Essential Amino Acid Leucine and Proteasome Inhibitor MG132 Attenuate Cigarette Smoke Induced Catabolism in C2 Myotubes

4

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Abstract

Exposure to cigarette smoke (CS) and cigarette smoking have been shown to promote catabolism of skeletal muscle. Previous studies and recent findings from our laboratory have demonstrated the involvement of the ubiquitin proteasome system and the muscle-specific E3 ubiquitin ligases MAFbx/atrogin-1 and MuRF1 in CS induced skeletal muscle catabolism. The essential amino acid leucine is a known anticatabolic agent that improves skeletal muscle metabolism in various atrophic conditions. To examine the protective effect of leucine and proteasome inhibition in CS induced muscle catabolism, C2 myotubes, from an *in vitro* skeletal muscle cell line, were exposed to CS in the presence or absence of leucine and a proteasome inhibitor, MG132. Diameter of myotubes, levels of the main contractile proteins – myosin heavy chain and actin, expression of MAFbx/atrogin-1 and MuRF1 were studied by microscopy, Western blotting, and qPCR. Leucine pretreatment prevented the CS-induced reduction in diameter of myotubes and degradation of myosin heavy chain by suppressing the upregulation of MAFbx/atrogin-1 and MuRF1. MG132 also attenuated the CS-induced decrease in diameter of myotubes and degradation of myosin

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heavy chain. Our findings demonstrate that supplementation with the essential amino acid leucine and inhibition of the proteasome may protect skeletal muscle from CS induced catabolism.

1 Introduction

Exposure to cigarette smoke (CS) and cigarette smoking have been shown to promote catabolism of skeletal muscle in clinical, *in vivo* and *in vitro* studies (Rom et al. 2012). In addition, epidemiological studies have identified smoking as risk factor for sarcopenia, the loss of muscle mass, strength and function in old age (Lee et al. 2007; Szulc et al. 2004; Castillo et al. 2003). CS is a complex aerosol containing over than 4,700 constituents, some of which have the potential to increase catabolism of skeletal muscle. These CS components include reactive oxygen species (ROS), reactive nitrogen species (RNS), and various aldehydes which can damage skeletal muscle by increasing oxidative and nitrative stress and activating muscle specific proteolytic pathways. Based on previous studies and recent findings from our laboratory, we have proposed a cellular model of CS induced catabolism of skeletal muscle (Rom et al. 2012). In this model, components of CS may reach skeletal muscle of smokers, increasing intracellular oxidative stress and activating the p38 mitogen-activated protein kinase (MAPK) and nuclear factor kappaB (NF-κB) pathways. Activation of these pathways results in up-regulation of muscle-specific E3 ubiquitin ligases (E3s) of the ubiquitin proteasome system (UPS), leading to increased degradation of muscle proteins and muscle catabolism (Rom et al. 2012).

The UPS is the primary pathway of intracellular protein degradation in skeletal muscle (Foletta et al. 2011). In this pathway 3 major enzymes are involved: E1 ubiquitin activating enzymes, E2 ubiquitin carriers, and E3s which mediate ubiquitination of target proteins (Herningtyas et al. 2008). E3s play the important role of determining which proteins are targeted for degradation by the proteasome (Rom et al. 2012). Two muscle-specific E3s have been identified: Muscle atrophy F-box (MAFbx/atrogin-1) and muscle RING finger-1 (MuRF1) (Foletta et al. 2011). Both muscle-specific E3s are considered to play a major role in muscle atrophy. Indeed, knockout mice lacking these E3s are prevented from muscle atrophy (Herningtyas et al. 2008). In a study recently submitted for publication we report that exposure of C2 myotubes, from an *in vitro* skeletal muscle cell line, to CS caused increased muscle catabolism mediated by upregulation of MAFbx/atrogin-1 and MuRF1.

Essential amino acid (EAA) leucine (Leu) stimulates protein synthesis in muscle by regulation of the mammalian target of the rapamycin (mTOR) signaling pathway. Leu stimulates phosphorylation of mTOR, leading to activation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase (S6K), resulting in increased muscle protein synthesis (Herningtyas et al. 2008). Pretreatment of C2C12 muscle cells with 5 mM Leu suppressed MAFbx/atrogin-1 and MuRF1 upregulation stimulated by starvation. In addition, Baptista et al. (2010) found that Leu supplementation to rats during hind limb immobilization attenuated loss of muscle mass and minimized gene expression of MAFbx/atrogin-1 and MuRF1. Therefore, in this study we aimed to examine the ability of Leu to reduce the catabolic effects of CS in C2 myotubes. In addition, the protective effects of proteasome inhibition were examined by pretreatment of myotubes with the proteasome inhibitor Z-Leu-Leu-Leu-aldehyde (MG132) prior to CS exposure (Kisselev and Goldberg 2001).

2   Methods

2.1   Cell Culture

The C2 mouse skeletal myoblast cell line was a generous gift from Prof. Bengal (Faculty of Medicine, Technion, Israel). C2 myoblasts were grown in 100 mm plates in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS), 1 % (v/v) penicillin/streptomycin and 1 % (v/v) L-glutamine at 37 °C in humidified 95 % air-5 % CO_2 atmosphere. For differentiation to myotubes, myoblasts were plated in 0.1 % gelatin-coated plates and grown to 90 % confluence. At this point, GM was replaced by differentiation medium (DM) consisting of DMEM supplemented with 2 % (v/v) heat-inactivated horse serum, 1 % (v/v) penicillin/streptomycin and 1 % (v/v) L-glutamine. During differentiation DM was replaced every 48 h for 6 days until cell fusion and multi-nucleated myotubes formation was achieved. Successful cell differentiation was determined by expression myosin heavy chain (MyHC) protein as measured by immunoblotting. Cell media and chemicals were purchased from Biological Industries, Israel.

2.2   CS Exposure Experiments

Experiments were held on day seven of differentiation when the cells have completed their differentiation into elongated multi-nucleated myotubes. Exposure to CS was performed by a system consisting of a chamber attached to a vacuum pump and a negative pressure gauge (up to 600 mmHg) at one end and a cigarette at the other end. Myotube plates were placed inside the chamber. Then, the vacuum pump was activated, valve B was closed and valve A opened until a desired level of negative pressure was created inside the chamber. Subsequently, a TIME commercial cigarette containing 14 mg of tar and 0.9 mg of nicotine and filter (Dubek Ltd., Israel) was lit, valve A was closed, and valve B

between the burning cigarette and chamber was opened for 10 s, allowing CS to enter the chamber. Creating reduced pressure inside the chamber allowed the drawing of CS from the burning cigarette into the chamber. Thus, the dose of CS entering the chamber equated the levels of negative pressure created inside the chamber. After exposure to CS the chamber with the myotube plates was sealed and transferred for different incubation times at 37 °C. The level of negative pressure used for CS exposure in this study was 50 mmHg. In a study recently submitted for publication, it was found that this level of CS did not cause a significant reduction in viability of myotubes until 24 h of exposure. Sham-air exposed myotubes were used as control. Control plates were subjected to the same incubation periods with exposure to air instead of CS.

2.3   Measurement of Myotube Diameters

Myotube plates were photographed after CS exposure experiments using a digital camera (Olympus UC30, Japan) mounted on a phase contrast microscope (Olympus CK40-SLP, Japan) (objective ×20). Following experiments, nine fields of view were chosen randomly and ten largest myotubes in each field were measured by Image J software (NIH, USA). Measurements were made in a blinded fashion without the knowledge of treatment. The mean values constituted a measure of 90 myotubes for each experiment. Results were expressed as percent of myotube diameters of the sham-air exposed control plates.

2.4   Cell Lysates and Western Blot Analysis

Following the CS exposure experiments, cells were washed twice by PBS and lysed for cytosolic proteins using 400 μl/plate lysis buffer consisting of 50 mM Tris HCl pH 7.4, 300 mM NaCl, 1.5 mM $MgCl_2$, 200 mM EDTA and 0.1 % Triton ×100. ×40 diluted protease inhibitor and

phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO) were added to lysis buffer just prior to use. Cells were scraped and transferred to micro-centrifuge tubes for incubation on ice for 10 min followed by centrifugation at 4 °C and 14,000 RPM for 10 min. Supernatants containing cytosolic proteins were collected and kept at −80 °C. Total protein concentrations were measured by Bradford assay (Bio-Rad, USA) using bovine serum albumin as standard. A total protein of 20 µg/lane was loaded and separated by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5 % non-fat milk powder in TBS-T (0.125 % Tween) (Sigma-Aldrich, St. Louis, MO) for 1 h and exposed overnight to primary antibody at 4 °C. Primary antibodies against MyHC (1:1,000) (Santa Cruz, USA), actin (1:4,000) (Millipore, USA) were used. The next day, membranes were washed with TBS-T followed by 1 h incubation at ambient temperature with appropriate secondary antibodies conjugated to horse-radish peroxidase (Jackson Immuno-Research, USA). Detection was performed by enzyme-linked chemiluminescence (ECL) (Biological Industries, Israel) using ImageQuant LAS 4,000 digital imager system (GE Healthcare, UK). Protein quantities were determined by densitometry and analyzed using Total Lab Software (version V2006C, Nonlinear Dynamics, UK).

2.5 Protein Loading Control by Ponceau S Staining

Actin protein could not be used as an internal control for protein loading because it has been found to be degraded by CS exposure. Therefore, we used quantitation of total proteins by Ponceau staining before antibody probing as an alternative to housekeeping protein blotting. Romero-Calvo et al. (2010) have shown that reversible Ponceau S staining can be used advantageously over actin detection for equal loading control in Western blotting. Ponceau S is a non-specific protein dye; all proteins in the membrane are colored.

After transfer of proteins to nitrocellulose membranes, the membranes were rinsed in Ponceau S solution (Bio-Rad, USA) for 10 min, followed by a brief rinse in double-distilled water (DDW) so that the lanes and bands were clearly visible. Membranes were then inserted in between transparency sheets and scanned using a standard scanner. Total protein quantity in each lane was determined by densitometry of the scanned membrane using Total Lab Software (version V2006C, Nonlinear Dynamics, UK), and was used for normalization. At each lane, ECL detected proteins were quantified relatively to total protein quantification found by densitometry of Ponceau S staining. Subsequently, membranes were rinsed once more in DDW until the staining was completely eliminated. From that point, the blocking and antibody incubation steps were continued as usual.

2.6 RNA Purification, Reverse Transcription and qPCR

Purification of total RNA from myotubes was performed by High Pure RNA Isolation Kit (Roche, Germany) according to the manufacturer's instruction. RNA concentrations were quantified at 260 nm by Nanodrop spectrophotometer (Nanodrop Technologies, USA). Samples were diluted to equal concentrations containing 1 µg of RNA. Samples were used to synthesize cDNA with High Capacity cDNA Reveres Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instruction for a final volume of 20 µl.

qPCR was performed using Corbett Rotor-Gene 6,000 (Qiagen, Germany) and qPCR SYBR Green ROX Mix (Thermo Scientific, UK). Before qPCR, the efficiency of amplification was determined for each primer set. All primer sets were tested for efficiency >90 % as required for the ΔΔCt relative quantification algorithm. 3 µl of diluted cDNA were used as template; 2 µl of forward and reverse primer mix (2 µM) were added to 5 µl of SYBR Green ROX Mix master. Reactions were performed in a 10 µl reaction volume under the following conditions:

Step 1–15 min at 95 °C; Step 2–5 s at 95 °C; Step 3–30 s at 60 °C, with 40 repeats of Steps 2 and 3. For each sample, a value of the threshold cycle (Ct) was calculated using Rotor Gene 6,000 series software (Qiagen, Germany) based on the time changes in mRNA expression level calculated subsequent to normalization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The abundance of target mRNA relative to GAPDH was determined by the $\Delta\Delta$Ct relative quantification method. Single products and specific melting temperatures were assessed by melting curve. The following primers (Sigma-Aldrich, St. Louis, MO) were designed by PrimerBank database: GAPDH forward: 5′–AGGTCGGTGTGAACGGATTTG–3′, reverse: 5′–TGTAGACCATGTAGTTGAGGTCA–3′; MAFbx/atrogin-1 forward: 5′–CAGCTTCG-TGAGCGACCTC–3′, reverse: 5′–GCAGT-CGAGAAGTCCAGTC–3′; MuRF1 forward: 5′–GTGTGAGGTGCCTACTTGCTC–3′, reverse: 5′–GCTCAGTCTTCTGTCCTTGGA–3′.

2.7   Leu and MG-132 Pretreatment

To examine the effects of Leu on CS induced muscle catabolism, myotubes were pretreated with 5 mM Leu (Fluka BioChemika, Switzerland) (Herningtyas et al. 2008) 24 h prior to CS exposure. Also, to examine the protective effect of MG132 in CS induced catabolism, myotubes were pretreated with 25 μM MG132 (Enzo, USA) 1 h prior to CS exposure. Diameter of myotubes, MyHC and actin protein levels and expression of muscle specific E3s were examined in CS exposed myotubes in the presence or absence of Leu or MG132.

2.8   Statistical Analysis

Statistical analysis was performed by a *t* test and one-way ANOVA followed by Tukey or Dunnett's test using SPSS Statistics 16 software (IBM, USA). $P < 0.05$ was considered statistically significant. Results were expressed as means $\pm$ SE of three independent experiments.

3   Results

3.1   Leu Prevents CS-Induced Myotube Wasting and MyHC Degradation

Recently we have reported that exposure of C2 myotubes to CS caused significant reductions in diameters of myotubes and in the level of MyHC protein (Rom et al. 2012). To examine the ability of Leu to prevent the catabolic effect of CS exposure, C2 myotubes were pretreated with 5 mM Leu 24 h before exposure to CS at 50 mmHg. Diameters and levels of MyHC and actin proteins were examined in myotubes exposed to CS followed by 6 h of incubation and in myotubes pretreated with 5 mM Leu prior to CS exposure. Control myotubes were treated in the same manner with exposure to air instead of CS with or without Leu pretreatment. Air exposed myotubes pretreated with Leu presented greater diameters and MyHC protein level when compared with control myotubes without Leu pretreatment, although these effects were not significant. Diameters of myotubes and MyHC level decreased significantly in the CS-exposed myotubes without Leu pretreatment when compared with the control myotubes. These effects were prevented in the CS-exposed myotubes pretreated with Leu 24 h prior to exposure. The level of actin protein remained stable and did not change under any treatment (Fig. 4.1a–g).

3.2   Leu Attenuates CS-Induced Upregulation of Muscle Specific E3s

Recently we have reported that exposure of C2 myotubes caused a significant increase in mRNA levels of MAFbx/atrogin-1 and MuRF1 (Rom et al. 2012). Also, MAFbx/atrogin-1 and MuRF1 are upregulated in mice chronically exposed to CS (Tang et al. 2010). To examine the effect of Leu on the expression levels of these muscle specific E3s following exposure to CS,

**Fig. 4.1 Leu prevents CS induced myotube wasting and MyHC degradation**. Myotubes were exposed to CS or air at negative pressure level of 50 mmHg with or without Leu pretreatment followed by incubation for 6 h. Following incubation, myotubes were photographed ($\times$20): (**a**) Control – myotubes exposed to air without Leu pretreatment followed by 6 h incubation. (**b**) Myotubes pretreated with 5 mM Leu 24 h prior to air exposure and 6 h incubation. (**c**) Myotubes exposed to CS without Leu pretreatment followed by 6 h incubation. (**d**) Myotubes pretreated with 5 mM Leu 24 h prior to CS exposure and incubation for 6 h. (**e**) Changes in myotube diameters are expressed as percent of the diameter of control myotubes. Results are relative to control and expressed as means $\pm$ SE of three different experiments. Following incubation, cell lysates were prepared and subjected to Western blot analysis using antibodies against MyHC and actin. (**f**) Representative Western blot is presented. (**g**) MyHC and actin protein levels were normalized by total protein densitometry detected by Ponceau S staining and expressed relative to the corresponding value of control myotubes. Results are expressed as means $\pm$ SE of three different experiments. *$p < 0.05$ vs. control myotubes
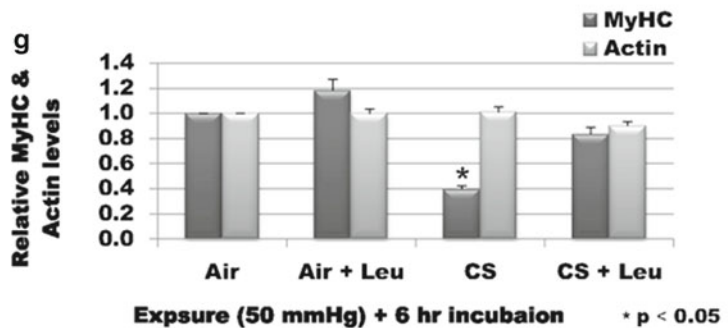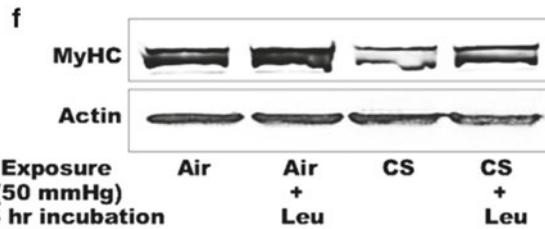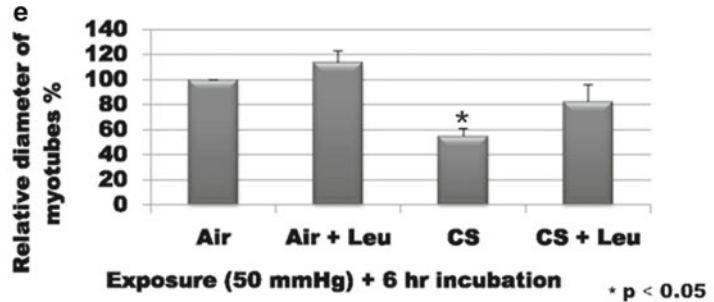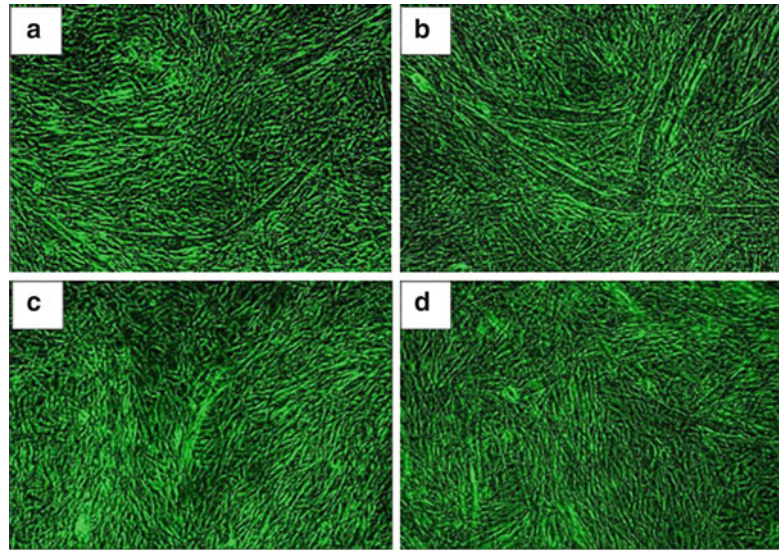
C2 myotubes were pretreated with 5 mM Leu 24 h before exposure to CS at 50 mmHg followed by 3 h of incubation. Following incubation mRNA levels of MAFbx/atrogin-1 and MuRF1 were examined in CS exposed myotubes without Leu pretreatment and in myotubes pretreated

with 5 mM Leu prior to CS exposure. Control myotubes were treated in the same manner with exposure to air instead of CS with or without Leu pretreatment. In air exposed myotubes pretreated with Leu, lower mRNA levels of MAFbx/atrogin-1 and MuRF1 were found, although this was not significant. Compared with the control myotubes, mRNA levels of MAFbx/atrogin-1 and MuRF1 increased significantly in the CS-exposed myotubes without Leu pretreatment. This upregulation was abolished in CS exposed myotubes pretreated with Leu 24 h prior to exposure (Fig. 4.2).

3.3   Proteasome Inhibition Prevents CS-Induced Myotube Wasting and MyHC Degradation

To examine the effect of proteasome inhibition on the CS-induced muscle catabolism, C2 myotubes were pretreated with 25 μM MG132 1 h prior to CS exposure. Diameters and levels of MyHC and actin proteins were examined in myotubes exposed to CS in negative pressure of 50 mmHg followed by 6 h of incubation and in myotubes pretreated with MG132 prior to CS exposure. Control myotubes were treated in the same manner with exposure to air instead of CS. Diameters of myotubes and MyHC level decreased significantly in CS exposed myotubes without MG132 pretreatment when compared with air exposed myotubes. These effects were prevented in CS exposed myotubes pretreated with MG132 1 h prior to exposure. The level of actin protein remained stable and did not change under any treatment (Fig. 4.3a–f).

4   Discussion

In this study, we reveal that the catabolic effects of CS were prevented in myotubes pretreated with EAA Leu. Leu pretreatment prior to CS exposure prevented CS induced reduction in myotube diameters and degradation of MyHC. Interestingly, myotubes exposed to air instead of CS and pretreated with Leu, presented greater diameters and higher protein level of MyHC. Although these
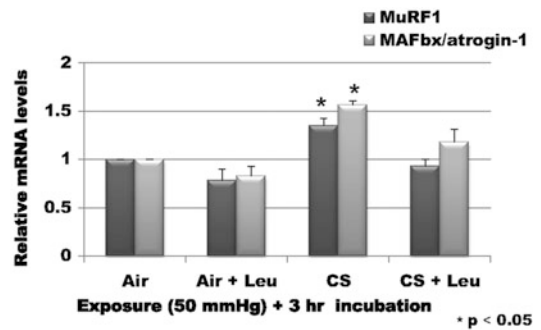

Fig. 4.2 Leu attenuates CS induced upregulation of muscle specific E3s. Myotubes were exposed to CS or air at negative pressure level of 50 mmHg with or without Leu pretreatment followed by incubation for 3 h. Control myotubes were exposed to air without Leu pretreatment. Following incubation, total RNA was isolated and subjected to reverse transcription and qPCR analysis to determine the expression of MAFbx/atrogin-1 and MuRF1. Data were normalized by GAPDH expression and are relative to the corresponding value of control myotubes. Results are expressed as means $\pm$ SE of three different experiments. $*p < 0.05$ vs. control myotubes

findings were not significant, they imply that Leu pretreatment increases anabolism of myotubes and thus may promote their resistance to the catabolic effects of CS. Also, Leu pretreatment prevented CS induced upregulation of the muscle specific E3s: MAFbx/atrogin-1 and MuRF1. Since these E3s play a major role in targeting muscle proteins for proteasomal degradation (Rom et al. 2012; Foletta et al. 2011; Herningtyas et al. 2008), prevention of their upregulation may be the key effect of Leu that protects myotubes from CS induced catabolism. These findings are consistent with previous studies reporting that Leu prevents muscle catabolism by suppressing upregulation of these E3s (Baptista et al. 2010; Herningtyas et al. 2008). Leu supplementation is known to improve muscle remodeling in various atrophic states including muscle disuse, sarcopenia, and cancer (Nicastro et al. 2011). Compared with non-smokers, skeletal muscle of smokers presented structural and metabolic damages (Montes de Oca et al. 2008). Lower fractional synthesis rate of muscle and higher expression level of MAFbx/atrogin-1 were found in smokers in comparison with non-smokers (Petersen et al. 2007). Also, chronic CS exposure
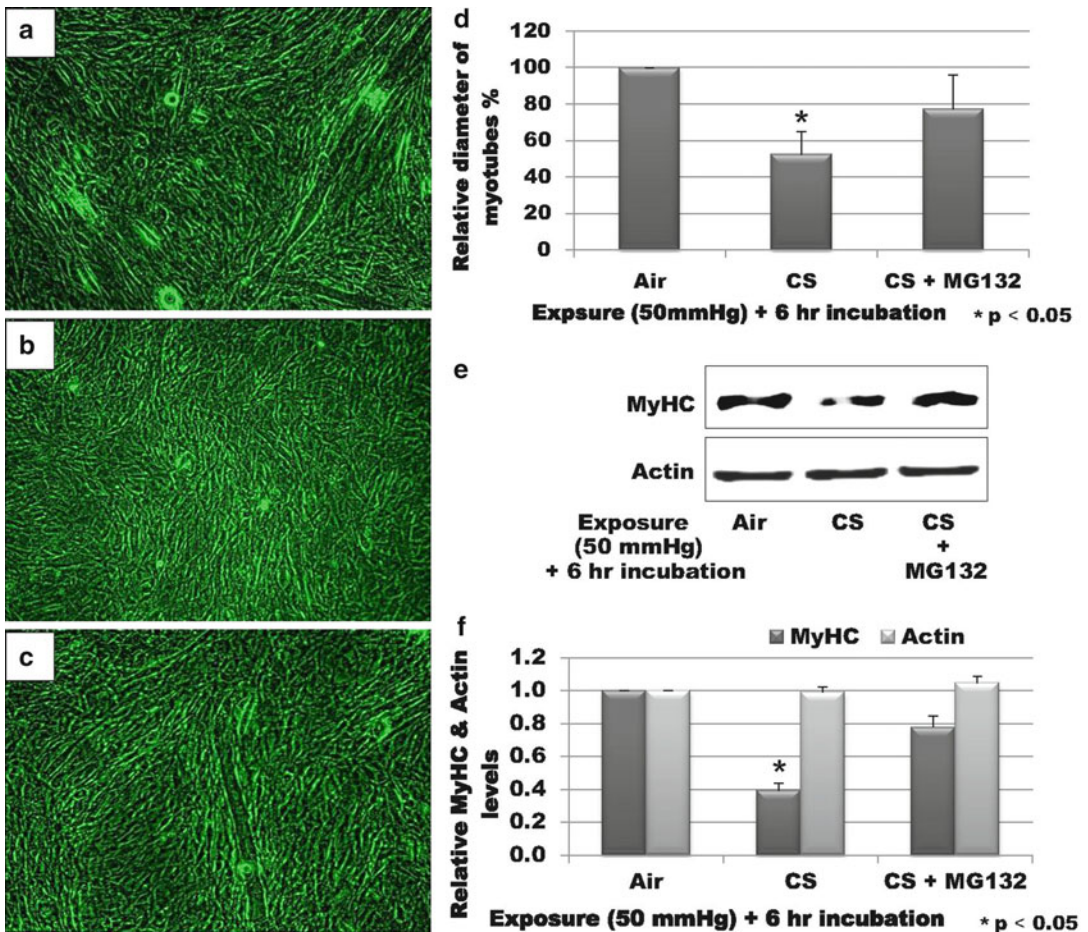
Fig. 4.3 Proteasome inhibition prevents CS induced myotube wasting and MyHC degradation. Myotubes were exposed to CS or air at negative pressure level of 50 mmHg with or without MG132 pretreatment followed by incubation for 6 h. Following incubation, myotubes were photographed (×20): (**a**) Control – myotubes exposed to air without MG132 pretreatment followed by 6 h incubation. (**b**) Myotubes exposed to CS without MG132 pretreatment followed by 6 h incubation. (**c**) Myotubes pretreated with 25 μM MG132 1 h prior to CS exposure and incubation for 6 h. (**d**) Changes in myotube diameters are expressed as percent of the diameter of control myotubes. Results are relative to control and expressed as means ± SE of three different experiments. Following incubation, cell lysates were prepared and subjected to Western blot analysis using antibodies against MyHC and actin. (**e**) Representative Western blot is presented. (**f**) MyHC and actin protein levels were normalized by total protein densitometry detected by Ponceau S staining and expressed relative to the corresponding value of control myotubes. Results are expressed as means ± SE of three different experiments. *p < 0.05 versus control myotubes

to mice resulted in loss of skeletal muscle mass and upregulation of MAFbx/atrogin-1 and MuRF1 (Tang et al. 2010). Therefore, we suggest that Leu supplementation may be effective in reducing skeletal muscle catabolism in smokers by suppressing muscle specific E3s. To further establish this, Leu supplementation should be examined in clinical and *in vivo* studies

investigating the protective effects of Leu on skeletal muscles of smokers and CS exposed animals.

Our findings also indicate the involvement of the proteasome in the CS-induced muscle catabolism. Pretreatment with the proteasome inhibitor MG132 prior to CS exposure prevented CS induced reduction in myotube diameters and degradation of MyHC. These findings imply that the

UPS is the primary pathway of CS-induced degradation of muscle proteins leading to reduced myotube diameters.

In conclusion, this study demonstrates that EAA Leu attenuates CS induced catabolism of C2 myotubes including reduction of myotube diameters and MyHC degradation. These effects were mediated by attenuating upregulation of the muscle specific E3s: MAFbx/atrogin-1 and MuRF1. Also, inhibition of the proteasome by MG132 prevented CS induced myotube catabolism, demonstrating the involvement of the UPS in CS induced skeletal muscle catabolism.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

References

Baptista, I. L., Leal, M. L., Artioli, G. G., Aoki, M. S., Fiamoncini, J., Turri, A. O., Curi, R., Miyabara, E. H., & Moriscot, A. S. (2010). Leucine attenuates skeletal muscle wasting via inhibition of ubiquitin ligases. *Muscle & Nerve, 41*, 800–808.

Castillo, E. M., Goodman-Gruen, D., Kritz-Silverstein, D., Morton, D. J., Wingard, D. L., & Barrett-Connor, E. (2003). Sarcopenia in elderly men and women: The Rancho Bernardo study. *American Journal of Preventive Medicine, 25*, 226–231.

Foletta, V. C., White, L. J., Larsen, A. E., Léger, B., & Russell, A. P. (2011). The role and regulation of MAFbx/atrogin-1 and MuRF1 in skeletal muscle atrophy. *Pflugers Archiv: European Journal of Physiology, 461*, 325–335.

Herningtyas, E. H., Okimura, Y., Handayaningsih, A. E., Yamamoto, D., Maki, T., Iida, K., Takahashi, Y.,

Kaji, H., & Chihara, K. (2008). Branched-chain amino acids and arginine suppress MAFbx/atrogin-1 mRNA expression via mTOR pathway in C2C12 cell line. *Biochimica et Biophysica Acta, 1780*, 1115–1120.

Kisselev, A. F., & Goldberg, A. L. (2001). Proteasome inhibitors: From research tools to drug candidates. *Chemistry & Biology, 8*, 739–758.

Lee, J. S., Auyeung, T. W., Kwok, T., Lau, E. M., Leung, P. C., & Woo, J. (2007). Associated factors and health impact of sarcopenia in older Chinese men and women: A cross-sectional study. *Gerontology, 53*, 404–410.

Montes de Oca, M., Loeb, E., Torres, S. H., De Sanctis, J., Hernández, N., & Tálamo, C. (2008). Peripheral muscle alterations in non-COPD smokers. *Chest, 133*, 13–18.

Nicastro, H., Artioli, G. G., Costa Ados, S., Solis, M. Y., da Luz, C. R., Blachier, F., & Lancha, A. H., Jr. (2011). An overview of the therapeutic effects of leucine supplementation on skeletal muscle under atrophic conditions. *Amino Acids, 40*, 287–300.

Petersen, A. M., Magkos, F., Atherton, P., Selby, A., Smith, K., Rennie, M. J., Pedersen, B. K., & Mittendorfer, B. (2007). Smoking impairs muscle protein synthesis and increases the expression of myostatin and MAFbx in muscle. *American Journal of Physiology. Endocrinology and Metabolism, 293*, E843–E848.

Rom, O., Kaisari, S., Aizenbud, D., & Reznick, A. Z. (2012). Sarcopenia and smoking: A possible cellular model of cigarette smoke effects on muscle protein breakdown. *Annals of the New York Academy of Sciences, 1259*, 47–53.

Romero-Calvo, I., Ocón, B., Martínez-Moya, P., Suárez, M. D., Zarzuelo, A., Martínez-Augustin, O., & de Medina, F. S. (2010). Reversible Ponceau staining as a loading control alternative to actin in Western blots. *Analytical Biochemistry, 401*, 318–320.

Szulc, P., Duboeuf, F., Marchand, F., & Delmas, P. D. (2004). Hormonal and lifestyle determinants of appendicular skeletal muscle mass in men: The MINOS study. *The American Journal of Clinical Nutrition, 80*, 496–503.

Tang, K., Wagner, P. D., & Breen, E. C. (2010). TNF-alpha-mediated reduction in PGC-1alpha may impair skeletal muscle function after cigarette smoke exposure. *Journal of Cellular Physiology, 222*, 320–327.