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Restorative effects of (+)-epicatechin in a rodent model of aging induced muscle atrophy: Underlying mechanisms

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Abstract

Sarcopenia is a progressive and generalized age-related skeletal muscle (SkM) disorder characterized by the accelerated loss of muscle mass (atrophy) and function. SkM atrophy is associated with increased incidence of falls, functional decline, frailty and mortality. In its early stage, SkM atrophy is associated with increased pro-inflammatory cytokine levels and proteasome-mediated protein degradation. These processes also link to the activation of atrophy associated factors and signaling pathways for which, there is a lack of approved pharmacotherapies. The objective of this study, was to characterize the capacity of the flavanol (+)-epicatechin (+Epi) to favorably modulate SkM mass and function in a rat model of aging induced sarcopenia and profile candidate mechanisms. Using 23 month old male Sprague-Dawley rats, an 8 week oral administration of the +Epi (1 mg/kg/day in water by gavage) was implemented while control rats only received water. SkM strength (grip), treadmill endurance, muscle mass, myofiber area, creatine kinase, lactate dehydrogenase, troponin, α-actin, tumor necrosis factor (TNF)-α and atrophy related endpoints (follistatin, myostatin, NFkB, MuRF 1, atrogin 1) were quantified in plasma and/or gastrocnemius. We also evaluated effects on insulin growth factor (IGF)-1 levels and downstream signaling (AKT/mTORC1). Treatment of aged rats with +Epi, led to significant increases in front paw grip strength, treadmill time and SkM mass vs. controls as well as beneficial changes in makers of myofiber integrity. Treatment significantly reversed adverse changes in plasma and/or SkM TNF-α, IGF-1, atrophy and protein synthesis related endpoints vs. controls. In conclusion, +Epi has the capacity to reverse sarcopenia associated detrimental changes in regulatory pathways leading to improved SkM mass and function. Given these results and its recognized safety and tolerance profile, +Epi warrants consideration for clinical trials.

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Conflicts of Interest
Dr. Villarreal (co-founder) and Dr. Ceballos are stockholders of Epirium Bio Inc.

Keywords

skeletal muscle; aging; sarcopenia; epicatechin; flavanols

Introduction

One of the most notable and debilitating age-associated changes is the progressive loss of fat-free skeletal muscle (SkM) mass and strength, a condition known as sarcopenia [1]. With normal aging, SkM mass is lost at ~8% per decade past the age of 40 and 15% past 70 [2]. Sarcopenia is usually accompanied by physical inactivity, low mobility, slow gait and poor exercise capacity, which are features of frailty [1, 2]. Up to 50% of people >80 years old have sarcopenia [3]. Reports indicate that SkM strength is inversely and independently associated with all cause mortality [4]. Thus, strategies that counter sarcopenia are seen as a means to reduce frailty. Currently, only exercise is recognized as effective. A review on resistance training validated its positive effects on SkM mass/strength but highlighted important limitations including the fact that training cannot be discontinued as effects quickly diminish, regular exercise can be difficult in the elderly and exercise alone, may not be sufficient to reverse sarcopenia [2]. Burton et al, discussed various non-exercise based treatments including hormone therapy [5]. With the exception of vitamin D, where a positive effect can be observed in subjects with a deficiency, other treatments including hormones were noted to be either risky or unproven. Thus, the pressing need to identify novel, safe and effective therapies.

Tumor necrosis factor (TNF)-α is a pro-inflammatory cytokine that plays key roles in immune system regulation. TNF-α has also been linked with the development of SkM atrophy and was originally termed “cachexin” in recognition to its catabolic actions as seen with cancer [6]. TNF-α levels increase with aging and are seen as a possible trigger of sarcopenia [7]. Atrophy inducing effects of TNF-α can be the results of direct and indirect actions. TNF-α can directly trigger SkM protein degradation via the induction of the ubiquitin-proteasome system (UPS) secondary to NFkB stimulated increases in atrophy-related gene transcription which encode two muscle-specific ubiquitin ligases, atrogin 1 and MuRF 1 [8]. TNF-α can also inhibit the AKT/mTORC1 signaling pathways leading to the reduced protein synthesis as a consequence of suppressing the stimulatory actions of insulin growth factor (IGF)-1 on SkM [8]. The peptide hormone myostatin, belongs to the transforming growth factor (TGF)-β superfamily and has been identified as the most potent suppressor of SkM growth via SMAD 2/3 pathway activation [9, 10]. Its muscle and/or blood levels are reported to increase with aging and in muscle wasting diseases [11]. Myostatin production can also be increased via TNF-α effects on NFkB [10, 12]. A physiological means of inhibiting myostatin action is to increase follistatin levels [13]. Follistatin is also a TGF-β family member of secreted peptides, yet it stimulates SkM growth by binding myostatin and thus, prevent attachment to its receptor [14]. Myostatin and follistatin shift in opposite directions with strength training [15, 16].

We reported that (−)-epicatechin, the primary cacao flavanol, enhances exercise capacity in middle aged mice to levels comparable to those triggered by treadmill training [17]. Two

week oral treatment with (−)-epicatechin yielded enhanced SkM function with increased myofiber cross sectional area in select muscles. In young and old mice, (−)-epicatechin decreases SkM protein levels of myostatin, while increasing follistatin [18]. In a rat model of SkM dysfunction mimicking Gulf War illness, (−)-epicatechin reverses SkM atrophy and increases exercise capacity while decreasing TNF-α levels [19]. Recently, we characterized the epimer (+)-epicatechin (+Epi) as 10 fold more potent vs. (−)-epicatechin in stimulating neurogenesis [20] but have not explored its SkM effects in the setting of aging.

The objective of this study was to examine the effects of 8 week treatment of +Epi on a rat model of sarcopenia. Blood and/or SkM levels of TNF-α, IGF-1, myostatin and follistatin were determined as well as muscle mass and function. Pathways related to protein synthesis/degradation (AKT, mTORC1), SMAD 2/3 and UPS associated endpoints including myostatin, MuRF 1 and atrogin 1 were evaluated in SkM.

Materials and Methods

Study design and animal model.

All experimental protocols were approved by UCSD's Institutional Animal Care and Use Committee (IACUC) and all methods were carried out in compliance with relevant institutional, Federal and ARRIVE guidelines and regulations. Our animal model of aging was implemented using 3 month old, male Sprague Dawley rats that aged to 23 months while provided regular chow and water at libitum. Blood samples were obtained from tail vein at required intervals and before and after treadmill tests and treatment and plasma isolated for subsequent analysis. +Epi was obtained as a gift from Epirium Bio, Inc. At 23 months of age, rats were randomly allocated in 2 groups: 1) control animals only given vehicle (water by gavage) (n=6) and, 2) +Epi (1 mg/kg/day in water by gavage) treated animals (n=6) for a period of 8 weeks. Group size was determined as per our previous experience in similar SkM focused studies [18] where groups of n=5 yielded significant results. Body weight was recorded every 3 days. After their final functional assessment, animals were euthanized and gastrocnemius muscles collected, weighed and processed for either histological (in 10% buffered formalin) or biochemical analysis (flash frozen).

Muscle strength.

Front limb muscle strength was measured once/week using a grip-strength meter device (BioSeb, Harvard Apparatus). Animals are placed on a metal grid with their front paws holding onto a T-bar. Upon the sudden application of a tail-pull, animals in a reflex manner pull the bar and tension is recorded digitally. Measurements were performed 3 times and values averaged.

Treadmill testing.

Rats were initially familiarized with the treadmill device at minimum speed (~14 m/min) for 5 min during 2 days previous to the actual test. Once familiarized, the exhaustion test (performed by a blinded operator) consisted of rats walking on the treadmill at minimum speed until the animal is no longer able to walk. Air jets at the back of the treadmill were used to discourage animals from stopping. Exhaustion was defined as when rats were no

longer able to maintain their normal walking position. Total walking time was recorded at this point. The test was implemented before treatment (23 months of age), at 4 weeks of treatment and at the end of treatment (1st trial). The test (2nd trial) was then repeated 48 h after the 1st trial in order to evaluate the recovery from fatigue.

Histomorphometry.

Gastrocnemius muscles were isolated, weighed and trimmed before formaldehyde fixing or immediate freezing for subsequent biochemical analysis. Fixed muscle samples were stained with Hematoxilin and Eosin and microscope imaged to digitally quantify myofiber cross sectional area (a total of ~450 fibers/group were measured).

Creatine kinase and lactate dehydrogenase activity.

As general measures of tissue/muscle integrity/stress creatine kinase (CK) (Abcam, 155901) and lactate dehydrogenase (LDH) (Sigma-Aldrich, MAK066) enzyme activity levels were measured using kits following manufacturer instructions.

TNF-α and IGF-1.

The assessment of plasma and/or SkM TNF-α (ThermoFisher Scientific KRC3011) and IGF-1 (ThermoFisher Scientific ERIGF1) levels was performed using ELISA kits as per manufacturer's instructions.

Protein degradation.

SkM protein degradation rate was measured as net tyrosine release from isolated samples. Gastrocnemius samples (10 mg) were pre-incubated for 30 min in Krebs Ringer buffer [NaCl 1.2 mmol/L; KCl 4.8 mmol/L; $NaHCO_3$ 25 mmol/L; $CaCl_2$ 2.5 mmol/L; KH_2PO_4 1.2 mmol/L and $MgSO_4$ 1.2 mmol/L; pH 7.4], supplemented with glucose [5.5 mmol/L], bovine serum albumin [1.0 g/L], insulin [5 U/mL], and cyclohexamide [5 mmol/L], saturated with 95% O_2/5% CO_2 gas mixture. Muscles were transferred into a fresh medium of the same composition and incubated for 2 h. At the end of the incubation, medium samples were used to measure released tyrosine by spectrophotometry.

Protein ubiquitylation.

Lysates were prepared by homogenizing gastrocnemius (15 mg) in a lysis buffer with protease inhibitors. Samples were centrifuged and supernatants collected. A total of 30 μg of protein were loaded onto a 4–15% gel and transferred to polyvinylidene fluoride membranes. An antibody (Cell Signaling, 3936) was used for the detection of ubiquitinated proteins by Westerns (see below).

Proteasome activity.

Proteasome activity was measured by using a Kit (Abcam, Ab107921) that detects chymotrypsin-like activity using a 7-amino-4-methylcoumarin tagged peptide substrate that generates a fluorescent product in the presence of proteolytic activity. Lysates of gastrocnemius muscle (15 mg) were used. Homogenates were centrifuged and supernatants collected. Proteasome activity was measured with fluorescent substrates of the tagged

peptide. The assay was conducted in the absence and presence of the specific proteasome inhibitor MG-132 to determine proteasome-specific activity. Released tagged peptide was measured using a fluorometer at an excitation wavelength of 350 nm and emission of 440 nm.

Western blotting.

To assess treatment effects on signaling pathways associated with the regulation of muscle growth relative protein levels for IGF-1, SMAD2/3, AKT, mTORC1, MuRF 1, atrogin 1, proteasome S20, protein ubiquitylation, TNF-α and NFkB were determined in gastrocnemius. For regulators of muscle growth as well as constitutive proteins, changes in protein levels, follistatin, myostatin, troponin and α1-actin were determined. A total of 30 μg of protein were loaded onto a 4–15% gel, electrotransferred, incubated for 1 h in blocking solution (5% non-fat dry milk in tween-Tris buffer saline) and followed by either 3 h incubation at room temperature or overnight at 4°C with primary antibodies. Primary antibodies were typically diluted 1:1,000 or 2,000 in Tween buffer plus 0.5% bovine serum albumin or 2% milk-based buffer. Membranes were washed with Tween buffer and incubated 1 h at room temperature in the presence of species-specific horseradish peroxidase-conjugated secondary antibodies diluted 1:5,000 in blocking solution. Membranes were again washed and immunoblots developed using chemiluminescence. Band intensities were digitally quantified using NIH Image J software and values normalized using GAPDH derived values as a loading control. Antibodies used from Cell Signaling, Inc. included GAPDH (catalog #, 5174), AKT (#9272), p-AKT (ser 473, #9271), mTORC1 (#2983), p-mTORC1 (ser 2448, #5536), SMAD 2/3 (#3102), p-SMAD 2/3 (ser 423, #9520), MuRF1 (#4305), ubiquitin (P4D1, #C3936), troponin I (#4002). From Abcam, Fbox40 (ab194362), follistatin (ab64490), myostatin (ab55106) proteasome 20S (ab242061). From Santa Cruz Biotechnology, MAFbx (atrogin 1) (sc-166806), α1-actin (sc-20641), TNF-α (sc-133192). NFkB (14-6731-81, ThermoFisher Scientific) and muscle creatine kinase (A01472–40, GenScript).

Statistical analysis

All results are presented as mean ± standard error of the mean (SEM) and analyzed by unpaired t-test to determine differences between each group means or by one-way ANOVA using multiple comparisons followed by Tukey’s test to determine differences. p values < 0.05 were considered statistically significant.

Results

Effects on morphometric, functional and biochemical endpoints

Figure 1A reports on the body weight of rats and denotes increases from 2 until ~18 months of age. Beyond 18 months, rat body weight showed a trend to decrease until the end of the study (25 months). Body weights recorded before (1B) and after (1C) treatment were comparable between control and Epi groups. Front limb strength was recorded once/week from 3 to 25 months of age and values are reported in figure 2. Animals demonstrate increases in strength from the 4 month time point, through month 11. Thereafter, animals

noted steady strength through month 17 followed by a progressive decline (2A). At 25 months of age (after treatment), +Epi treatment yielded a significant increase in strength of ~25% vs. controls (2B). Treadmill walking time is reported in figure 3. Panel A shows walking times at 23, 24, 25 months of age. At the end of treatment, a significant increase of ~28% occurred in the +Epi group vs. control. +Epi also yielded a sustained improved performance in the 2nd treadmill trial vs. controls which yielded a poorer treadmill time (A, B). Figure 4 reports on fold changes in CK (A) and LDH (B) activity in plasma of aged rats before and after the treadmill 1st and 2nd trials. After the 1st trial, in control and +Epi treated rats CK and LDH levels increased as compared vs. before. However, the +Epi group demonstrated a significant ~15% decrease vs. controls in both enzymes while after the 2nd trial +Epi treated animals demonstrated a significant ~25% decrease suggesting improved SkM recovery after exercise. Figure 5 reports on changes in gastrocnemius mass (A, B) and myofiber cross-sectional area (C, D). A significant gain of ~24% in muscle mass was recorded with +Epi vs. controls, which correlated with similar increases in myofiber cross-sectional area. Analysis of the percentage of fiber size distribution (E) demonstrate a higher population of small fibers in controls and higher population of medium size fibers with +Epi.

Effects on modulators of muscle mass

Figure 6 reports on the involvement of IGF-1 a key stimulator of muscle growth. Plasma IGF-1 levels demonstrate progressive decreases with aging (A). Following treatment, plasma IGF-1 levels were significantly higher in +Epi treated animals vs. controls (A), which is in agreement with SkM IGF-1 levels as per Westerns (B). IGF-1 associated protein synthesis pathway signaling as measured by AKT and mTORC1 phosphorylation (activation) levels were assessed by Westerns (C). +Epi treatment led to significantly higher levels of p-AKT and p-mTORC1 vs. controls. Figure 7A reports on age and treatment dependent changes in plasma myostatin levels. With aging, myostatin levels increased modestly over time. Following +Epi treatment, levels were significantly reduced vs. controls. Relative muscle protein levels for follistatin and myostatin were compared post-treatment (B). +Epi was able to significantly increase muscle follistatin levels while reducing those of myostatin. Myostatin associated downstream signaling was evaluated by assessing p-SMAD 2/3 relative protein levels in gastrocnemius (C). +Epi led to significantly decreased p-SMAD 2/3 levels vs. controls.

Effects on pro-inflammatory cytokines

Figure 8A documents aging and treatment associated effects on plasma TNF-α levels. With aging, a progressive increase in plasma levels was documented. With +Epi treatment, a significant decrease of ~33% was noted (B). Relative protein levels of TNF-α and NFkB were determined in gastrocnemius by Westerns (C) and ELISA (D and E) demonstrating significant decreases ranging from ~35–60% following +Epi treatment.

Effects on indicators of protein degradation and atrophy modulators

Gastrocnemius protein degradation was assessed by the analysis of protein ubiquitylation, proteasome subunit 20S, proteasome activity and tyrosine release (degradation) before and after treatment and results are reported in figure 9. Panel A shows a representative

Western blot image used to document changes in protein ubiquitylation which decreased significantly with +Epi treatment. +Epi treatment also resulted in significant decreases in proteasome subunit 20 (B), proteasome activity (C) and protein degradation rates (D). Figure 10 documents the involvement of two key protein factors known to trigger muscle atrophy, MuRF 1 and atrogin 1. Significant decreases in relative protein content of these two factors were observed by Westerns in Epi treated animals (A). Panel B reports on two SkM structural proteins troponin I and α1-actin which noted significant increases with +Epi treatment.

Discussion

Aging associated SkM atrophy and frailty represents a growing and pressing public health problem in need of the identification of safe and effective long-term treatments given its link to serious outcomes such as falls and death [1, 2]. Flavonoids are a class of polyphenolic compounds present in plants and their fruits that have been linked to many of the beneficial health effects attributed to them [21]. Cacao beans have one of the highest concentrations by weight of flavanols a subclass, of flavonoids. The regular consumption of modest amounts of cacao products high on flavanol (e.g., dark chocolate) content has been linked to beneficial effects on cardiovascular and metabolic systems [22]. On the basis of the evidence generated so far, the FDA recently allowed for limited health claims linking the consumption of products high in cacao flavanol content with improved cardiovascular outcomes. Investigations have documented beneficial effects of cacao flavanols on neurological and SkM systems [23, 24]. Epicatechin is the most abundant flavanol present in cacao and can be found in its two epimer (+) or (−) configurations [25]. In contrast to (−)-epicatechin, which is readily found in raw or processed cacao beans and cocoa powder (up to 82% of flavanols present), the natural occurrence of +Epi is undetectable and its appearance (secondary to epimerization reactions) is believed to only occur after processing of beans in such small amounts, that have proven difficult to detect and quantify [26, 27]. Whereas a large body of published reports have focused on (−)-epicatechin, significantly less has been explored regarding its (+) epimer form. In a pilot study, we characterized the favorable PK and safety profile of +Epi in normal human subjects and in a mouse model of high fat diet induced obesity and insulin resistance where favorable effects were noted on cardiometabolic endpoints [28]. In a separate study, we also reported the capacity of +Epi to increase C2C12 myotube ATP levels hinting at positive effects on SkM [29].

Aging in rodent models is known to lead to a decline in muscle mass and myofiber quality which parallels changes reported in humans thus, they have become a model to explore effects of candidate treatments [30]. While life expectancy of Sprague Dawley rats can range up to 36 months, significant mortality is noted above 20 months of age. It is estimated that two year old rats are equivalent to ~60-year-old humans [30]. In this study using a rat model of aging induced SkM atrophy, we examined the potential of +Epi to positively impact recognized measures of SkM function and link such changes to improvements in muscle mass.

As a means to track SkM function over time, we relied on grip strength which was tracked in animals from 3 to 25 months of age. Grip strength increased progressively from ~14 N at 3 months peaking at ~20 N at 11 months declining, from 17 months onward reaching in controls, a force of ~12 N at 25 months. Thus, we were able to document a progressive loss of SkM strength with aging. Two months of +Epi treatment was able to stabilize strength at ~15 N (~25% greater strength vs. controls) preventing the further decline noted. This observation is of interest as it suggests, that +Epi may preserve either muscle mass and/or retain muscle quality. Post-mortem analysis confirmed that +Epi was able to induce a higher muscle weight that also yielded a positive effect on myofiber cross sectional area and myofiber size towards a larger distribution. Previous studies have reported on the positive effects that natural compounds have on SkM mass and strength in aged rodents. In a study by Wang et al., treatment of 16 month old mice with apigenin (a flavonoid abundant in many plants and fruits) at a dose of 50 mg/kg/day for 9 months led to a significant recovery in grip strength and muscle mass similar in magnitudes to those reported by us including, a shift in myofibers towards a larger size [31]. Liao et al., reported on the positive effects of resveratrol (a polyphenol found in fruits such as grapes) in 24 month old rats treated with 150 mg/kg/day for 6 weeks on grip strength while not increasing SkM mass [32]. Thus, there is precedent for the beneficial effects that natural products have on such endpoints.

In this study, we also used treadmill walking time to assess the effects of +Epi on SkM function and implemented a double trial to further stress the SkM of aged rats. In the 1st trial, results documented increased total walking time effects of +Epi vs. controls an effect, similar in magnitude to that reported with apigenin in aged mice. Of interest is that following the 2nd trial, control animals covered less distance vs. the 1st trial while +Epi treated animals, retained their performance at 1st trial levels. CK and LDH are enzymes found in most cell types including myofibers and are released into the bloodstream when SkM damage or injury occurs such as during intense exercise or with muscle disease [33]. As expected, the activity of both enzymes increased similarly ~2–3 fold after the 1st and 2nd trials and +Epi was able to mitigate such increases by up to 25%. Of interest is that in the control group, the 2nd trial did not yield even higher levels of CK and LDH. Possible explanations include the fact that the 2nd test was done 48 h after the initial one and levels may have already decreased or that peak levels occurred at a time different than those used to sample blood. Altogether, results from grip strength measurements and treadmill tests document the capacity of +Epi to counter age induced loss of strength and such effects are linked to a preservation of muscle mass and lesser degrees of organ stress.

IGF-1 is a peptide secreted by many organs including the liver and SkM [34]. Circulating IGF-1 is relatively stable and young adults have greater concentrations vs. older individuals [34]. Apparently, intrinsic IGF-1 secretion by SkM and not circulating IGF-1, is a key determinant for switching on anabolic pathways in myofibers although it remains controversial. In SkM, IGF-1 is stimulated by mechanical loading and contraction [34]. Once secreted, IGF-1 activates its receptor to which intracellular signaling processes driving SkM protein synthesis can occur. The AKT pathway is recognized as the primary driver between mechanical contraction induced IGF-1 release and activation of protein synthesis and has been implicated as an important site of dysregulation between old and younger individuals [34]. AKT activates mTOR signaling leading to the formation of phosphorylated

mTORC1 protein complexes which then, activate protein synthesis. Thus, the stimulation of increases in SkM IGF-1 and potentially of plasma levels by non-mechanical means may be of great interest in countering aging induced sarcopenia. In this study, we report on progressive decreases in plasma IGF-1 levels with aging. Interestingly, plasma IGF-1 levels increased with +Epi but more importantly, SkM IGF-1 levels also increased. As expected, increased IGF-1 levels were associated with the activation of the AKT and mTORC1 protein synthesis pathway. Other natural compounds have been reported to stimulate IGF-1 levels in aged rodents. In a study by Niu et al., feeding of 21 month old mice an oral jelly for 3 months attenuated SkM atrophy and grip strength and increased serum IGF-1 levels vs. controls [35].

The second major humoral pathway that controls SkM growth involves myostatin which can be produced by many organs including SkM [10, 14]. Myostatin is one of the most well-known member of the TGF-β superfamily because of the dramatic hypermuscularity that develops in myostatin null mice [36]. Myostatin levels increase modestly with aging and with diseases associated with the development of sarcopenia such as heart failure and cancer [10, 14]. Binding of myostatin to its receptor induces SMAD 2/3 phosphorylation to promote the formation of a complex with SMAD 4 triggering its downstream atrophy inducing effects [10]. Notably, inhibition of SMAD 2/3 is sufficient to promote SkM growth suggesting that genes involved in protein turnover are the target of these transcription factors. Reports also indicate the presence of an inhibitory cross-talk between myostatin and the AKT/mTOR axis to reduce protein synthesis [10]. Follistatin also a member of the TGF-β superfamily, is produced by many organs including SkM and promotes increases in muscle mass by binding to myostatin and thus, preventing its attachment to its receptor [13]. We previously reported that follistatin levels decrease with aging [18]. In this study, relative SkM protein levels for follistatin and myostatin were compared post-treatment. +Epi was able to significantly increase follistatin levels while reducing those of myostatin. Myostatin associated downstream signaling was evaluated by assessing p-SMAD 2/3 relative protein levels in gastrocnemius. +Epi led to significantly decreased p-SMAD 2/3 levels vs. controls. In a study by Wijawa et al., a natural product in ginseng provided at 10 mg/kg for 5 weeks to 23 month old mice yielded increases in SkM mass and function while suppressing atrogin 1, MuRF 1 and myostatin levels vs. controls [37].

Several cytokines have been implicated in the pathogenesis of muscle wasting, most notably TNF-α, which is often elevated in the circulations of patients with sepsis or cancer, contributing to negative nitrogen balance that can promote SkM atrophy [6]. With aging, TNF-α levels increase in circulation and in SkM and thus, have been suspected of driving protein degradation in myofibers [6, 7]. TNF-α binds to receptors on cell membranes and activates the transcription factor NFkB.

NF-kB can stimulate atrogin 1 and MuRF 1 production by binding to their respective promoters and increasing their transcription [8]. Atrogin 1 is a muscle-specific E3 ubiquitin ligase, which targets muscle proteins for degradation via the UPS pathway. MuRF 1 is also a muscle-specific E3 ubiquitin ligase, and it works together with atrogin 1 to promote muscle atrophy by targeting different muscle proteins for degradation. Thus, NFkB upregulates the expression of atrogin 1 and MuRF 1, which leads to increased muscle protein breakdown

and ultimately muscle atrophy [8]. In our study, aging increased plasma TNF-α levels and +Epi treatment significantly decreased by ~33%. SkM TNF-α and NFkB protein levels also demonstrated significant decreases of ~35–60% with +Epi. Significant decreases in MuRF 1 and atrogin 1 relative protein levels were observed in +Epi treated animals. SkM protein ubiquitylation decreased significantly with +Epi as well as proteasome subunit 20, proteasome activity and protein degradation rates. Furthermore, the analysis of two SkM structural proteins troponin I and α1-actin noted significant increases with +Epi treatment. Thus, the effects triggered by +Epi yielded reduced levels of atrophy associated pathway activity and myofiber proteolysis while increasing the levels of contractile proteins. There is precedent for the potential of flavonoids to reduce circulating TNF-α levels. In the study by Wijawa et al., the ginseng extract provided to aged mice reduced TNF-α induced dysregulation of SkM oxidative stress levels [37].

Summary and Conclusions

Results from this study demonstrate that there is a progressive loss in SkM function that is known to be associated with loss of SkM mass and increases in pro-inflammatory cytokines and inhibitors of muscle growth. The analysis of SkM samples document aging induced detrimental changes in those that positively regulate muscle growth (follistatin, protein synthesis pathway, AKT mTOR, IGF-1) and structure/function (α1-actin and troponin I), while stimulating those that are associated with SkM protein degradation including TNF-α, myostatin, protein ubiquitination and proteosome activity levels. As illustrated in the graphical abstract, eight week treatment of aged animals with +Epi yielded significant positive effects on key pathways providing evidence for the capacity of this flavanol to stimulate the recovery of SkM mass and function. It can be hypothesized that in elderly subjects the continuous supplementation of +Epi is likely to increase SkM mass and function while at the same time, promoting a greater tolerance to fatigue upon repeated bouts of physical activity. Altogether, results support consideration towards the implementation of clinical trials to test the above stated hypothesis in older subjects.

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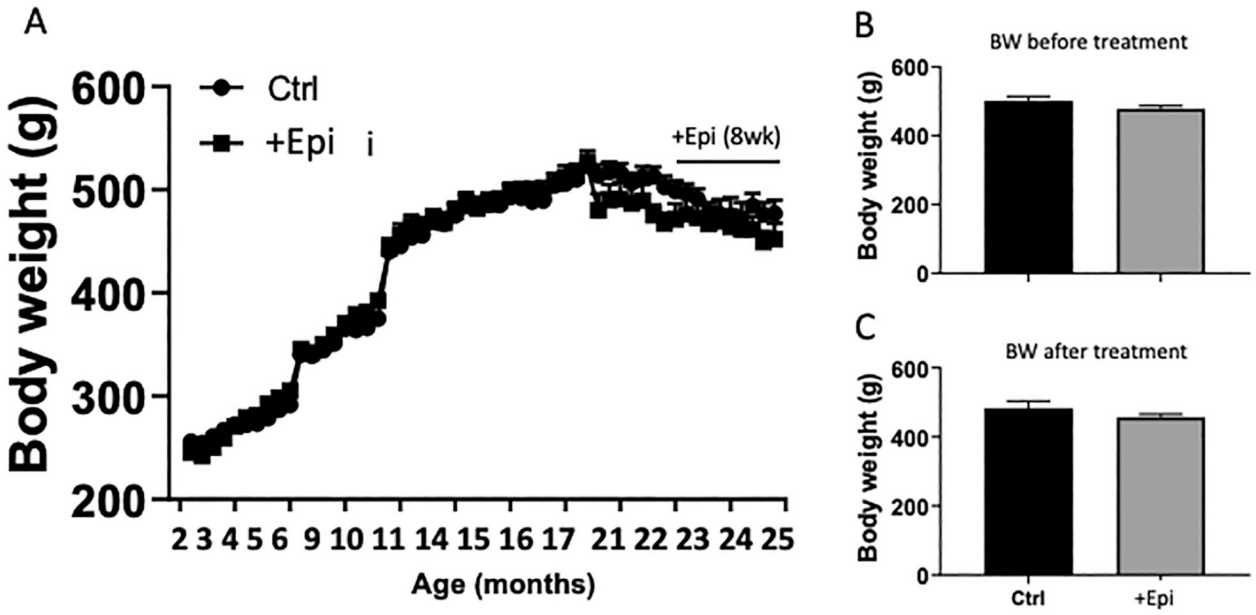

Figure 1.

Body weights recorded during aging and treatment. Panel A reports on body weight during the time course of the study. Panel B reports weights before treatment and panel C after treatment. Data are mean ± SEM, n=6/group.

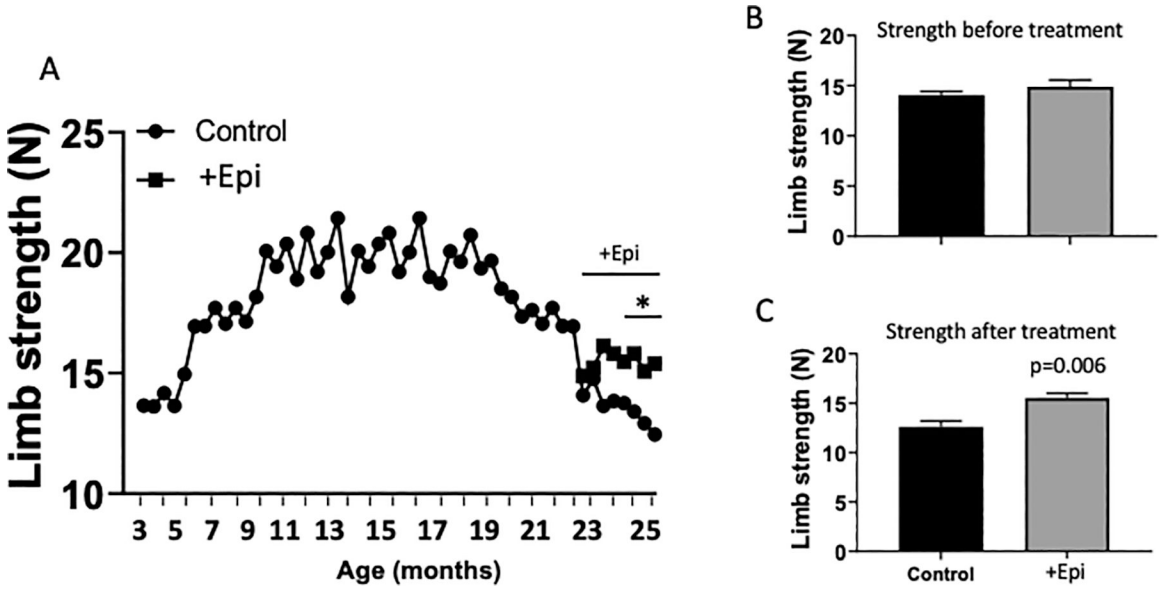

Figure 2.

Front limb strength recorded during aging and +Epi treatment. Panel A reports on limb strength during the time course of the study. Panel B reports strength recorded before +Epi treatment and panel C after treatment. Data are mean ± SEM, n=6/group, *p ≤0.05.

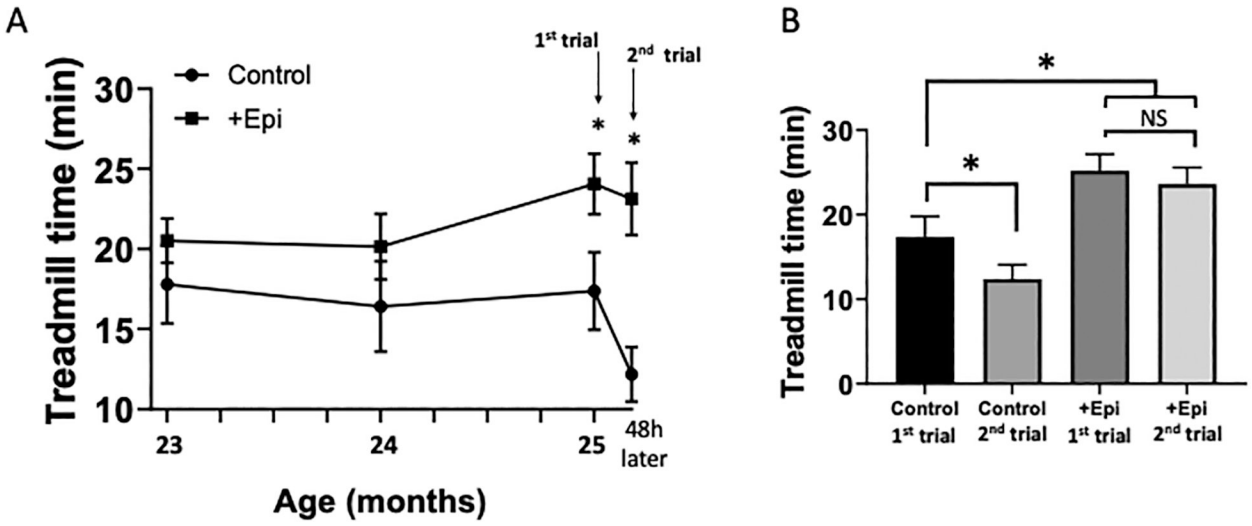

Figure 3.

Treadmill times recorded in control and +Epi treated rats at 23, 24 and 25 months (A). Panel B denotes comparisons within and among groups at 1st and 2nd trials. Data are mean ± SEM, n=6/group, *p ≤ 0.05, NS = not significant.

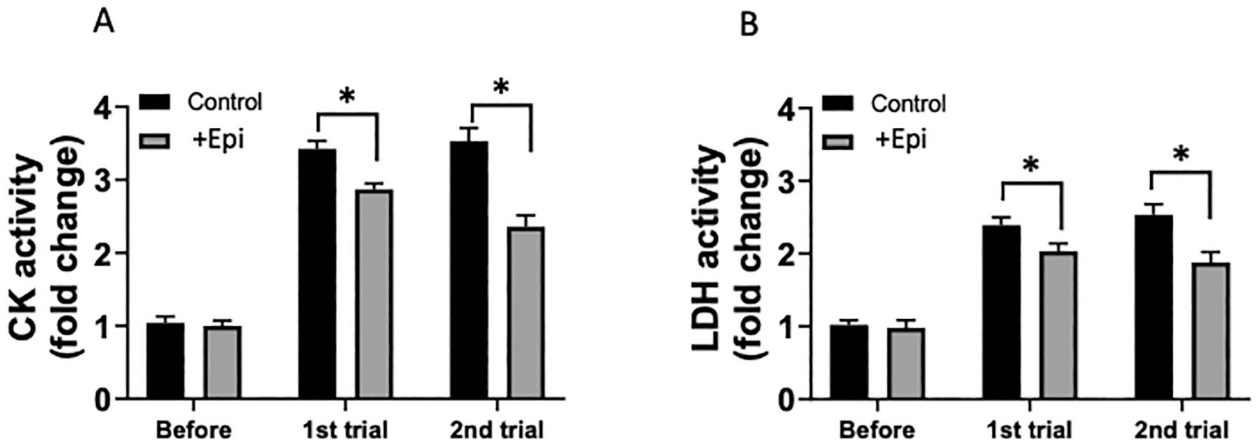

Figure 4.

Creatine Kinase (CK) and lactate dehydrogenase (LDH) enzyme activity in aged rats before and after treadmill testing. Values are reported as fold changes vs. before treadmill testing. CK (A) and LDH (B) were evaluated in control and +Epi treated plasma samples before and after treadmill in the 1st and 2nd trials. Data are mean ± SEM, n=6/group, *p values ≤ 0.05.

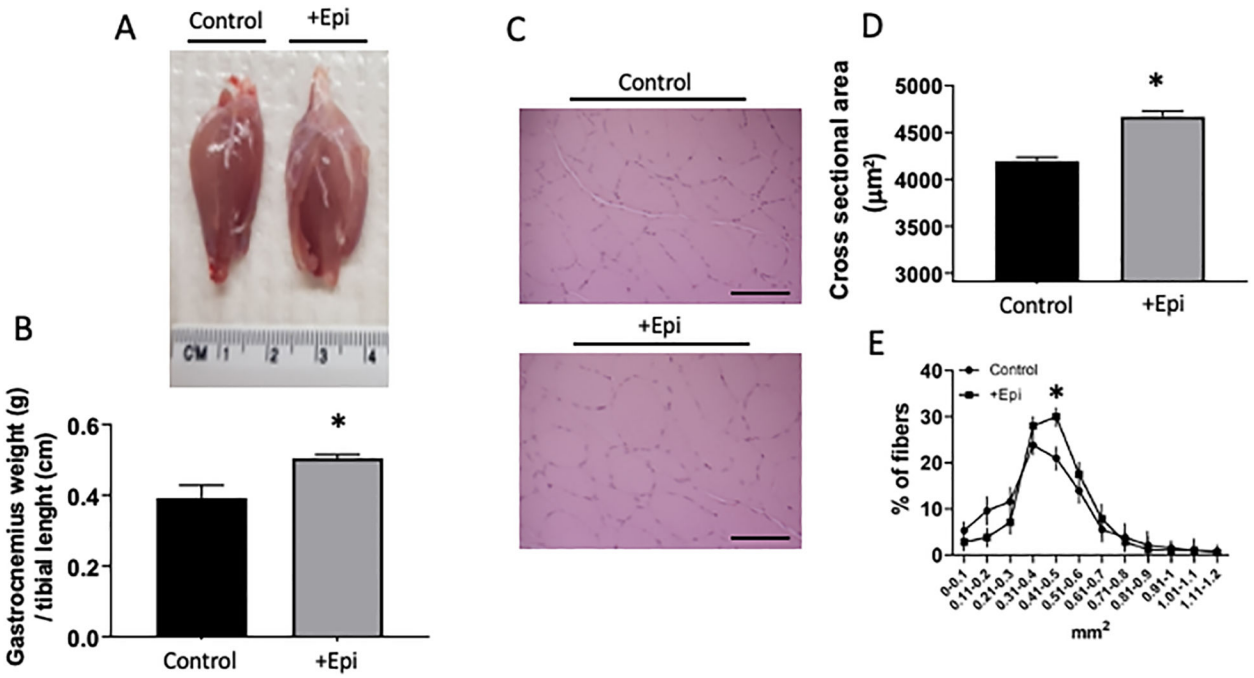

Figure 5.

Effect of +Epi treatment on gastrocnemius mass. Panels A and B illustrate and report on normalized muscle weight recorded in control and Epi groups. Panel C are representative images from samples stained with hematoxilin and eosin (scale bar = 50 µm). Panel D reports on myofiber cross sectional area measured and (E) on average myofiber size distribution. Data are mean ± SEM, n=6/group, *p values ≤ 0.05.

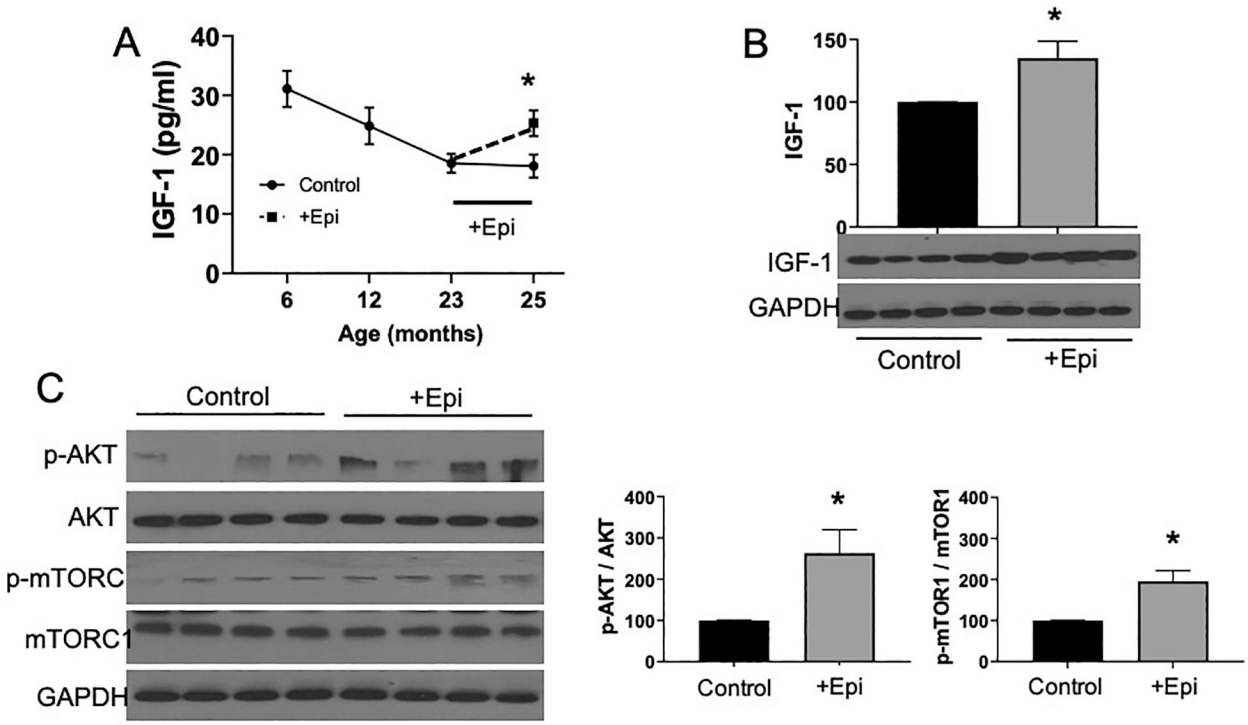

Figure 6.

Effects of aging and +Epi treatment on plasma and gastrocnemius IGF-1 and pathway related endpoints. Panel A plasma illustrates IGF-1 time course values. Panel B reports post +Epi treatment levels of IGF-1 in gastrocnemius (relative protein levels by Westerns vs. control). Panel C reports post-treatment changes in AKT/mTORC1 protein levels (relative vs. control). Data are mean ± SEM, n=6/group, *p values ≤ 0.05.

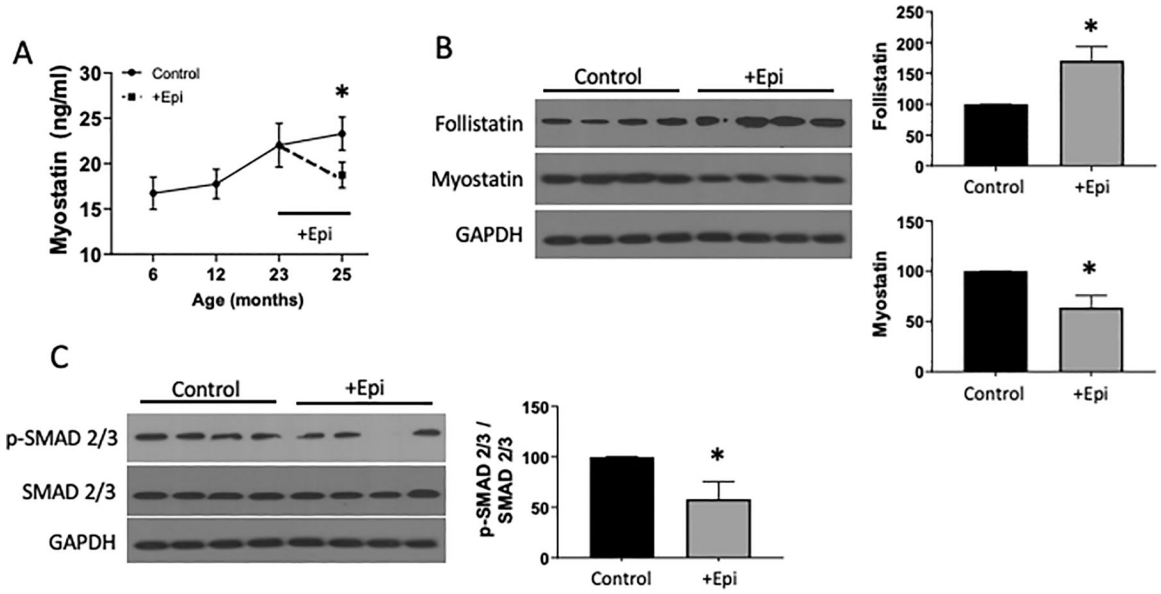

Figure 7.

Effects of aging and +Epi treatment on plasma and/or gastrocnemius follistatin and myostatin pathway related endpoints. Panel A illustrates myostatin plasma levels with aging and +Epi treatment. Relative gastrocnemius protein level changes (vs. controls) for follistatin and myostatin following treatment are shown in panel B. Panel C reports post-treatment changes in p-SMAD 2/3 protein levels. Data are mean ± SEM, n=6/group, *p values ≤ 0.05.

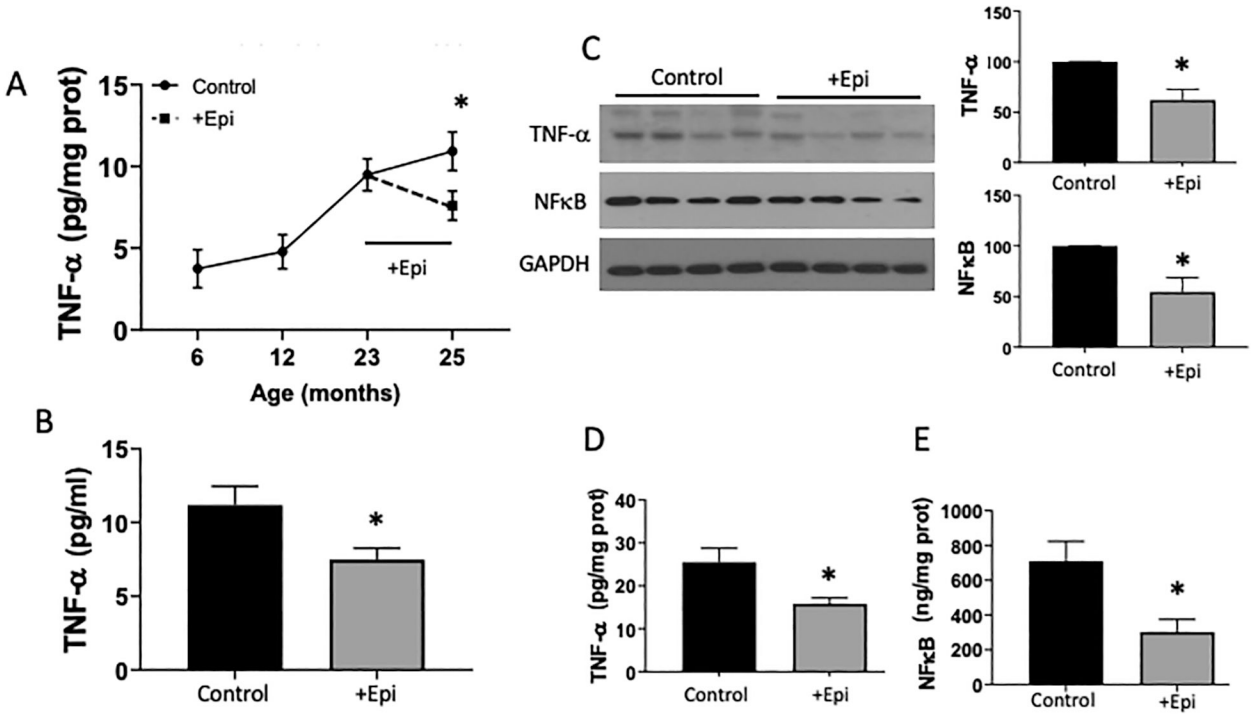

Figure 8.

Effects of aging and +Epi treatment on plasma and gastrocnemius TNF-α and NFkB levels. Changes in plasma TNF-α levels are shown in panel A. Panel B reports plasma TNF-α levels before and after +Epi treatment. Panel C reports treatment changes in gastrocnemius relative protein levels (by Westerns) for TNF-α and NFkB while panels D and E reports values quantified by ELISA. Data are mean ± SEM, n=6/group, *p values ≤ 0.05.

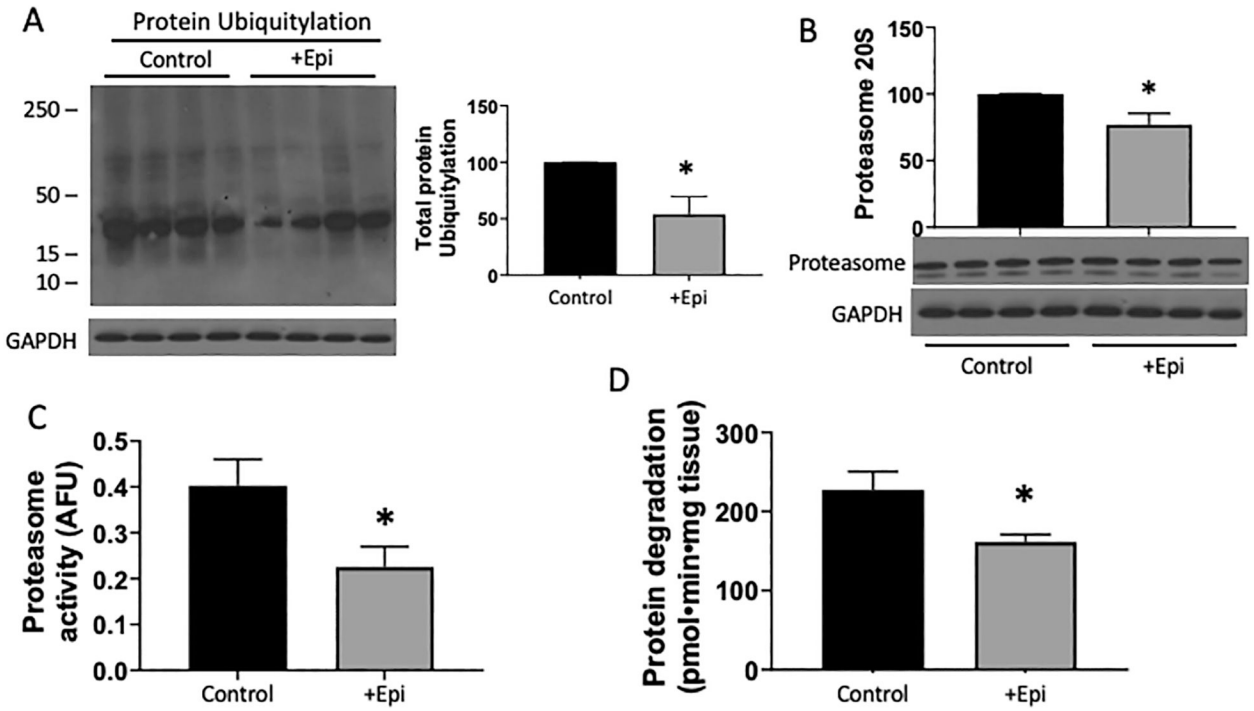

Figure 9.

Effects of +Epi treatment on protein degradation endpoints. Panel A reports on relative protein changes for total protein ubiquitylation as detected by Westerns, (B) reports on changes in proteasome 20S levels, (C) on proteasome activity (kit) and (D), on protein degradation rates (kit) before and after +Epi treatment. Arbitrary fluorescent unit (AFU). Data are mean ± SEM, n=6/group, *p values ≤ 0.05.

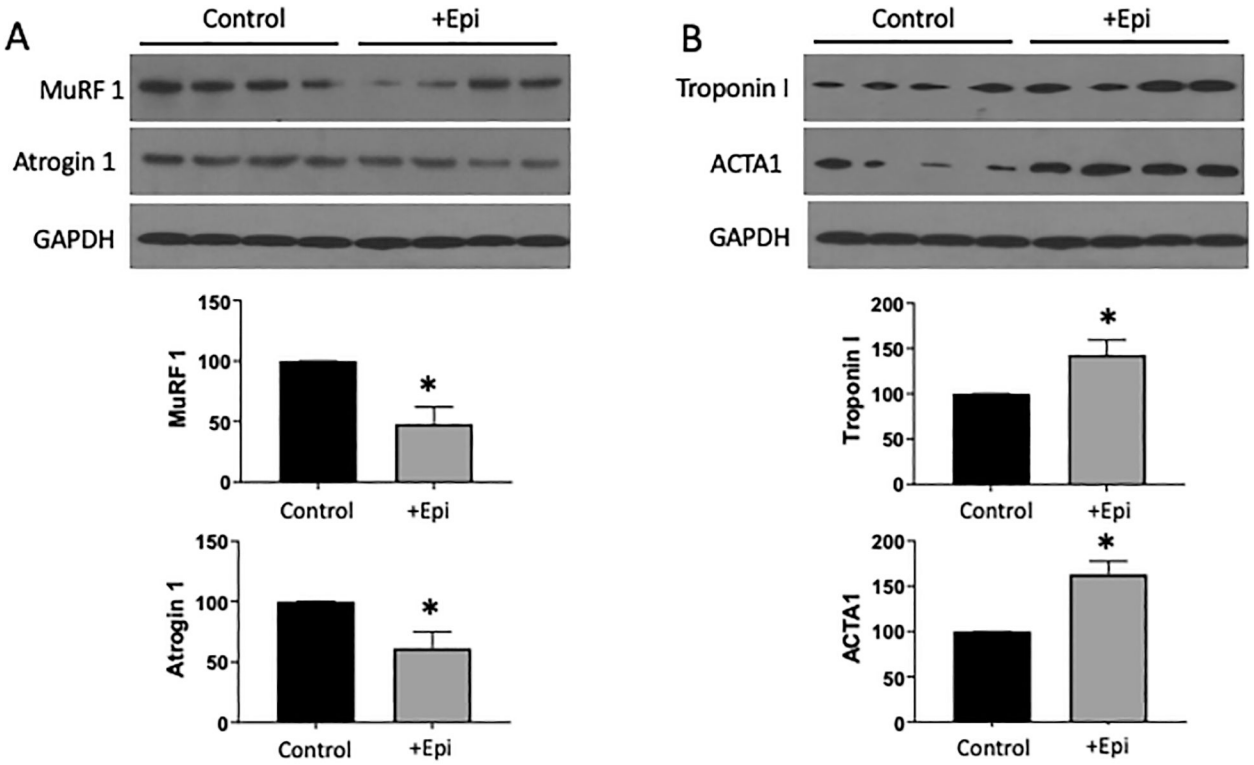

Figure 10.

Effect of +Epi treatment on regulators of muscle atrophy and structural proteins levels. Panel A reports on relative protein levels changes (by Westerns) for MuRF1 and atrogin 1 while panel B, reports on those for troponin I and α1-actin before and after +Epi treatment. Data are mean ± SEM, n=6/group, *p values ≤ 0.05.