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Angiotensin-(1-7) improves skeletal muscle regeneration

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Abstract

Skeletal muscle possesses regenerative potential via satellite cells, compromised in muscular dystrophies leading to fibrosis and fat infiltration. Angiotensin II (Ang-II) is commonly associated with pathological states. In contrast, Angiotensin (1-7) [Ang-(1-7)] counters Ang-II, acting via the Mas receptor. While Ang-II affects skeletal muscle regeneration, the influence of Ang-(1-7) remains to be elucidated. Therefore, this study aims to investigate the role of Ang-(1-7) in skeletal muscle regeneration. C2C12 cells were differentiated in the absence or presence of 10 nM of Ang-(1-7). The diameter of myotubes and protein levels of myogenin and myosin heavy chain (MHC) were determined. C57BL/6 WT male mice 16-18 weeks old) were randomly assigned to injury-vehicle, injury-Ang-(1-7), and control groups. Ang-(1-7) was administered via osmotic pumps, and muscle injury was induced by injecting barium chloride to assess muscle regeneration through histological analyses. Moreover, embryonic myosin (eMHC) and myogenin protein levels were evaluated. C2C12 myotubes incubated with Ang-(1-7) showed larger diameters than the untreated group and increased myogenin and MHC protein levels during differentiation. Ang-(1-7) administration enhances regeneration by promoting a larger diameter of new muscle fibers. Furthermore, higher numbers of eMHC (+) fibers were observed in the injured-Ang-(1-7), which also had a larger diameter. Moreover, eMHC and myogenin protein levels were elevated, supporting enhanced regeneration due to Ang-(1-7) administration. Ang-(1-7) effectively promotes differentiation *in vitro* and improves muscle regeneration in the context of injuries, with potential implications for treating muscle-related disorders.

Key Words: Angiotensin (1-7); regeneration; myogenesis.

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The skeletal muscles can remarkably adapt to physiological demands such as growth, training, and injury.[1] After an injury, skeletal muscle repair occurs by a small population of cells that reside in adult skeletal muscle and are referred to as satellite cells (SC).[2] When an injury occurs, regeneration begins with the activation, proliferation, and differentiation of SC, generating new muscle fibers either by fusing with each other or pre-existing fibers.[3] Because most muscle diseases involve fiber damage and degeneration, regeneration is a feature of all muscle diseases. The regenerative process fails in several skeletal muscular dystrophies, which present continuous fiber damage, and the normal skeletal muscle architecture is lost.[4] In these cases, increased connective tissue in the form of interstitial fibrosis and fatty replacement are evidence of persistence of disease .[5]

The classical Renin–angiotensin system (RAS) axis mainly comprises Angiotensin II (Ang-II), Angiotensin Converting Enzyme (ACE), and the transducer receptors for Ang-II AT-1 and AT-2. The Ang-II-AT-1 receptor axis has been associated with pathological states and is related to harmful effects on skeletal muscle.[6] Ang-II and AT-1 receptor participation in skeletal muscle fibrosis associated with dystrophic diseases has been studied.[7] Interestingly, experimental evidence indicates an

atrophic effect of Ang-II on skeletal muscle.[8,9] In several pathologic states, such as chronic heart failure and chronic kidney failure, muscle wasting has been observed concomitantly with increased levels of Ang-II.[10]

The non-classical RAS axis is mainly composed of Angiotensin (1-7) [Ang-(1-7)], an endogenous heptapeptide generated by actions of ACE-2 in Ang-II.[6] Ang-(1-7) functions are often opposite to those attributed to Ang-II.[11] Ang-(1-7) binds and signals through its transducer receptor Mas, a G protein-coupled receptor. Interestingly, administering Ang-(1-7) to dystrophic mice decreases the fibrosis associated with DMD.[12] Moreover, we have previously reported the anti-atrophic effect of Ang-(1-7) on skeletal muscle in models of cachexia induced by Ang-II, LPS, or during disuse atrophy.[8] There are antecedents that classical RAS modulates skeletal muscle regeneration. Thus, administering AT-1 receptor blockers (ARB) to injured normal or dystrophic muscles reduced fibrosis and inflammation and increased regeneration.[13] In the same

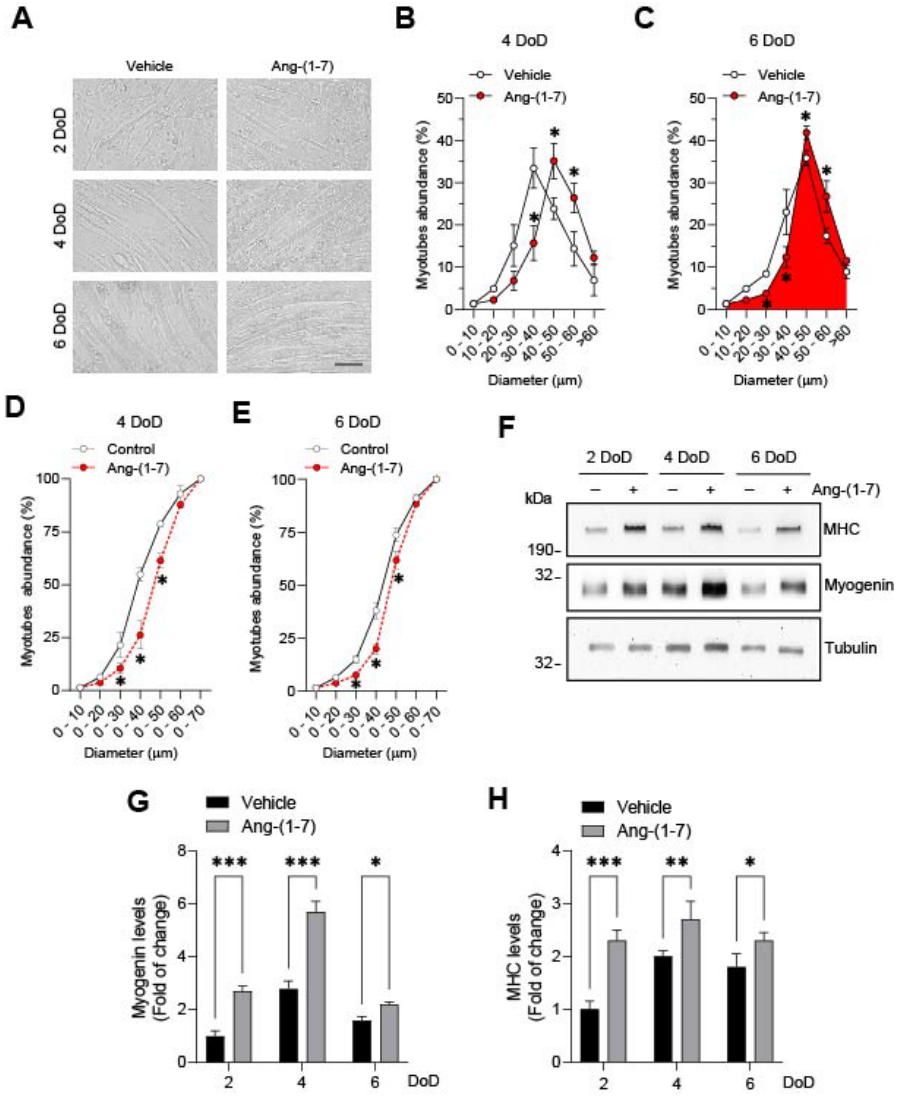

Fig 1. *Angiotensin-(1-7) promotes differentiation in C2C2 cells. C2C12 cells were incubated with angiotensin-(1-7) at day 0 of differentiation for 2, 4, or 6 days of differentiation (DoD). (A) Representative images of C2C12 cells incubated with vehicle or Ang-(1-7) for 2, 4, and 6 DoD. The diameter of the myotubes at days 4 (B) and 6 (C) of differentiation was quantified. (D-E) Accumulative frequency analysis was plotted to the same groups and times points. (F) C2C12 cells were homogenized to evaluate myogenin and MHC protein levels by western blot analysis using tubulin as a loading control. Molecular weights are indicated in kDa. Densitometric analysis of myogenin (G) and MHC (H) were performed. The values are shown as the mean ± SEM of three independent experiments. Two-way ANOVA and Tukey's multiple comparisons test are shown. *p<0.05, **p<0.001, ***p<0.0001 vs vehicle. SEM, standard error of the mean; MHC, myosin heavy chain; DoD, days of differentiation.*

line, Ang-II infusion decreases muscle regeneration by reducing the SC number and proliferation via an AT-1 receptor.[14] AT-2 receptor has a contrary effect to AT-1. Moreover, the AT-2-dependent signaling in SC is critical for muscle regeneration.[15]

Despite this evidence, the effect of the non-classical RAS axis, specifically Ang-(1-7), on skeletal muscle regeneration has yet to be studied.

Materials and Methods

Animals

C57BL/6J WT male mice (16-18 weeks old) were grouped in polycarbonate cages, maintained under controlled temperature and relative humidity, and fed with a standard diet and water ad libitum. Mice were randomized and separated into experimental groups (5 to 6 mice for each group): injured with barium-chloride

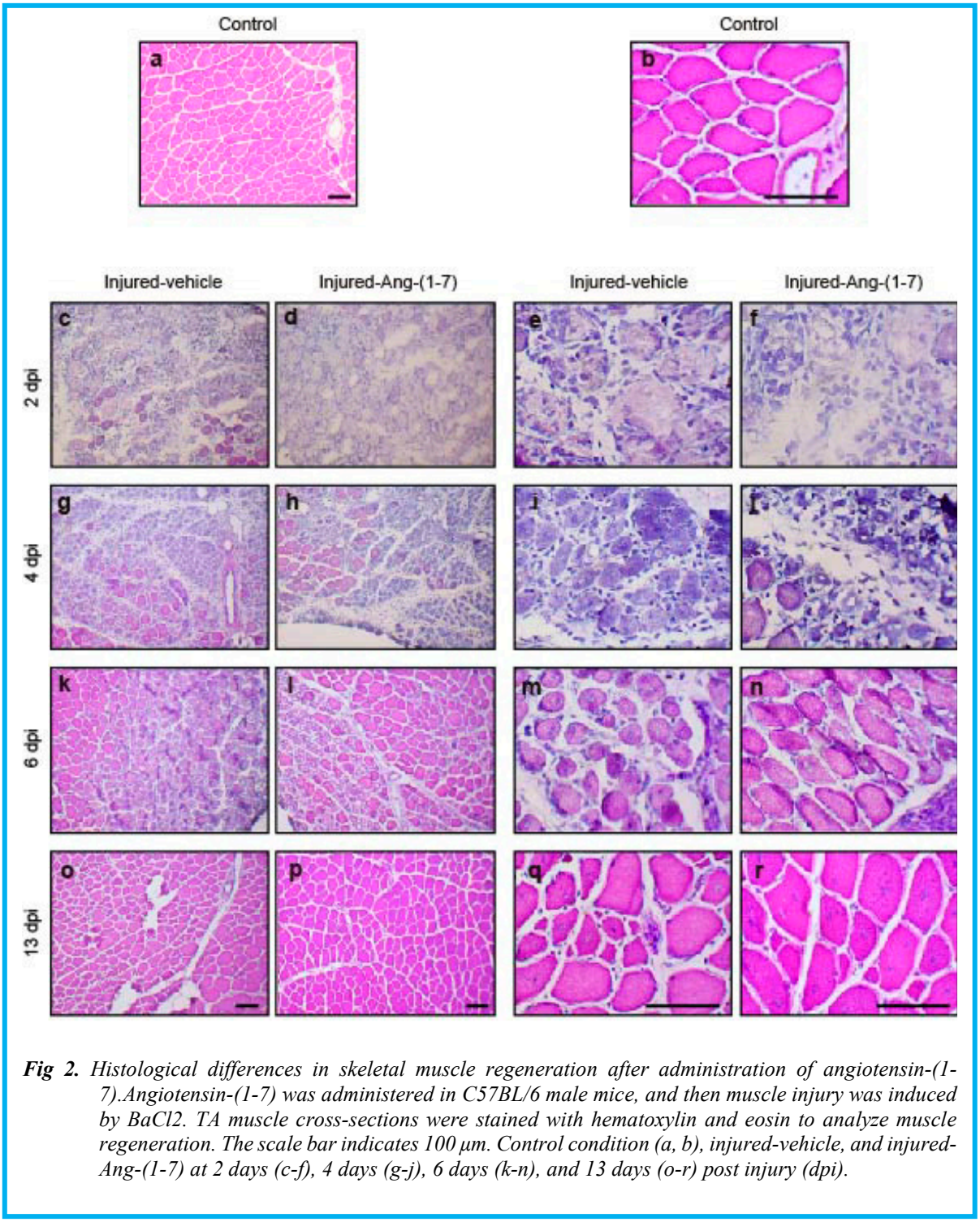

Fig 2. *Histological differences in skeletal muscle regeneration after administration of angiotensin-(1-7).Angiotensin-(1-7) was administered in C57BL/6 male mice, and then muscle injury was induced by BaCl2. TA muscle cross-sections were stained with hematoxylin and eosin to analyze muscle regeneration. The scale bar indicates 100 µm. Control condition (a, b), injured-vehicle, and injured-Ang-(1-7) at 2 days (c-f), 4 days (g-j), 6 days (k-n), and 13 days (o-r) post injury (dpi).*

(BaCl2) (Injured-vehicle), injured with BaCl2 plus Ang-(1-7) administration [Injured-Ang-(1-7)], and no injured (Control). Ang-(1-7) (Sigma-Aldrich, St Louis, MO, USA) was administered to the animals through osmotic mini pumps (Alzet-Durect, Cupertino, CA, USA) with a 100 ng/kg/min dose.[16] Injury of tibial anterior (TA) muscles in mice was performed by BaCl2 injection under anesthesia (3 % isoflurane in O2). Briefly, 60 µl of 1.2% BaCl2 diluted in sterile saline (NaCl 0.9%) was injected along the whole length of the muscle,[17] and 2-, 4-, 6-, and 13-days post-injury (dpi), the animals were euthanized. Immediately, TA muscles were obtained, weighed, frozen in isopentane, and stored at -80 C° until processing.

All animal procedures complied with international, national, and institutional animal care guidelines and were approved by the Animal Ethics Committee at the Universidad Andrés Bello Committee (approval number 030/2012).

Histological Analysis

Cryosections (8 µm) of the TA muscles were washed with distilled water and stained with hematoxylin for 9 min and eosin for 4 min. Samples were dried in ethanol, fixed in neo-clear, and mounted with Neo-Mount (Sigma-Aldrich,

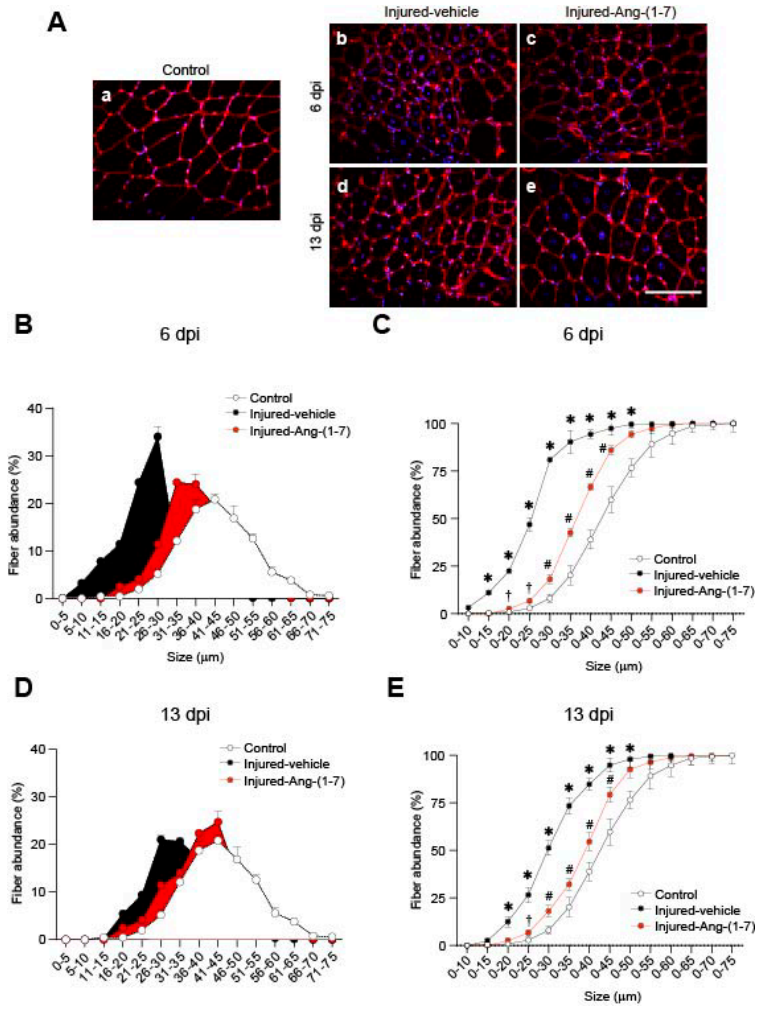

Fig 3. *Angiotensin-(1-7) administration enhances fiber diameter during skeletal muscle regeneration. Angiotensin-(1-7) was administered in C57BL/6 male mice, and then muscle injury was induced by BaCl2. (A) TA muscle cross-sections were stained with WGA to delimit the sarcolemma. The scale bar indicates 100 µm. Control condition (a), injured-vehicle, and injured-Ang-(1-7) at 6 days (b-c) and 13 days (d-e) post injury (dpi). Fiber diameters were grouped from 5 to 75 µm to quantify the total fiber percentage by each group at 6 (B) and 13 dpi (D). Accumulative frequency analysis was plotted to the same groups and times points (C-E). The mean ± SEM for each group (n=5-6 mice per group), Two-way ANOVA, and Tukey's multiple comparisons test are shown. *p<0.05 injured-vehicle vs control; #p<0.05 injured-Ang-(1-7) vs control/injured-vehicle; †p<0.05 injured-Ang-(1-7) vs injured-vehicle. SEM, standard error of the mean; TA, tibialis anterior.*

St. Louis, MO, USA) for histological observation to analyze muscle fiber regeneration.

Immunofluorescence microscopy

Cryosections (8 µm) of the TA muscles were fixed in 4% paraformaldehyde overnight at 4 °C, permeabilized with 0.05 % Triton X-100 for 10 min, and blocked with 1 % BSA for 30 min. Fibers were immunostained with 1:100 mouse anti-eMHC (F.1652s; Developmental Studies, Hybridoma Bank, University of Iowa, Iowa, IA, USA) in PBS-1 % BSA. Bound antibodies were detected with 1:200 affinity-purified Alexa Fluor dye-conjugated goat anti-mouse antibody (Thermo Fisher Scientific, Waltham, MA, USA). In addition, another cryosection was stained with wheat germ agglutinin (WGA) attached to Alexa-Fluor 594 (Thermo Fisher Scientific, Waltham, MA, USA) according to standard procedures.[8] The samples were marked with 1 µg/ml Hoechst 33258 for 10 min and then mounted with a fluorescent dye-stained FluoromontTM Aqueous Mounting Medium (Sigma-Aldrich, St. Louis, MO, USA) under glass coverslips. Fiber sizes were determined by the minimal Feret diameter of each fiber, and values were grouped in 5-µm diameter ranges. Images were acquired in a Motic BA310 epifluorescence microscope (Motic, Hong Kong), and the quantification was done by Image J software [National Institutes of Health (NIH), Bethesda, MD, USA].

Cell culture

The skeletal muscle cell line ATCC C2C12 (American Type Culture Collection, ATCC, Manassas, VA, USA) was grown and differentiated on Dulbecco's modified

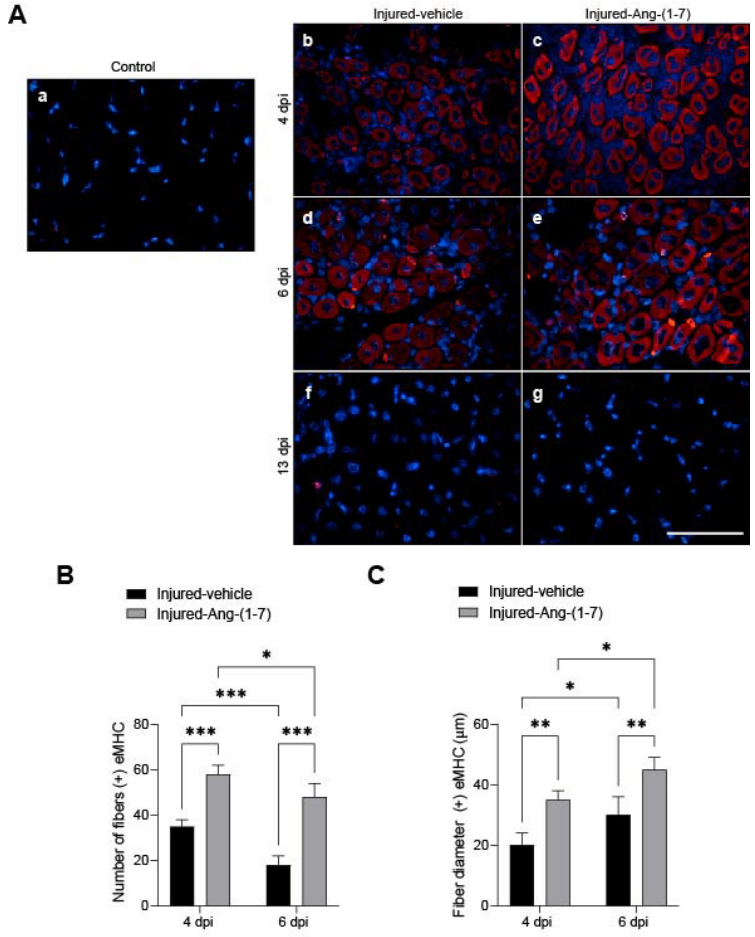

Fig 4. *Angiotensin-(1-7) administration promotes increased eMHC (+) fibers during skeletal muscle regeneration.Angiotensin-(1-7) was administered in C57BL/6 male mice and BaCl2 induced muscle injury. (A) eMHC immunofluorescence in TA cross-sections was performed to evaluate muscle regeneration. The scale bar indicates 100 µm. Control condition (a), injured-vehicle, and injured-Ang-(1-7) at 4 days (b-c), 6 days (d-e), and 13 days (f-g) post injury (dpi). The number of eMHC (+) fibers (B) and their diameter (C) were quantified. The values are shown as the mean ± SEM for each group (n=5-6 mice per group, Student t-test. *p<0.05, **p<0.001, ***p<0.0001 vs injured-vehicle). SEM, standard error of the mean; TA, tibialis anterior; eMHC, embryonic myosin.*

eagle's medium (DMEM) 4% horse serum until day 6 to obtain myotubes.[18] The myotubes were incubated with 10 nM of Ang-(1-7) (Sigma-Aldrich, St. Louis, MO, USA) at day 0 for 2, 4, and 6 days of differentiation (DoD). The medium was changed every 2 days and fresh Ang-(1-7) was added. Samples were observed in the Floid Cell Imaging Station (ThermoFisher Scientific, Waltham, MA, USA). Photographs obtained were analyzed (ImageJ, NIH, Bethesda, MD, USA), and the minimal Feret diameters were measured in approximately 50 myotubes from 10 random fields from each condition.

Western Blot

For the protein extracts, myotubes or TA muscles were homogenized in a RIPA buffer containing phosphatase inhibitors to protect the phosphorylation status and with 1 mM of a cocktail of protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA) and 1 mM of phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO, USA). 30 µg of proteins determined by Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Thermo Fisher Scientific, Waltham, MA, USA). The immunoblotting was developed with the following primary antibodies: mouse anti-MHC (1:1000 MF-20; Developmental Studies, Hybridoma Bank, University of Iowa, Iowa, IA, USA), rabbit anti-phospho-AKT (1:1000 9271 Cell Signaling, Danvers, MA, USA), rabbit anti-AKT (1:1000 92972, Cell Signaling, Danvers, MA, USA), mouse anti-eMHC (1:1000; F.1652s; Developmental Studies,

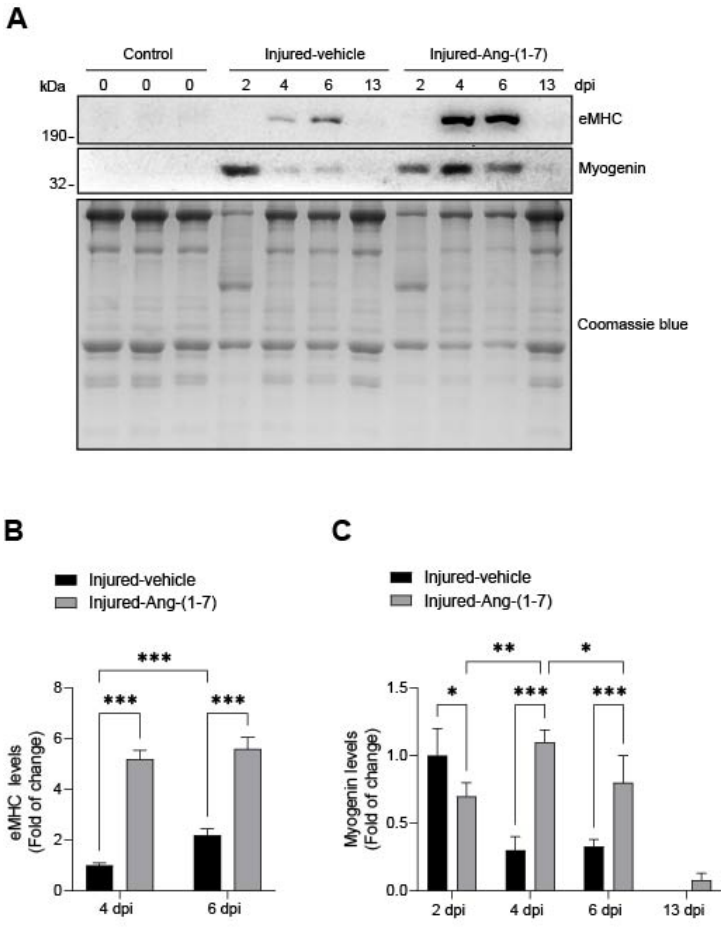

Fig 5. *Figure 5. Angiotensin-(1-7) administration increases eMHC and Myogenin protein levels during regeneration. Angiotensin-(1-7) was administered in C57BL/6 male mice, and then muscle injury was induced by BaCl2. (A) TA muscle were extracted in the indicated days post injury (dpi) and were homogenized to evaluate eMHC and myogenin protein levels and were detected by western blot analysis using Coomassie blue as a loading control. Molecular weight is indicated in kDa. Densitometric analysis of eMHC (B) and myogenin (C) were performed. The values are shown as the mean ± SEM for each group (n=5-6 mice per group, Student t-test. *p<0.05, **p<0.001, ***p<0.0001 vs injured-vehicle). SEM, standard error of the mean; TA, tibialis anterior; eMHC, embryonic myosin; dpi, days post injury.*

Hybridoma Bank, University of Iowa, Iowa, IA, USA), mouse anti-myogenin (1:1000; F5D-S; Developmental Studies, Hybridoma Bank, University of Iowa, Iowa, IA, USA), mouse anti-Mas (1:500; SC-54848; Santa Cruz, Dallas, TX, USA). Furthermore, the membranes were incubated with the respective secondary antibody [goat anti-mouse IgG-HRP (1:10,000; Santa Cruz, Dallas, TX, USA) and mouse anti-rabbit IgG-HRP (1:10,000; Santa Cruz, Dallas, TX, USA)]. The protein levels were normalized to Coomassie blue and used as load control. 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 [National Institutes of Health (NIH), Bethesda, MD, USA].

Statistical analysis

The Shapiro–Wilk test was used to assess the data distribution, and the analysis showed that all dependent variables were normally distributed. Two-way analysis of variance (ANOVA) was used. Data are presented as the mean ± standard error of the mean (SEM). All statistical analyses were performed with Prism 9.0 analysis software (GraphPad Software, San Diego, CA, USA). The p-values < 0.05 were considered statistically significant.

Results

Angiotensin-(1-7) enhances the diameter of myotubes and protein levels of MHC and myogenin during skeletal muscle differentiation.

C2C12 cells were treated with Ang-(1-7) and the effect on their diameter was evaluated at 4 days and 6 days of differentiation (Figure 1A). On days 4 (Figure 1B) and 6 (Figure 1C) of differentiation, the myotubes incubated with Ang-(1-7) presented a displacement to the right of the curve of abundance vs diameter, which indicates that the population of myotubes incubated with Ang-(1-7) are greater than myotubes treated with vehicle. When the accumulative frequency graph was analyzed, we observed that the Ang-(1-7) treatment accumulated a lower amount of myotubes in small sizes on day 4 compared to the vehicle. Thus, the most differences are in the ranges of 0-30 μm ($p<0.05$, 10.4 ± 2.5 vs 21.4 ± 5.7 %), 0-40 μm ($p<0.05$, 26.8 ± 6.6 vs 54.8 ± 3.3 %), and 0-50 μm ($p<0.05$, 61.4 ± 3.3 vs 78.7 ± 1.5 %) (Figure 1D). The same effect was observed on day 6 of differentiation, with a significant number of myotubes in the same size ranges (0-30 μm: $p<0.05$, 7.4 ± 0.8 vs 14.8 ± 1.7 %; 0-40 μm: $p<0.05$, 19.8 ± 2.5 vs 37.8 ± 3.6 %; 0-50 μm: $p<0.05$, 61.7 ± 4.1 vs 73.7 ± 3.4 %) (Figure 1E). Besides, protein levels of myogenin, a myogenic regulatory factor associated with terminal differentiation, and myosin heavy chain (MHC), a marker of myotubes maturation, were evaluated (Figure 1F). The results show an increase in the protein levels of myogenin at 2 ($p<0.001$, 2.7 ± 0.2 vs 1.0 ± 0.2 fold of change), 4 ($p<0.0001$, 5.7 ± 0.4 vs 2.8 ± 0.3 fold of change), and 6 ($p<0.05$, 2.2 ± 0.1 vs 1.6 ± 0.15 fold of change) days of differentiation in the Angio-(1-7) group (Figure G). Figure 1H shows similar results when MHC protein levels were determined (Day 2, $p<0.0001$, 2.3 ± 0.2 vs 1.0 ± 0.15 fold of change), (Day 4, $p<0.001$, 2.7 ± 0.24 vs 2.0 ± 0.1 fold of change), (Day 6, $p<0.05$, 2.3 ± 0.15 vs 1.8 ± 0.25 fold of change).

These results demonstrate that Ang-(1-7) promotes differentiation of C2C12 cells.

Angiotensin-(1-7) administration promotes a larger diameter of muscle fibers during regeneration.

Figure 2 shows the results obtained from hematoxylin and eosin (H&E) staining of TA sections. The upper images correspond to the contralateral control of the injured TA. The analyses demonstrate the integrity of muscle fibers and the peripheral location of nuclei in control TA muscles (Figure 2a-b). H&E staining was performed at four-time points following barium chloride-induced damage in the absence or presence of Ang-(1-7). Muscle injury was observed in vehicle- and Ang-(1-7)-treated groups on day 2 (Figure 2c-f) and 4 (Figure 2g-j) after intramuscular injection of BaCl2. However, staining at day 6 dpi (Figure 2k-n) reveals the formation of new muscle fibers due to the regeneration process, characterized by centralized nuclei that persist until day 13 (Figure 2o-r). Interestingly, an apparent difference in the size of regenerating fibers was observed, showing larger fiber in the injured-Ang-(1-7) group compared to the injured-vehicle.

To confirm whether the muscles in the injured-Ang-(1-7) group exhibited an improved regeneration, the minimum Feret´s diameter of the new regenerating fibers was evaluated using WGA staining. Figure 3A-a shows the control group that was not injured. The injured-vehicle and injured-Ang-(1-7) groups are shown at 6 days (Figure 3A-b, 3A-d) and 13 days (Figure 3A-c, 3A-e) of TA muscle regeneration. The plot of the diameter vs fiber abundance shows that at 6 (Figures 3B) and 13 (Figures 3D) days, injured-vehicle TA muscles present a higher proportion of fiber with little size, evidenced by a displacement to the left of the curve (black area) compared to control (white area) and injured-Ang-(1-7) muscles (red area). In Figure 3C, the graph shows that the accumulated frequency of fibers between 0-40 μm at day 6 in control and injured-vehicle groups was 38.9 ± 4.9 % and 94.4 ± 2.2 %, respectively ($p < 0.001$). This result demonstrates that injured-vehicle muscles have a high proportion of fibers in this size range. In addition, Figure 3C also shows that Ang-(1-7) partially prevents this accumulation induced by injured, reaching 66.6 ± 1.5 % of fiber abundance in the range 0-40 μm ($p < 0.001$ injured-vehicle vs injured-Ang-(1-7)). Similar results were obtained at day 13 of TA muscle regeneration, showing a displacement of the frequency curve to the left in injured-vehicle muscles (black area) compared to control muscles (white area), and partially prevented by Ang-(1-7) in injured muscles (red area) (Figure 3D).

When comparing the accumulated frequency, we observed that the abundance of fibers in the range of 0-40 μm was 35.7 ± 3.5 % (control), 84.8 ± 3.4 % (injured-vehicle), and 54.6 ± 4.7 % (injured-Ang-(1-7) (Figure 3E).

These results suggest that Ang-(1-7) administration affects skeletal muscle by enhancing its capacity to regenerate following injury with BaCl2 on TA muscles.

Angiotensin-(1-7) administration promotes increased numbers of eMHC (+) fibers during regeneration.

Embryonic myosin (eMHC), a key marker of the regeneration process that is transiently expressed in new regenerating muscle fibers, was detected by indirect immunofluorescence. The images show that the control group was not injured; therefore, no muscle fiber expressing eMHC was observed (Figure 4A-a). The injured-vehicle and injured-Ang-(1-7) groups are shown at 4 days (Figure 4A-b, and 4A-e), 6 days (Figure 4A-c, 4A-f), and 13 days (Figure 4A-c, 4A-g) of muscle regeneration. Figure 4B shows the number of eMHC (+) fibers in both conditions. The injured-Ang-(1-7) group exhibits more eMHC (+) fibers than the injured-vehicle group at both 4 ($p<0.0001$, 58 ± 4 vs 35 ± 3) and 6 dpi with BaCl2 ($p<0.0001$, 48 ± 6 vs 18 ± 4). Additionally, each condition, injured-vehicle and injured-Ang-(1-7), significantly differed between day 4 and day 6, indicating a transition in eMHC expression as the number of positive fibers began to decrease (Figure 4B).

Figure 4C illustrates the diameter analysis of eMHC (+) fibers to determine differences in the size of the new fibers at 4 and 6 dpi. The Ang-(1-7) administration to the injured group presents a larger diameter compared to the fibers of the injured-vehicle group after 4 ($p=0.0024$, 35 ± 3 vs 20 ± 4 μm) and 6 dpi ($p=0.0023$, 45 ± 4 vs 30 ± 6 μm). Furthermore, in the injured-Ang-(1-7) group at 6 dpi, the diameter of the fibers is higher than the same group on day 4 ($p=0.043$, 45 ± 4 vs 35 ± 3 μm). Similar results were observed in the injured-vehicle group ($p=0.043$, 30 ± 6 vs 20 ± 4 μm).

These results confirm that Ang-(1-7) administration increases eMHC expression, promoting muscle regeneration after a BaCl2.-induced injury.

Angiotensin-(1-7) administration increases eMHC and myogenin protein levels during muscle regeneration.

Subsequently, protein extraction and western blot analysis from the TA muscles were performed to confirm the results obtained from the histological studies (Figure 5A). Specifically, Figure 5B shows a difference in eMHC protein levels between injured-Ang-(1-7) compared to the injured-vehicle groups at 4 ($p<0.0001$, 5.2 ± 0.3 vs 1.0 ± 0.1 fold of change) and 6 dpi ($p<0.0001$, 5.6 ± 0.5 vs 2.2 ± 0.3 fold of change). Additionally, the injured-vehicle group showed an increased eMHC level on day 6 compared to day 4 after injury ($p=0.0008$, 2.0 ± 0.3 vs 1.0 ± 0.1 fold of change). On the contrary, Ang-(1-7) administration does not produce changes in eMHC levels at day 6 compared to day 4. Therefore, these results confirm the findings obtained through histological analyses, suggesting that Ang-(1-7) improves regenerative capacity. Also, myogenin protein levels were analyzed since it is an essential marker of terminal differentiation that allows regeneration. On day 2 post-injury, myogenin levels in the injured-Ang-(1-7) group are significantly lower than in the injured-vehicle group (Figure 5C; $p=0.028$, 0.70 ± 0.10 vs 1.00 ± 0.20 fold of change). However, these levels increase in the injured-Ang-(1-7) group on days 4 ($p<0.0001$, 1.10 ± 0.09 vs 0.30 ± 0.10 fold of change) and 6 post-injury ($p=0.0002$, 0.80 ± 0.20 vs 0.33 ± 0.05 fold of change), with levels on day 4 even higher than those on day 2 ($p=0.0017$, 1.10 ± 0.09 vs 0.70 ± 0.10 fold of change) and day 6 ($p=0.028$, 1.10 ± 0.09 vs 0.80 ± 0.20 fold of change) (Figure 5C).

Together, these results suggest that administering Ang-(1-7) in mice promotes the myogenic program, favoring muscle regeneration.

Discussion

Skeletal muscle is a dynamic tissue capable of generating a response and adapting to different stimuli. Some of these stimuli can be harmful and influence muscle mass. An example of this is the detrimental effects of Ang-II on skeletal muscle in terms of its atrophic and fibrotic effects, which have been extensively demonstrated.[7,8] Despite the opposing effect of Ang-(1-7), the peptide has been studied in some contexts related to muscle atrophy, preventing the decrease in the diameter of fibers and sarcomeric proteins and the increase of the ubiquitin-proteasome system (UPS) and reactive oxygen species (ROS),[19] to date, it had not yet been explored their role in muscle regeneration. Skeletal muscle can be damaged by sports or traumatic injuries, degenerative diseases, and disorders, which can cause severe disability and functional impairment.[20-22] The process of muscle regeneration is complex and relies on various cell types and signaling molecules. Treating injuries is challenging as they heal slowly and often result in incomplete functional recovery.[23] Therefore, revealing the effects of Ang-(1-7) on skeletal muscle regeneration is crucial to understand if this peptide affects this process and, in this manner, could be used to treat injuries or muscle-related disorders. This study demonstrated the potential of Ang-(1-7) administration for the first time in promoting muscle regeneration following injury.

Our research revealed that muscle cells respond to Ang-(1-7) *in vitro*, which increased myotubes diameter and the level of proteins associated with myogenic differentiation and maturation, such as myogenin and MHC, respectively (Figure 1). These results indicate an improvement in myogenic differentiation, a crucial process for muscle regeneration. On the other hand, it is worth noting that these results on myotube diameter and MHC protein levels indicate a hypertrophic effect.[24,25] However, future studies should also examine the effects of Ang-(1-7) on satellite cell proliferation and fusion. As depicted in Figures 2 and 3, the histological analyses revealed a significant difference in the size of regenerated

muscle fibers between the vehicle and Ang-(1-7) groups. It is known that centralized nuclei in muscle fibers are indicative of the regeneration process.[26] Therefore, the minimum Feret´s diameter of these fibers with central nuclei was assessed through WGA staining (Figure 3), which provided quantitative support for the morphological changes observed. One of the main mechanisms that regulate muscle mass is the degradation of sarcomeric proteins through the UPS due to an increase in atrogin-1 and MuRF-1 expression.[27]) Research has shown that Ang-(1-7) can block this pathway through the Mas receptor depending on the IGF-1/IGF-1R/AKT pathway.[27] This could prevent the increase of atrogin-1 and MuRF-1, which can lead to the degradation of sarcomeric proteins. As a result, this process can help to avoid a decrease in fiber diameter. Therefore, the activation of the UPS pathway during BaCl2-induced muscle injury could have been prevented in the presence of Ang-(1-7), which could have generated a larger fiber diameter during regeneration. However, it should be noted that IGF-1 is an anabolic growth factor that is induced in the regeneration process.[28] Interestingly, previous studies have shown that the IGF-1 signaling pathway is activated by Ang-(1-7).[29] In conjunction with inhibiting the degradative pathway, Ang-(1-7) via IGF-1 could also favor the greater diameter of the new regenerating fibers.

Another critical aspect of muscle regeneration is the expression of eMHC. It is a marker for regeneration, indicating the formation of new fibers. eMHC expression is transient and not expressed in mature fibers. Our immunofluorescence results (Figure 4) show a higher number of eMHC(+) fibers in the injured-Ang-(1-7) group compared to injured-vehicle at both 4- and 6-days post-injury. This finding suggests that Ang-(1-7) administration promotes an earlier onset and increased duration of eMHC expression, which is indicative of enhanced muscle regeneration. To substantiate the histological findings, protein levels of essential proteins associated with muscle regeneration were assessed. The increase in eMHC protein levels at 4- and 6 days post-injury in the injured Ang-(1-7) group corroborates the histological evidence of enhanced regeneration (Figure 4B). No literature directly links Ang-(1-7) to eMHC expression. However, it has been described that Ang II can suppress it.[15] Ang-(1-7) may counteract Ang II's effects on skeletal muscle.[8] Therefore, the inhibition of Ang II signaling by administering Ang-(1-7) would allow greater expression of eMHC during regeneration.

The process of muscle regeneration involves various factors and signaling pathways. However, this process is mainly carried out by skeletal muscle stem cells, also known as SC, through a process called myogenesis that includes SC activation, proliferation, and differentiation.[3] In this context, the effect of Ang-(1-7) on the transcription factors that control the fate of SC during myogenesis is relevant. One of them that is critical to regulating terminal myogenic differentiation and the formation of new muscle fibers is myogenin.[30] Therefore, myogenin protein levels were also determined. Figure 5C shows the increase in myogenin levels over the same time frame that eMHC, which suggests that Ang-(1-7) administration promotes the activation of the myogenic program, a crucial aspect of muscle tissue repair. The mechanism by which Ang-(1-7) could promote differentiation during regeneration through myogenin is unknown. However, other studies have observed that Ang-(1-7) administration affects mRNA expression of this myogenic regulatory factor, showing that muscle fibers were protected from atrophy due to this treatment with the peptide.[31] Another mechanism that could explain the enhancement of regeneration induced by Ang-(1-7) is related to SC and ROS because a higher amount of ROS can cause senescence of SC.[32]

Studies have shown that increased ROS in muscle cells results in increased expression of MuRF-1 and atrogin-1,[33] which could lead to impaired muscle regeneration. However, Abrigo et al. 2016 demonstrated that Ang-(1-7) can prevent atrophy induced by the administration of TGF-β by reducing increased ROS.[34] Therefore, the administration of Ang-(1-7) could act on the production of ROS during the muscle regeneration process, disfavoring the senescence of SC and inducing MuRF-1 and atrogin-1.

The results obtained in the study support the beneficial effect of Ang-(1-7) in regeneration because the process of forming new fibers to repair muscle damage is more efficient and faster than the group that was not administered the peptide. However, there is a lack of evidence that determines how Ang-(1-7) could promote muscle regeneration since understanding this phenomenon is very useful to give a therapeutic approach to Ang-(1-7) associated with skeletal muscle disorders. In addition, the information obtained on the increase in the diameter of fibers regenerated, the diameter and quantity of eMHC+ fibers, and the increase in the protein levels of eMHC and myogenin, future studies could incorporate the evaluation of muscle fiber maturation in a final temporal frame.[35] This analysis could help to understand whether the improvement in regeneration translates into earlier mature muscle fibers than in the condition that did not have Ang-(1-7) administration. Additionally, these antecedents could be related to functional parameters of skeletal muscle. Once muscle regeneration is completed, it would be interesting to evaluate whether the improvement in this process leads to an enhancement in muscle function through evaluations of physical performance and maximum strength.[35]

Regarding signaling, we observed that the Mas receptor increased compared to the control muscle. This result is interesting because other models of muscle injury that induce atrophy (by LPS, immobilization) also increase the Mas receptor.[36] In our model of muscle regeneration, once the injury induction, we observed a gradual

decrease in Mas receptor levels from 2 to 13 days after injury.

A similar receptor expression pattern was observed in the injured Ang (1-7) group. However, on day 13 post-injury, this group showed lower levels of Mas receptor than the vehicle group, reaching a value near the control muscle (Figure S1). Ang-(1-7) via Mas receptor has an endogenous protective effect on myostatin-induced muscle atrophy and Duchenne muscular dystrophy.[37,38] Therefore, the gradual decrease in the Mas receptor may represent a compensatory mechanism activated early after muscle damage to offset the signaling. Similarly, Mas internalization has been described as a physiological mechanism for desensitization of its signaling by prolonged stimulation. Once internalized, it can be degraded via lysosome or proteasome.[39,40]

Although the Injured-Ang-(1-7) muscles showed a decrease in Mas receptor at 13 dpi, the levels of AKT phosphorylation remained elevated. Thus, Ang-(1-7) administration enhanced Mas/AKT signaling activation even when Mas receptor levels declined (Figure 1S). Further studies could investigate if Ang-(1-7) could bind to AT-2 receptors and activate AKT.[41] Therefore, this study describes the favorable effect of Ang-(1-7) on muscle regeneration for the first time. However, the possible molecular mechanisms involved still need to be clarified. These results give rise to future studies to evaluate molecular pathways of IGF-1/IGF-1R/AKT, ROS production, and expression of MuRF-1 and atrogin-1, which are assessed as targets by which Ang-1-7 would improve muscle regeneration. It is crucial to ascertain the impact of Ang-(1-7) on the myogenesis process associated with markers of SC proliferation and differentiation. Finally, it will be essential to contribute to studies incorporating functional tests that determine whether the favored muscle regeneration improves the skeletal muscle function.

In summary, our study provides evidence that angiotensin (1-7) promotes differentiation in vitro and enhances muscle regeneration following injury. The observed morphological changes, increased eMHC expression, and elevated myogenin levels collectively indicate that angiotensin-(1-7) is pivotal in promoting muscle tissue repair and regeneration. These findings significantly impact the development of therapeutic interventions to improve skeletal muscle-related disorders.

List of acronyms

ACE - Angiotensin Converting Enzyme
AKT - Protein kinase B
Ang-(1-7) - Angiotensin (1-7)
Ang-II - Angiotensin II
ANOVA - Two-way analysis of variance
ARB - AT-1 receptor blockers
BaCl2 - Barium-chloride
BSA - Bovine serum albumin
DMD - Duchenne muscular dystrophy
Dpi - Days post-injury
eMHC - Embryonic myosin
H&E - Hematoxylin and eosin
IGF-1 - Insulin-like growth factor 1
IGF-1R - Insulin-like growth factor 1 receptor
LPS - Lipopolysaccharide
MHC - Myosin heavy chain
RAS - Renin–angiotensin system
RIPA - Radioimmunoprecipitation assay
ROS - Reactive oxygen species
SC - Satellite cells
SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM - Standard error of the mean
TA - Tibial anterior
TGF-β - Transforming growth factor-beta
UPS - Ubiquitin-proteasome system
WGA - Wheat germ agglutinin

Contributions of Authors

Conceptualization, M.V-B., J.A., F.S., D.C., F.T., and C.C-V.; Methodology, F.T., M.V-B., J.A., and C.C-V.; Validation, M.V-B., and J.A.; Investigation, M.V-B., J.A., F.T., D.C., F.S., and C.C-V.; Visualization, F.T., F.S., and C.C-V.; Supervision, C.C-V.; Project administration, C.C-V.; Formal Analysis, M.V-B., F.T., J.A., and C.C-V.; Writing – Original Draft Preparation, M.V-B., D.C., F.T., J.A., and C.C-V.; Writing – Review & Editing, M.V-B., F.T., D.C., J.A., F.S., and C.C-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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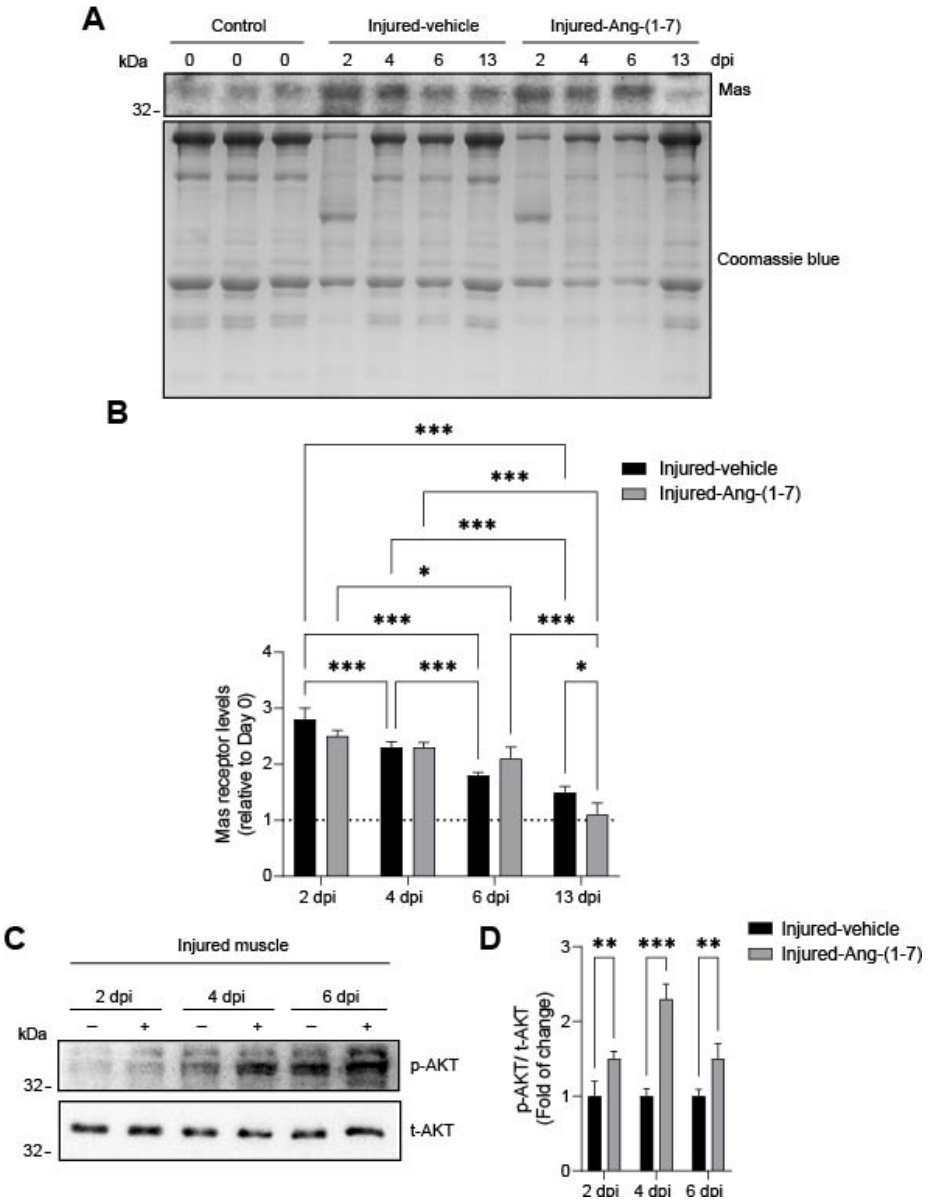

*Supplementary Figure S1. Levels of Mas receptor and AKT phosphorylation in regenerating muscles treated with Angiotensin-(1-7). Angiotensin-(1-7) was administered in C57BL/6 male mice, and then muscle injury was induced by BaCl2. (A, C) TA muscle was homogenized to evaluate Mas, AKT phosphorylation, and AKT total levels detected by western blot analysis using Coomassie blue or AKT total levels as a loading control. Molecular weights are indicated in kDa. Densitometric analysis of Mas (B), phosphorylated AKT (p-AKT), and total AKT (t-AKT) (D) were performed. In (B), dotted line represents control group. The values are shown as the mean ± SEM for each group (n=5-6 mice per group Student t-test. *p<0.05, **p<0.001, ***p<0.0001 vs injured-vehicle). SEM, standard error of the mean; TA, tibialis anterior; AKT, protein kinase B; dpi, days post injury.*