



## Original Article

## Alterations in renin-angiotensin receptors are not responsible for exercise preconditioning of skeletal muscle fibers



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## ABSTRACT

Endurance exercise training promotes a protective phenotype in skeletal muscle known as exercise preconditioning. Exercise preconditioning protects muscle fibers against a variety of threats including inactivity-induced muscle atrophy. The mechanism(s) responsible for exercise preconditioning remain unknown and are explored in these experiments. Specifically, we investigated the impact of endurance exercise training on key components of the renin-angiotensin system (RAS). The RAS was targeted because activation of the classical axis of the RAS pathway via angiotensin II type I receptors (AT1Rs) promotes muscle atrophy whereas activation of the non-classical RAS axis via Mas receptors (MasRs) inhibits the atrophic signaling of the classical RAS pathway. Guided by prior studies, we hypothesized that an exercise-induced decrease in AT1Rs and/or increases in MasRs in skeletal muscle fibers is a potential mechanism responsible for exercise preconditioning. Following endurance exercise training in rats, we examined the abundance of AT1Rs and MasRs in both locomotor and respiratory muscles. Our results indicate that endurance exercise training does not alter the protein abundance of AT1Rs or MasRs in muscle fibers from the diaphragm, plantaris, and soleus muscles compared to sedentary controls ( $p > 0.05$ ). Furthermore, fluorescent angiotensin II (AngII) binding analyses confirm our results that exercise preconditioning does not alter the protein abundance of AT1Rs in the diaphragm, plantaris, and soleus ( $p > 0.05$ ). This study confirms that exercise-induced changes in RAS receptors are not a key mechanism that contributes to the beneficial effects of exercise preconditioning in skeletal muscle fibers.

## Introduction

It is established that endurance exercise training results in numerous biochemical adaptations in skeletal muscle fibers. Collectively, these exercise-induced changes result in a protective phenotype in muscle that is labeled “exercise-preconditioning”. Indeed, abundant evidence reveals that exercise preconditioning defends against several threats to skeletal muscle including exercise-induced oxidative stress, cancer chemotherapy-induced muscle wasting, and inactivity-induced muscle atrophy.<sup>1–5</sup> Research investigating molecular mechanism(s) responsible for exercise preconditioning is an ongoing area of research and many questions remain unanswered. In this regard, evidence indicates that increases in both muscle antioxidant capacity and/or heat shock proteins are potential contributors to exercise preconditioning.<sup>6–8</sup> In addition, it is also feasible that exercise-induced alterations in signaling components of

the renin-angiotensin system (RAS) also contribute to exercise-preconditioning.<sup>9–11</sup> However, this prediction has received limited attention, and this forms the rationale for the current experiments.

The RAS system consists of 2 signaling arms identified as the classical and non-classical RAS pathways. Activation of the classical RAS pathway occurs by stimulation of the angiotensin II type I receptor (AT1Rs); activation of the classical RAS pathway in muscle fibers promotes both oxidative stress and accelerated proteolysis, leading to fiber atrophy.<sup>12–16</sup> In contrast, activation of the non-classical RAS pathway, via stimulation of the Mas receptor (MasRs), inhibits signaling through the classical RAS pathway.<sup>17,18</sup> Previous work has implicated the role of the RAS signaling pathway in inactivity-induced muscle atrophy, whereby pharmacological inhibitors of AT1Rs and/or MasRs activation by ligand binding protects muscle from inactivity-induced muscle wasting.<sup>19,20</sup> Therefore, in theory, an exercise-induced decrease in AT1Rs and/or increased abundance of MasRs can contribute to exercise preconditioning and confer

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### Abbreviations

Ang1-7	Angiotensin 1-7
AngII	Angiotensin II
AT1Rs	Angiotensin II type I receptors
CON	Sedentary control
EX	Endurance exercise trained
MasR	Mas receptors
RAS	Renin-angiotensin system
VO <sub>2max</sub>	Maximal oxygen consumption

protection against inactivity-induced muscle atrophy. To date, there are only a few published reports that have investigated the impact of exercise on AT1Rs and MasRs in skeletal muscle.<sup>21–23</sup> Unfortunately, these studies suffer from major experimental limitations including the use bundles of muscle tissue and non-specific antibodies. Specifically, the antibodies used in these experiments were purchased from Santa Cruz Biotechnology which have been shown to have non-specific binding in knockout models of AT1R.<sup>24,25</sup> Thus, additional research is required to establish if exercise training alters the abundance of AT1Rs and MasRs in skeletal muscle fibers. Therefore, to determine if exercise-induced changes in key components of the RAS are a potential contributor to exercise-preconditioning, we used a rigorous experimental approach to test the hypothesis that endurance exercise training decreases the abundance of AT1Rs and increases the abundance of MasRs in skeletal muscle fibers.

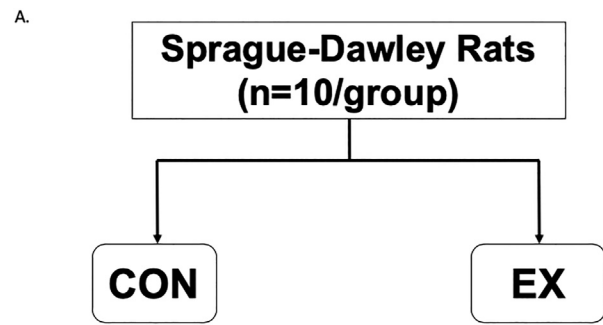
## Materials and methods

### Experimental animals

Adult (around 4–6 months old) female Sprague-Dawley rats were used in these experiments. These experiments were approved by the University of Florida Animal Care and Use Committee. All animals were housed at the University of Florida Animal Care Services Center according to guidelines set forth by the Institute of Animal Care and Use Committee. The experimental animals were maintained on a 12:12 h light-dark cycle with food and water provided *ad libitum* throughout the experimental protocol. This study meets the ethical standards of the International Journal of Sports Medicine.<sup>26</sup>

### Experimental design

To test our experimental hypothesis, we used a well-established animal model of endurance exercise training and studied both respiratory and locomotor skeletal muscles. Importantly, to rigorously test our hypothesis, we studied isolated single muscle fibers and used a multi-technique approach to identify the abundance of AT1Rs and MasRs. Specifically, to study the abundance of both AT1Rs and MasRs in muscle, we isolated individual muscle fibers from 3 skeletal muscles (i.e., diaphragm, plantaris, and soleus). The diaphragm was selected for analysis because RAS signaling plays a required role in ventilator-induced diaphragm atrophy.<sup>19,20,27</sup> The soleus and plantaris muscles were studied because these hindlimb muscles atrophy in response to atrophic stimuli such as angiotensin II (AngII)<sup>28</sup> and these muscles differ in fiber type composition; the soleus muscle in rats is dominated by slow, type I fibers whereas the plantaris muscle contains primarily fast, type II fibers.<sup>29</sup> On the basis of a report indicating that endurance exercise training lowers the AT1Rs abundance in the heart,<sup>30</sup> we formulated the hypothesis that endurance exercise training results in both a decrease in AT1Rs and an increase in MasRs in diaphragm and locomotor skeletal muscle fibers. A brief description of the experimental design and general methods follows.



**Fig. 1.** Experimental Design. Groups: 1) sedentary control and 2) endurance exercise training  $n = 10/\text{group}$ . CON = control; EX = endurance exercise training.

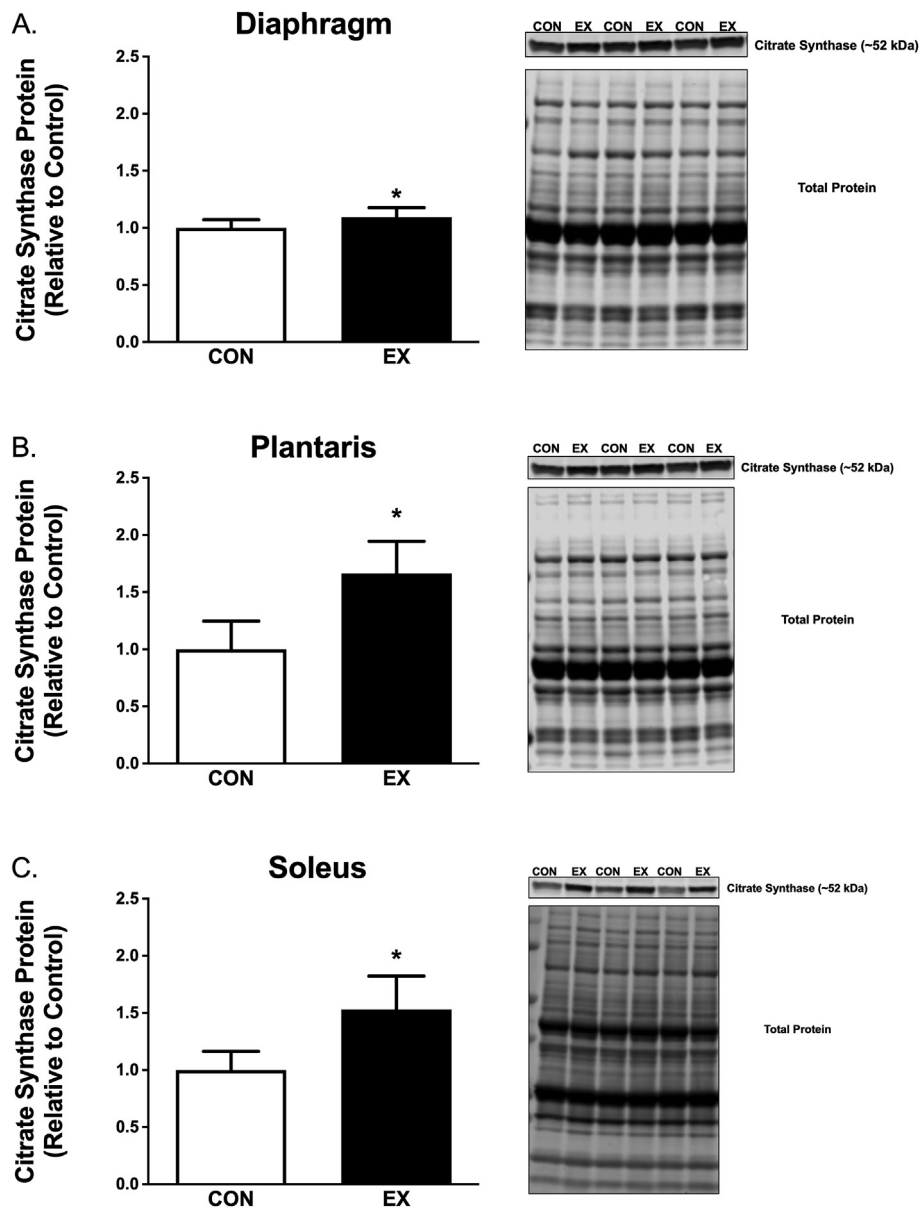
Female rats (around 4–6 months old) were randomly assigned to experimental groups consisting of: 1) Sedentary Control (CON); or 2) Endurance Exercise Training (EX) ( $n = 10/\text{group}$ ) Fig. 1A. CON animals remained sedentary in their cages and were not exposed to endurance exercise training throughout the duration of the experiments.

### Endurance exercise treadmill training protocol

Animals in the EX group performed 5 consecutive days of progressive habituation to treadmill running prior to endurance exercise training (Fig. 1B). Following 2 days of rest, each animal performed an endurance exercise training program consisting of 10 total days of treadmill exercise (60 min per day) at a predicted work rate of 70% maximal oxygen consumption (VO<sub>2max</sub>). Based on previous work describing the VO<sub>2max</sub> and oxygen cost of treadmill exercise in untrained rats, 70% VO<sub>2max</sub> in untrained animals corresponds to treadmill settings of 30 m/min at 0% grade.<sup>31</sup> Each exercise training session consisted of 4 separate 15-min intervals of treadmill running with 3 min rest between each interval for a total of 60 min of endurance exercise. The EX group were exercise trained for 5 consecutive days with 2 days of rest followed by additional 5 consecutive days of training. Importantly, this endurance exercise training protocol has shown to result in a diaphragmatic phenotype that is protected against the detrimental effects of prolonged mechanical ventilation.<sup>4</sup> In addition, exercise preconditioning has shown to attenuate skeletal muscle atrophy after hindlimb unloading.<sup>5,32,33</sup> Twenty-four hours after the animal's last bout of endurance exercise training, animals were sacrificed for tissue harvest and muscle sections of the costal diaphragm, soleus, and plantaris muscles were collected, frozen in liquid nitrogen, and stored in  $-80\text{ }^{\circ}\text{C}$  for subsequent analysis.

### Single fiber isolation

To eliminate the risk of contamination of other tissue types that may be abundant in RAS proteins (i.e., vascular tissue), individual muscle fibers (~3 mm in length) were isolated from bundles of costal diaphragm, soleus, and plantaris muscles as previously described by Deminice et al.<sup>34</sup> Briefly, bundles of muscle fibers were placed in a relax solution (in mM: 100 KCl, 20 imidazole, 4 ATP, 2 EGTA, and 7 MgCl<sub>2</sub>; pH adjusted to 7.0 using KOH) on ice where ~80 individual muscle fibers were carefully isolated under a stereomicroscope and placed into Eppendorf tubes prepared for Western blot analysis.

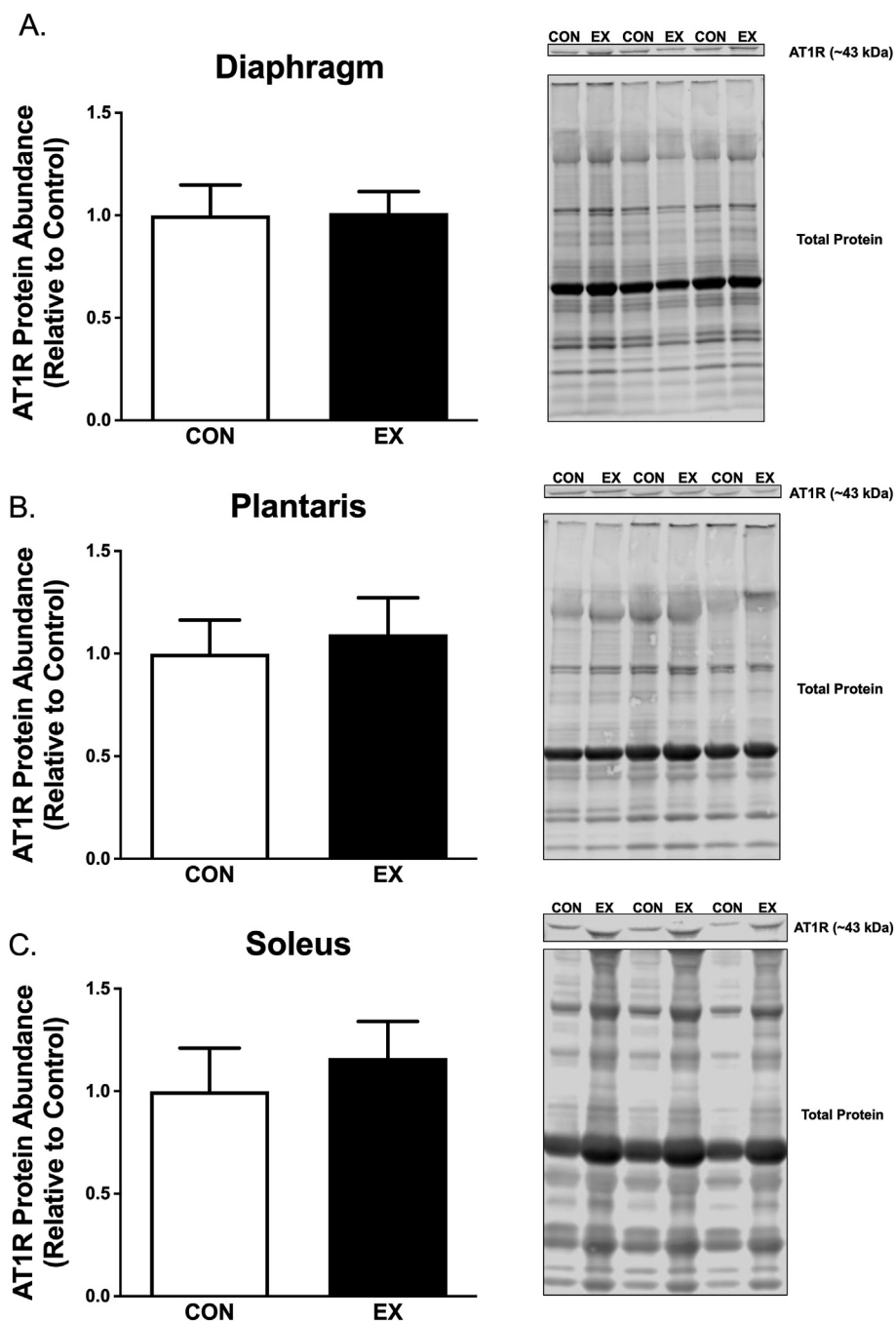


**Fig. 2.** Citrate synthase (CS) was measured via Western blot as a biomarker of mitochondrial volume. Compared to sedentary control (CON), endurance exercise training (EX) significantly increased citrate synthase protein abundance in the A) diaphragm ( $p = 0.0168$ ), B) plantaris muscles ( $p < 0.0001$ ), and C) soleus ( $p < 0.0001$ ). Values are mean  $\pm$  SD. Data given \* significant difference between CON and EX ( $p < 0.05$ ). CON = Sedentary control; EX = Endurance exercise training.

#### Western blot analysis

Western blot samples were prepared as followed. Around 80 muscle fibers were placed in Eppendorf tubes containing Tris-EDTA homogenization buffer (5 mM Tris, 5 mM EDTA at a pH of 7.4). Protease inhibitor cocktail was then added at 1:20 vol/vol (Sigma-Aldrich, St. Louis MO) with 1% Triton X-100. Laemmli sample buffer (1610747, Bio Rad Hercules, CA) containing 5% dithiothreitol was added to Western blot samples. Single fiber Western blot samples then completed 3 freeze/thaw cycles and homogenized with a small pestle. After, the muscle fiber samples were boiled for 5 min and single fiber homogenate samples were cooled on ice and loaded on a 4%–20% gradient Criterion TGX gels (Bio-Rad) and electrophoresed for 60 min at 150 V. After completion of electrophoresis, proteins were then transferred to a LF-PVDF membrane (Millipore Burlington, MA). Membranes were incubated with antigen pretreatment buffer (ThermoFisher Scientific) for 10 min at room temperature. Following pretreatment, membranes were blocked with SuperBlock blocking buffer (ThermoFisher Scientific) for 1 h at room

temperature. Primary antibodies of interest were anti-AT1Rs (Abcam ab124734 1:1000), anti-MasRs (Novus NBP1-78444 1:1000), anti-CD31 (Abcam ab24590 1:1000), and anti-citrate synthase (Santa Cruz sc-390693 1:1000). Membranes were incubated with Alexa Fluoro 800 IgG secondary, scanned, and analyzed using an infrared imager (LI-COR Bioscience) using Odyssey 2.1 software. All Western blot images were normalized to total protein using REVERT Total Protein (Li-core) which has been shown to be superior for Western blot normalization as compared with the use of housekeeping proteins.<sup>35</sup> Note that anti-CD31, a biomarker of vascular tissue was used to ensure that single muscle fiber samples were not contaminated with vascular tissue. Importantly, membranes imaged for AT1R and MasR protein were then probed for CD31 to ensure that the single fiber homogenates were not contaminated with vascular tissue. Furthermore, anti-citrate synthase was used as a biomarker of mitochondrial density to confirm the exercise-induced increase in mitochondrial density in skeletal muscle fibers.

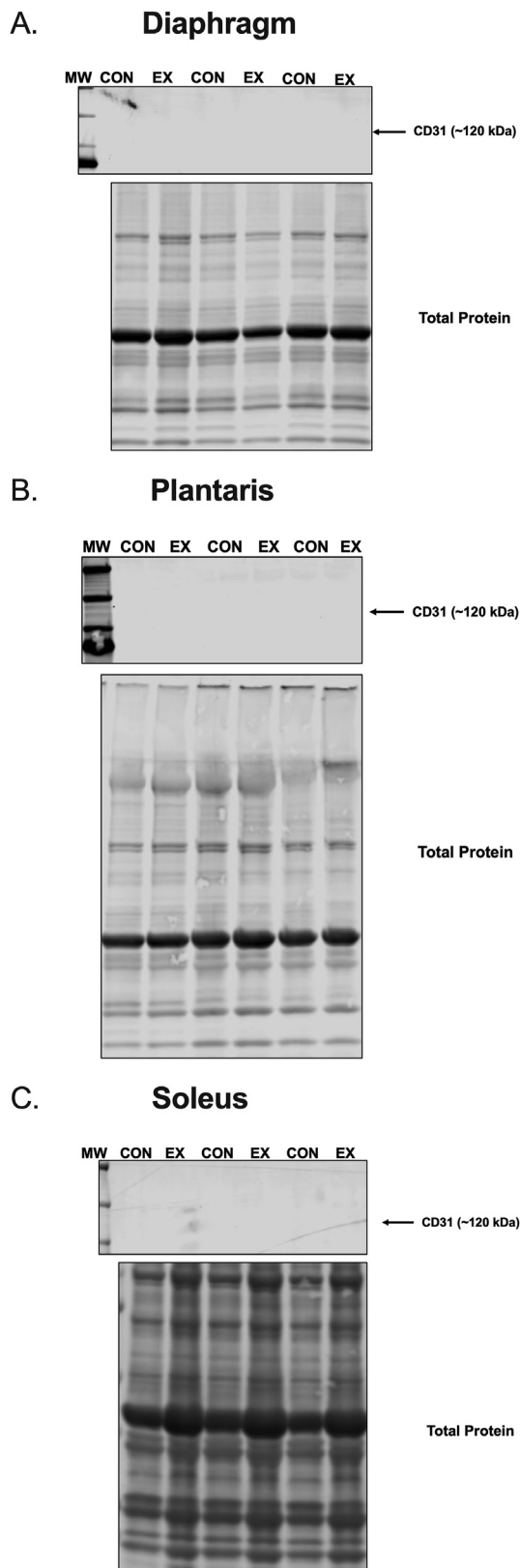


**Fig. 3.** AT1Rs protein abundance was measured via Western blotting. Compared to sedentary control, endurance exercise training does not significantly decrease AT1R protein abundance in isolated single muscle fibers of the A) diaphragm, B) plantaris, and C) soleus muscles ( $p > 0.05$ ). Values are mean  $\pm$  SD. AT1R = angiotensin II type I receptors; CON = Sedentary control; EX = Endurance exercise training.

*AT1Rs fluorescence-based binding assay*

Measurement of fluorescent AngII ligand-binding to AT1Rs provides a powerful experimental tool to determine the abundance of AT1Rs in tissue samples. Therefore, fluorescently labeled AngII methods were adapted from previous reports to evaluate AT1R abundance.<sup>36,37</sup> Skeletal muscle cross sections from the costal diaphragm, plantaris, and soleus muscles were evaluated for AT1Rs, as previously demonstrated by Deminice et al.<sup>34</sup> Briefly, diaphragm, plantaris, and soleus muscle samples were embedded in OCT and placed in  $-80^{\circ}\text{C}$ . Samples were then transversely cut to 7 separate 10  $\mu\text{m}$  thick sections using the cryotome (Shandon, Pittsburg, PA). Then, slides containing the tissue sections were incubated at  $4^{\circ}\text{C}$  to dry for 45 min. After drying, all sections of muscle

tissue were incubated in Hanks' Balanced Salt solution (Thermo Fisher, #14025076) supplemented with protease inhibitor buffer (containing 0.1% bacitracin, Sigma B0125), 0.002% phenylmethylsulfonyl fluoride (Sigma 78830), and 0.01% 1,10-phenanthroline (Sigma 131377) for 30 min on ice. Each slide was then dried for 5 min and prepared for staining. The 7 sections from each muscle tissue sample were divided and incubated in 3 different incubation medias. Three sections were incubated with the fluorescently labeled AngII (TAMRA-labeled Ang II peptide; Anaspec AS-60275-1) for 1 h. The other 3 sections were incubated with fluorescently labeled AngII and the AT1Rs blocker losartan (Sigma CAS 124750-99-8) for 1 h. The last section of tissue served as the negative control and was incubated with only non-fluorescently labeled AngII (Sigma A9525) for 1 h. Following incubation, each of the sections were



**Fig. 4.** To eliminate the risk of contamination of vascular tissue, we isolated single muscle fibers to determine RAS receptor abundance. CD31 was used as a biomarker for vascular tissue via Western blot. CD31 was not detected in isolated single fiber muscles of A) diaphragm, B) plantaris, and C) soleus. The expected molecular weight of CD31 is approximately 120 kDa. MW = molecular weight marker; CON = control; EX = exercise.

washed with buffer assay and dried for imaging. An Axiovert 200 Inverted Fluorescence Microscope (Zeiss, Germany) was used to image each section at 100x and 200x magnification using a rhodamine excitation fluorescence filter. For AT1R quantification, the auto local thresholding technique<sup>38</sup> was used to quantify relative fluorescence density in the AT1R binding sections using ImageJ software (National Institutes of Health, USA).<sup>34</sup> Relative AT1R values were generated by subtracting background fluorescence (the value of threshold fluorescence detected in the presence of losartan and co-incubated with TAMRA-labeled Ang II from sectioned tissues from the same sample) from total threshold fluorescence (fluorescence detected with TAMRA-labeled Ang II without losartan). All measurements were made in triplicate. This technique was established in previous experiments confirming the existence of AT1Rs in both human and rat skeletal muscles compared with other tissues known to express high levels of AT1R.<sup>34</sup>

#### Statistical analysis

The group size ( $n = 10$ ) was determined by a statistical power analysis using data from previous studies. Data were analyzed by an independent *t*-test and Shapiro-Wilk normality test conducted separately on each dependent measure. Data are presented as mean  $\pm$  standard deviation (*SD*). Significance was established at  $p < 0.05$ .

#### Results

##### Exercise training and body weights

All animals in the exercise group successfully completed the 10-days of treadmill exercise training. Similar to previous studies utilizing this exercise training protocol,<sup>4</sup> our results demonstrated no mean differences in animal body weights between experimental groups (CON =  $313.2 \pm 6.4$  g, EX =  $303.1 \pm 3.4$  g).

##### Mitochondrial volume increased with exercise training

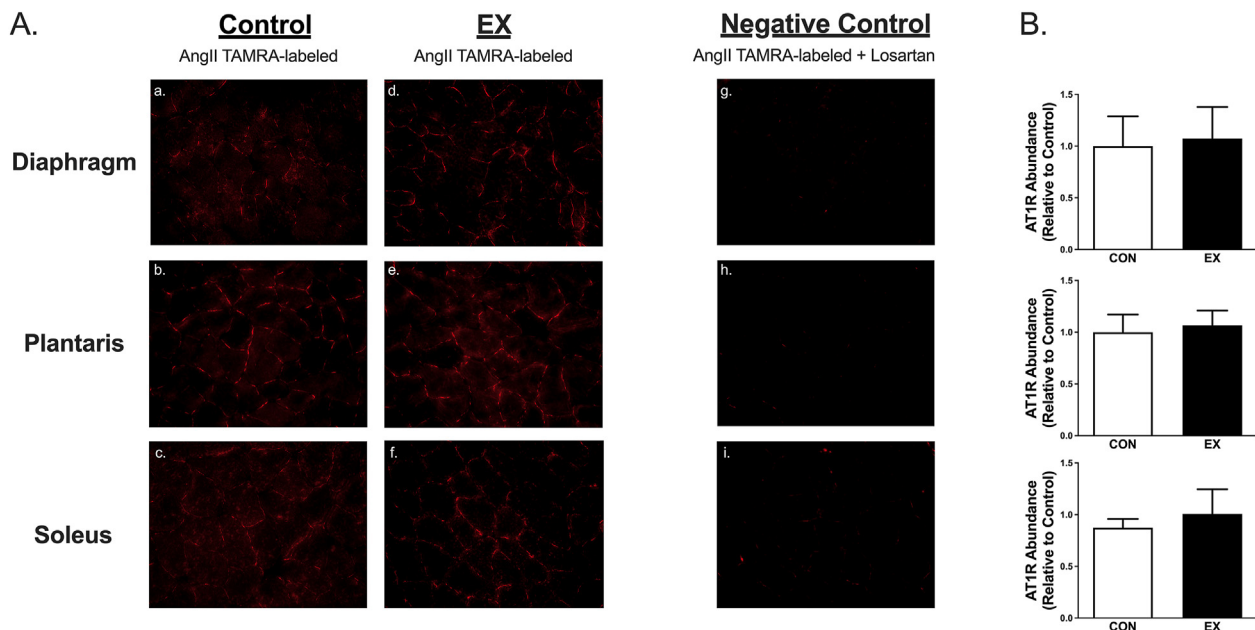
To confirm that our endurance exercise training program was sufficient to promote training adaptations in skeletal muscle, we measured citrate synthase as a biomarker of mitochondrial density. Compared to sedentary control, 10 days of endurance exercise training significantly increased citrate synthase protein abundance in the diaphragm ( $p = 0.0168$ ), plantaris ( $p < 0.0001$ ), and soleus muscles ( $p < 0.0001$ ) (Fig. 2).

##### Endurance exercise training does not alter the protein abundance of AT1Rs in skeletal muscle fibers

To determine if endurance exercise training decreases AT1Rs protein abundance in skeletal muscle, we measured the protein abundance of AT1Rs via Western blot in isolated skeletal muscle fibers. Compared to sedentary control animals, 10 days of endurance exercise training did not alter the abundance of AT1Rs in isolated diaphragm, plantaris, and soleus muscle fibers (Fig. 3).

Importantly, preliminary experiments optimizing the single fiber technique utilized the heart, liver, and kidney as positive controls for CD31 (protein biomarker unique in vascular tissue; around 120 kDa) demonstrating a strong single band at the expected molecular weight and confirmed that the single muscle fiber homogenates were not contaminated with vascular tissue (data not shown). Similar to our preliminary experiments, vascular contamination did not exist in the homogenate from isolated fibers when measuring the abundance of CD31 in isolated single muscle fiber homogenate. Our single fiber isolation technique was successful in eliminating vascular contamination as CD31 was not detectable in homogenate of isolated diaphragm, plantaris, and soleus muscle fibers (Fig. 4).





**Fig. 5.** Fluorescently labeled AngII was used as a functional measurement of AT1R abundance in skeletal muscle fibers. Panel (A) contains representative images compared to sedentary control, endurance exercise training does not significantly decrease the abundance of AT1Rs in diaphragm, plantaris, and soleus muscles. Images a-f depict representative images of AngII TAMRA-labeled cross sections of diaphragm, plantaris, and soleus. Images g-i depict negative control images of AngII TAMRA-labeled + Losartan cross sections of diaphragm, plantaris, and soleus. Panel (B) contains AT1R quantification of fluorescence of AngII TAMRA-labeled cross sections of control and exercise diaphragm, plantaris, and soleus relative to control. AngII = angiotensin II; AT1Rs = angiotensin II type I receptor; CON = Sedentary control; EX = Endurance exercise training.

#### *Endurance exercise training does not alter AngII binding capacity in skeletal muscle fibers*

In addition to Western blotting for AT1Rs protein abundance in isolated muscle fibers, we employed a quantitative fluorescent-ligand binding assay to determine the abundance of AT1Rs in skeletal muscle fibers. This sensitive binding assay provides a physiological biomarker of the functional presence of AT1Rs. First, TAMRA-labeled AngII was used to determine the location and abundance of AT1Rs in the diaphragm, plantaris, and soleus muscles. Our results confirm that, compared to sedentary control animals, endurance exercise training does not significantly alter the abundance of AT1Rs in diaphragm, plantaris, and soleus muscle fibers (Fig. 5). To confirm that the fluorescent signal in the muscle was due to the TAMRA-labeled AngII, muscle sections were incubated with losartan to prevent the binding of TAMRA-labeled AngII to AT1Rs which resulted in diminished intensity of the fluorescence (Fig. 5).

#### *Endurance exercise training does not alter the protein abundance of MasRs in skeletal muscle fibers*

To determine if endurance exercise training alters the abundance of MasRs in skeletal muscles, we measured the protein abundance of MasRs in skeletal muscle fibers via Western blotting. Compared to sedentary controls, endurance exercise training does not significantly increase the abundance of MasRs protein in diaphragm, plantaris, and soleus muscle fibers (Fig. 6). Note that an angiotensin 1-7 (Ang1-7) binding assay for MasRs was not performed because a fluorescent label for Ang1-7 does not exist and the commercially available antibody against MasRs has been validated as being specific for the MasRs protein.

## Discussion

### *Overview of principal findings*

These experiments provide important information about the mechanisms responsible for exercise preconditioning of skeletal muscles.

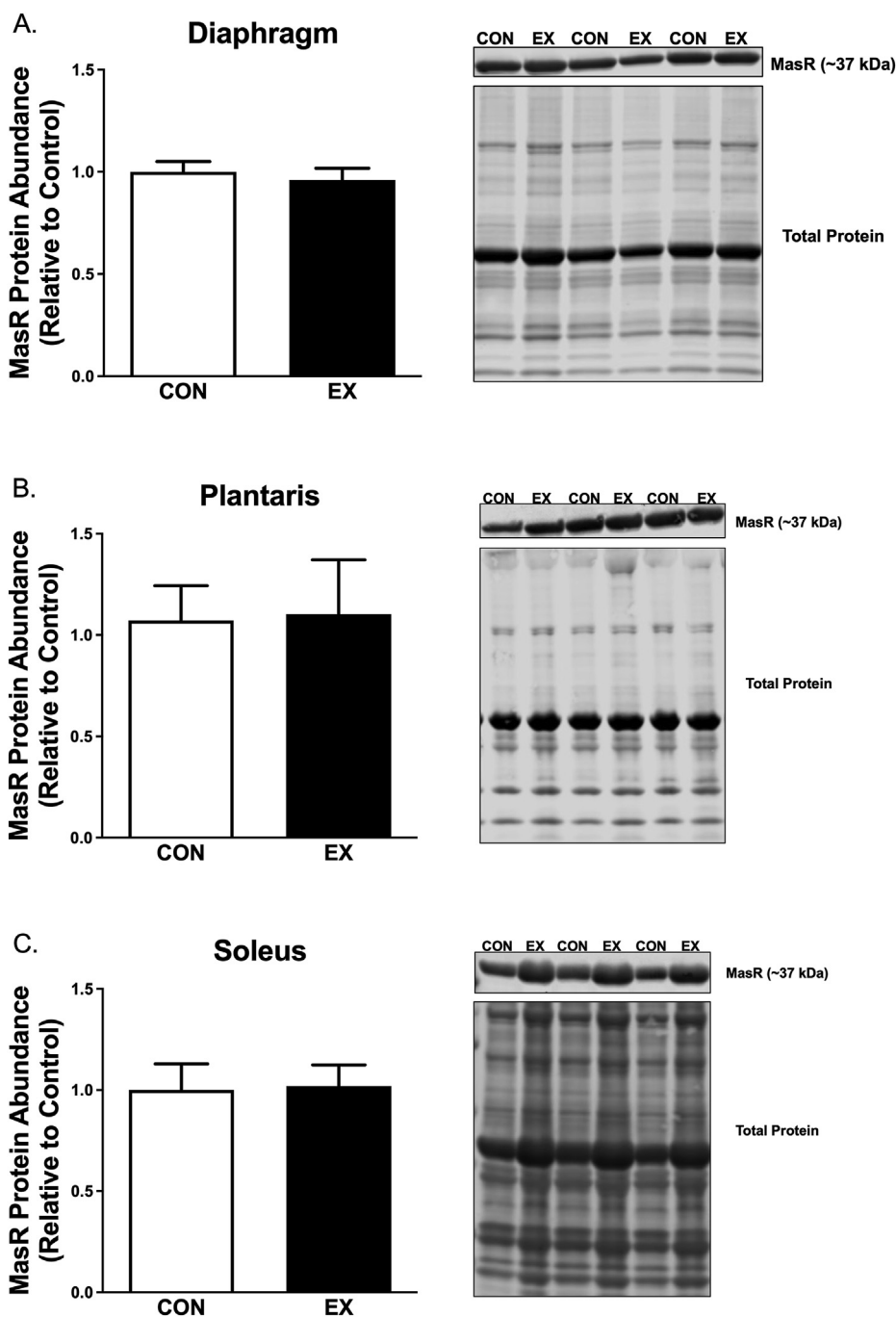
Specifically, our findings establish that exercise preconditioning of skeletal muscles is not dependent upon changes in the abundance of both AT1Rs and MasRs. A critique of our experimental approach and a discussion of the significance of these findings follows.

### *Critique of experimental model*

The current experiments differ significantly than the studies previously mentioned investigating the effect of exercise on RAS receptors in skeletal muscles. In regard to sex differences, the previous studies investigating the exercise-induced adaptations to RAS receptors utilized adult male rodents while the current experiment utilized female rodents.<sup>21-23</sup> It is important to consider possible sex differences in RAS receptor abundance in skeletal muscle due to numerous reports demonstrating sex differences in RAS receptors in the kidneys of females and males, with males having greater AT1R expression than females.<sup>39,40</sup> Therefore, it is also possible that sex differences exist in skeletal muscle fibers and sex must be considered when investigating skeletal muscle RAS receptors. We arbitrarily selected adult female Sprague-Dawley rats as a model to investigate the effects of endurance exercise training on the RAS in skeletal muscle because this species/strain is a well-established model to investigate exercise-induced adaptations in skeletal muscles.<sup>41</sup>

The exercise protocol in this experiment also differs in exercise duration, intensity, and type. Animals in previous studies were prescribed voluntary wheel running for 6 weeks<sup>21,22</sup> while others utilized 8–12 weeks of treadmill exercise running at 60%  $VO_{2max}$  60min/day 5 days/week.<sup>9,23</sup> The exercise protocol used in this experiment was selected because previous experiments confirm that this training program results in the rapid acquisition of an exercise preconditioned phenotype in skeletal muscle that is protected against both ventilator-induced diaphragm wasting and contraction-induced oxidative stress.<sup>4,42,43</sup> However, as seen in the previous studies, a longer training duration may be necessary to facilitate adaptations of RAS receptors in skeletal muscle fibers.

To prevent contamination of muscle fibers with non-muscle tissue, we measured the abundance of AT1Rs and MasRs within isolated single



**Fig. 6.** MasR protein abundance was measured via Western blotting. Compared to sedentary control, endurance exercise training does not significantly increase MasR protein abundance in isolated single muscle fibers of the A) diaphragm, B) plantaris, and C) soleus muscles ( $p > 0.05$ ). CON = Sedentary control; EX = Endurance exercise training; MasR = Mas receptor.

muscle fibers. Importantly, we confirmed that our single fiber muscle homogenate was not contaminated with vascular tissue by probing for the vascular biomarker, CD31; notably, CD31 was absent from all single fiber muscle homogenates. Indeed, the abundance of RAS receptors within the microvasculature of skeletal muscles has been established and may play an important role in skeletal muscle function.<sup>44–46</sup> Evidence in the heart demonstrates an important paracrine signaling between the microvasculature and cardiomyocytes on regulating and maintaining cardiac function.<sup>47</sup> Therefore, it is possible that the skeletal muscle microvasculature may also play an important role in skeletal muscle function and could be an important system in exercise preconditioning of skeletal muscles.

When measuring the abundance of AT1Rs in skeletal muscle, cautious selection of AT1Rs antibodies is essential because numerous commercially available AT1Rs antibodies lack specificity.<sup>24,25</sup> To avoid this pitfall, we carefully screened available AT1Rs antibodies and selected an antibody that provided a clear, single Western blot band at the expected molecular weight of AT1Rs (43 kDa). Moreover, to further confirm the specificity of the antibody, we performed experiments on single muscle fibers and tissue types with an abundant amount of AT1R. (i.e., heart, liver, and kidney) from animals with decreased expression of AT1Rs in skeletal muscles. AT1R expression was silenced via adeno-associated virus containing a short-hairpin RNA specifically targeting skeletal muscle tissue using a muscle specific promoter. These studies confirmed a

reduction of AT1Rs protein abundance in only skeletal muscles from the AT1Rs knock-down animals compared to wild type in a dose-dependent manner and did not impact AT1R abundance in the heart, liver, and kidney (unpublished). Together, these data support the specificity of the AT1R antibody utilized in these experiments.

In addition to Western blot analysis of AT1Rs abundance in muscle fibers, we also used an *in vitro* AngII binding assay as a secondary technique to assess AT1Rs abundance in skeletal muscle fibers. The specificity of the binding assay was confirmed by incubation of muscle sections with the AT1Rs blocker, losartan. Importantly, the inclusion of a binding assay provides additional strength to our experimental approach to determine if endurance exercise training alters the abundance of AT1Rs in skeletal muscle.

We measured the abundance of AT1Rs in 3 skeletal muscles including the primary inspiratory muscle (diaphragm) and 2 limb muscles (plantaris and soleus). The diaphragm is a mixed fiber type muscle that contains a blend of fast and slow fibers<sup>29,48</sup> and was studied because activation of the classical arm of the RAS promotes diaphragmatic atrophy during prolonged mechanical ventilation.<sup>20</sup> Furthermore, activation of the non-classical RAS pathway via Ang1-7 infusion attenuates ventilator-induced diaphragm dysfunction.<sup>19</sup> It follows that an exercise-induced change in the abundance of AT1Rs and/or MasRs is a potential mechanism to explain why exercise training prevents ventilator-induced diaphragmatic wasting. In addition, evidence suggest dysregulated RAS signaling may play a role in skeletal muscle wasting conditions (i.e., hindlimb immobilization), therefore, the plantaris and soleus muscles were also studied.<sup>28</sup>

#### *Exercise-induced adaptations does not include changes in RAS receptor abundance in skeletal muscle*

Currently, there are only 2 reports that demonstrated a decrease in AT1Rs and an increase in MasRs protein expression in rat skeletal muscle.<sup>9,23</sup> Gomes-santos et al. performed exercise training in a model with a pathological increase in classical RAS signaling (i.e., chronic heart failure), however, healthy exercised control animals demonstrated an increase in MasR mRNA in only soleus muscles which may be due to adaptations within the vascular beds within the soleus.<sup>9</sup> Similarly, Frantz et al. suggested that exercise training results in a shift of the RAS axis to favor the non-classical RAS in skeletal muscles from obese animals, however, they did not include a healthy exercise trained group of animals.<sup>23</sup> Ultimately, it remains unclear if exercise preconditioning results in a shift of RAS receptors in healthy skeletal muscle tissues and not due to adaptations within the microvasculature.

#### Conclusions

This study provides the first robust evidence that endurance exercise training does not modify the expression of AT1Rs or MasRs in skeletal muscle fibers. These results are important because they reject the postulate that exercise-induced changes in RAS receptors are a mechanism to explain exercise preconditioning in skeletal muscles. By confirming that endurance exercise training does not change the abundance of RAS receptors in skeletal muscle, these results direct future studies toward alternative mechanisms that contribute to exercise preconditioning in skeletal muscles.

#### Ethical approval statement

These experiments were approved by the University of Florida Animal Care and Use Committee (Protocol 201810432). All animals were housed at the University of Florida Animal Care Services Center according to guidelines set forth by the Institute of Animal Care and Use Committee.

#### Submission statement

This manuscript has not been published and is not under consideration for publication elsewhere.

#### Authors' contributions

BLN and SKP synthesized experimental design. BLN collected and analyzed data, wrote animal use protocol, implemented experiments, and edited manuscript. RD, TY, JL, HH, and MO assisted with animal experiments. SKP acquired funding. BLN, SKP, RD, TY, JL, HH, and MO edited and revised manuscript.

#### Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### References

- Lawler JM, Rodriguez DA, Hord JM. Mitochondria in the middle: exercise preconditioning protection of striated muscle. *J Physiol.* 2016;594(18):5161–5183. <https://doi.org/10.1113/JP270656>.
- Powers SK, Deminice R, Ozdemir M, Yoshihara T, Bomkamp MP, Hyatt H. Exercise-induced oxidative stress: friend or foe? *J Sport Health Sci.* 2020;9(5):415–425. <https://doi.org/10.1016/j.jshs.2020.04.001>.
- Smuder AJ, Kavazis AN, Min K, Powers SK. Exercise protects against doxorubicin-induced markers of autophagy signaling in skeletal muscle. *J Appl Physiol (1985).* 2011;111(4):1190–1198. <https://doi.org/10.1152/jappphysiol.00429.2011>.
- Smuder AJ, Min K, Hudson MB, et al. Endurance exercise attenuates ventilator-induced diaphragm dysfunction. *J Appl Physiol (1985).* 2012;112(3):501–510. <https://doi.org/10.1152/jappphysiol.01086.2011>.
- Fujino H, Ishihara A, Murakami S, et al. Protective effects of exercise preconditioning on hindlimb unloading-induced atrophy of rat soleus muscle. *Acta Physiol (Oxf).* 2009;197(1):65–74. <https://doi.org/10.1111/j.1748-1716.2009.01984.x>.
- Powers SK, Duarte JA, Le Nguyen B, Hyatt H. Endurance exercise protects skeletal muscle against both doxorubicin-induced and inactivity-induced muscle wasting. *Pflugers Arch.* 2019;471(3):441–453. <https://doi.org/10.1007/s00424-018-2227-8>.
- Wiggs MP. Can endurance exercise preconditioning prevent disuse muscle atrophy? *Front Physiol.* 2015;6:63. <https://doi.org/10.3389/fphys.2015.00063>. Published 2015 Mar 11.
- Smuder AJ, Morton AB, Hall SE, et al. Effects of exercise preconditioning and HSP72 on diaphragm muscle function during mechanical ventilation. *J Cachexia Sarcopenia Muscle.* 2019;10(4):767–781. <https://doi.org/10.1002/jcsm.12427>.
- Gomes-Santos IL, Fernandes T, Couto GK, et al. Effects of exercise training on circulating and skeletal muscle renin-angiotensin system in chronic heart failure rats. *PLoS One.* 2014;9(5):e98012. <https://doi.org/10.1371/journal.pone.0098012>. Published 2014 May 23.
- Frantz EDC, Prodel E, Braz ID, et al. Modulation of the renin-angiotensin system in white adipose tissue and skeletal muscle: focus on exercise training. *Clin Sci (Lond).* 2018;132(14):1487–1507. <https://doi.org/10.1042/CS20180276>.
- Powers SK, Morton AB, Hyatt H, Hinkley MJ. The renin-angiotensin system and skeletal muscle. *Exerc Sport Sci Rev.* 2018;46(4):205–214. <https://doi.org/10.1249/JES.0000000000000158>.
- Brink M, Price SR, Chrast J, et al. Angiotensin II induces skeletal muscle wasting through enhanced protein degradation and down-regulates autocrine insulin-like growth factor I. *Endocrinology.* 2001;142(4):1489–1496. <https://doi.org/10.1210/endo.142.4.8082>.
- Kadoguchi T, Takada S, Yokota T, et al. Deletion of NAD(P)H oxidase 2 prevents angiotensin II-induced skeletal muscle atrophy. *BioMed Res Int.* 2018;2018, 3194917. <https://doi.org/10.1155/2018/3194917>.
- Du Bois P, Pablo Tortola C, Lodka D, et al. Angiotensin II induces skeletal muscle atrophy by activating TFEB-mediated MuRF1 expression. *Circ Res.* 2015;117(5):424–436. <https://doi.org/10.1161/CIRCRESAHA.114.305393>.
- Sukhanov S, Semprun-Prieto L, Yoshida T, et al. Angiotensin II, oxidative stress and skeletal muscle wasting. *Am J Med Sci.* 2011;342(2):143–147. <https://doi.org/10.1097/MAJ.0b013e318222e620>.



16. Rezk BM, Yoshida T, Semprun-Prieto L, Higashi Y, Sukhanov S, Delafontaine P. Angiotensin II infusion induces marked diaphragmatic skeletal muscle atrophy. *PLoS One*. 2012;7(1), e30276. <https://doi.org/10.1371/journal.pone.0030276>.
17. Cisternas F, Morales MG, Meneses C, et al. Angiotensin-(1-7) decreases skeletal muscle atrophy induced by angiotensin II through a Mas receptor-dependent mechanism. *Clin Sci (Lond)*. 2015;128(5):307–319. <https://doi.org/10.1042/CS20140215>.
18. Meneses C, Morales MG, Abrigo J, Simon F, Brandan E, Cabello-Verrugio C. The angiotensin-(1-7)/mas Axis reduces myonuclear apoptosis during recovery from angiotensin II-induced skeletal muscle atrophy in mice. *Pflugers Arch*. 2015;467(9):1975–1984. <https://doi.org/10.1007/s00424-014-1617-9>.
19. Zambelli V, Sigurta A, Rizzi L, et al. Angiotensin-(1-7) exerts a protective action in a rat model of ventilator-induced diaphragmatic dysfunction. *Intensive Care Med Exp*. 2019;7(1):8. <https://doi.org/10.1186/s40635-018-0218-x>.
20. Kwon OS, Smuder AJ, Wiggs MP, et al. AT1 receptor blocker losartan protects against mechanical ventilation-induced diaphragmatic dysfunction. *J Appl Physiol (1985)*. 2015;119(10):1033–1041. <https://doi.org/10.1152/jappphysiol.00237.2015>.
21. Surapongchai J, Rattanavichit Y, Buniam J, Saengsirisuwan V. Exercise protects against defective insulin signaling and insulin resistance of glucose transport in skeletal muscle of angiotensin II-infused rat. *Front Physiol*. 2018;9:358. <https://doi.org/10.3389/fphys.2018.00358>.
22. Rattanavichit Y, Buniam J, Surapongchai J, Saengsirisuwan V. Voluntary exercise opposes insulin resistance of skeletal muscle glucose transport during liquid fructose ingestion in rats. *J Physiol Biochem*. 2018;74(3):455–466. <https://doi.org/10.1007/s13105-018-0639-8>.
23. Frantz EDC, Giori IG, Machado MV, et al. High, but not low, exercise volume shifts the balance of renin-angiotensin system toward ACE2/Mas receptor axis in skeletal muscle in obese rats. *Am J Physiol Endocrinol Metab*. 2017;313(4):E473–E482. <https://doi.org/10.1152/ajpendo.00078.2017>.
24. Benicky J, Hafko R, Sanchez-Lemus E, Aguilera G, Saavedra JM. Six commercially available angiotensin II AT1 receptor antibodies are non-specific. *Cell Mol Neurobiol*. 2012;32(8):1353–1365. <https://doi.org/10.1007/s10571-012-9862-y>.
25. Herrera M, Sparks MA, Alfonso-Pecchio AR, Harrison-Bernard LM, Coffman TM. Lack of specificity of commercial antibodies leads to misidentification of angiotensin type 1 receptor protein. *Hypertension*. 2013;61(1):253–258. <https://doi.org/10.1161/HYPERTENSIONAHA.112.203679>.
26. Harriss DJ, MacSween A, Atkinson G. Ethical standards in sport and exercise science research: 2020 update. *Int J Sports Med*. 2019;40(13):813–817. <https://doi.org/10.1055/a-1015-3123>.
27. Green HJ, Plyley MJ, Smith DM, Kile JG. Extreme endurance training and fiber type adaptation in rat diaphragm. *J Appl Physiol (1985)*. 1989;66(4):1914–1920. <https://doi.org/10.1152/jappphysiol.1989.66.4.1914>.
28. Cabello-Verrugio C, Cordova G, Salas JD. Angiotensin II: role in skeletal muscle atrophy. *Curr Protein Pept Sci*. 2012;13(6):560–569. <https://doi.org/10.2174/138920312803582933>.
29. Delp MD, Duan C. Composition and size of type I, IIA, IID/X, and IIB fibers and citrate synthase activity of rat muscle. *J Appl Physiol*. 1996;80(1):261–270. <https://doi.org/10.1152/jappphysiol.1996.80.1.261>.
30. Li X, Wang K. Effects of moderate-intensity endurance exercise on angiotensin II and angiotensin II type I receptors in the rat heart. *Mol Med Rep*. 2017;16(3):2439–2444. <https://doi.org/10.3892/mmr.2017.6864>.
31. Criswell D, Powers S, Dodd S, et al. High intensity training-induced changes in skeletal muscle antioxidant enzyme activity. *Med Sci Sports Exerc*. 1993;25(10):1135–1140.
32. Theilen NT, Jeremic N, Weber GJ, Tyagi SC. Exercise preconditioning diminishes skeletal muscle atrophy after hindlimb suspension in mice. *J Appl Physiol (1985)*. 2018;125(4):999–1010. <https://doi.org/10.1152/jappphysiol.00137.2018>.
33. Petrini AC, Ramos DM, Gomes de Oliveira L, Alberto da Silva C, Pertille A. Prior swimming exercise favors muscle recovery in adult female rats after joint immobilization. *J Phys Ther Sci*. 2016;28(7):2072–2077. <https://doi.org/10.1589/jpts.28.2072>.
34. Deminice R, Hyatt H, Yoshihara T, et al. Human and rodent skeletal muscles express angiotensin II type 1 receptors. *Cells*. 2020;9(7):1688. <https://doi.org/10.3390/cells9071688>.
35. Kirshner ZZ, Gibbs RB. Use of the REVERT® total protein stain as a loading control demonstrates significant benefits over the use of housekeeping proteins when analyzing brain homogenates by Western blot: An analysis of samples representing different gonadal hormone states. *Mol Cell Endocrinol*. 2018;473:156–165. <https://doi.org/10.1016/j.mce.2018.01.015>.
36. Falcon BL, Stewart JM, Bourassa E, et al. Angiotensin II type 2 receptor gene transfer elicits cardioprotective effects in an angiotensin II infusion rat model of hypertension. *Physiol Genomics*. 2004;19(3):255–261. <https://doi.org/10.1152/physiolgenomics.00170.2004>.
37. Bragina ME, Stergiopoulos N, Fraga-Silva RA. Fluorescence-based binding assay for screening ligands of angiotensin receptors. *Methods Mol Biol*. 2017;1614:165–174. [https://doi.org/10.1007/978-1-4939-7030-8\\_13](https://doi.org/10.1007/978-1-4939-7030-8_13).
38. Phansalkar NMS, Sabale A, Joshi A. Adaptive local thresholding for detection of nuclei in diversity stained cytology images. In: *Proceedings of the 2011 International Conference on Communications and Signal Processing*. 10–12 February 2011. 2011: 218–220. <https://doi.org/10.1109/ICCSP.2011.5739305>.
39. Fischer M, Baessler A, Schunkert H. Renin angiotensin system and gender differences in the cardiovascular system. *Cardiovasc Res*. 2002;53(3):672–677. [https://doi.org/10.1016/s0008-6363\(01\)00479-5](https://doi.org/10.1016/s0008-6363(01)00479-5).
40. Sandberg K, Ji H. Sex and the renin angiotensin system: implications for gender differences in the progression of kidney disease. *Adv Ren Replace Ther*. 2003;10(1): 15–23. <https://doi.org/10.1053/jarr.2003.50006>.
41. Farhat F, Amerand A, Simon B, Gueguenat N, Moisan C. Gender-dependent differences of mitochondrial function and oxidative stress in rat skeletal muscle at rest and after exercise training. *Redox Rep*. 2017;22(6):508–514. <https://doi.org/10.1080/13510002.2017.1296637>.
42. Vincent HK, Powers SK, Stewart DJ, Demirel HA, Shanelly RA, Naito H. Short-term exercise training improves diaphragm antioxidant capacity and endurance. *Eur J Appl Physiol*. 2000;81(1–2):67–74. <https://doi.org/10.1007/PL00013799>.
43. Vincent HK, Powers SK, Demirel HA, Coombes JS, Naito H. Exercise training protects against contraction-induced lipid peroxidation in the diaphragm. *Eur J Appl Physiol Occup Physiol*. 1999;79(3):268–273. <https://doi.org/10.1007/s004210050505>.
44. Zhang L, Du J, Hu Z, et al. IL-6 and serum amyloid A synergy mediates angiotensin II-induced muscle wasting. *J Am Soc Nephrol*. 2009;20(3):604–612. <https://doi.org/10.1681/ASN.2008060628>.
45. Agoudemos MM, Greene AS. Localization of the renin-angiotensin system components to the skeletal muscle microcirculation. *Microcirculation*. 2005;12(8): 627–636. <https://doi.org/10.1080/10739680500301664>.
46. Li X, Wang K. Effects of moderate-intensity endurance exercise on angiotensin II and angiotensin II type I receptors in the rat heart. *Mol Med Rep*. 2017;16(3):2439–2444. <https://doi.org/10.3892/mmr.2017.6864>.
47. Brutsaert DL. Cardiac endothelial-myocardial signaling: its role in cardiac growth, contractile performance, and rhythmicity. *Physiol Rev*. 2003;83(1):59–115. <https://doi.org/10.1152/physrev.00017.2002>.
48. Tikunov BA, Mancini D, Levine S. Changes in myofibrillar protein composition of human diaphragm elicited by congestive heart failure. *J Mol Cell Cardiol*. 1996; 28(12):2537–2541. <https://doi.org/10.1006/jmcc.1996.0245>.