Original Paper

Angiotensin-(1-7) Prevents Skeletal Muscle Atrophy Induced by Transforming Growth Factor Type Beta (TGF- β) via Mas Receptor Activation

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Key Words

Muscle wasting • TGF- β • ng-(1-7) • Mas receptor • MHC • MuRF-1

Abstract

Background: Transforming growth factor type beta 1 (TGF- β 1) produces skeletal muscle atrophy. Angiotensin-(1-7) (Ang-(1-7)), through the Mas receptor, prevents the skeletal muscle atrophy induced by sepsis, immobilization, or angiotensin II (Ang-II). However, the effect of Ang-(1-7) on muscle wasting induced by TGF- β 1 is unknown. **Aim:** To evaluate whether Ang-(1-7)/Mas receptor axis could prevent the skeletal muscle atrophy induced by TGF- β 1. **Methods:** This study assessed the atrophic effect of TGF- β 1 in C₂C₁₂ myotubes and mice in absence or presence of Ang-(1-7), and the receptor participation using A779, an antagonist of the Mas receptor. The levels of myosin heavy chain (MHC), polyubiquitination, and MuRF-1 were detected by western blot. Myotube diameter was also evaluated. *In vivo* analysis included the muscle strength, fibre diameter, MHC and MuRF-1 levels by western blot, and ROS levels by DCF probe detection. **Results:** The results showed that Ang-(1-7) prevented the increase in MuRF-1 and polyubiquitinated protein levels, the decrease of MHC levels, the myotubes/fibre diameter diminution, and the increased production of reactive oxygen species (ROS) induced by TGF- β 1. Utilizing A779 inhibited the anti-atrophic effect of Ang-(1-7). **Conclusion:** The preventive effect of Ang-(1-7) on skeletal muscle atrophy induced by TGF- β 1 is produced through inhibition of ROS production and proteasomal degradation of MHC.

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Introduction

Skeletal muscle atrophy is a pathological condition that affects skeletal muscle, producing loss of strength and muscle mass and leading to weakness [1, 2]. This condition can be produced by disuse, aging, bacterial infections, and chronic diseases [3-9]. There are

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different soluble factors that can be involved in the development of muscle atrophy, among them transforming growth factor type beta 1 (TGF- β 1) [10, 11]. TGF- β 1 is a growth factor that regulates different processes in the cell such as proliferation, differentiation, regeneration, and apoptosis [12]. Studies indicate that the levels of TGF- β 1 primarily increase in cases of chronic diseases such as cancer, heart failure, or diabetes [13-18], and this increase could cause skeletal muscle atrophy [19]. Our recent research elucidated the mechanism involved in the atrophic effect of TGF- β 1 in skeletal muscle, which depends on an increase in reactive oxygen species (ROS) [11, 20]. We have shown the over-activation of ubiquitin-proteasome pathway (UPP), specifically an increase in the expression of E3 ubiquitin ligase MuRF-1. This occurs concomitant with a decrease in myosin heavy chain (MHC) protein levels and muscle fibre diameters.

Another important regulator of skeletal muscle mass is the renin-angiotensin system (RAS) [21-24]. Previous antecedents had demonstrated that Ang-II - a vasoactive peptide of the classical RAS axis - induces skeletal muscle atrophy by an over-activation of UPP and myonuclear apoptosis [25-27]. The alternative arm of RAS or non-classical RAS, is formed by ACE-2 that induces the synthesis of Ang-(1-7) peptide that acts via Mas receptor producing contrary effects than classical RAS [24, 28]. In this context, our previous studies have shown that Ang-(1-7) prevents muscle atrophy induced by Ang-II, lipopolysaccharides, and disuse through the Mas receptor [29-32].

Based on this information, this study was designed to evaluate the effect of the Ang-(1-7)/Mas receptor axis on the TGF- β 1-dependent atrophic effect utilizing an *in vitro* and *in vivo* model of skeletal muscle atrophy.

Materials and Methods

Animals studies

Male C57BL/10J mice (12 weeks old) were used for the analysis. The mice were kept at room temperature on a 12:12 h light-dark cycle with access to food and water *ad libitum* [33]. Four groups of animals (with four mice per group) were randomly separated for the experiments: control, TGF- β 1 (10 ng) (Shenandoah Biotechnology Inc., PA, USA), Ang-(1-7) (100 ng/kg per min) (Sigma-Aldrich, MO, USA), and TGF- β 1 plus Ang-(1-7). Independent experiments were performed in triplicate. Mice under ketamine/xylazine anaesthesia were injected with TGF- β 1 in the tibialis anterior (TA) muscle using the contralateral muscle as a control (injected with PBS) [34]. For the infusion of Ang-(1-7), two groups were implanted with osmotic micropumps (Alzet-Durect, CA, USA) 24 hours prior to TGF- β 1 injection following manufacturer instructions [34]. The mice were killed under anaesthesia 72 h post-injection, and the TA muscles were dissected, removed, rapidly frozen with isopentane, and stored at -80 °C until use. All protocols were conducted in strict accordance with and under the formal approval of the Animal Ethics Committee of the Universidad Andrés Bello.

Cell cultures

The mouse skeletal muscle cell line C₂C₁₂ (American Type Culture Collection, VA, USA) were grown and used until passage 10. The cells were differentiated into myotubes for five days, as described previously [35-39]. The myotubes were pre-incubated for 30 min with Ang-(1-7) (10 nM) in presence or absence of Mas Inhibitor A779 (10 μ M) (CPC Scientific, CA, USA) and then incubated with TGF- β 1 (10 ng/ml) for the times indicated in each figure.

Measurement of intracellular ROS levels

The C₂C₁₂ myotubes were grown, differentiated, and treated on glass coverslips. After each treatment, the myotubes were washed with Hank's balanced salt solution (HBSS) and incubated with 10 μ M of the cell-permeant dye dichlorodihydrofluorescein (Invitrogen, CA, USA) for 30 min at 37 °C. Then, after duplicate washes with HBSS and one wash with phosphate-buffered saline (PBS), the myotubes were fixed with 4% paraformaldehyde for 10 min and washed with PBS. Nuclear staining was performed with 1 μ g/ml Hoechst 33258 in PBS for 10 min. After rinsing the cells were mounted with a fluorescent mounting medium (Dako

Corporation, CA, USA), viewed, and photographed with the Motic BA310 epifluorescence microscope (Motic, Hong Kong).

Western blot analysis

After each treatment, the protein extracts of myotubes were obtained by homogenizing the sample in radioimmunoprecipitation assay buffer, and the protein extracts of TA were obtained by homogenizing the muscle with TRIS/EDTA buffer, with a cocktail of protease inhibitors and 1 mM of phenylmethylsulfonyl fluoride in each case. Proteins were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Germany). The proteins were detected using the follow antibodies: mouse anti-MHC (1:3,000) (MF-20, University of Iowa Hybridoma Bank, IA, USA), rabbit anti-MuRF-1 (1:500) (ECM Biosciences, KY, USA), mouse anti-tubulin (1:5,000) (Santa Cruz Biotechnology, CA, USA). All immunoreactions were visualized by enhanced chemiluminescence (Thermo Fischer Scientific, MA, USA) using the FOTODYNE FOTO/Analyst Luminary Workstation Systems (FOTODYNE Inc., WI, USA).

Contractile properties

After treatment, the mice were anesthetized and the tibialis anterior (TA) muscles were removed and placed in a dish containing oxygenated Krebs-Ringer solution. Then the muscles were tied firmly with surgical silk, and transferred to a custom-built Plexiglas bath filled with oxygenated Krebs-Ringer solution thermostatically maintained at 37 °C for optimal oxygen diffusion. The muscles were tied to a MLT 1030/D force transducer (AD Instruments, Dunedin New Zealand) and further were stimulated by supramaximal square wave pulses using a S48 Stimulator (Grass Research Instruments, RI, USA), controlled and measured using a Power Lab 4/35 (AD Instruments) and computed with Lab Chart analysis software (AD Instruments). Maximum isometric tetanic force was determined as previously reported, by calculating in addition, the specific net force (force normalized per total muscle fiber cross-sectional area, mN/mm²).

Indirect Immunofluorescence

The C₂C₁₂ cells were grown, differentiated for five days, and treated on plastic coverslips. Then, each treated myotubes was washed twice in ice-cold HBSS, fixed in 4% paraformaldehyde, and permeabilized with 0.05% Triton X-100. Afterward, treatments were blocked for 1 h with a buffer containing 50 mM Tris-HCl (pH of 7.7), 0.1 M NaCl, and 2% bovine serum albumin and incubated for 1 h with 1:100 mouse anti-MHC in the same buffer. After antibody removal and several washes with HBSS, bound antibodies were detected by incubating the cells for 1 h with 1:500 affinity-purified Alexa Fluor dye-conjugated goat anti-mouse antibody (Life Technologies, CA, USA). Nuclear staining was performed with 1 µg/ml Hoechst 33258 in PBS for 10 min. After rinsing, the cells were mounted with a fluorescent mounting medium, viewed, and photographed with the Motic BA310 epifluorescence microscope.

Wheat germ agglutinin staining

Wheat germ agglutinin (WGA) staining labels glycoproteins at the sarcolemma [29, 31]. To stain the samples, freshly-frozen TA muscles were cryosectioned (8 µm) and placed on glass slides. The samples were fixed in 4% paraformaldehyde and incubated with Alexa-594-conjugated WGA (1:200) (Molecular Probes, OR, USA) and 1 µg/ml Hoechst 33258 in PBS. Triplicate PBS washes were performed between each step. After rinsing, the TA sections were mounted with a fluorescent mounting medium under glass coverslips, viewed, and photographed with the Motic BA310 epifluorescence microscope.

Myotube/Fibre diameter determination and quantification

Photographs obtained of immunofluorescent myotubes for MHC in different zones of the coverslip were used to obtain the myotube diameters. These measurements were determined through a blinded analysis with the ImageJ software (National Institutes of Health, MD, USA) of the images captured from each experimental condition. Myotube diameter was measured at three different points of the individual myotube, and then the values were averaged. Myotubes were defined as all multinucleated (containing three or more nuclei) cells positive for MHC stain. For the fibre diameter measurements, photographs obtained of TA sections stained with WGA were analysed from each experimental condition. The fibres were manually selected, and the minimal Feret's diameter of each fibre was quantified by the ImageJ software.

Statistics

Data were analysed statistically using one-way analysis of variance with a post-hoc Bonferroni multiple-comparison test (Sigma Stat Systat Software, CA, USA). Differences were considered statistically significant at $P < 0.05$.

Results

Ang-(1-7)/Mas receptor axis prevents the atrophic effects induced by TGF- β in C2C12 myotubes by averting the decrease in myotube diameter and MHC levels

The effects of Ang-(1-7) on the atrophic effect of TGF- β 1 on skeletal muscle were evaluated in C₂C₁₂ myotubes. First, myotube diameter was determined after pre-incubating myotubes with Ang-(1-7) for 30 min followed by a co-incubation with TGF- β 1 for 72 h. Myotubes treated with TGF- β 1 were qualitatively smaller than control myotubes, but in the presence of Ang-(1-7) shrinkage was prevented and myotubes were similar in size to the control (Fig. 1A). The effect of Ang-(1-7) incubation depended on the Mas receptor; as demonstrated by the inhibition of the Ang-(1-7)-dependent anti-trophic effect when the Mas receptor antagonist A779 was used (Fig. 1A). Quantification of myotube diameters showed that TGF- β 1 treatment increased the proportion of myotubes with small diameters ([0-5 μ m] and [5-10 μ m]) and decreased the proportion with big diameters ([20-30 μ m] and [30-40 μ m]) compared to the control (Fig. 1B). However, the myotubes treated with TGF- β 1 in the presence of Ang-(1-7) showed a curve of myotube diameters similar to the control (Fig. 1B).

Fig. 1. The Ang-(1-7)/Mas receptor axis prevents TGF- β 1-induced decrease in diameters of myotubes (A) C₂C₁₂ myoblasts differentiated for five days (myotubes) were pre-incubated in absence or presence of Ang-(1-7) (10 nM), A779 (10 μ M), or both for 30 min, and then incubated with TGF- β 1 (10 ng/mL) for 72 h. MHC was detected by indirect immunofluorescence assay (green). Nuclei were labelled with Hoechst staining (blue). The bar scale represents 100 μ m. The myotube diameters from (A) were measured, quantified and plotted. The graphics

shows the effect of Ang-(1-7) (B), and the effect of Ang-(1-7) plus A779 (c) on the distribution of myotube diameters in presence or absence of TGF- β 1. The values are expressed as a percentage of the total myotubes and correspond to the mean $\pm$ SD from three independent experiments (*, $P < 0.05$ vs control).

Fig. 2. Ang-(1-7) through the Mas receptor prevents TGF- β 1-induced decrease of MHC levels in myotubes. C₂C₁₂ myotubes were incubated with TGF- β 1 (10 ng/mL) for 72 h in presence or absence of Ang-(1-7) (10 nM), A779 (10 μ M) (pre-incubated for 30 min), or both. (A) The MHC protein levels were determined by Western blot analysis. Tubulin was used as loading control. Molecular weights are shown in kDa. (B) Densitometric analysis for MHC protein levels with values normalized to tubulin and expressed as fold of induction relative to the control (myotubes without treatment). All values correspond to the mean $\pm$ SD from three independent experiments (*, $P < 0.05$ vs control without treatment; #, $P < 0.05$ vs TGF- β 1 treatment).

Fig. 3. Ang-(1-7) through the Mas receptor averts the rise in MuRF-1 levels induced by TGF- β 1 in C₂C₁₂ myotubes. C₂C₁₂ cells differentiated for five days were incubated with TGF- β 1 (10 ng/mL) for 48 h in presence or absence of Ang-(1-7) (10 nM), A779 (10 μ M) (pre-incubated for 30 min), or both. (A) The protein levels of MuRF-1 were determined by Western blot analysis. Tubulin was used as loading control. Molecular weights are shown in kDa. (B) The graph shows the quantitative analysis for MuRF-1 levels with values normalized to tubulin and expressed as fold of induction relative to the control (myotubes without treatment). All values correspond to the mean $\pm$ SD from three independent experiments (*, $P < 0.05$ vs control without treatment; #, $P < 0.05$ vs TGF- β 1 treatment).

Interestingly, Ang-(1-7) alone slightly displaced the curve of myotube diameters towards the right, with a significant difference in the range of 30–40 μ m (Fig. 1B). Co-incubation with A779, a Mas receptor antagonist, in the presence of Ang-(1-7) and TGF- β 1 produced myotube diameters similar in size to those displayed in the presence of TGF- β 1 (Fig. 1C).

The levels of myofibrillar proteins, such as MHC, were assessed in the presence or absence of TGF- β 1, Ang-(1-7), and A779. The MHC levels decreased in the presence of TGF- β 1 treatment, displaying a 60% drop in comparison to the control (Fig. 2A). Co-incubation of cells with TGF- β 1 and Ang-(1-7) displayed MHC levels similar to the control condition, while myotubes treated with Ang-(1-7), A779, and TGF- β 1 displayed results similar to those of cells treated with TGF- β 1 alone (Fig. 2A, B). These results indicate that the Ang-(1-7)/Mas receptor axis prevented the atrophic effect of TGF- β 1 induced in C₂C₁₂ myotubes.

Ang-(1-7), through the Mas receptor, prevents the over-activation of ubiquitin proteasome pathway (UPP) induced by TGF- β 1 in C2C12 myotubes

The UPP, the most studied catabolic pathway of sarcomere proteins such as MHC [40–42], is over-activated in several types of skeletal muscle atrophy. Recently, TGF- β 1 was found

Fig. 4. The Ang-(1-7)/Mas receptor axis prevents an increase in the polyubiquitination of protein induced by TGF- β 1 in myotubes. C₂C₁₂ cells differentiated for five days (myotubes) were pre-incubated in absence or presence of Ang-(1-7) (10 nM), A779 (10 μ M), or both and then incubated with TGF- β 1 (10 ng/mL) for 48 h as indicated by the figure. (A) The polyubiquitinated protein levels were determined by Western blot analysis using tubulin as loading control. Molecular weights are shown in kDa. (B) Densitometric analysis for polyubiquitinated proteins levels with values normalized to tubulin and expressed as fold of induction relative to the control (myotubes without treatment). The values correspond to the mean $\pm$ SD from three independent experiments (*, $P < 0.05$ vs control without treatment; #, $P < 0.05$ vs TGF- β 1 treatment).

to produce an increase in the E3 ubiquitin ligase MuRF-1 in myotubes. The effect of Ang-(1-7)/Mas axis on TGF- β 1-induced UPP over-activation was evaluated by determining the MuRF-1 levels and profile of the polyubiquitinated protein. To accomplish this, myotubes were incubated in the presence or absence of Ang-(1-7), A779, and TGF- β 1 for 48 h. Levels of MuRF-1 protein increased by 3.8-fold due to TGF- β 1 incubation. In contrast, in the presence of Ang-(1-7) these levels decreased to amounts similar to the control, but co-incubation with A779 blocked the effect of Ang-(1-7) in the presence of TGF- β 1, allowing for a 3.1-fold rise in MuRF-1 levels (Fig. 3A, B).

The effect of Ang-(1-7) on the polyubiquitinated proteins was similar to that of MuRF-1. Polyubiquitinated proteins increased by 2.7-fold when incubated with TGF- β 1, and the increase was prevented by Ang-(1-7). In the presence of A779, the preventive effect of Ang-(1-7) was impeded, and the levels of polyubiquitination were similar to those in samples with only TGF- β 1 (Fig. 4A, B).

Together, these results indicate that Ang-(1-7)/Mas axis prevented the over-activation of UPP induced by TGF- β 1 in C₂C₁₂ myotubes, specifically, the increase in MuRF-1 expression and polyubiquitinated proteins.

TGF- β 1-dependent ROS production is prevented by Ang-(1-7)/Mas receptor axis in C2C12 myotubes

Previously, we have demonstrated that ROS production is a key regulator in the skeletal muscle atrophy induced by TGF- β 1. To evaluate the effect of Ang-(1-7) on ROS induced by TGF- β 1, myotubes were co-incubated with TGF- β 1, Ang-(1-7), and A779, and the ROS levels were measured using the fluorescent dye CM-DCF (Fig. 5A). The DCF-positive myotubes were

Fig. 5. The increase of reactive oxygen species (ROS) induced by TGF- β 1 is prevented by Ang-(1-7) through the Mas receptor in myotubes. (A) C₂C₁₂ myotubes were incubated with TGF- β 1 (10 ng/mL) for 24 h in presence or absence of Ang-(1-7) (10 nM), A779 (10 μ M) (pre-incubated for 30 min), or both. The ROS levels were then determined by loading the cells with a CM-DCF-DA probe, and the nuclei were labelled with Hoechst. Fluorescence was determined by microscopy. Scale bar represents 100 μ m. (B) The graph shows the quantification of DCF-positive myotubes for the different treatments. The values are expressed as fold of induction relative to the myotubes without treatment and correspond to the mean $\pm$ SD from three independent experiments (*, $P < 0.05$ vs control without treatment; #, $P < 0.05$ vs TGF- β 1 treatment).

quantified for each condition. TGF- β 1-dependent ROS production (2.8-fold of induction relative to control) was partially decreased due to the effect of Ang-(1-7) (1.65-fold relative to control). The use of A779 hindered the effect of Ang-(1-7), and the ROS levels were similar to those of TGF- β 1 alone (Fig. 5B).

These results indicate that Ang-(1-7) through Mas receptor decreased the ROS production induced by TGF- β 1 in C₂C₁₂ myotubes.

Ang-(1-7) prevented the atrophic effect of TGF- β 1, specifically muscle strength, fibre diameter and myosin level diminution in the tibialis anterior muscle of mice.

We evaluated the effect of Ang-(1-7) on the decline in the muscle strength induced by TGF- β . The measures of isometric force showed that Ang-(1-7) is able to recover the decline in muscle strength produced by TGF- β (Fig. 6A). In a previous report, we showed that TGF- β 1 injected into TA muscle produces a diminution in the diameter of the fibres and decreases the MHC protein levels [11]. To test the anti-atrophic effect of Ang-(1-7) in this model, the peptide was systemically administered to mice that were then injected with TGF- β 1 in the TA. The results indicated that diminution in muscle fibre size induced by TGF- β 1 was prevented when Ang-(1-7) was administered (Fig. 6B). The TGF- β 1 injections produced a shift towards fibre of a lesser diameter than the control condition, while in the presence of Ang-(1-7) this effect was lost and fibre diameters were similar to the control condition (Fig. 6C). When the MHC levels were evaluated, Ang-(1-7)-treated samples did not display the decrease in MHC levels induced by TGF- β 1 (Fig. 6D, E).

These results show that the anti-atrophic effects of Ang-(1-7) were observable in an *in vivo* model of TGF- β 1-induced muscle wasting in mice.

Fig. 6. Ang-(1-7) prevents the decrease of the fibre diameters and MHC levels induced by TGF- β 1 in the tibialis anterior (TA) of mice. (A) TA muscles of male C57/BL10 mice were injected with either PBS (control) or TGF- β 1 (10 ng) at day 1 and 3. To the correspondent group, osmotic micropumps with Ang-(1-7) (100 ng/kg per min) or vehicle were implanted 24 h prior to first injection with TGF- β 1. After 5 days of the first TGF- β injection, muscles were excised and maximal isometric strength (mN/mm²) was evaluated. Values represent the mean $\pm$ SD of triplicate independent experiments and are expressed as percentage of the net force relative to mice treated with vehicle and injected with PBS. In each experiment, three mice were used for each experimental condition (*, $P < 0.05$ vs control vehicle; #, $P < 0.05$ vs TGF- β 1 vehicle). (B) TA

muscles of male C57/BL10 mice were injected with either PBS (control) or TGF- β 1 (10 ng) for once. To the correspondent group, osmotic micropumps with Ang-(1-7) (100 ng/kg per min) or vehicle were implanted 24 h prior to the injection with TGF- β 1. After 72 h, the muscles were removed. Cryosections of TA muscles were stained with WGA and the fibre Feret's diameter was determined. The bar scale corresponds to 100 μ m. Quantitative analysis for the fibre diameters is shown in (C). Values are expressed as the percentage of the total fibres quantified and correspond to the mean $\pm$ SD from three independent experiments (*, $P < 0.05$). The MHC protein levels were determined by Western blot analysis. Tubulin was used as loading control. Molecular weights are shown in kDa (D). The quantitative analyses for MHC levels are shown (E). The values were normalized to tubulin and are expressed as fold of induction relative to the control incubated without TGF- β 1. Values correspond to the mean $\pm$ SD from three independent experiments (*, $P < 0.05$ vs control vehicle; #, $P < 0.05$ vs TGF- β 1 vehicle).

Discussion

The present study demonstrates that Ang-(1-7)/Mas receptor axis averted the atrophic effects induced by TGF- β 1 in C₂C₁₂ myotubes. The results show that the use of Ang-(1-7) prevented an increase in ROS levels and the over-activation of UPP, specifically the increased MuRF-1 protein levels and polyubiquitinated proteins. Concomitantly, Ang-(1-7) prevented the decrease in MHC protein levels and myotube and fibre diameters in skeletal muscle.

Despite that this study has several limitations, mainly by the *in vitro* experiments and the mechanisms that were derived from them, our findings must be corroborated in a pathological status in which an increase of TGF- β can be observed.

The beneficial effects of Ang-(1-7) have been described in different tissues and cell types [43-48]. In skeletal muscle, previous research performed on rodent muscle wasting

induced by disuse [32], lipopolysaccharides [31], and Ang-II [29, 30], indicates that Ang-(1-7) prevents muscle atrophy through the Mas receptor. This study is the first to demonstrate the protective effect of Ang-(1-7) in muscle atrophy induced by TGF- β 1.

Our previous study establishes that one of the primary mechanisms involved in the development of skeletal muscle atrophy induced by TGF- β 1 is the increase in ROS production [11]. This study demonstrated that Ang-(1-7)/Mas axis prevented the rise of ROS levels induced by TGF- β 1 in C₂C₁₂ myotubes. These results concur with previous evidence indicating that Ang-(1-7) via the Mas receptor decreases oxidative stress in different cell types, such as adipocytes [49], cerebral endothelial cells [50], and, interestingly, in pathologies associated with skeletal muscle atrophy such as diabetes [51], cardiac failure [52], and hepatic damage [53]. Additionally, previously published results by us shows that Ang-(1-7)/Mas receptor prevents ROS induction by Ang-II in skeletal muscle cells [54]. Therefore, Ang-(1-7) could have an antioxidant effect in skeletal muscle atrophy conditions induced by TGF- β 1.

Among the main sources of intracellular ROS is NADPH oxidase (NOX) [55, 56]. Previous work indicates that the TGF- β 1-induced rise in ROS production is dependent on NOX in skeletal muscle cells [20]. Other groups demonstrate that Ang-(1-7) decreases mRNA levels of NOX in adipocytes [49] or the NOX activation [57, 58]. Based on this research, Ang-(1-7) could block the NOX-dependent ROS production. Further investigation is necessary in order to study this possible Ang-(1-7) effect.

An increase in TGF- β 1 levels as well as in its signalling is observed in various skeletal muscle disorders [19, 59-61]. One of these is Duchenne muscular dystrophy, in which *mdx* mice has serve as murine models [62]. Interestingly, the muscle wasting associated with *mdx* mice is decreased by an Ang-(1-7) infusion that inhibits TGF- β 1 signalling; thus, reducing fibrosis and restoring muscle strength [34]. TGF- β 1 signaling also increases during muscle atrophy induced by Ang-II or disuse [63-66]. In these muscle wasting models, Ang-(1-7) displays an anti-atrophic effect evidenced by the prevention of decreases in muscle strength, UPP over-activation, and decreases in MHC levels [32, 54]. However, other complementary studies must be performed to evaluate the direct relation between the Ang-(1-7) mediated inhibition of TGF- β 1 signalling and the anti-atrophic effect in these models.

One of the possible mechanisms regulated by Ang-(1-7) is the signalling pathway activated by TGF- β 1. The canonical TGF- β 1 pathway is mediated by the phosphorylation of Smad2/3 proteins [67] and further transcription of target genes. In this pathway, Smad7 is an inhibitory protein, acting through negative feedback by blocking the Smad2/3 phosphorylation [67]. In dystrophic *mdx* mice, previous research demonstrated that Ang-(1-7) increases Smad7 expression [34]. This study, we have demonstrated that TGF- β 1 induces the phosphorylation of both Smad proteins and that this pathway is involved in the atrophic effect induced by TGF- β 1 (unpublished result) in C₂C₁₂ myotubes. However, further studies are necessary to evaluate the possible participation of Smad7 on the anti-atrophic effect mediated by Ang-(1-7).

The present study shows that Ang-(1-7), through the Mas receptor, prevented the atrophic effects induced by TGF- β 1 in skeletal muscle cells and tissue. In this context, the clinical importance of our results is that could be extrapolated to diseases in which TGF- β levels are increased (such as Duchenne muscular dystrophy) and evaluate the effect on muscle atrophy. Thus, Ang-(1-7) could be used as a therapeutic alternative for the treatment of skeletal muscle atrophy induced by different chronic diseases

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Disclosure Statement

The authors declare no conflict of interest.

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