Captopril treatment induces hyperplasia but inhibits myonuclear accretion following severe myotrauma in murine skeletal muscle

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Johnston AP, Bellamy LM, De Lisio M, Parise G. Captopril treatment induces hyperplasia but inhibits myonuclear accretion following severe myotrauma in murine skeletal muscle. Am J Physiol Regul Integr Comp Physiol 301: R363–R369, 2011. First published June 1, 2011; doi:10.1152/ajpregu.00766.2010.—The role of ANG II in skeletal muscle and satellite cell regulation is largely unknown. Cardiotoxin (CTX) was used to investigate whether muscle injury activates a local ANG II signaling system. Following injury, immunohistochemistry (IHC) analysis revealed a robust increase in the intensity of angiotensinogen and angiotensin type 1 (AT1) receptor expression. As regeneration proceeded, however, AT1 and angiotensinogen were downregulated. Nuclear accretion and fiber formation were also assessed during muscle regeneration in mice treated with captopril (an angiotensin-converting enzyme inhibitor). When ANG II formation was blocked through the use of captopril, we observed a significantly reduced accretion of nuclei into myofibers (−25%), while tibialis anterior total fiber number was significantly increased +37%. This phenotype appeared to be due to alterations in satellite cell differentiation kinetics; captopril treatment led to sustained mRNA expression of markers associated with quiescence and proliferation (Myf5, Pax7) and simultaneously delayed or inhibited the expression of myogenin. IHC staining supported these findings, revealing that captopril treatment resulted in a strong trend (P = 0.06) for a decrease in the proportion of myogenin-positive myoblasts. Furthermore, these observations were associated with a delay in muscle fiber maturation; captopril treatment resulted in sustained expression of embryonic myosin heavy chain. Collectively, these findings demonstrate that localized skeletal muscle angiotensin signaling is important to muscle fiber formation, myonuclear accretion, and satellite cell function.

satellite cell; angiotensin II; muscle growth; differentiation; myonuclear accretion; renin-angiotensin system

THE GROWTH AND MAINTENANCE of skeletal muscle are largely dependent on a population of myogenic progenitor cells commonly referred to as satellite cells (22). These cells are maintained in a state of quiescence under basal conditions, residing in the satellite cell niche between the basal lamina and sarcolemma (22). In response to myotrauma, satellite cells activate, migrate to the site of injury, proliferate, and terminally differentiate (11). It is now understood that adult myogenesis proceeds in a stepwise fashion. During the first wave of differentiation, myoblasts fuse to one another to form new myotubes; during the second wave of differentiation, myoblasts fuse to nascent myotubes and promote their growth. (13, 14).

The transition of a satellite cell from quiescence to terminal differentiation is referred to as the myogenic program and is orchestrated by a series of transcription factors known as the myogenic regulatory factors [Myf5, MyoD, myogenic regulatory factor-4 (MRF-4), and myogenin], as well as the paired box transcription factor Pax7 (3). Although much is known about the function of these transcriptional factors, little is known regarding the cytokines and growth factors that serve to regulate their activity. We believe that one such growth factor may be ANG II.

We have recently described a local ANG II signaling system in cultured primary and C2C12 myoblasts (17). The presence of this system was defined by the expression of a large number of ANG II-signaling family members (17). Furthermore, we also demonstrated that cultured C2C12 cells possess the ability to secrete both ANG I and ANG II in vitro (17). We have also demonstrated that inhibition of ANG II signaling [through captopril treatment or angiotension type 1a (AT1a) receptor ablation] impaired the growth of skeletal muscle following cardiotoxin (CTX)-induced injury (16). Finally, we established that ANG II serves a functional role in satellite cell regulation, inducing activation and promoting chemotaxis (16). These findings are in agreement with other published works (7, 35) that have demonstrated that angiotensin-converting enzyme (ACE) inhibition significantly represses skeletal muscle hypertrophy, as evidenced by a decrease in total protein content, fiber cross-sectional area, and myonuclear accretion by new muscle fibers.

Although we have described a role for ANG II in regulating the early response of satellite cells to muscle injury, ANG II may also play an important role in other satellite cell processes. Indeed, ANG II has been implicated in the regulation of numerous cellular functions, such as cell proliferation, protein synthesis (15, 20), protein degradation (39), and cell survival (27). In this study, we further define skeletal muscle as possessing a local ANG II signaling system and demonstrate that it functions to regulate myonuclear accretion and myofiber hyperplasia following severe myotrauma. These results further support the capacity for ANG II to act as a pleiotropic factor in skeletal muscle.

MATERIALS AND METHODS

Animals and experimental procedures. Data were generated from two separate time-course experiments using 10-wk-old C57BL/6 male mice (Jackson Laboratories, Bar Harbor, ME). The first experiment (n = 5 per group) was designed to describe the effect of muscle regeneration on the activation pattern of a local ANG II signaling system. Animals were subjected to bilateral injections of CTX (25 μl at 10 μM) into their tibialis anterior (TA) muscle. The second experiment (n = 10 per group) was designed to assess the role of ANG II in regulating skeletal muscle fiber formation and myonuclear accretion. Animals were supplemented with either normal drinking water or captopril (0.5 mg/ml; Sigma, Kootenay Plains, Alberta, Alberta, Canada).
myogenin were chosen as markers representative of satellite cells.

Table 1. Prime sequences and annealing temperatures

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myf5</td>
<td>TGAAGGATGGAGCATGAGCGG</td>
<td>TGTGTCGCTACGAAAGCACTTA</td>
<td>59°C</td>
</tr>
<tr>
<td>MyoD</td>
<td>TACCAAGGCGATGAGCTCTG</td>
<td>CATCATGGGACTCAGAGAGCT</td>
<td>59°C</td>
</tr>
<tr>
<td>Myogenin</td>
<td>CATACAGCAGGATTCACTCTG</td>
<td>AGATTGTGGGCTGCTGAG</td>
<td>59°C</td>
</tr>
<tr>
<td>Pax7</td>
<td>CGATTCAGGATCGATGAGCTT</td>
<td>GGTAGTCCTGCTGCTGCTTTA</td>
<td>59°C</td>
</tr>
<tr>
<td>eMHC</td>
<td>ACGGATCGATGGGACAGG</td>
<td>CAGCTCCTGATCCTGCTTCTC</td>
<td>60°C</td>
</tr>
<tr>
<td>L32</td>
<td>TCCACAGAATGTGAAGGACCTC</td>
<td>ACTGATTCTCTGCCGCTGCT</td>
<td>59°C</td>
</tr>
</tbody>
</table>

RESULTS

CTX-induced injury activates a local skeletal muscle angiotensin signaling system. We previously demonstrated that, in vitro, skeletal muscle myoblasts and myotubes possess a local angiotensin signaling system and the ability to produce ANG II (17). Therefore, we assessed the response of components of this local system to muscle damage in vivo using immunohistochemistry (IHC). Under basal conditions AT1 and angiotensin (pan-angiotensin antibody) displayed weak, diffuse staining, suggesting low protein expression (Fig. 1, A and C). However, 7 days following injury, robust staining of angiotensin and AT1 was observed in newly regenerated fibers (fibers displaying central nuclei), demonstrating a clear activation of this system (Fig. 1, B and D). Interestingly, as regeneration proceeded, AT1 was downregulated, resulting in a mosaic staining pattern, whereby some fibers appeared completely positive, while others appeared completely negative for AT1 (Fig. 1, E and F). These results indicate that ANG II signaling through AT1 may play a role in the regenerative response to muscle injury.

Captopril treatment inhibits myonuclear accretion and induces hyperplasia. On the basis of the IHC results and our previous findings that inhibition of ANG II signaling resulted in decreased myofiber size following injury (16), we then investigated the role of ANG II signaling in the formation of new fibers and the accretion of nuclei into myofibers during regeneration. Analysis of H&E-stained cross sections demonstrated that, compared with controls, angiotensin-converting enzyme (ACE) inhibition significantly reduced the myonuclei-fiber ratio by 28%, 21 days following CTX injury (Fig. 2C). No differences were observed 10 days following injury (data not shown). Interestingly, when the total number of myofibers per TA was analyzed, it was discovered that captopril treatment also induced substantial hyperplasia with a 37% increase in total fiber number compared with controls (P < 0.05, Fig. 2D) 21 days following injury, while no differences were observed 10 days following injury (data not shown). This also translated into a 56% increased density of myofibers/mm² (Fig. 2E). Therefore, while controls appeared to reach a plateau in fiber number by 10 days, the captopril-treated mice continued to form myofibers until day 21. Interestingly, because of this robust increase in fiber content, the absolute estimated number of myoblasts (37, 40) and fusion-competent (differentiating) myoblasts, respectively (33, 38).

Statistical analysis. The number of nuclei and fibers, fiber density, and total number of muscle fibers per TA were calculated by two-way ANOVA. The number of myogenin-positive and Pax7-positive cells per 100 fibers, as well as all PCR analysis, were compared between groups by Student’s t-test. Significance was accepted at P < 0.05.
in control and captopril-treated mice. Ten days following injury, captopril treatment appeared to retain the expression of myoblast proliferative markers with significant 1.7- and 1.9-fold increases in Myf5 and Pax7 (Fig. 3, A and B), respectively, while repressing the expression of markers of differentiation (myogenin: 2.9-fold decrease; \( P < 0.05 \), Fig. 3D). We believe this gene expression profile is indicative of delayed differentiation in the captopril-treated animals.

**ACE inhibition decreases satellite cell content following CTX-induced injury.** We performed IHC staining of satellite cells to assess the effect of captopril treatment on muscle satellite cell content. Although no significant differences were observed in total satellite cell content (Pax7-positive cells, Control: 12.1 ± 1.2, Captopril: 11.7 ± 1.5 Pax7-positive cells/100 fibers; \( P = 0.9 \)), a strong trend (\( P = 0.063 \)) for a reduction in the proportion of myogenin-positive (differentiating) myoblasts was evident in the captopril-treated animals 10 days post-CTX injection (Fig. 4C) in agreement with the myogenin expression analysis. To lend further support to the idea that captopril treatment induced delayed differentiation, we assessed the expression of the embryonic isoform of myosin heavy chain (eMHC), which becomes rapidly downregulated upon fiber maturation and is only expressed in newly formed fibers (4). qRT-PCR analysis revealed a significant 3.5-fold higher expression of eMHC in the captopril-treated mice compared with the controls (Fig. 3E) 10 days post-CTX injection, demonstrating that the inhibition of myoblast differentiation resulted in delayed fiber maturation. We then examined whether captopril affected the basal content of satellite cells 21 days following injury. The proportion of Pax7-positive cells following captopril treatment significantly decreased by 25% compared with controls (Fig. 5C). In agreement, we also observed a significant reduction in the expression of MyoD (Fig. 3C) at the same time point. However, it should be noted that the absolute number of satellite cells/myoblasts in the TA may not have decreased with captopril treatment. For example, when estimating the total number of Pax7-positive cells (by multiplying the total number of fibers per section by the % of

of nuclei within the cross section (by calculating the nuclei per fiber multiplied by the total number of fibers) may not have been different between groups 21 days postinjury (control: 9214.8 ± 1352.4 vs. captopril: 9938.6 ± 284.3, \( P = 0.6 \)). Because the loss of the transforming growth factor-\( \beta \) family member myostatin has been strongly implicated in the hyperplasia of skeletal muscle fibers (8, 10, 25), we assessed the mRNA expression of myostatin-related genes, including myostatin, follistatin, activin IIb receptor, and follistatin-like protein-3. Surprisingly, with the exception of a trend for a decrease in the activin IIb receptor with captopril treatment 21 days postinjection (\( P = 0.067 \)), no significant differences were observed in the expression of the genes analyzed between groups (Supplemental Fig. S1). Although these data are not definitive, they indicate that ANG II may regulate muscle cell number through mechanisms independent of myostatin- and myostatin-related proteins. Collectively, these results indicate that a lack of ANG II signaling during muscle regeneration resulted in the de novo synthesis of additional myofibers that contained fewer myonuclei, likely through mechanisms independent of myostatin signaling.

**Blockade of ANG II production alters MRF gene expression.**

The formation and growth of myofibers following injury is dependent on appropriate function of the satellite cell pool, including adequate expansion, efficient differentiation, and the subsequent growth of de novo fibers (11). Therefore, we assessed the mRNA expression of the MRFs and the content of satellite cells/myoblasts 10 and 21 days following CTX injury.
Pax7-positive cells per fiber), no significant differences between control and captopril-treated mice were observed (192.44 ± 32.75, 188.23 ± 24.50, respectively, P = 0.96). Collectively, these results suggest that the observed reduction in myonuclei per fiber and altered fiber formation observed 21 days following injury with captopril treatment is due to alterations in myogenic precursor content and function.

**DISCUSSION**

The current data demonstrate that skeletal muscle possesses a local ANG II signaling system that functions in vivo to regulate muscle fiber formation, myonuclear addition, and fiber

![Fig. 3. ACE inhibition alters MRF gene expression following injury. qRT-PCR analysis of the expression of Myf5 (A), Pax7 (B), MyoD (C), myogenin (D), and eMHC (E) 10 and 21 days following CTX injection (n = 10 mice per group).](http://ajpregu.physiology.org/)

![Fig. 4. Captopril treatment inhibits myogenin expression. Representative IHC stains of myogenin 10 days following CTX injection within the TA of control (A) and captopril-treated (B) mice. C: analysis the proportion of myogenin-positive cells 10 days following injury (n = 10 mice per group).](http://ajpregu.physiology.org/)

![Fig. 5. ACE inhibition decreases in vivo satellite cell content. Representative IHC stains of Pax7, 21 days following CTX injection of control (A) and captopril-treated (B) mice. C: graphical representation of the proportion of Pax7-positive cells in control and captopril-treated mice 21 days following CTX injection (40× magnification, scale bar = 50 μm; n = 10 mice per group).](http://ajpregu.physiology.org/)
maturation during muscle regeneration. The myogenic effects of ANG II appear to be related to regulation of satellite cell differentiation. These findings implicate ANG II as a pleiotropic growth factor in skeletal muscle, with functions separate and independent of its classical role as a regulator of blood pressure and fluid homeostasis. We have previously reported that isolated primary and C2C12 myoblasts and myotubes express a local ANG II signaling system that is responsive to mechanical stimulation (17). We extend these findings by reporting that injection of CTX, and the subsequent regenerative response, activates key members of this local RAS. We report an increased production of angiotensinogen, and/or its metabolites, at the onset of regeneration using IHC. The absence of angiotensinogen in uninjured muscle followed by a robust upregulation and progressive downregulation over time suggests that the intramuscular angiotensin signaling system is not constitutively active under basal conditions and requires an appropriate cue for activation. This notion was further supported by the observation that the AT1 receptor shared the same temporal expression pattern as pan-angiotensin following injury. An intriguing observation was that the mosaic staining pattern of AT1 observed 21 days following injury was similar in injury. Although the exact molecular mechanisms underlying altered fiber formation and nuclear accretion are not understood, it is possible that an ACE inhibitor to the observed phenotype is the repression of satellite cell function. Adequate expansion of myogenic precursors is necessary to provide a sufficient source of nuclei to fuse with growing myofibers (3). This may potentially be limited by impaired ANG II signaling, as captopril treatment significantly decreased the proportion of Pax7-positive satellite cells 21 days following CTX-induced injury. We think that this is not the case, however; at 21 days following injury, the regenerative response was largely complete. Supporting this theory, we observed no difference in the number of Pax7-positive cells 10 days following injury, a more relevant time point during muscle regeneration. Therefore, we focused our analysis on the role of ANG II in the process of myoblast differentiation for a number of reasons. First, data presented in the current manuscript demonstrated that a defect in myoblast fusion could account for both the alterations observed in nuclear content and fiber formation. Second, differences in MyoD mRNA expression were only observed 21 days following CTX injection when myoblast proliferation was primarily completed. Third, our previous investigation (16) demonstrated that although ANG II treatment of quiescent cells can alter myogenic regulatory factor gene expression, ANG II does not directly affect myoblast proliferation, a process largely regulated by MyoD expression. This theory is supported by both qRT-PCR and IHC staining data, indicating that captopril-inhibited markers of satellite cell differentiation and decreased the number of differentiating myoblasts, while maintaining the expression of proliferative markers. How ANG II functions to regulate myoblast differentiation is currently unknown. However, ANG II has been demonstrated to activate signaling pathways necessary for myoblast differentiation in other cell types. For example, ANG II signaling in cardiomyocytes has recently been demonstrated to increase the activation and binding of members of the nuclear factor of the activated T-cell family (24, 30, 31), which have been identified as indispensable in regulating myoblast fusion (26). Moreover, ANG II increases the mRNA and protein content of vascular cell adhesion molecule-1 in rat aortic smooth muscle cells (34),...
which is expressed at sites of second-wave myogenesis in vivo and regulates myoblast differentiation in culture (29).

Perhaps most surprisingly, captopril treatment resulted in a significant increase in the total number of fibers in the TA 21 days after injury. Though skeletal muscle fiber hyperplasia has been previously observed using load-induced stretch of avian skeletal muscle, (1, 2), as well as strength training of cats (5, 6) and rodents (32), no reports exist documenting the induction of hyperplasia following severe injury. To our knowledge, this is the first report describing the induction of hyperplasia due to captopril treatment following CTX-induced injury. The functional significance and cellular and molecular mechanisms underlying captopril-induced hyperplasia are currently unknown. However, it is conceivable that the altered cell differentiation observed in the present study could result in hyperplasia. Alternatively, ANG II may not directly impact myoblast differentiation per se; the deficiency in myoblast fusion may ultimately be a consequence of the lack of ANG II-induced migration (16). For example, if myoblasts are unable to efficiently migrate and fuse with new myofibers, it is plausible that aberrant cell-to-cell fusion could occur, resulting in the synthesis of more fibers. This hypothesis is in agreement with our in vivo IHC staining, demonstrating the increased production of angiotensin following injury. We propose that ANG II may serve as the chemotactic signal directing the differentiation of myoblasts and allowing the subsequent growth of myofibers. This is also in line with results suggesting that the total estimated number of myonuclei within the cross section was not different between groups 21 days postinjection; similar numbers of myoblasts in each group ultimately underwent terminal differentiation. Although hyperplasia may be seen as a potentially beneficial adaptation, it should be considered that under the current experimental conditions, hyperplasia was accompanied by an inability to add nuclei to existing fibers, an inhibited maturation of newly formed myofibers, and a disrupted satellite cell response. As our study design only monitored regeneration until 21 days, it is unknown whether this captopril-induced hyperplasia is a persistent phenotype or one that will fade with time. The functional relevance of this observation is unclear at this time.

Clinically, ACE inhibitors and angiotensin receptor blockers (ARBs) are among the most commonly prescribed antihypertensive medications (9), particularly amongst older adults. Although generally considered safe, few studies have examined the direct effects of ACE inhibitors or ARBs on skeletal muscle function, adaptation to exercise, and muscle regeneration in humans. This is a particularly relevant issue, given that the majority of individuals on these drugs are already at risk of skeletal muscle loss as a result of aging. Delineating all potential secondary effects of these widely prescribed pharmaceuticals in “nonsignificant” tissues, such as skeletal muscle is an important endeavor.

In summary, the current investigation provides evidence that the activation of a local ANG II signaling system is an important component of skeletal muscle fiber formation and nuclear accretion following CTX-induced injury. This local system functions to regulate the differentiation of muscle satellite cells and maturation of new myofibers.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

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