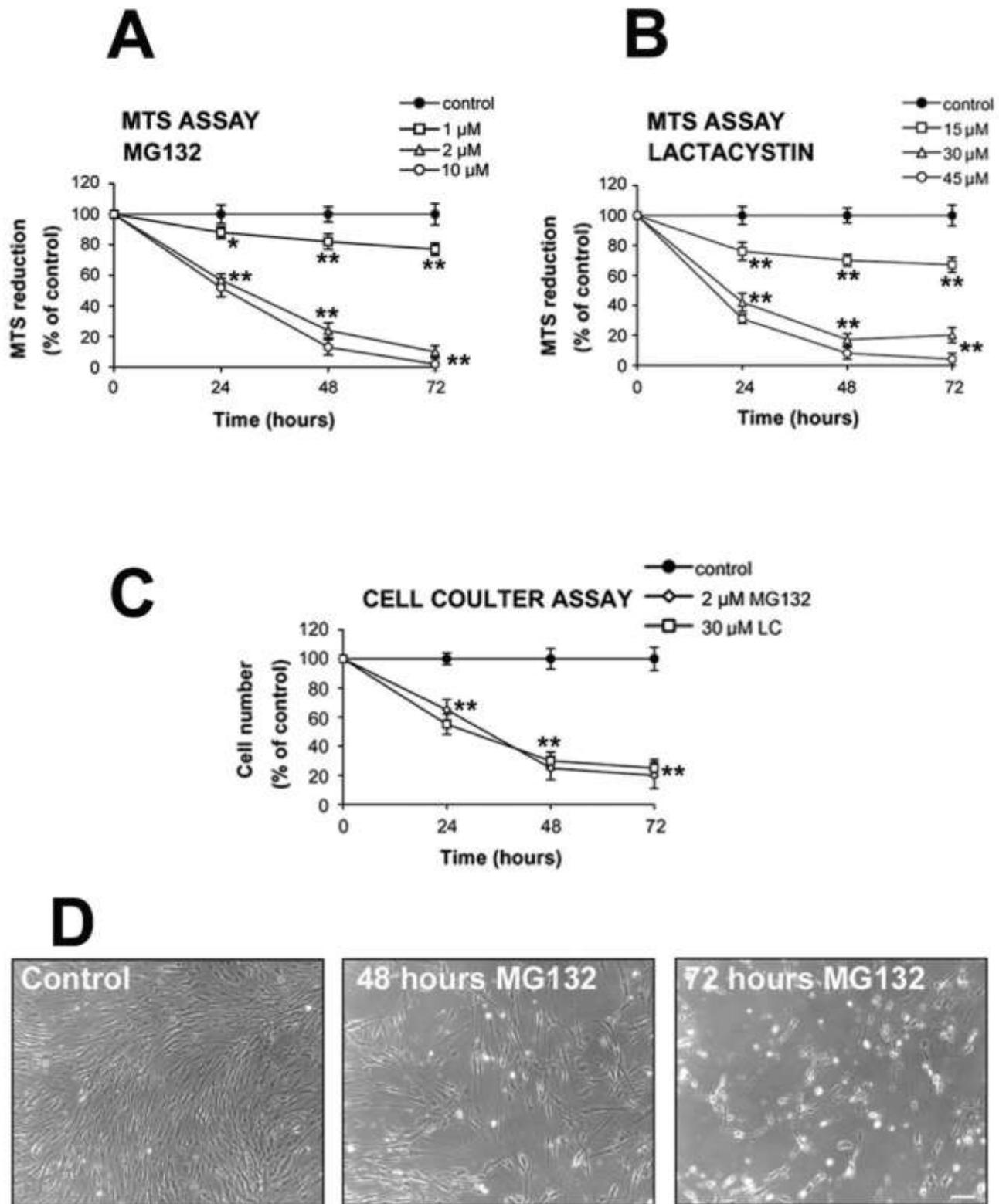


Figure 3. Myoblasts were exposed to different concentrations of MG132. A) or lactacystin B) for the indicated time intervals and survival was assessed by MTS assay (A, B) or cell coulter assay. C) Photomicrographs (2 μM MG132) are representative of four independent experiments. D) Values are mean \pm SEM; ** $p < 0.01$, * $p < 0.05$ vs control.

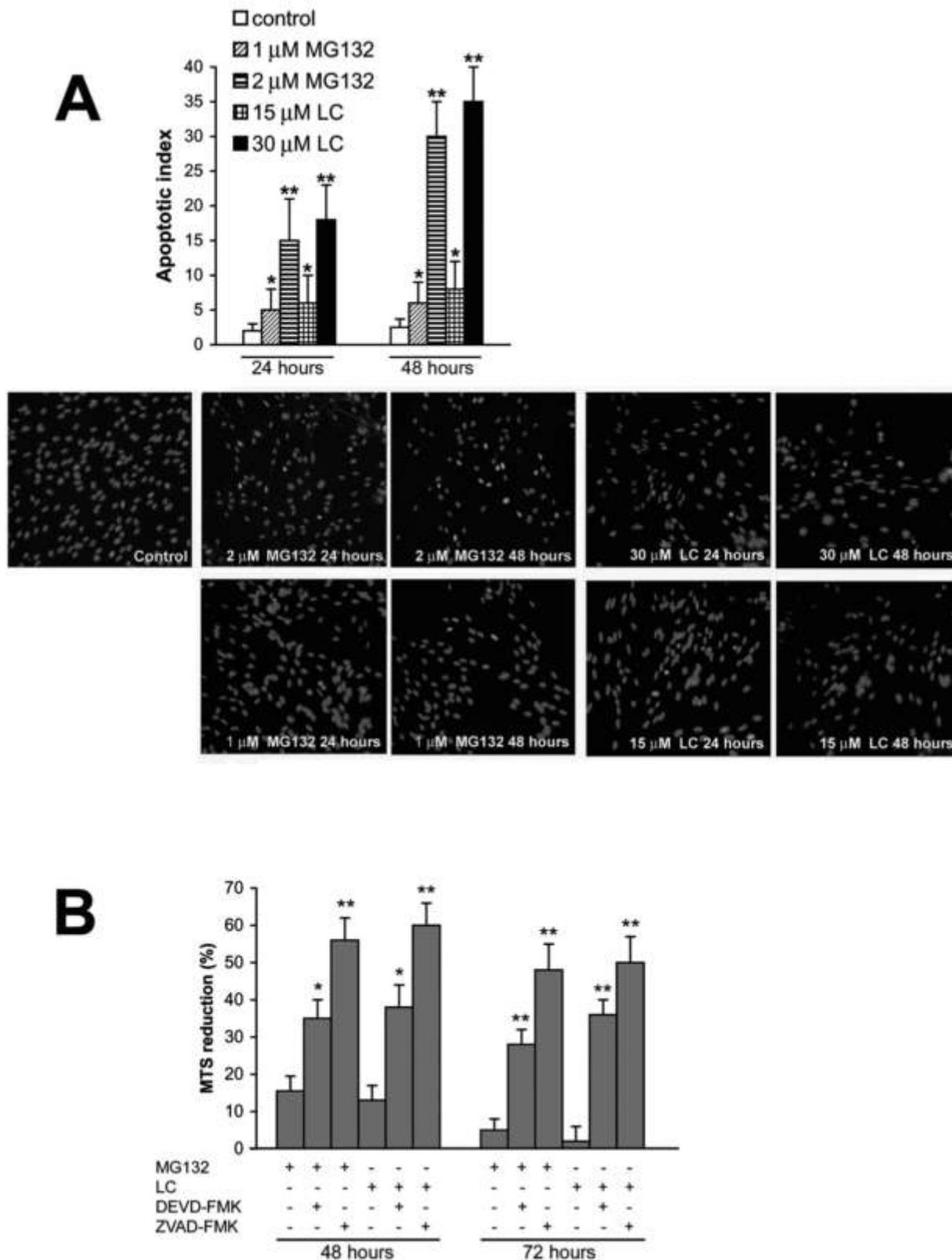


Figure 4. A) Apoptotic index in myoblasts treated with proteasome inhibitors (1-2 μM MG132, 15-30 μM LC) for 24 or 48 hours. Values are mean \pm SEM from three independent experiments; * p <0.05, ** p <0.01 vs control. Photomicrographs are representative of three independent experiments B) Protective effect of 100 μM ZVAD-FMK and 100 μM DEVD-FMK on myoblast death induced by proteasome inhibitors (2 μM MG132, 30 μM LC) was evaluated with MTS assay. Values are mean \pm SEM (** p <0.01, * p <0.05 vs proteasome inhibitor alone; values were normalized to untreated samples).

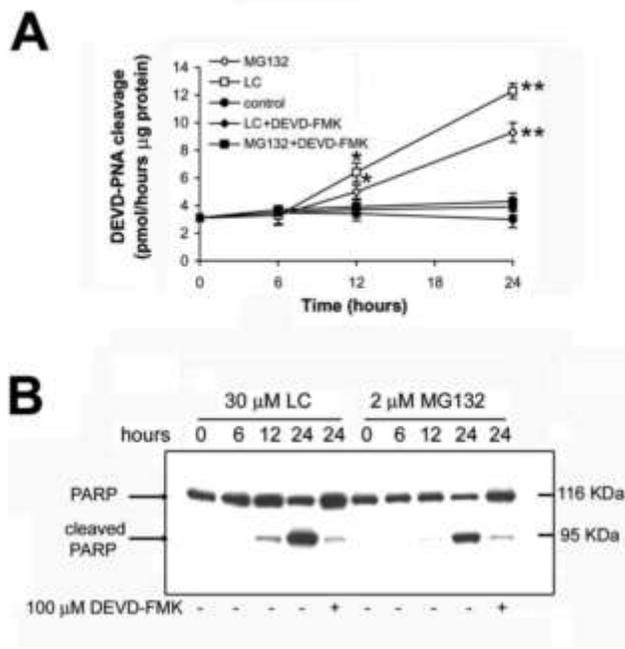


Figure 5. A) Caspase-3 like activity was examined by DEVD-PNA cleavage at 6, 12, 24 hours, under 2 μM MG132 or 30 μM lactacystin treatment. In the experiments, a negative control performed by adding DEVD-FMK to the myoblasts was included. Data are expressed as units of enzymatic activity (pmol of p-nitroaniline liberated/hours×μg protein). Values are mean ± SEM; ** $p < 0.01$, * $p < 0.05$ vs control. B) PARP-cleavage was assayed by western blot analyses in lysates from control myoblasts and myoblasts treated with proteasome inhibitors. The blot also shows PARP in sample co-treated with DEVD-FMK.

myoblasts with the mitochondrial chain inhibitor Azide and the caspase-8 activation after treating them with TNF- α . Interestingly, myoblasts presented a basal caspase-8 and -9 activity which never increased under MG132 or LC treatment (figure 6C). These data suggest that increased caspase-3 activity by proteasome inhibition isn't likely dependent from the proteolytic cleavage of p32 to p17 fragment.

Proteasome inhibition induces accumulation of both ubiquitinated and un-ubiquitinated caspase-3 fragments.

Recent reports suggested that active caspase-3 is ubiquitinated and then degraded by UPS (Chen, 2003; Suzuki, 2001). As Western blot analysis for the active caspase-3 showed accumulation of p17 as well as of higher molecular weight fragments (Figure 6A), we assessed if these last fragments might have resulted from the ubiquitination of the p17/p20 subunits. Lysates were prepared from myoblasts treated for 24 hours with MG132 and then active caspase-3 protein was collected by immunoprecipitation with anti-cleaved caspase-3 antibody. When the blot was probed with anti-ubiq-

uitin antibody or with anti-cleaved caspase-3, a signal corresponding to higher molecular weight species of caspase-3 was detected (Figure 7). Taken together, this result demonstrated that endogenous caspase-3 is partially ubiquitinated in myoblasts.

Discussion

Previous studies suggest that proteasome dysfunction is involved in the accumulation of misfolded proteins and cytotoxicity occurring in various myodegenerative conditions (Abu-Baker, 2003; Fratta, 2004; Ferrer, 2004). In this context human primary myoblast cultures treated with proteasome inhibitors may represent a useful in vitro model to understand how dysfunction of the UPS could contribute to muscle degeneration.

In the present study we showed that UPS impairment leads to human skeletal myoblast death. Since myoblasts are indispensable for growth, repair of skeletal muscle, and for the maintenance of bulk in adult skeletal muscle (Chargè, 2004), myoblast death could contribute to muscle atrophy in myodegenerative diseases characterized by UPS impairment. Likewise, the decreased proteasome function, recently described in aging (Ferrington, 2005; Husom, 2004), could lead to a reduction of myoblast population, explaining the impaired regenerative capacity of muscle tissues observed in advanced age (Bockhold, 1998; Webster, 1990; Heslop, 2000).

Interestingly, we observed a progressive accumulation of cytoplasmic inclusions after proteasome inhibition. Similar aggregates, containing ubiquitinated proteins and UPS components, have been observed in several myodegenerative and neurodegenerative disorders (De Blecker, 1996; Calado, 2000; Askanas, 1991; Alves-Rodrigues, 2003) but their role in the pathogenic process leading to cell death is still controversial. In human myoblasts in vitro, we observed that activation of the apoptotic process (i.e. caspase-3 activation) precedes the identification of aggregates. Although we cannot exclude the presence of early intermediates of protein aggregates toxic to the cell (Bucciattini, 2002), our data suggest that aggregate formation and apoptotic system activation might be independent and parallel processes.

Caspase-3 activation by proteasome inhibition was previously reported (Drexler, 1997; Zhang, 1999), however the detailed mechanisms, through

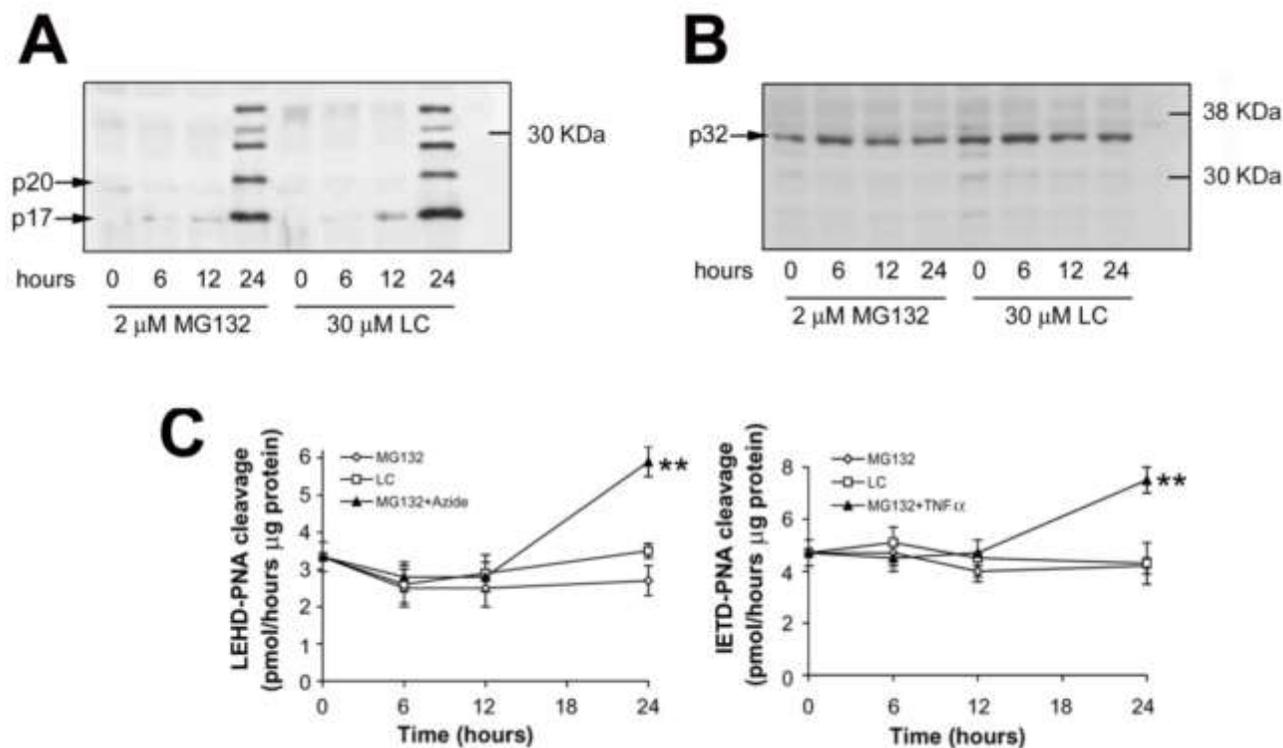


Figure 6. Lysates from control myoblasts and myoblasts treated with proteasome inhibitors were separated on polyacrylamide gel electrophoresis. The signal corresponding to caspase-3 was detected with an antibody specific for active caspase-3 subunit **A**) and an antibody specific for p32-caspase-3 proform **B**) The two figures represent the same blot that was first probed with anti p32 antibody and then stripped and reprobed with anti active caspase-3. The figure is representative of three independent experiments. **C**) Caspase-9 like and caspase-8 like activities were respectively examined by LEHD-PNA and IETD-PNA cleavage at 6, 12, 24 hours, after 2 μ M MG132 or 30 μ M lactacystin treatment. Data are expressed as units of enzymatic activity (pmol of p-nitroaniline liberated/hours \times μ g protein). Values are mean \pm SEM; ** $p < 0.01$, vs untreated samples. MG132+Azide (5 μ M)-treated myoblasts were used as positive control for caspase-9. MG132+TNF- α (2 ng/mL)-treated myoblasts were used as positive control for caspase-8.

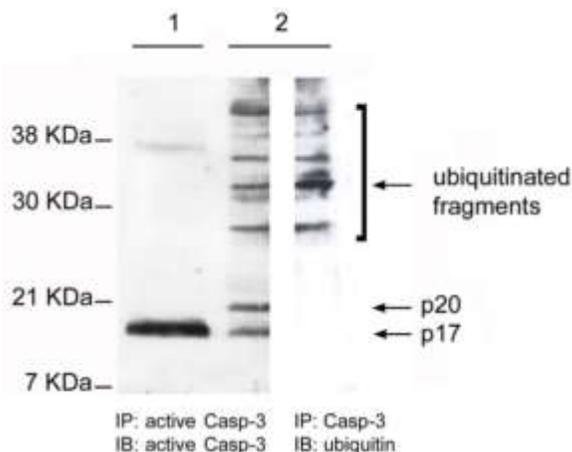


Figure 7. Active caspase-3 was immunoprecipitated (IP) with the antibody specific for p17/p20 subunits. Immunoprecipitates were then subjected to Western blot analysis (IB) and probed with the antibody specific to p17/p20 subunits and the antibody for ubiquitin (lane 2). Lane 1 represent a positive control (human active caspase-3 recombinant). The figure is representative of three independent experiments.

which UPS dysfunction activates caspase-3, are still debated and appear to be strongly dependent on the cell type (Wagenknecht, 2000; Mitsiades,

2002; Chen, 2003). Notably, our data show that in human skeletal myoblast cultures the caspase-3 activation was not accompanied by an activation of initiator caspase-8 and -9. These findings indicate that caspase-3 activation by proteasome inhibitors in myoblasts could be independent from the classical apoptotic pathways (i.e. extrinsic pathways mediated by activation of caspase-8 and intrinsic pathway which leads to activation of caspase-9), even if they leave open the involvement of other caspases (Lu, 2003).

Proteasome inhibition in human myoblasts leads to accumulation of ubiquitinated caspase-3 fragments. We therefore speculated that in myoblasts caspase-3 is degraded by UPS. Consistently with our results, recent reports showed that active caspase-3 was ubiquitinated and then degraded by UPS in HL-60 and 293T cells (Chen, 2003; Suzuki, 2001). Concomitantly the important accumulation of un-ubiquitinated p17 subunit suggests that proteasome dysfunction could also partially inhibit caspase-3 ubiquitination.

In conclusion, the present study does demonstrate that UPS dysfunction leads to aggregate formation and apoptosis in human skeletal myoblast cultures. Moreover, the data suggest that an inefficient removal by UPS of activated caspase-3 might be the mechanism underlying the apoptosis. Thus a similar mechanism might promote cell death in myodegenerative disease characterized by UPS impairment.

Acknowledgements

This work was supported by grants from IRCCS Istituto Auxologico Italiano, Dino Ferrari Centre, and the Italian Ministry of Health (Malattie Neurodegenerative ex art. 56 legge finanziaria 2004).

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