Disruption of muscle renin-angiotensin system in AT$_{1a}^{-/-}$ mice enhances muscle function despite reducing muscle mass but compromises repair after injury

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Murphy KT, Allen AM, Chee A, Naim T, Lynch GS. Disruption of muscle renin-angiotensin system in AT$_{1a}^{-/-}$ mice enhances muscle function despite reducing muscle mass but compromises repair after injury. Am J Physiol Regul Integr Comp Physiol 303: R321–R331, 2012. First published June 6, 2012; doi:10.1152/ajpregu.00007.2012.—The role of the renin-angiotensin system (RAS) in vasoregulation is well established, but a localized RAS exists in multiple tissues and exerts diverse functions including autonomic control and thermogenesis. The role of the RAS in the maintenance and function of skeletal muscle is not well understood, especially the role of angiotensin peptides, which appear to contribute to muscle atrophy. We tested the hypothesis that mice lacking the angiotensin type 1A receptor (AT$_{1A}^{-/-}$) would exhibit enhanced whole body and skeletal muscle function and improved regeneration after severe injury. Despite 18- to 20-wk-old AT$_{1A}^{-/-}$ mice exhibiting reduced muscle mass compared with controls ($P < 0.05$), the tibialis anterior (TA) muscles produced a 25% higher maximum specific force ($P < 0.05$). Average fiber cross-sectional area (CSA) and fiber oxidative capacity was not different between groups, but TA muscles from AT$_{1A}^{-/-}$ mice had a reduced number of muscle fibers as well as a higher proportion of type IIa fibers and a lower proportion of type IIb fibers ($P < 0.05$). Measures of whole body function (grip strength, rotarod performance, locomotor activity) were all improved in AT$_{1A}^{-/-}$ mice ($P < 0.05$). Surprisingly, the recovery of muscle mass and fiber CSA following myotoxic injury was impaired in AT$_{1A}^{-/-}$ mice, in part by impaired myoblast fusion, prolonged collagen infiltration and inflammation, and delayed expression of myogenic regulatory factors. The findings support the therapeutic potential of RAS inhibition for enhancing whole body and skeletal muscle function, but they also reveal the importance of RAS signaling in the maintenance of muscle mass and for normal fiber repair after injury.

angiotensin type 1 receptor; wasting; atrophy; regeneration

THE RENIN-ANGIOTENSIN SYSTEM (RAS) is typically associated with the regulation of blood pressure and water balance and has well-characterized effects on vascular control and signaling, including vasoconstriction. RAS inhibition is used widely for the treatment of hypertension. However, local RAS exist in multiple tissues including the nervous system, digestive tract, reproductive system, skin, adipose tissue, and lymphatic system and has diverse effects such as autonomic control, fertility, and thermogenesis (33). Although conclusive evidence supporting the synthesis of all components of the RAS within skeletal muscle is not available, it is clear that angiotensin peptides are produced within this tissue (14, 24). Skeletal myocytes express AT$_1$ receptors and are thus responsive to locally formed angiotensin (ANG) II (27). Stimulation of RAS may contribute to skeletal muscle breakdown, either directly or indirectly, by myriad mechanisms including inflammation (49), insulin resistance (46), apoptosis (9), reduced protein synthesis (36), and enhanced protein degradation (38).

Skeletal muscle atrophy is a devastating consequence of numerous conditions, including aging, cancer, the muscular dystrophies, chronic renal failure, chronic heart failure, denervation, and disuse (29). Atrophy impairs muscle function and mobility leading to a loss of independence and a reduction in overall quality of life. Death may result from impaired respiratory and cardiac muscle function (29). Skeletal muscle atrophy can impair regenerative capacity, which is especially relevant for the muscular dystrophies characterized by continuous cycles of degeneration and regeneration, as well as for sarcopenia, which is associated with an increased incidence of fall-related injuries (29). RAS levels and/or receptor sensitivity are increased in many conditions associated with skeletal muscle atrophy, including cancer cachexia (25), sarcopenia (47), chronic renal failure (48), and chronic heart failure (35), and may contribute to their pathophysiology. Although exercise can attenuate muscle atrophy in some cases, it is not an option for patients with severe muscle wasting and so pharmacological interventions are needed to enhance whole body and skeletal muscle function and improve muscle regenerative capacity. RAS inhibition therefore represents a potential therapeutic strategy for attenuating skeletal muscle wasting and weakness.

RAS inhibition with the angiotensin type I receptor (AT$_1$) antagonist losartan improved skeletal muscle pathology and function associated with muscle wasting, including laceration injury (2), sarcopenia (8), and the muscular dystrophies (13), but some studies reported no improvement in the functional capacity of dystrophic muscle (3, 42). The conflicting findings may reflect the limited efficacy of long-term RAS inhibitors (6, 30). AT$_1$ antagonists and angiotensin-converting enzyme (ACE) inhibitors cause a compensatory rise in plasma renin, increasing the conversion of ANG I to ANG II and therefore limiting the efficacy of RAS inhibition (1). Elevated plasma renin with AT$_1$ antagonists and ACE inhibitors may also increase ANG II generation by ACE-independent pathways including chymases (20). Mice lacking the angiotensin type I receptor (AT$_{1A}^{-/-}$) have reduced ANG II levels (43) and provide an excellent platform for the comprehensive investigation of long-term RAS inhibition. We therefore compared whole body and skeletal muscle function as well as muscle regenerative capacity in healthy wild-type mice and AT$_{1A}^{-/-}$ mice, testing the hypothesis that AT$_{1A}^{-/-}$ mice would exhibit...
improved whole body strength (improved grip strength, rotarod performance, and locomotor activity) and skeletal muscle function (increased peak tetanic force and reduced fatigability of tibialis anterior, TA, muscles in situ), and enhanced regeneration after injury (muscle mass and muscle fiber CSA). Support for this hypothesis would provide important proof-of-principle evidence for the therapeutic potential of RAS inhibition for muscle wasting and weakness.

**METHODS**

Ethical approval. All experiments were approved by the Animal Ethics Committee of The University of Melbourne and conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes as stipulated by the National Health and Medical Research Council (Australia).

Experimental animals. Experiments were performed on 18- to 20-wk-old male AT1A\(^{-/-}\) (\(n = 23\)) and wild-type (AT1A\(^{+/+}\)) mice (\(n = 18\)), which were bred in the Biological Resources Facility of The University of Melbourne. The generation of these mice has been described in detail previously (11). Mice were maintained under a 12:12-h light-dark cycle with water and standard laboratory chow available ad libitum.

Grip strength. Front limb strength was assessed by means of a grip strength meter (Columbus Instruments, Columbus, OH) (15). Briefly, mice were anesthetized to the testing room for a minimum of 15 min. The mouse grasped a triangular metal ring connected to a force transducer and was pulled gently by the tail until the grip was broken. The test was performed five times within 2 min. Peak force (in kg) during the grip exercise was normalized to body mass.

Rotarod test. Whole body mobility and coordination was assessed by rotarod performance (Rotamex-5, Columbus Instruments). After 15 min acclimatization in the test room, the mice were placed on the rod, which was rotated at an initial speed of 4 rpm. The speed was increased gradually by 1 rpm every 8 s and latency-to-fall on to a soft recovery on a heating pad. Five or 10 days after the notexin injection, mice were anesthetized with Nembutal (60 mg·kg\(^{-1}\) ip; Sigma-Aldrich), and the left (uninjured) and right (injured) TA muscles were surgically excised, blotted on filter paper, and weighed on an analytical balance. They were then mounted in embedding medium and frozen in thawing isopentane for histochemical and biochemical analyses. The TA muscle was chosen for these analyses because the contractile properties of this muscle were specifically assessed in situ. The anesthetized mice were killed by cardiac excision.

Skeletal muscle histology. Serial sections were cut transversely through the TA muscle using a refrigerated cryostat (–20°C; Microm cryostat, Microm International GmbH, Walldorf, Germany). Sections were stained (or reacted) with: hematoxylin and eosin (H&E) to determine general muscle architecture; laminin (no. L9393, Sigma-Aldrich) for determination of total fiber number per cross section as well as mean myofiber CSA; DAPI (no. D9542, Sigma-Aldrich) to determine the total number of nuclei, number and proportion of centrally located nuclei, and number and proportion of peripherally located nuclei. Sections were stained (or reacted) with the following: succinate dehydrogenase (SDH) to determine activity of oxidative enzymes (5); van Gieson’s for assessment of collagen infiltration (31); myosin ATPase activity to determine the percentage of type I fibers (37); and N2.261 (both developed by Dr. Helen M. Blau, obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA) to assess the percentage of myosin IIa isoforms (45). Since myosin ATPase (mATPase) histochemistry revealed a virtual absence of type I fibers, contractile properties were exclusively determined in type II fibers.

**Table 1. Primer sequences used**

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<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Sense Primer, 5’-3’</th>
<th>Antisense Primer, 5’-3’</th>
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<td>IL-6</td>
<td>NM_031168</td>
<td>CCGAGAGGAGACCTCAAGAG</td>
<td>TCCAGGTTTCCAGAGAAC</td>
</tr>
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<td>TNF-α</td>
<td>NM_013693</td>
<td>AGCGCTCTTCTGAGGCAAGC</td>
<td>AGGAAAGAAGGAGCAAGA</td>
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<tr>
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<td>LIF</td>
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<tr>
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<td>AAGCAGAGCCTGCGAAGAG</td>
<td>AGGTGAGACGAGGCTTTTA</td>
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<td>Myogenin</td>
<td>NM_031189</td>
<td>CACTCCCTCTGCTGAGG</td>
<td>CAAGGAGACGCCCCTAAAA</td>
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<tr>
<td>Renin</td>
<td>NM_031192</td>
<td>AGGGCTCTGTGAGCACTAT</td>
<td>GTGAATGCCAGAAAGAGT</td>
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IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; LIF, leukemia inhibitory factor. Primers were designed using Invitrogen software from gene sequences obtained from GenBank. Primer specificity was determined using a BLAST search.
all non-N2.261 reacting fibers were assumed to represent type IIx/b fibers. Optical density (OD) of SDH was determined after 6 min of reactivity for all samples, and sections were captured in full color using brightfield light microscopy and analyzed, as described previously (31). Digital images were obtained using an upright microscope with camera (Axio Imager D1, Carl Zeiss, Wrek, Göttingen, Germany), controlled, and quantified by AxiosVision AC software (AxioVision AC Rel. 4.7.1, Carl Zeiss).

Real-time RT-PCR analyses. Total RNA was extracted from 10 to 20 mg of left TA muscle using a commercially available kit, according to manufacturers' instructions (PureLink RNA Mini Kit, Invitrogen). RNA concentration was determined spectrophotometrically at 260 nm, and the samples were stored at −80°C. RNA was transcribed into cDNA using the Invitrogen SuperScript VILO cDNA Synthesis Kit, and the resulting cDNA was stored at −20°C for subsequent analysis. Real-time RT-PCR was performed as described previously (31). Primer sequences for the myosin heavy chains (MHC) MHCIIa, MHCIIx, and MHCIIb were as detailed previously (31), and sequences of the other primers used are listed in Table 1. IL-6, TNF-α, and IFN-γ were chosen as markers of inflammation and regeneration because TNF-α and IFN-γ are involved in macrophage activation (12, 44) and all three cytokines stimulate muscle cell proliferation (12, 40, 44) and are involved in mediating ANG II-induced muscle wasting (17, 46, 49). LIF and Myf5 are important factors in satellite cell proliferation, and myogenin is important for differentiation (10). Furthermore, ANG II signaling has been shown to modulate the

Fig. 1. Angiotensin type 1A knockout mice (AT1A−/−) mice exhibit reduced muscle mass but have increased whole body strength, mobility, and movement speed compared with wild-type (WT) mice. A: absolute mass; B: normalized mass of selected hindlimb muscles. C: grip strength; and D: latency-to-fall during rotarod test. Mice were placed in an open field metabolic chamber for 24 h and locomotor activity including movement distance (E), duration (F), and speed were assessed (G), as well as rest duration (H). Results are shown for 6 h each of the light and dark cycles and for total of the light and dark cycles (12-h period). EDL, extensor digitorum longus; Plant, plantaris; TA, tibialis anterior; Gastroc, gastrocnemius; Quad, quadriceps. Data are means ± SE; n = 6–23. *P < 0.05 vs. WT.
expression of all three factors (23, 39). The content of single-stranded DNA (ssDNA) in each sample was determined using the Quanti-iT OliGreen ssDNA Assay Kit (Molecular Probes, Eugene, OR), as described previously (28, 31). Gene expression was quantified by normalizing the logarithmic cycle threshold (CT) value ($2^{-\Delta\Delta CT}$) to the cDNA content of each sample to obtain the expression $\Delta\Delta CT/cDNA$ content (in ng/ml).

**Statistical analyses.** All values are expressed as means ± SE, unless stated otherwise. Groups were compared using a Student’s t-test or a two-way ANOVA, where appropriate. A Student’s t-test was used to compare muscle mass, grip strength, rotarod performance, twitch characteristics, peak tetanic force, average number of centrally located nuclei per fiber, and MHCII isoform mRNA expression. Fiber-type proportions are presented as 95% confidence intervals of the mean. Differences were considered significant when no overlap existed between the 95% confidence intervals (41). A two-way ANOVA was used to compare locomotor activity (group, time), frequency-force relationship (group, frequency), fatiguing stimulation protocol (group, time), total fiber and nuclei number per cross section, proportion of centrally located and peripherally located nuclei, proportion of fibers with specific numbers of centrally located nuclei (group, number of centrally located nuclei per fiber), collagen infiltration and cytokine, and MRF mRNA expression in the notexin study (group, time). When significant group main effects were found, a Bonferroni’s post hoc test was used to determine where the differences existed. The level of significance was set at $P < 0.05$ for all comparisons.

**RESULTS**

**$AT_{1A}^{-/-}$ mice exhibit reductions in muscle and heart mass.** Body mass was similar between $AT_{1A}^{-/-}$ mice (28.4 ± 0.6 g; $n = 18$) and wild-type controls (28.9 ± 0.6 g; $n = 18$). Absolute mass of the soleus, EDL, TA, and quadriceps muscles was reduced in the $AT_{1A}^{-/-}$ mice compared with wild-type

<table>
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<tr>
<th>Characteristic</th>
<th>WT</th>
<th>$AT_{1A}^{-/-}$</th>
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<tr>
<td>$P$, mN</td>
<td>384.5 ± 32.7</td>
<td>343.4 ± 12.7</td>
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<tr>
<td>TPT, ms</td>
<td>16.0 ± 0.5</td>
<td>16.1 ± 0.5</td>
</tr>
<tr>
<td>$1/2$ RT, ms</td>
<td>12.6 ± 1.0</td>
<td>10.8 ± 0.4</td>
</tr>
<tr>
<td>$dP/dt$, mN/ms</td>
<td>60.7 ± 3.1</td>
<td>54.7 ± 1.9</td>
</tr>
<tr>
<td>CSA, mm²</td>
<td>7.3 ± 0.2</td>
<td>6.2 ± 0.2*</td>
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$AT_{1A}^{-/-}$, angiotensin type 1A receptors; WT, wild-type; TA, tibialis anterior; $P$, peak twitch tension; TPT, time-to-peak twitch tension; $1/2$ RT, one-half relaxation time; $dP/dt$, maximum rate of force development during a twitch contraction; CSA, cross-sectional area. Data are means ± SE; $n = 13$. *$P < 0.01$ versus WT.

**Fig. 2. Functional properties of TA muscles from $AT_{1A}^{-/-}$ and WT assessed in situ.** Frequency-force relationship in absolute values (A) and normalized for muscle CSA (B), peak tetanic force production (C), specific (normalized) force production (D), and relative force production during and following 4 min of fatiguing, intermittent stimulation (E) are shown. Data are means ± SE; $n = 11–13$. *$P < 0.03$ vs. WT; $a$ $P < 0.02$ group main effect (post hoc tests did not reveal any statistically significant differences between groups at specific stimulation frequencies).
controls (P < 0.05, Fig. 1A). When normalized for body mass, muscle mass was lower in the soleus, EDL, and TA muscles of the AT1A−/− mice (P < 0.05, Fig. 1B). AT1A−/− mice exhibited reductions in both absolute (-22%, P < 0.001) and normalized heart mass (-18%, P < 0.001), but there was no difference in epididymal fat mass compared with controls (data not shown).

Enhanced whole body strength, mobility, and locomotor activity in AT1A−/− mice. Both absolute grip strength (+26%, data not shown) and grip strength normalized for body mass were higher in AT1A−/− mice compared with controls (P < 0.05, Fig. 1C). AT1A−/− mice also demonstrated a 49% longer latency-to-fall during the rotarod test than controls (P < 0.001, Fig. 1D). AT1A−/− mice covered a greater distance than controls during the dark cycle and total of the light and dark cycles (P < 0.05, Fig. 1E). The duration of movement and rest were not different between groups (Fig. 1, F and H), but average movement speed was higher during all cycles in the AT1A−/− mice (P < 0.05, Fig. 1G).

TA muscles from AT1A−/− mice have normal contractile characteristics. There was no difference between groups for peak twitch force or twitch characteristics (Table 2) but TA muscle CSA was 19% smaller in AT1A−/− mice than controls (P < 0.01, Table 2). Examination of the frequency-force relationship showed only a very minor difference in absolute force and force normalized to muscle CSA (specific force) between TA muscles from WT and AT1A−/− mice (P < 0.02 group main effect, Fig. 2, A and B). As the effects were in opposite directions, the lower absolute force in the AT1A−/− mice was due to the smaller CSA of their muscles. Similarly, for peak tetanic force, TA muscles from AT1A−/− mice were marginally weaker than controls when expressed as absolute force (Fig. 2C) but slightly stronger when expressed as specific force (P < 0.03, Fig. 2D). There was no difference between groups in relative force produced during and after a 4-min, fatiguing stimulation protocol (Fig. 2E).

Altered muscle fiber type proportions in AT1A−/− mice. TA muscle cross sections were reacted for mATPase to detect type I fibers (Fig. 3A), which revealed a very low abundance of type I fibers and no difference in the proportion of type I fibers between groups (Fig. 3A). TA muscle cross sections were also reacted for laminin (red), myosin IIa (N2.261, green), and SDH activity (blue) to visualize all fibers, identify type IIA fibers, and indicate activity of oxidative enzymes (SDH), respectively (Fig. 3A). Since mATPase histochemistry revealed a virtual absence of type I fibers, all non-N2.261-reacting fibers were assumed to represent type IIX/b fibers. TA muscles from AT1A−/− mice had an 11% lower proportion of type IIA fibers and an 11% higher proportion of type IIX/b fibers compared with controls (P < 0.05, Fig. 3B). The area of the type IIA fibers was 18% smaller in the AT1A−/− mice (P < 0.01), but there were no differences between groups in the area of the type IIX/b fibers or in average fiber area (Fig. 3C). SDH intensity in all fiber types was not significantly different between groups (Fig. 3D).

Real-time RT-PCR analysis was used to determine whether the lower proportion of type IIA fibers in the AT1A−/− mice detected with N2.261 corresponded with a lower mRNA expression of MHCIIa and a concomitant higher mRNA expression of MHCIIx and MHCIIb isoforms (Fig. 3E). TA muscles from AT1A−/− mice had a 76% lower MHCIIa mRNA expression compared with controls (P < 0.03). There was a tendency toward lower MHCIIa mRNA expression in AT1A−/− mice, but this was not statistically significant (P < 0.06),
and there was no significance difference between groups in MHCIIx mRNA expression \( (P<0.32) \). However, MHCIIb mRNA expression was two times higher in AT1A\(^{-/-}\) mice compared with controls \( (P<0.05) \).

**AT1A\(^{-/-}\) mice have impaired muscle fiber regeneration after injury.** Representative H&E-stained sections revealed similar degeneration at 5 days postnotexin injury in AT1A\(^{-/-}\) mice and controls but impaired regeneration at 10 days postnotexin injury in AT1A\(^{-/-}\) mice (Fig. 4A). AT1A\(^{-/-}\) mice had a 23% and 46% lower normalized mass of the TA muscle at 5 and 10 days postnotexin injury compared with controls, respectively \( (P<0.05, \text{ Fig. 4B}) \). Mass of the injured TA muscle normalized to the uninjured TA muscle was not different between groups at 5 days postnotexin injury but was 22% lower in AT1A\(^{-/-}\) mice at 10 days postnotexin injury \( (P<0.001, \text{ Fig. 4C}) \). Median fiber CSA was similar between groups at 5 days postnotexin injury but was 22% lower in AT1A\(^{-/-}\) mice at 10 days postnotexin injury \( (P<0.001, \text{ Fig. 4D}) \). A similar pattern of effects was evident from laminin and DAPI staining to determine the total number of fibers per cross section (Fig. 5), with the AT1A\(^{-/-}\) mice having fewer fibers than controls \( (P<0.05, \text{ Fig. 5C}) \). There were no significant differences between groups for the total number of nuclei per cross section, the number of myonuclei per cross section, the number of peripherally located nuclei per cross section, or the proportion of fibers with peripherally located nuclei (Table 3). However, AT1A\(^{-/-}\) mice had 72% fewer centrally located nuclei per cross section and a smaller proportion of fibers with centrally located nuclei compared with controls \( (P<0.01, \text{ Table 3}) \).

Examination of muscle fiber cross sections at 10 days postnotexin injury showed impaired myoblast fusion in AT1A\(^{-/-}\) mice, as evidenced by clumping of small regenerating fibers with a single centrally located nucleus (Fig. 5B). To assess myoblast fusion, the number of centrally located nuclei per fiber at 10 days postnotexin injury was determined, and AT1A\(^{-/-}\) mice had a greater proportion of fibers with a single central nucleus and a smaller proportion of fibers with two or three central nuclei \( (P<0.001, \text{ Fig. 5D}) \). Regenerating muscles from AT1A\(^{-/-}\) mice also had a 32% lower average number of centrally located nuclei per fiber compared with controls \( (P<0.01, \text{ Fig. 5E}) \).

To investigate the mechanisms responsible for the impaired regeneration after myotoxic injury in AT1A\(^{-/-}\) mice, we examined the infiltration of collagen and the expression of genes involved in inflammation (IL-6, TNF-\(\alpha\), IFN-\(\gamma\)) and muscle regeneration (leukemia inhibitory factor, LIF, Myf5, and myogenin). Representative sections stained for assessment of collagen infiltration are shown in Fig. 6A. The effect of AT1A deletion on collagen infiltration was consistent with the previous results with muscle mass and fiber CSA, with the AT1A\(^{-/-}\) mice having higher collagen infiltration than controls at 10 days postinjury \( (P<0.01, \text{ Fig. 6B}) \). The elevated IL-6 mRNA expression after injury was exaggerated in the AT1A\(^{-/-}\) mice (Fig. 7A), and there was an increase in TNF-\(\alpha\) mRNA expres-
Fig. 5. Total fiber number per cross section in uninjured TA muscles and at 5 and 10 days postnotexin injury and the average number of centrally located nuclei per fiber at 10 days postnotexin injury from AT1A−/− and WT. Representative images (×40) and inset magnified of muscle sections reacted for laminin (red) and DAPI (blue, A) are shown. C: quantification of laminin reaction to determine the total fiber number per cross section. Representative sections (×20) at 10 days postnotexin injury indicate impaired myoblast fusion in AT1A−/− mice, as evidenced by clumping of small regenerating fibers with a single central nucleus (indicated by arrows) (B). To assess myoblast fusion, the number of centrally located nuclei per fiber at 10 days postnotexin injury was determined, and AT1A−/− mice had a greater proportion of fibers with a single central nucleus and a smaller proportion of fibers with multiple central nuclei (D), as well as a lower average number of centrally located nuclei per fiber compared with controls (E). Data are means ± SE (C, E) or means ± 95% CI (D); n = 4–6. *P < 0.05 vs. WT.

**DISCUSSION**

RAS inhibition is a potential strategy for ameliorating the muscle wasting and weakness associated with conditions such as aging (sarcopenia), cancer, and the muscular dystrophies and may therefore improve quality of life and reduce mortality in affected patients. This proof-of-principle study investigated whether mice lacking the angiotensin type 1A receptor (AT1A−/−), the main receptor mediating RAS signaling in mice (21), had improved whole body and skeletal muscle function and im-

### Table 3. Nuclei number and localization in cross sections from uninjured TA muscles and at 5 and 10 days postnotexin injury from AT1A−/− and WT

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Uninjured WT</th>
<th>AT1A−/−</th>
<th>5 days postnotexin WT</th>
<th>AT1A−/−</th>
<th>10 days postnotexin WT</th>
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<tr>
<td>No. nuclei/cross section</td>
<td>9,454 ± 2,339</td>
<td>9,849 ± 1,209</td>
<td>18,687 ± 5,854</td>
<td>21,084 ± 1,083</td>
<td>17,832 ± 4,111</td>
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<tr>
<td>No. myonuclei/cross section</td>
<td>2,917 ± 1,224</td>
<td>2,815 ± 270</td>
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<td>4,295 ± 271</td>
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<td>No. peripherally located nuclei/cross section</td>
<td>2476 ± 1223</td>
<td>2631 ± 265</td>
<td>5073 ± 1712</td>
<td>3903 ± 281</td>
<td>5116 ± 1407</td>
<td>3683 ± 694</td>
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<tr>
<td>No. centrally located nuclei/cross section</td>
<td>126 ± 27</td>
<td>202 ± 97</td>
<td>571 ± 182</td>
<td>392 ± 57</td>
<td>1755 ± 494</td>
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<td>% peripherally located nuclei/total nuclei</td>
<td>25.3 ± 5.1</td>
<td>27.3 ± 2.2</td>
<td>18.5 ± 6.9</td>
<td>18.6 ± 1.1</td>
<td>28.0 ± 5.4</td>
<td>18.7 ± 2.1</td>
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<tr>
<td>% centrally located nuclei/total nuclei</td>
<td>1.4 ± 0.3</td>
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<td>2.4 ± 1.6</td>
<td>1.9 ± 0.4</td>
<td>10.0 ± 2.8</td>
<td>2.5 ± 0.3*</td>
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Data are means ± SE; n = 4–6. *P < 0.01 versus WT.
proved muscle regenerative capacity compared with wild-type controls. The most important findings were that despite AT1A−/− mice having smaller muscles, they had increased whole body strength and locomotor activity and increased skeletal muscle function. Another important, but surprising, finding was that muscle fiber regenerative capacity was impaired in AT1A−/− mice. While the findings support the therapeutic potential of RAS inhibition for enhancing whole body and skeletal muscle function, they also reveal that RAS inhibition could compromise muscle size and muscle fiber regeneration after severe injury.

The functional capacity of skeletal muscle is one of the most important indicators of quality of life and morbidity (32). A reduction in muscle function can impair mobility and cause severe fatigue, leading to a loss of independence and an overall reduction in quality of life. More severe reductions in muscle function can result in death, mainly due to respiratory or cardiac failure. Therefore, improving the functional capacity of skeletal muscle has important implications for preserving and enhancing the quality of life. Several studies have investigated the efficacy of the AT1 antagonist losartan for improving muscle function in dystrophic mdx mice with conflicting findings (3, 13, 42). Whereas one study reported an increased absolute and normalized tetanic force of EDL muscles in vitro (13), others found no improvements in grip strength, rotarod performance, locomotor activity, and tetanic (absolute and normalized) force of EDL (3, 42), soleus, and diaphragm muscles in vitro (3). These conflicting findings may be attributed to the limited efficacy of long-term RAS inhibitors, with AT1 antagonists and ACE inhibitors causing compensatory elevations in plasma renin (1, 6, 30). The improvements in whole body and skeletal muscle function were despite most of the muscles examined being smaller in AT1A−/− mice. Interestingly, only absolute mass of the muscles predominantly comprising type IIb fibers, the plantaris, and gastrocnemius was not different between controls and AT1A−/− mice, indicating a preservation of the fast, glycolytic type IIb fibers. This is consistent with AT1A−/− mice having selective atrophy of the type IIa but not type IIx/b fibers and increased MHCIIb mRNA expression. Despite the lower muscle mass, AT1A−/− mice had similar body mass to controls but this was probably due to AT1A−/− mice having a 40–45% higher bone mass than controls (26). The surprising reduction in muscle mass in AT1A−/− mice contrasts with the reported increased muscle mass after acute RAS inhibition in mdx mice (13) and with other studies in mdx mice finding no effect of acute RAS inhibition on muscle mass (3, 42). The differences in findings between these studies and those of the current study may reflect
possible compensatory changes as a consequence of life-long RAS inhibition that ultimately reduces muscle mass. Average fiber CSA was not different between groups, but total fiber number was reduced in AT1A\(^{-/-}\)/H11002 mice so the decrease in muscle mass was attributed to the reduction in fiber number. The improvements in muscle function are due, at least in part, to the shift toward a higher proportion of the high force-producing, fast, glycolytic type IIb fibers. Despite the shift toward a greater proportion of type IIb fibers, there was no concomitant reduction in average fiber oxidative enzyme capacity (SDH) or increase in fatigue. To our knowledge, the present study is the first investigation of whole body and skeletal muscle function in AT1A\(^{-/-}\)/H11002 mice, and although our proof-of-principle findings support the therapeutic potential of RAS inhibition for enhancing whole body and skeletal muscle function, the reduction in muscle mass with life-long RAS inhibition is problematic. Studies using AT1 antagonists or ACE inhibitors have not found reductions in muscle mass.

Fig. 7. Expression of genes involved in inflammation and regeneration in uninjured TA muscles and at 5 and 10 d postnotoxin injury from AT1A\(^{-/-}\) and WT. Genes involved in inflammation include interleukin-6 (IL-6, A), tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\), B), and interferon-\(\gamma\) (IFN-\(\gamma\), C). Genes involved in muscle fiber regeneration include leukemia inhibitory factor (LIF, D), Myf5 (E), and myogenin (F). Gene expression of renin was also examined (G). Data are means \(\pm\) SE; \(n = 5–6\). *\(P < 0.05\) vs. WT; \(aP < 0.01\) group main effect (post hoc tests did not reveal any statistically significant differences between groups at specific time points).
13, 42), but as discussed above, these studies were limited by potential compensatory increases in renin levels. Since the combination of AT₁ inhibitors and renin inhibitors have been shown to maximize the efficacy of RAS inhibition (50), future studies should determine whether the combination of AT₁ and renin inhibitors can enhance both skeletal muscle mass and function.

The weakness associated with skeletal muscle atrophy can render muscles more susceptible to injury. Duchenne muscular dystrophy is characterized by degenerating muscle fibers (4), and sarcopenia is associated with an increased risk of falls (29). Strategies to enhance muscle regeneration are significant for improving the long-term outcome and for minimizing functional disabilities in affected patients (19). Both cardiotoxicity and laceration injury are associated with increased expression of AT₁ in mice (2, 23), and losartan reduced fibrosis and enhanced regeneration in injured muscles from healthy mice, dystrophic mdx mice, and aged mice (2, 8, 13, 42). Based on these findings, we hypothesized that regeneration after notexin-induced injury in AT₁A⁻/⁻ mice would be enhanced. Contrary to our hypothesis, muscles from AT₁A⁻/⁻ mice exhibited impaired regeneration after myotoxic injury, with a reduction in normalized muscle mass (compared with uninjured muscle) and muscle fiber CSA at 10 days postinjury. This was due to a reduction in the total number of muscle fibers and associated with a reduced proportion of centrally located fibers, increased collagen infiltration, an exaggerated and prolonged inflammatory response (IL-6, TNF-α, IFN-γ), and delayed expression of myogenic regulatory factors (Myf5, myogenin). An interesting observation was that at 10 days postnotexin injury, muscles from control mice had large fibers with multiple centrally located nuclei indicating successful myoblast fusion, whereas muscles from AT₁A⁻/⁻ mice had clumping of small fibers with a single centrally located nuclei indicating impaired myoblast fusion. Indeed, muscles from AT₁A⁻/⁻ mice had a greater proportion of fibers with a single central nucleus and a smaller proportion of fibers with multiple central nuclei, confirming impaired myoblast fusion after myotoxic injury. Although our findings conflict with those reported for losartan, they are consistent with a demonstrated reduction in muscle fiber CSA after cardiotoxin injury in AT₁A⁻/⁻ mice (22). Whether the differences between studies reflect the problems of sustained efficacy of AT₁ antagonists or because losartan has other effects besides AT₁ inhibition is unknown, but renin mRNA expression was not different between groups in the present study. Since AT₁-dependent ANG II signaling increases the expression of cytokines including IL-6 (49) and TNF-α (46), it was surprising that the mRNA expression of these cytokines was elevated postinjury in AT₁A⁻/⁻ mice. The delayed regeneration following notexin injury in AT₁A⁻/⁻ mice, as assessed by Myf5 and myogenin mRNA expression, is consistent with the elevated and prolonged inflammatory response (19). Taken together, these findings indicate that AT₁ signaling is necessary for the normal regenerative response following myotoxic injury.

Perspectives and Significance

Lifelong RAS inhibition enhanced whole body and skeletal muscle function in healthy mice. However, lifelong RAS inhibition also reduced muscle mass and compromised muscle fiber regeneration after injury, findings that limit the therapeutic potential of chronic RAS inhibition. Since acute RAS inhibition does not reduce muscle mass but is limited in its efficacy because of compensatory increases in plasma renin levels, future studies should investigate whether combination treatment with AT₁ and renin inhibitors can increase both skeletal muscle mass and function.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

RAS INHIBITION AND SKELETAL MUSCLE FUNCTION


