

Ang-(1-7) Protects Skeletal Muscle Function in Aged Mice

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Abstract

Background: The angiotensin-converting enzyme 2 (ACE2)/angiotensin 1-7 (Ang-(1-7)) axis has been shown to perform a protective task in the decline of the function of skeletal muscle correlated with the process of aging. In the present investigation, the protective effects of ACE2 in mitigating the age-associated decline of skeletal function and identified the potential underlying molecular mechanism mediating the process have been extensively evaluated.

Methods: We measured the expression levels of Ang-(1-7) in C57BL/6J mice of different ages and correlated these levels with measures of skeletal muscle function. Also, we determine the expression of myocyte enhancer factor 2A (MEF2A) were detected in ACE2 knockout (ACE2KO) and correlated with muscle function. We then treated ACE2KO aged mice for 4 weeks with Ang-(1-7) and characterized the levels of MEF2A and skeletal muscle function before and after treatment. We assessed the impact of Ang-(1-7) on the growth and differentiation of C2C12 cells in vitro and assessed changes in the glucose transporter type 4 (Glut4) expression.

Results: Aged mice showed reduced skeletal muscle function and levels of Ang-(1-7) expression in comparison to young and middle-aged mice. In ACE2KO mice, skeletal muscle function and MEF2A protein expression were significantly lower than in age-matched WT mice. After 1 month of the treatment of Ang-(1-7), the function of skeletal muscle related to the aged ACE2KO mice improved, however, the expression of MEF2A protein was similar to that in the untreated group. In C2C12 cells, Ang-(1-7) was shown to increased cell growth and differentiation characteristics along with the upregulated expression of Glut4.

Conclusions: The axis of ACE2/ Ang-(1-7) has a protective task in skeletal muscle and the administration of exogenous Ang-(1-7) can delay the age-related decline in the functions of skeletal muscle.

Introduction

The process of aging is correlated with pronounced decreases in skeletal muscle mass, reduced muscular function and a general decline in physical fitness. In the clinic, these manifestations are commonly associated with sarcopenia and frailty [1]. Frailty and Sarcopenia severely affect the standard of living in elderly people and constitute major risk factors for falls, disability, and even death. Selective androgen agonists can be prescribed to partially increase muscle mass, however, these agents fail to improve muscle function. Myostatin-neutralizing antibody and activin IIB receptor blockers can significantly increase muscle mass and partially improve muscle function but their clinical benefits are unclear [2, 3]. The identification of safe and clinically effective drugs capable of managing the age-related loss of muscle function and mass represents a significant challenge with a high potential for clinical impact.

The Renin-angiotensin system (RAS) is widely expressed in the system of cardiovascular, kidneys, and the lung. The prevalent pathway of the RAS via the angiotensin-converting enzyme (ACE)-angiotensin

(Ang II) axis is well established. However, the pathway of angiotensin-converting enzyme 2 (ACE2) - angiotensin 1–7 (Ang-(1–7)) has been indicated to compete with the activity of the ACE/Ang II pathway.

The main pathological features of sarcopenia are directly related to the aging-related decline in the quantity and structure of skeletal muscles and the ACE/Ang II pathway is closely associated with skeletal muscle loss [4]. By attaching to the angiotensin II type 1 receptor (AT1R), Ang II reduces microvascular perfusion, the uptake of glucose, and the sensitivity of insulin in the muscle fibers of mice [5, 6]. Attempts have been made to ameliorate the function of skeletal muscle and delay muscle atrophy in the old age by pharmacological antagonism of the ACE-Ang II-AT1R pathways using AT1R antagonists or ACE inhibitors (ACEIs). However, randomized controlled clinical trials have so far demonstrated unsatisfactory results. Shrikrishna and colleagues showed that ACEI could not significantly improve lower extremity skeletal muscle function in elderly patients with severe obstructive pulmonary illness [7]. Similarly, the TRAIN trial demonstrated that ACEI had no significant effects on the activity capacity of elderly patients [8].

Animal investigations have revealed that the pathway of ACE2/Ang-(1–7) can increase the uptake of glucose in the skeletal muscles and promote the expression of key transcription factors that regulate the growth of skeletal muscles [9, 10]. The main aim of the current research is to analyze the impacts of the ACE2/Ang-(1–7) pathway on aging-related skeletal function decline and to identify the possible underlying regulatory molecular mechanisms.

Methods

Experimental design

C57BL/6J mice of different ages were used for in vivo experiments. An ACE2-KO mouse model (C57BL/6J background) was also used for functional studies. All mice were bought from the Laboratory Animal Research Institute of the Chinese Academy of Medical Sciences. In vitro assays were performed using C2C12 cells. Experiments were performed to determine skeletal muscle function and the expression of Ang (1-7) and Ang II in old (20 months), middle-aged (12 months), and young (3 months) C57BL/6J mice. Further, the skeletal muscle function and the expression of myocyte enhancer factor 2A (MEF2A) were determined in young (4 months) and old (16 months) ACE2-KO mice and their wild-type aged-matched controls.

Old (16 months) ACE2-KO mice were subcutaneously injected with 400 ng/kg/min of Ang-(1-7) (APEXBio, Houston, TX, USA, product No. A1041) for 4 weeks. Skeletal muscle function and the expression of MEF2A were compared before and after treatment. In the control group, ACE2-KO mice were subcutaneously injected with 0.9% normal saline. C2C12 cells (prepared from the bank of the cell of the Chinese Academy of Sciences) were grown for 48 hours in DMEM milieu including 2% serum of horse (Gibco, Grand Island, NY, USA), supplemented with 10^{-8} mol/L of Ang-(1–7) or the same volume of PBS. Following the period of incubation, the cellular differentiation levels and the expression of glucose transporter type 4 (Glut4), MEF2A, myosin heavy chain (MHC), and creatine kinase, muscle

(CKM) proteins were evaluated. All in vivo protocols were confirmed through the Animal Committee of West China Hospital, Sichuan University.

Testing of skeletal muscle function

The function of skeletal muscle was evaluated by measuring the grip forelimb strength and the number of falls in a treadmill test. The grip strength of the mouse forelimb was measured using a tester of grip strength (YLS-13A, Jinan Yiyan Technology Development Co., Ltd, Jinan, China.). For each mouse, 5 measurements were performed at 5-second intervals and the average value recorded. The treadmill test used an experimental animal treadmill (ZH-PT, Shanghai Kehuai Instrument Co., Ltd, Shanghai, China., Shanghai, China) with the track speed set at 9 m/min. The number of falls from the track within 5 minutes was recorded. For each mouse, measurements were recorded in triplicate and performed at 5-minute intervals. The average number of falls was recorded.

Preparation of mouse skeletal muscle

Mice were anesthetized by using pentobarbital (45 mg/kg) and the gastrocnemius and soleus muscles were readily anatomized and frozen in liquid nitrogen for subsequent analyses.

Determination of the levels of Ang-(1-7) and Ang II in mouse skeletal muscles

Frozen skeletal muscle samples were thawed and precooled. A homogenate buffer was added to the samples before being homogenization. Specimens were then incubated at 4°C for 1 hour and centrifuged. The supernatant was utilized to measure the concentrations of Ang II and Ang-(1-7) using the kits of ELISA (Cloud-Clone Corp., Houston, TX, USA) Cat. No. CEA005Mu (Ang II) and CES085Mi (Ang-(1-7)). The levels of Ang II and Ang-(1-7) were measured in muscle samples obtained from five mice in each experimental group.

Determination of MEF2A protein levels in mouse skeletal muscle

The expression of MEF2A was determined by Western blotting. Frozen skeletal muscle samples were thawed, lysed by using a lysis buffer, and centrifuged. The protein concentration of the supernatant was quantified utilizing the BCA assessment. Proteins were dissociated via SDS-PAGE and transferred to the membranes of nitrocellulose. The incubation of the membranes was carried out with a blocking solution at 37°C for 1 hour. Primary antibody was then added directed against MEF2A (Proteintech, Rosemont, IL,

USA, Cat. No. 12382-1-AP) and the incubation of the membranes was carried out during the night at 4°C. Secondary antibody (HRP Goat Anti-Rabbit IgG) was then increased to membranes and the protein levels detected using a chemiluminescence detection kit. The absorbance of each protein band was determined using the Image-Pro Plus 6.0 computer program for a quantitative study.

C2C12 cell culture, proliferation, and differentiation

C2C12 cells were cultivated in the DMEM milieu including 2% serum of horse. To analyze cell proliferation and differentiation, Ang-(1-7) was enhanced and the incubation of the cells was performed at 37°C for an additional 48 h. PBS was added to the control cultures.

Protein expression in differentiating C2C12 cells

The expression of MHC, creatine kinase, muscle CKM, Glut4, MEF2A proteins was determined by Western blotting in C2C12 cells. Following the differentiation assessment, the cells were collected, rinsed with PBS, incubated on ice by using the RIPA as the lysis buffer and centrifuged. The concentration of protein in the supernatant was quantified employing the BCA assessment. Proteins were dissociated via SDS-PAGE and transferred to the membranes of nitrocellulose. The membranes were blocked for 1 hour at 37°C and subsequently incubated during the night at 4°C using antibodies against MHC (Proteintech, Rosemont, IL, USA, Cat. No. 10799-1-AP), CKM (Proteintech, Cat. No. 18712-1-AP), Glut4 (Proteintech, Rosemont, IL, USA, Cat. No. 21048-1-AP), and MEF2A (Proteintech, Rosemont, IL, USA, Cat. No. 12382-1-AP). In addition, the incubation of the membranes was carried out by taking advantage of a secondary antibody and exposed using a chemiluminescence detection kit. The absorbance of each protein band was determined using the Image-Pro Plus 6.0 computer program for a quantitative study.

Statistical methods

All achieved outcomes were expressed as mean \pm SD and analyzed utilizing the SPSS 20.0 computer program package. A Student's t-test or a t-test was utilized for comparison among two groups. One-way ANOVA or the Kruskal-Wallis test were implemented for comparison between several groups. A P-value threshold of 0.05 was utilized to ascertain statistical significance.

Results

The grip strength of old mice decreased compared to young and middle-aged mice (young: 1.93 ± 0.14 g/g, middle-aged: 2.34 ± 0.29 g/g, old: 1.58 ± 0.06 g/g, all $P < 0.05$) (Figure 1A). In the treadmill test, the number of falls in old mice (17.86 ± 3.85) was higher than in the young (13.14 ± 2.60) and middle-aged (12.71 ± 2.29) mice ($P < 0.05$) (Fig. 1B). The levels of Ang-(1-7) in the skeletal muscle of old

mice were found to be lower compared to the young and middle-aged mice. The levels of Ang II were greater than in young and middle-aged mice compared to old mice ($P < 0.05$ in all cases) (Fig. 1C). These data indicated that the function of mouse skeletal muscle and the expression of Ang-(1-7) both reduction with aging. In contrast, the expression of Ang II increases with aging in mice.

We compared the motor abilities of ACE2-KO and WT mice at various ages and found that the grip strength of ACE2-KO mice was considerably lower than age-matched WT animals ($P < 0.05$). Moreover, the grip strength of young ACE2-KO mice was similar to that of old WT mice (2.80 ± 0.34 g/g vs. 2.63 ± 0.13 g/g, $P > 0.05$) (Fig. 2A).

In the treadmill experiments, the number of falls was significantly higher in the ACE2-KO mice in comparison to the age-matched WT animals ($P < 0.05$). The number of treadmill falls in young ACE2-KO was comparable to that in old WT mice (15.33 ± 2.16 vs. 14.00 ± 1.41 , $P > 0.05$) (Fig. 2B).

The changes in the levels of MEF2A protein expression in the mice skeletal muscle at different ages indicated that the level of MEF2A in WT and ACE2-KO mice both decreased with age (both $P < 0.05$). However, the expression of MEF2A was lower in ACE2-KO than in WT mice ($P < 0.05$) (Fig. 2C).

After 4 weeks of Ang-(1-7) injections, the grip strength of aged ACE2-KO increased ($P < 0.05$) (Fig. 3A) and the number of falls in the treadmill test decreased ($P < 0.05$) (Fig. 3B). Although, the expression of MEF2A protein in skeletal muscle remained similar to that in the normal saline group ($P > 0.05$) (Fig. 3C). Skeletal muscle function and the expression of MEF2A protein decreased in aged ACE2-KO mice. The administration of Ang-(1-7) in ACE2-KO mice increased motor ability without producing a significant change in the level of MEF2A protein.

The influences of Ang-(1-7) on the differentiation of C2C12 cells in vitro were evaluated. After the exposure to Ang-(1-7) for 48 hours, C2C12 cells formed tentacle-like extensions that had fibroblast-like morphologies. Importantly, tubular connections between cells were formed (Fig. 4A). Western blotting exhibited that the treatment of Ang-(1-7) upregulated the expression of the Glut4 protein but down-regulated the levels of MEF2A, MHC, and CKM (Fig. 4B). Treatment with Ang-(1-7) promoted the differentiation and growth of C2C12 cells and enhanced the expression of the Glut4 protein.

Discussion

In this research, it was illustrated that with increasing age, the function of the skeletal muscle and the tissue levels of Ang-(1-7) decreases in mice. The skeletal muscle function in old ACE2-KO mice decreased and the motor ability of young ACE2-KO mice was equivalent to that of the old wild-type mice. However, the motor function of aged ACE2-KO increased after Ang-(1-7) treatment. We investigated the possible underlying mechanisms of these effects and illuminated that the treatment of Ang-(1-7) resulted in the upregulated expression of Glut4 but did not affect the expression of MEF2A. The obtained outcomes indicate that Ang-(1-7) enhances skeletal muscle motor function in aging mice by promoting cellular glucose metabolism.

Our experiments demonstrated that the older mice had reduced grip strength and experienced a higher number of falls compared to young mice in the treadmill test. The skeletal muscles of aged animals showed decreased levels of Ang-(1–7) and an improved level of Ang II compared to young mice. Previous studies showed that the ACE/Ang II axis is correlated with skeletal muscle insulin resistance, atrophy and fibrosis and antagonizes the ACE2/Ang-(1–7) axis [11, 12]. The ACE/Ang II axis reduces microvascular perfusion, the uptake of glucose, and the sensitivity of insulin is strongly implicated in skeletal muscle atrophy [5, 6].

On the contrary, the axis of ACE2/Ang-(1–7) is beneficial to skeletal muscles as it acts to improve insulin resistance and prevent fibrosis [13, 14]. The current work revealed that the decline in skeletal muscle function in aged mice was accompanied by a decrease in the tissue concentration of Ang-(1–7) and an enhancement in the concentration of Ang II. These findings suggest that as mice age, an imbalance in the RAS system may develop in the skeletal muscle. These changes cause decreased performance of the protective ACE2/Ang-(1–7) axis and an increase in deleterious activity of the ACE/Ang II pathway.

Further analysis showed that at any age, the skeletal muscle performance in ACE2-KO mice was worse than in WT animals, and the muscle performance in young ACE2-KO mice was similar to aged WT mice. Continuous injection of Ang-(1–7) in old ACE2-KO mice increased motor capacity which was consistent with the previous findings of Takeshita and Hikari [15, 16]. The deletion of the ACE2 gene accelerated the age-associated damage of skeletal muscle function in mice whilst the administration of Ang-(1–7) alleviated this type of damage.

In animal models of heart failure, ACE2 improves cardiac function and ventricular remodeling by the production of Ang-(1–7) resulting in improved survival [17]. In another study, the treatment of ACE2-KO mice by exogenous Ang-(1–7) reduced cardiac lipid toxicity and atherosclerosis caused by a high-fat diet and prevented the development of heart failure [18]. Similarly, in the system of skeletal muscle, Ang-(1–7) has been demonstrated to enhance microvascular perfusion and the uptake of glucose and improves insulin resistance [9, 13].

In a mouse model of Duchenne muscular dystrophy, the upregulation of ACE2 expression or the administration of Ang-(1–7) reduces the fibrosis of skeletal muscle and ameliorates skeletal muscle structure and function [19–21]. Our data is also supported by the work from the Yamamoto laboratory that shows ACE2 deficiency accelerates the age-related loss of skeletal muscle mass and functional decline. However, the Yamamoto group found that the protective effect of ACE2 on skeletal muscles may be achieved through the non-Ang-(1–7)-MAS pathway [16, 22]. Furthermore, the ACE2/Ang-(1–7) pathway, ACE2 has several functions in other biological pathways including the ACE2/Apelin pathway that acts to reverse sarcopenia and age-related muscle loss and functional decline [23]. Further in vivo studies are required to identify the pathways through which ACE2 achieves its protective effect on skeletal muscle during aging.

Although ACE2 may exert biological effects in ACE2-KO mice through a variety of pathways, the delivery of exogenous Ang-(1–7) was shown to slow the process of age-associated skeletal muscle decline and resulted in increased muscle function. We then explored the specific mechanism responsible underlying

the impacts of exogenous Ang-(1–7) was explored in vitro. After the exposure of C2C12 cells to Ang-(1–7), the expression of Glut4 increased and the expression of MEF2A decreased. Takeda and coworkers previously explained that Ang-(1–7) upregulates the Glut4 protein in myoblasts and increases insulin-stimulated glucose uptake [10]. Glut4 is responsible for the transport of glucose transport in skeletal muscle cells and regulates glucose homeostasis to reduce insulin resistance [24, 25]. Regular exercise can improve the expression of Glut4 in the muscles thereby increasing glucose uptake capacity and improving muscle function [24]. The results presented in the current study also support the hypothesis that Ang-(1–7) enhances skeletal muscle motor function in mice by promoting glucose metabolism in muscle cells.

Conclusions

The imbalance of the RAS system may be responsible for the aging-associated decline in the function of skeletal muscle. The ACE2/Ang-(1–7) axis has a protective task in skeletal muscles and the administration of exogenous Ang-(1–7) can delay the reduction in skeletal muscle function. This study provides new perspectives for the treatment of age-related sarcopenia with Ang-(1–7).

Abbreviations

ACE:angiotensin-converting enzyme;

Ang:angiotensin; MEF2A:myocyte enhancer factor 2A ;ACE2KO:ACE2knockout; Glut4:glucose transporter type 4 ; RAS:Renin-angiotensin system;ACEI:Angiotensin converting enzyme inhibitor; MHC:myosin heavy chain; CKM:creatine kinase muscle

Declarations

Acknowledgements:

Not applicable.

Author contributions:

YL and BRD led the development of the study concept. XLH, JS, YYJ, XY and LC drafted the manuscript. YL, CX, and SLL performed the experiments.

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Availability of data and materials:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate:

The in vivo protocols were approved by the Animal Committee of the West China Hospital, Sichuan University.

Statement:

We confirmed that all methods were performed in accordance with the relevant regulations of the Animal Committee of the West China Hospital, Sichuan University (reference number : 2019277A), and the study was carried out in compliance with the ARRIVE guideline.

Consent for publication:

Not applicable.

Competing interests:

The authors declare that they have no competing interests.

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Figures

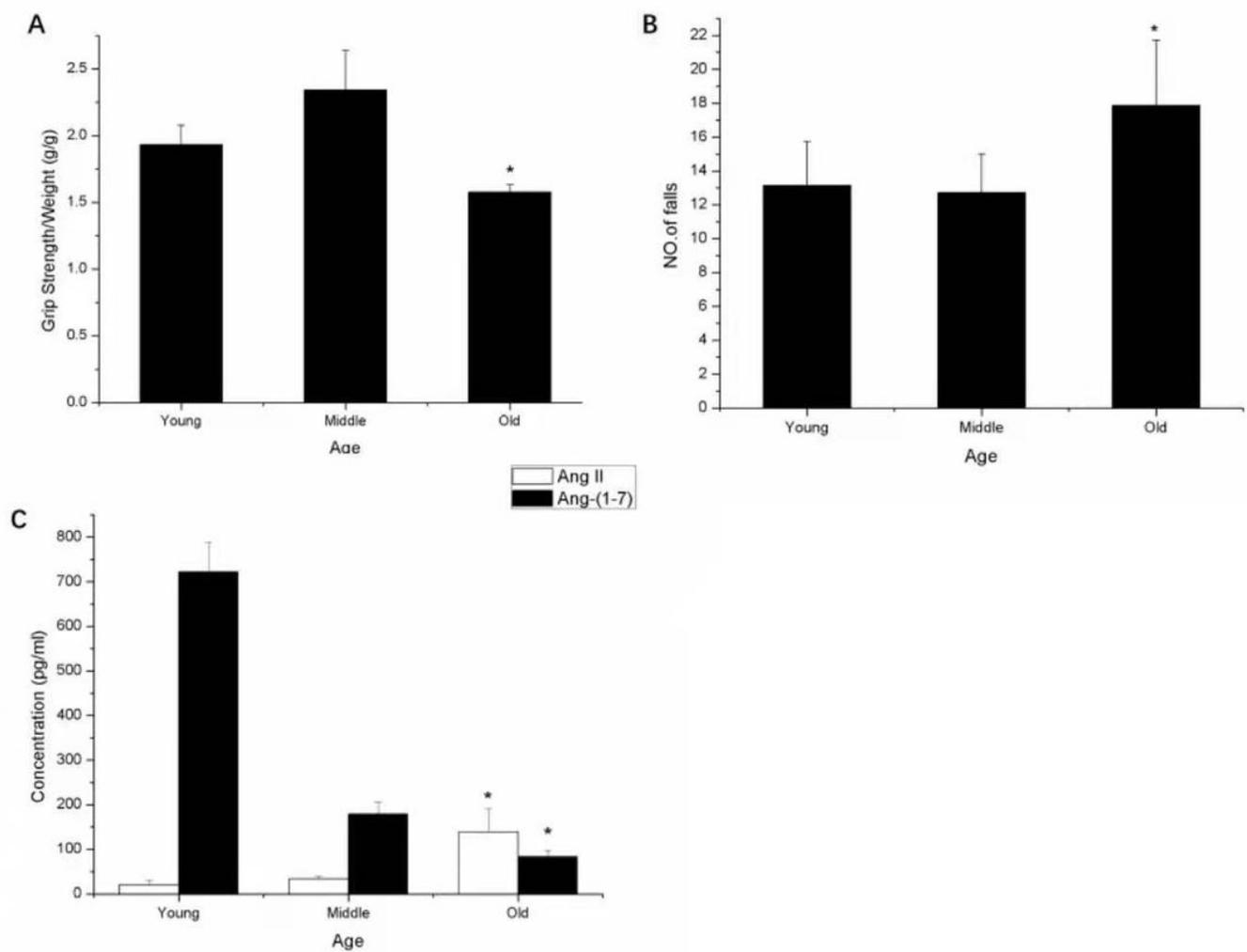


Figure 1

(A) grip strength, (B) fall numbers, and (C) Ang-(1-7) and Ang II expression in the treadmill test in different age mice. * $P < 0.05$, t-test between different age group.

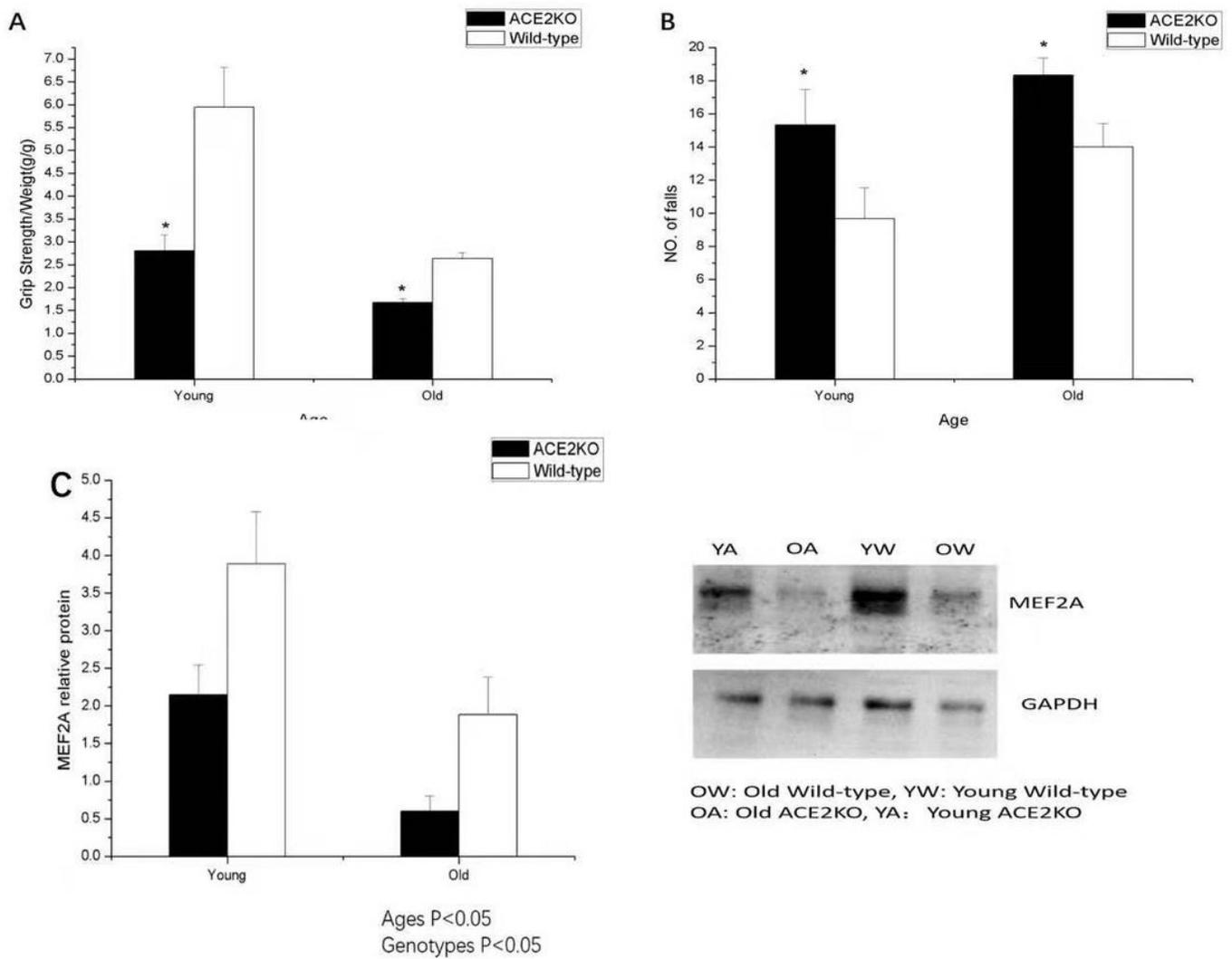


Figure 2

(A) grip strength, (B) fall numbers, and (C) MEF2A expression in ACE2-KO and WT mice. * $P < 0.05$, t-test between same age ACE2-KO and WT mice.

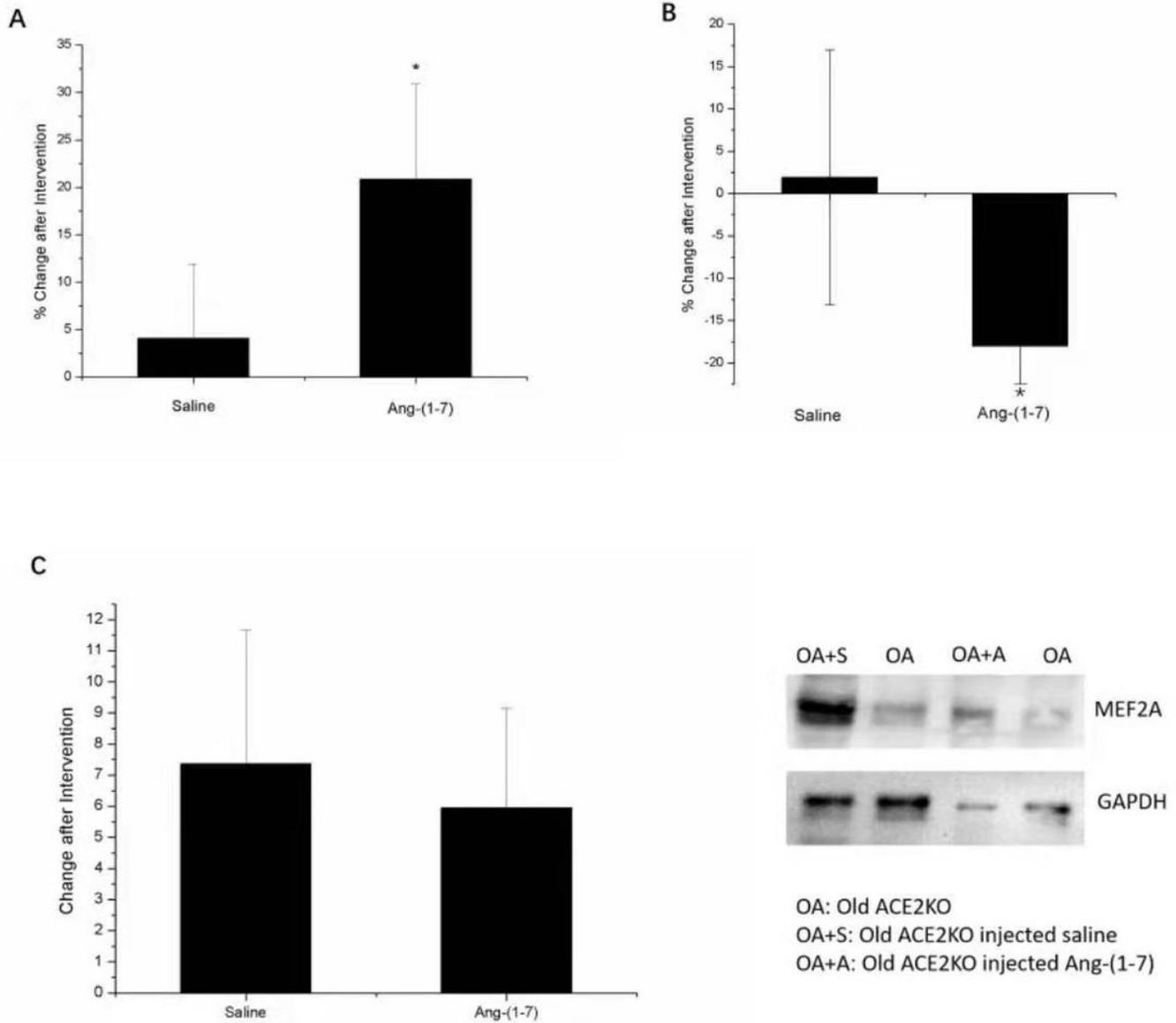
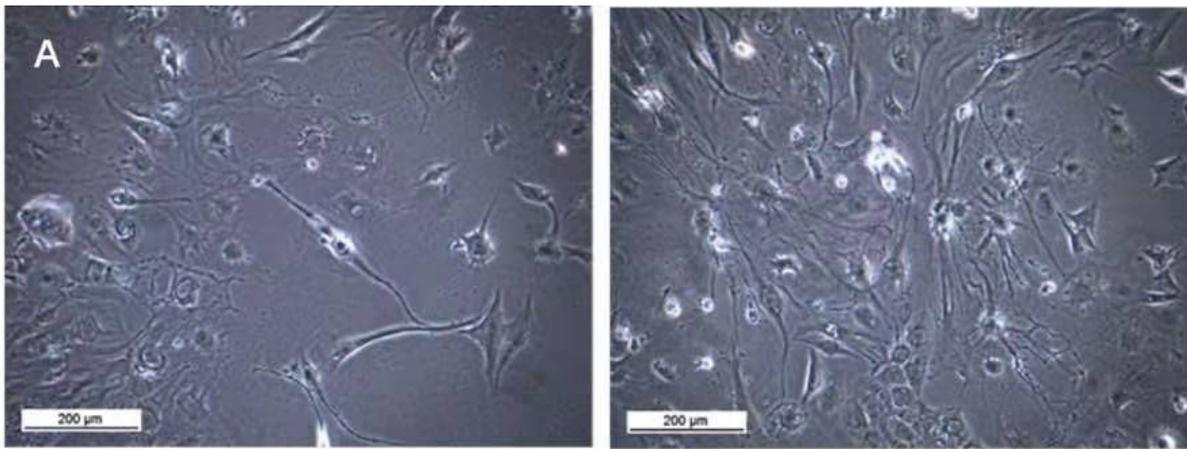


Figure 3

Changes of (A) grip strength, (B) fall numbers, and (C) MEF2A expression after Ang-(1-7) intervention in older ACE2-KO mice. * $P < 0.05$, t-test between intervention and control.



PBS

Ang (1-7)

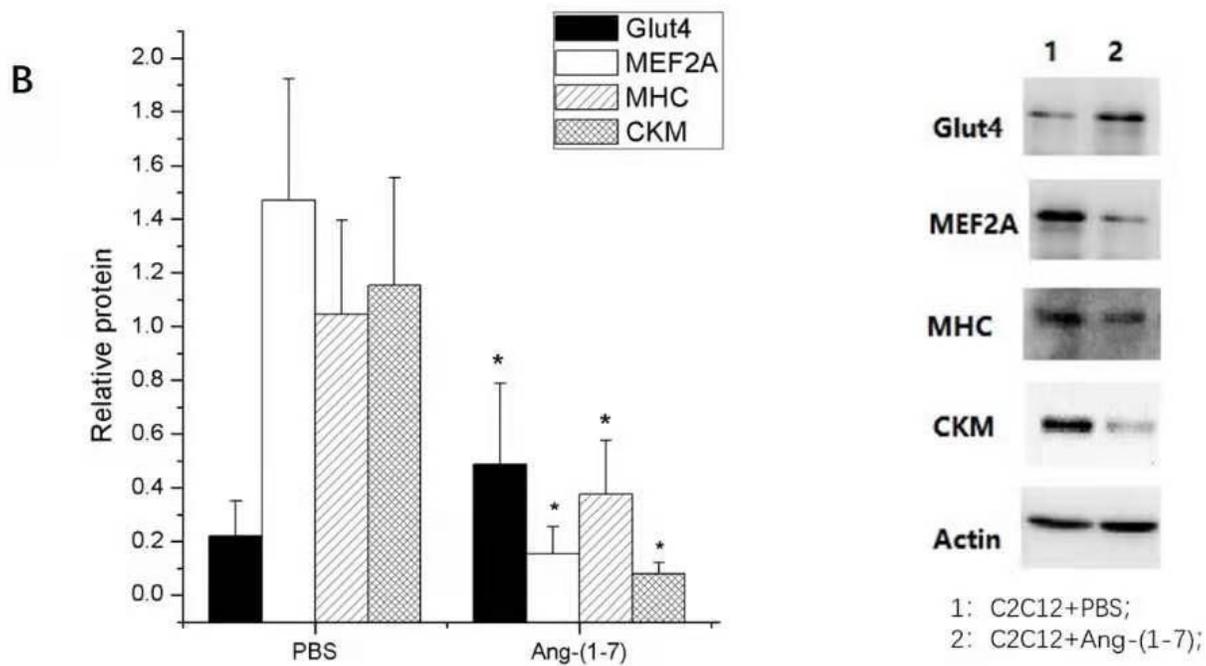


Figure 4

(A) the differentiation and (B) Glut4, MEF2A, MHC, and CKM expression of C2C12 cells after Ang-(1-7) intervention. * $P < 0.05$, t-test between intervention and control.