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Role of the ubiquitin-proteasome system in the sarcopenic-like phenotype induced by CCL5/RANTES

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Abstract

Sarcopenia is characterized by reduced muscle strength and mass, and a decline in muscle fiber diameter and amount of sarcomeric proteins. Sarcopenia involves the activation of the ubiquitin-proteasome system (UPS). MuRF-1 and atrogin-1 are E3 ubiquitin ligases belonging to UPS, leading to proteolysis mediated by the PSMB 5, 6, and 7 subunits of 20S proteasome. CCL5/RANTES induces a sarcopenic-like effect in muscle cells. The present work explored the impact of CCL5 on UPS components and the influence of UPS on its sarcopenic-like effect. We demonstrated that CCL5 increased MuRF-1 and atrogin-1 protein levels and mRNA levels of subunits PSMB 5, 6, and 7. We used the MG132 inhibitor to elucidate the role of the 20S proteasome in the CCL5-induced sarcopenic-like effect. This inhibitor prevented the decrease in troponin and MHC protein levels and partially stopped the reduction in the diameter of single-isolated flexor digitorum brevis (FDB) muscle fibers induced by CCL5. These findings indicate that CCL5 actively modulates the UPS. Moreover, our results show the direct participation of UPS in the sarcopenic-like phenotype induced by CCL5.

Key Words: Irisin, saliva, serum, muscle, physical exercise.

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The skeletal muscle is distinguished by its plasticity and organized structure. The sarcomere, the structural unit of the muscle, comprises thick and thin filaments, mainly composed of myosin and alpha-actin proteins, along with the troponin protein complex.[1] The ubiquitin-proteasome system (UPS) is a central complex that modulates protein catabolism in the organism. This process involves the ubiquitination of target proteins and their subsequent proteolysis by the proteasome.[2] Ubiquitination involves the covalent bind of the polyubiquitin (Ub) chain to the target protein, which is mediated by E3 ubiquitin ligases. This polyubiquitination acts as a tag the proteasome recognizes.[3] The proteasome is composed of catalytic subunits that include caspase-like activity (PSMB6/β1 subunit), trypsin-like wang activity (PSMB7/β2 subunit), and chymotrypsin-like activity (PSMB5/β5 subunit), allowing the degradative activity of the targeted proteins.[4] Muscle RING-finger protein-1 (MuRF-1) and muscle-specific F-box protein 1 (atrogin-1/MAFbx1) are the primary ubiquitin ligases targeting sarcomeric proteins.[2] The upregulation of atrogin-1 and MuRF-1 results in an imbalance in muscle proteostasis, a phenomenon observed in pathological conditions such as sarcopenia.

Sarcopenia is a syndrome characterized by losing muscle mass, strength, and physical function.[5,6] Proteostasis imbalance, reduced fiber diameter, and diminished sarcomeric proteins like myosin heavy chain (MHC) and troponin I are characteristic of sarcopenia. [7]

Specific cytokines have been implicated in inducing sarcopenia. Recently, CCL5/RANTES was identified as

a myokine.[8] CCL5/RANTES expresses two receptors, CCR1 and CCR5, in skeletal muscle.[9] In cells different from skeletal muscle, CCL5/RANTES modulates the activation of the NF-κB and FoxO transcription factors, which regulate the muscular expression of UPS components.[10,11] However, it remains unexplored whether CCL5/RANTES modulates UPS components and the specific participation of UPS in the sarcopenia induced by CCL5/RANTES.

This study demonstrates the critical involvement of the ubiquitin-proteasome system in the CCL5/RANTES-mediated proteolysis of sarcomeric proteins.

Materials and Methods

Mice

Male mice of the C57BL/6 strain, 4 months old, were used and had access to water and food. The animals were anesthetized with 5 % isoflurane and sacrificed by cervical dislocation. Subsequently, flexor digitorum brevis (FDB) muscle extraction was performed to obtain isolated muscle fibers.

Cell culture

Myoblast C2C12 were maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with fetal bovine serum 10 % at 37 ºC and CO2 5 % for 2 days at 90 % of confluency and then differentiated to myotubes using DMEM supplemented with horse serum 4 % for 5 days. Later, the myotubes were incubated with rCCL5 at 200 ng/mL (ProSpec, East Brunswick, NJ, USA) according to the time indicated in each experiment.

Table 1. List of the primer sequences used for qPCR in Eco Real-Time.

Gene	Primers
PSMB5/β5	Forward: GAGATCAACCCGTACCTTCTG Reverse: GACCGAGATGCGTTCCTTAT
PSMB6/β6	Forward: CCCTATCCACGATCACATCTTC Reverse: GGAGGCTCGTTCAGTTCAATA
PSMB7/β7	Forward: CTGGACTTTCTTCGTCCATTCT Reverse: CAGTCTCTTCTAGCACCTCAATC
β-actin	Forward: GTGACGTTGACATCCGTAAAGA Reverse: GCCGGACTCATCGTACTCC

Pre-incubation with 40 µM of MG132 was performed 1 h before incubation with rCCL5.

Single-isolated flexor digitorum brevis (FDB) muscle fiber cultures

The flexor digitorium brevis (FDB) muscle from C57BL6J was extracted and enzymatically digested with collagenase I in DMEM supplemented with horse serum 10 %. Once the muscle was mechanically disaggregated, the medium was replaced, and then the fibers obtained were separated and incubated in plates previously treated with Matrigel. The treatments with rCCL5 200 ng/mL started when the fibers were attached to the plates. Pre-incubation with 40 µM of MG132 was performed 1 h before incubation with rCCL5.

RNA extraction and RT-qPCR.

RNA was extracted from myotubes C2C12 using Chomczynski's solution. The RNA was obtained and resuspended in nuclease-free water. 2 mg of RNA was used to obtain the cDNA for retrotranscription using the M-MLV enzyme (Invitrogen, Carlsbad, CA, USA). Then, cDNA was used to analyze the catalytic subunits of proteasome PSMB5/β5, PSMB6/β1, and PSMB7/β2 by qPCR in Eco Real-Time PCR System using β-actin as housekeeping (Table 1). The mRNA expression of target genes was calculated using the ΔΔCt method, normalized relative to *β-actin* gene expression.

Western blot analysis.

The protein extracts were obtained from myotubes at 72 h after incubation with rCCL5 200 µg/mL. The cells were homogenized with radioimmunoprecipitation assay (RIPA) buffer containing phosphatase inhibitors sodium orthovanadate 1mM, sodium pyrophosphate 1 mM, a Protease Inhibitor Cocktail 1 mM (Cat# P8340 Sigma-Aldrich, St. Louis, MO, USA), and phenylmethylsulfonyl fluoride 1 mM. The protein quantification was performed with the MicroBCA Protein Assay Kit. Protein extracts were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes (PVDF). The immunoblotting was developed with the following primary antibodies: mouse anti-MHC (1:1,000 MF-20; Developmental Studies, Hybridoma Bank, University of Iowa, Iowa, IA, USA), mouse anti-troponin I (1:1,000; Cell Signaling, Danvers, MA, USA), mouse anti-MuRF-1 (1:1,000, Abcam, Cambridge, MA, USA), rabbit anti-atrogin-1 (1:1,000, Abcam, Cambridge, MA, USA) mouse anti-α-Tubulin (1:1,000; Santa Cruz, Dallas, TX, USA), mouse anti-β-actin (1:2,000, Abcam, Cambridge, MA, USA). The following secondary antibodies were used: anti-mouse IgG-HRP (1:10,000; Santa Cruz, Dallas, TX, USA) and anti-rabbit IgG-HRP (1:10,000; Santa Cruz, Dallas, TX, USA). The immunoreaction was visualized by enhanced chemiluminescence (Thermo Scientific, Waltham, MA, USA). Images were acquired using the Fotodyne FOTO/Analyst Luminary Workstation Systems (Fisher Scientific, St. Waltham, MA, USA), and the quantification of the bands was performed utilizing densitometric analysis using ImageJ software. Then, each

condition was normalized with the loading control and further represented as a fold of change relative to the vehicle group.

Determination of single-isolated FDB fiber diameter. The single-isolated FDB fibers were maintained in 24-well plates and treated with rCCL5 200 ng/mL for 72 h. For MG132, fibers were pre-treated for 1 h with this inhibitor (40 µM). The medium was removed, fibers were fixed with PFA 4%, permeabilized with PBS-Triton X-100 0.1 %, and then blocked with PBS-BSA 1 % for 1 h. To delimit the isolated fibers, we used antibody anti-Caveolin-3 (1:500 Santa Cruz Biotechnology, Dallas, TX, USA), and then, an affinity-purified Alexa Fluor dye-conjugated anti-mouse antibody (1:1,000 Life Technologies, Waltham, MA, USA). After, the fibers were incubated with Hoechst-PBS and finally with Fluoromont mounting solution. Samples were observed on a FLoid® Cell Imaging Station and analyzed using ImageJ software (NIH, Bethesda, MD, USA), measuring the diameter of five fiber sections in 50 fibers from each experimental condition. The five diameter measurements were averaged per fiber and plotted by diameter ranks of 10 µm, which also were analyzed and expressed as the accumulative frequency of these ranks.

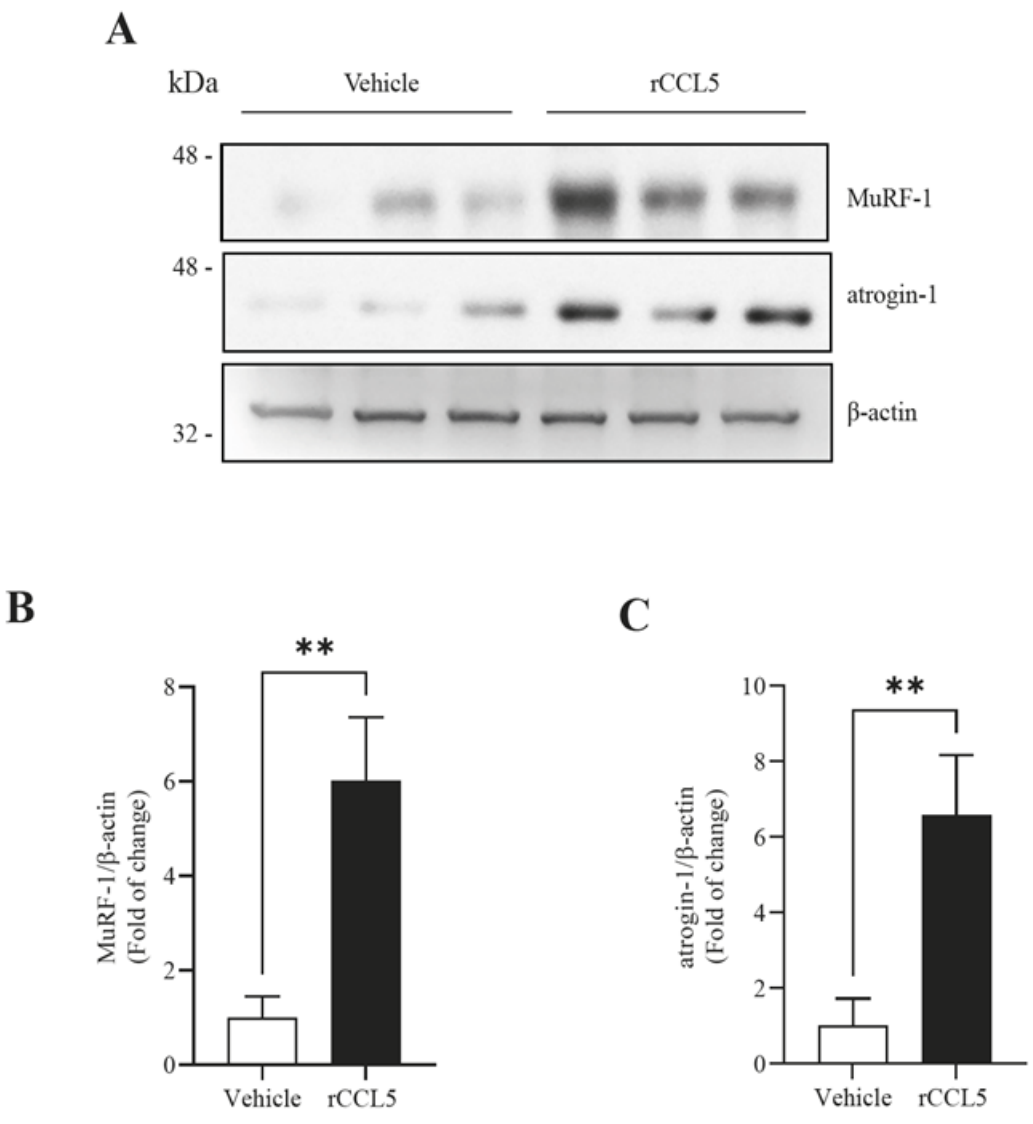

Fig 1. CCL5/RANTES increases atrogin-1 and MuRF-1 protein levels in C2C12 myotubes. *C2C12 myoblasts were differentiated for 5 days. Then, myotubes were incubated with 200 ng/mL of recombinant CCL5/RANTES (rCCL5) or vehicle for 24 h. (A) MuRF-1 and (B) atrogin-1 protein levels were detected for western blot analysis using β-actin as a loading control. Molecular weights are shown in kDa. Densitometry analysis for MuRF-1 (C) and atrogin-1 (D) is represented as a fold of change relative to the vehicle and expressed as the mean ± SD of three independent experiments. (t-test, * $p < 0.05$ vs. vehicle group).*

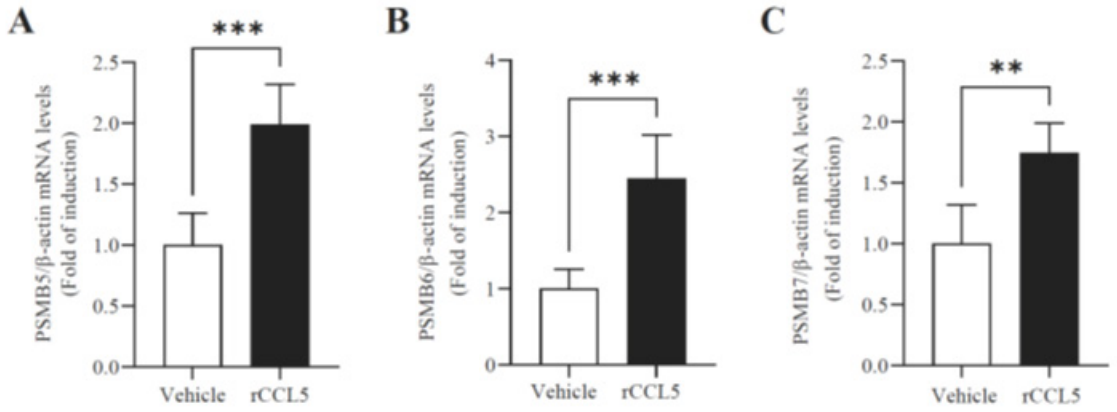

Fig 2. CCL5/RANTES increases mRNA levels of 20S proteasome subunits in C2C12 myotubes. C2C12 myoblasts were differentiated for 5 days until forming myotubes. Then, cells were incubated with 200 ng/mL of recombinant CCL5/RANTES (rCCL5) or vehicle for 24 h. The gene expression of (A) PSMB 5, (B) PSMB 6, and (C) PSMB 7 was detected for RT-qPCR using β-actin as the housekeeping gene. Analysis for PSMB 5, 6, and 7 is represented as a fold of change relative to the vehicle and expressed as the mean ± SD of three independent experiments. (t-test, *$p < 0.05$ vs. vehicle group).

Statistics

The statistical analysis of the data was performed with the Prism 8.0 analysis software (GraphPad Software, San Diego, CA, USA). The assumption of normality distribution was tested using the Shapiro–Wilk test. Data were analyzed with a t-test, or one-way or two-way ANOVA, with a Dunnet post-hoc test. Differences were considered significant when $p < 0.05$.

Results

CCL5/RANTES increases MuRF-1 and atrogin-1 protein expression.

The ubiquitin-proteasome system (UPS) is a mechanism of protein degradation that undergoes alterations in sarcopenia, as evidenced by the increased expression of MuRF-1 and atrogin-1. These proteins are responsible for the polyubiquitination of muscle proteins, marking them for subsequent degradation via the proteasome.[3] To assess the impact of recombinant CCL5/RANTES (rCCL5) incubation on the protein levels of MuRF-1 and atrogin-1 in muscle cells, we treated C2C12 myotubes with rCCL5. We observed a significant increment in the protein levels of MuRF-1 and atrogin-1 (Figure 1A). Specifically, MuRF-1 protein levels increased approximately sixfold compared to vehicle-treated cells (Figure 1B), while atrogin-1 exhibited a roughly sevenfold increase (Figure 1C).

These findings demonstrate that CCL5/RANTES enhances the protein levels of MuRF-1 and atrogin-1, critical components of the UPS.

CCL5/RANTES upregulates mRNA levels of 20S proteasome subunits in skeletal muscle cells.

The 20S proteasome complex, a part of UPS, is essential in breaking down proteins through different enzymatic activities such as chymotrypsin-like, caspase, and trypsin-like. Studies on sarcopenia showed increased expression in E3 ligases MuRF-1, atrogin-1, and all three subunits of the 20S proteasome.[11] To investigate how CCL5/RANTES potentially regulates the subunits of the 20S proteasome, we analyzed the mRNA levels in C2C12 myotubes incubated with rCCL5. As shown in Figure 2, treatment with rCCL5 led to double the mRNA levels of PSMB5 subunit associated with chymotrypsin-like activity (Figure 2A), around 2.5-fold higher mRNA levels of PSMB6 subunit associated with caspase-like activity (Figure 2B) and a corresponding increase in mRNA levels of PSMB7 subunit associated with trypsin-like activity (Figure 2C) compared to the group treated with vehicle.

Overall, these findings suggest that CCL5/RANTES promotes an increase in production for the subunits of the 20S proteasome, thereby influencing processes involved in protein breakdown.

Mitigation of CCL5/RANTES-induced fiber diameter reduction by suppressing UPS activity

Considering prior findings indicating that CCL5/RANTES decreases the diameter of flexor digitorum brevis (FDB)-isolated fibers, we evaluated the role of UPS in this size reduction by using the MG132 inhibitor, which impedes the proteolytic activity of the 20S proteasome. Thus, MG132 was a crucial tool in understanding the mechanism involved in the CCL5/RANTES-induced sarcopenic-like effect. As depicted in Figure 3A, incubation with rCCL5 led to a notable reduction in the diameter of single-isolated muscle fibers. When scrutinizing the diameter across different ranges, it became apparent that proteasome inhibition with MG132 partially counteracted the decrease in fiber diameter within the 30-40 µm range (Figure 3B). This protective effect consistently manifested with MG132, resulting in a roughly 20 %

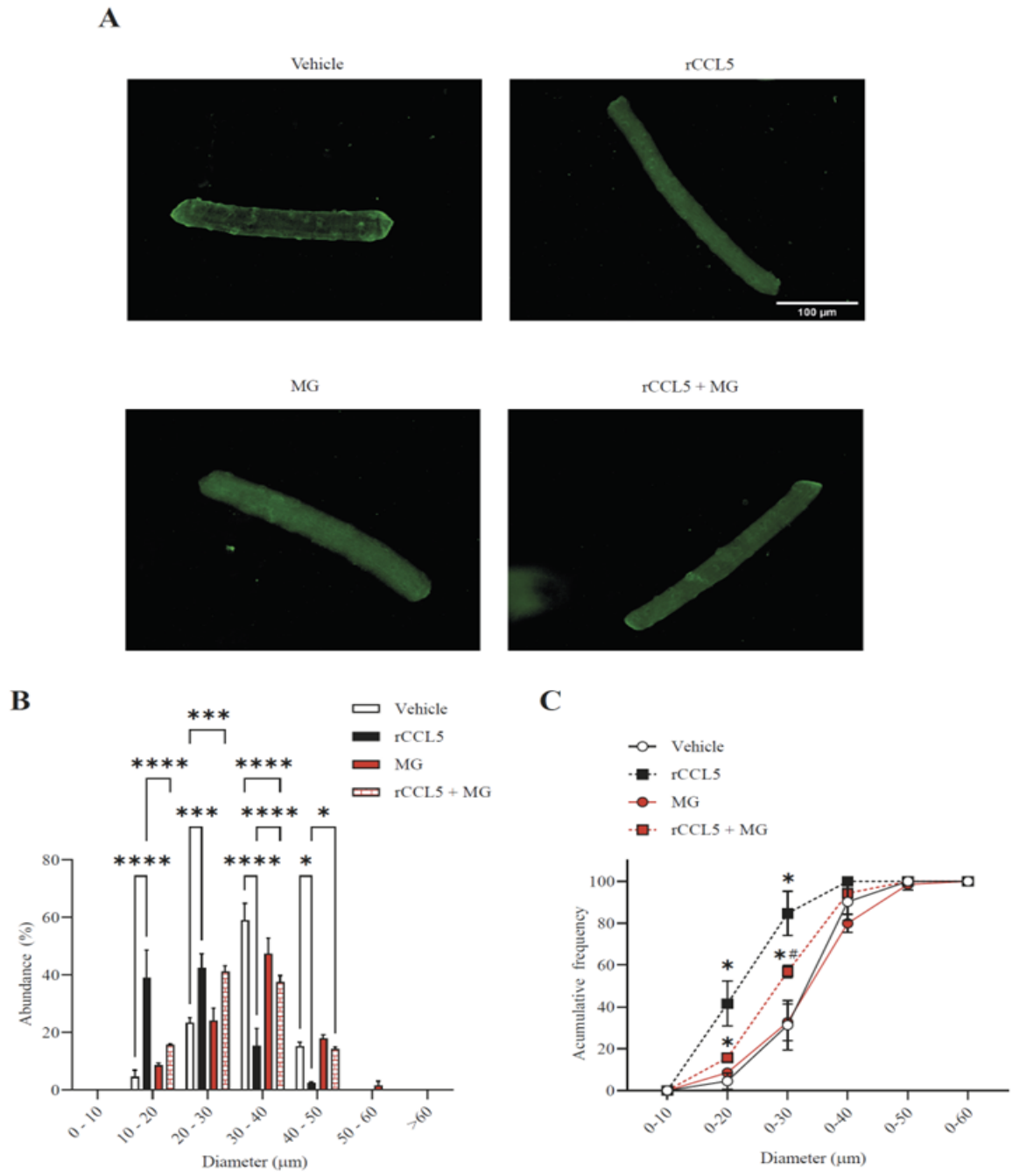

Figure 3. Inhibition of UPS prevents the decrease in diameter of isolated muscle fiber induced by CCL5/RANTES. Isolated-FDB muscle fibers were obtained from C57BL/6 male mice, pre-treated with MG132 (MG) 40 µM for 1 h, and incubated with 200 ng/mL of recombinant CCL5/RANTES (rCCL5) for 72 h. **(A)** The diameter of isolated muscle fiber was delimited by indirect immunofluorescence for caveolin 3. The scale bar indicates 100 µm. The quantification was performed using ImageJ software. The fiber diameters were plotted through accumulative frequency analysis **(B)** and ranks **(C).** Results were represented as the mean ± SD for each group (n = 50 FDB muscle fibers. Two-way ANOVA post hoc Dunnet, *p < 0.05 vs. vehicle group).

reduction in fibers within the 20-30 µm range (Figure 3B). Accumulative frequency analysis showed a 20 % prevention in the decrease in fiber diameter within the 0-30 µm range when the UPS was inhibited during CCL5/RANTES treatment (Figure 3C). These findings strongly suggest the involvement of UPS in the CCL5/RANTES-induced reduction in the diameter of single-isolated FDB fibers.

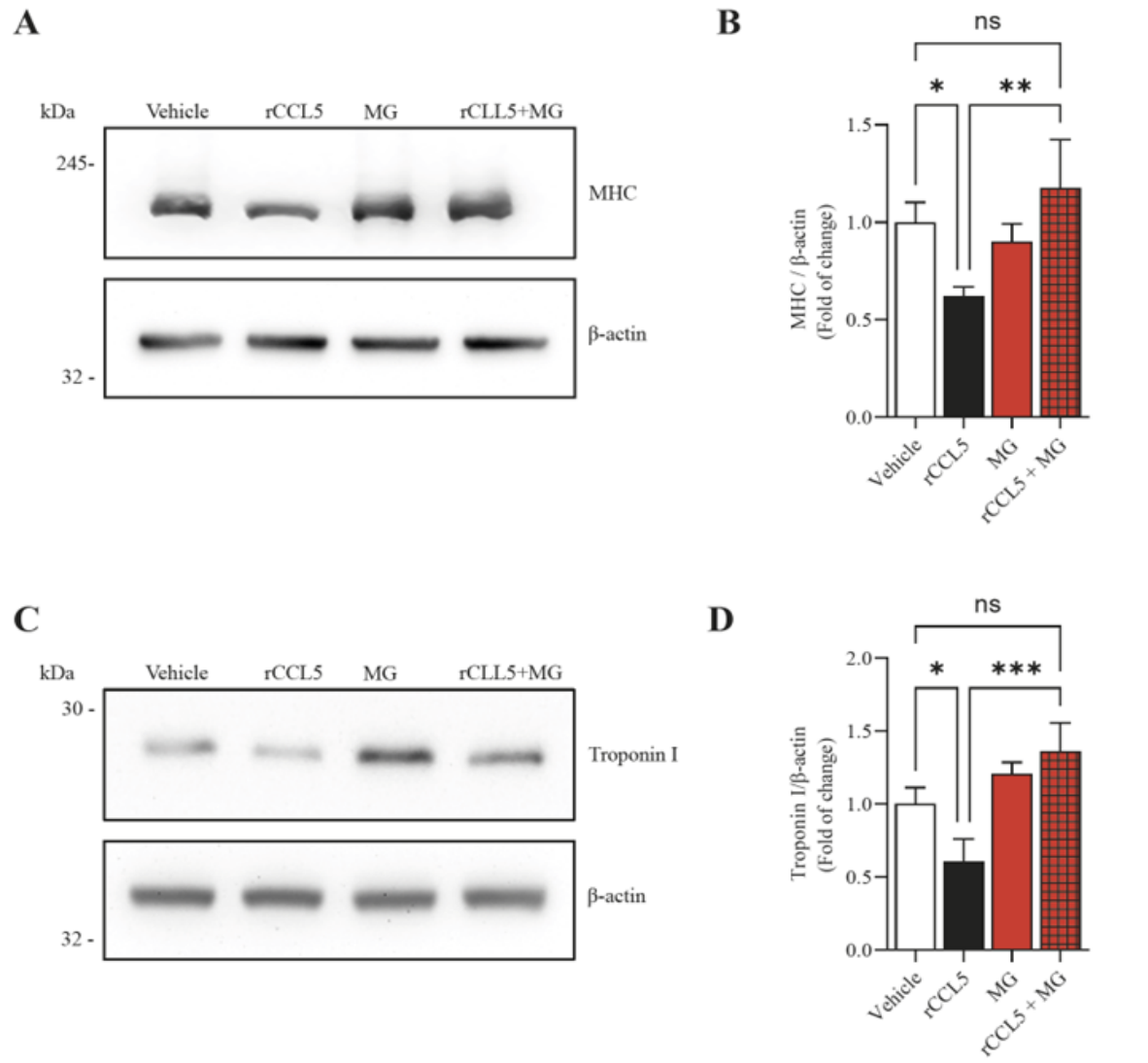

Figure 4. Inhibition of UPS prevents the decrease of sarcomeric proteins in C_2C_{12} myotubes. C2C12 myoblasts were differentiated for 5 days until forming myotubes, which were pre-treated with MG132 inhibitor (MG) 40 µM for 1 h and then incubated with 200ng/mL of recombinant CCL5/RANTES (rCCL5) or vehicle for 72 h. **(A)** MHC and **(B)** troponin I protein levels were detected by western blot analysis, using β-actin as loading control. Molecular weights are shown in kDa. Densitometric analysis was performed for MHC **(C)** and troponin I **(D)**. The results were represented as a fold of change relative to the vehicle and expressed as the mean ± SD of three independent experiments. (One way ANOVA, post hoc Dunnet, *p < 0.05 vs. vehicle group).

Preventing CCL5/RANTES-induced reduction in sarcomeric protein levels through UPS inhibition

To better understand the impact of the MG132 inhibitor on the role of UPS in the CCL5/RANTES-induced sarcopenic effect, we conducted a study about the reduction of sarcomeric protein levels caused by CCL5/RANTES in C2C12 myotubes. Our investigation focused on myosin heavy chain (MHC) levels and troponin I proteins. As shown in Figure 4A, CCL5/RANTES led to a 50 % decrease in MHC protein levels compared to the control group. However, this reduction was wholly prevented when we inhibited UPS using MG132 (as depicted in Figures 4A and 4B). Moreover, CCL5/RANTES caused a decline of 40 % in troponin I protein levels. These levels were fully restored when we inhibited UPS with MG132 (as seen in Figures 4C and 4D).

These findings highlight that the decrease observed in proteins like MHC and troponin I due to CCL5/RANTES is directly influenced by UPS activity within muscle cells.

Discussion

The ubiquitin-proteasome system (UPS) plays a role in sarcopenia by degrading components associated with muscle contraction. Our research shows that CCL5/RANTES increases UPS components, specifically MuRF-1, atrogin-1, and 20S proteasome subunits in skeletal muscle cells. We also demonstrated that the sarcopenic-like effect induced by CCL5/RANTES is dependent on UPS activity. Our findings about CCL5/RANTES induce an increase of the MuRF-1 and atrogin-1, two E3s ligases extensively associated with sarcopenia,[14] and the enhancement of 20S proteasome subunits (PSMB 5, 6, and 7) expression, aligns with observations made in denervation studies and cancer-induced sarcopenia,[12-15] showing higher levels of these subunits correlate with increased breakdown of muscle proteins. An interesting future aspect to study is elucidate signaling pathways associatedwi ho the effect of CCL5/RANTES on UPS components. CCL5/RANTES signals through the CCR1 and CCR5 receptors, which are expressed in skeletal muscle.[14] Thus, we must perform future experiments to thoroughly explore which receptor, CCR5 and/or CCR1, influences the gene expression of proteasome 20S subunits, atrogin-1, and MuRF-1. Regarding signaling pathways downstream of the receptor for CCL5/RANTES involved in the sarcopenic-like phenotype, there is evidence that NF-κB pathway can be activated through its receptors CCR1 and CCR5. For instance, CCL5/RANTES, via the CCR5 receptor, increases NF-κB pathway activity, inducing a synthetic atherogenic phenotype in smooth muscle cells.[11] Additionally, in human lung adenocarcinoma cells, CCL5, through the CCR5 receptor, augments the phosphorylation of p65 at Ser536 to enhance the transactivation of the NF-κB.[16] Similarly, CCL5 induces polarization to M1 macrophages in liver cells through both receptors, CCR1 and CCR5, activating NF-κB.[16] In addition, the expression of proteasome subunits decreases when NF-κB activity is inhibited. These observations suggest that CCL5/RANTES has the potential to modulate the NF-κB signaling pathway through its interactions with CCR1 and CCR5 receptors in skeletal muscle. Thus, other experiments could be conducted to elucidate the role of NF-κB in the CCL5/RANTES-dependent sarcopenic-like effect and specifically on the increase of UPS components. Our investigation also focused on the role of the 20S proteasome in the CCL5/RANTES-induced sarcopenic-like phenotype by using the MG132 inhibitor. When cells were co-treated with CCL5/RANTES and MG132, we observed prevention of the reduced MHC and troponin I protein levels. These results confirm that the inhibition of degradative activity associated to UPS attenuates the decrease in sarcomeric proteins caused by CCL5/RANTES. Interestingly, MG132 inhibitor partially prevented the decrease in the fiber diameter in single-isolated FDB fibers. This result can be explained because UPS is not the only degradative pathway that recognizes polyubiquitinated proteins and causes their degradation in skeletal muscle. The process of lysosomal degradation via autophagy shares with the UPS the recognition of protein targets via ubiquitination.[17] This similarity could explain the partial prevention observed in the diameter of FDB fibers. An imbalance in autophagy could also play a role in developing and promoting the sarcopenic phenotype.[2] Thus, new experiments must be performed to determine the possible contribution of the autophagy/lysosome pathway in the proteolysis induced by CCL5/RANTES and how it could be involved in the degradation of other components in muscle cells.

This study, for the first time, demonstrates the critical role of the UPS as a pivotal catabolic mechanism in CCL5/RANTES-dependent sarcopenic-like phenotype, particularly in the degradation of sarcomeric proteins.

List of acronyms

CCL5 - Chemokine (CC) ligand 5
CCR5 - Chemokine (CC) receptor type 5
FDB - Flexor digitorium brevis
FoxO - Forkhead box O
MHC - Myosin heavy chain
MuRF-1 - Muscle RING-finger protein1
MAFbx1/atrogin-1: Muscle-specific F-box protein 1
NF-kB: - Nuclear factor kappa B
PSMB5 - Proteasome 20S Subunit Beta 5
PSMB6 - Proteasome 20S Subunit Beta 6
PSMB7 - Proteasome 20S Subunit Beta 7
UPS - Ubiquitin-proteasome system

Contributions of Authors

Conceptualization, SC-L and CC-V; Methodology, SC-L, FA, DC, LP, and CC-V; Validation, SC-L, FA and CC-V; Investigation, SC-L, FA, DC, FS and CC-V; Visualization, SC-L, and CC-V; Supervision, CC-V; Project administration, DC, FS, LP and CC-V.; Formal Analysis, SC-L, FA and CC-V; Writing – Original Draft Preparation, SC-L, FA, FS, LP and CC-V; Writing – Review and Editing, FA, DC, FS, LP and CC-V.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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