Angiotensin type 2 receptor potentiates skeletal muscle regeneration*

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*Running title: AT2 receptor potentiates skeletal muscle regeneration

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Background: Angiotensin II induces skeletal muscle atrophy, but the function of other renin-angiotensin system components in skeletal muscle physiology is poorly understood.

Results: Angiotensin II type 2 receptor (AT2R) blockade inhibits skeletal muscle regeneration and myoblast differentiation.

Conclusion: AT2R positively regulates skeletal muscle regeneration.

Significance: Stimulation of AT2R signaling may be beneficial in muscle wasting conditions.

ABSTRACT

Patients with advanced congestive heart failure (CHF) or chronic kidney disease (CKD) often have increased angiotensin II (Ang II) levels and cachexia. Ang II infusion in rodents causes sustained skeletal muscle wasting and decreases muscle regenerative potential through Ang II type 1 receptor (AT1R)-mediated signaling, likely contributing to the development of cachexia in CHF and CKD. However, the potential role of Ang II type 2 receptor (AT2R) signaling in skeletal muscle physiology is unknown. We found that AT2R expression was robustly increased in regenerating skeletal muscle after cardiotoxin (CTX)-induced muscle injury in vivo and differentiating myoblasts in vitro, suggesting that the increase in AT2R played an important role in regulating myoblast differentiation and muscle regeneration. To determine the potential role of AT2R in muscle regeneration, we infused C57BL/6 mice with AT2R antagonist PD123319 (PD) during CTX-induced muscle regeneration. PD reduced the size of regenerating myofibers and expression of the myoblast differentiation markers myogenin and eMyHC. On the other hand, AT2R agonist CGP42112 (CGP) infusion potentiated CTX injury-induced myogenin and eMyHC expression and increased the size of regenerating myofibers. In cultured myoblasts, AT2R knockdown by siRNA suppressed myoblast differentiation marker expression and myoblast differentiation via upregulation of phospho-ERK1/2, and ERK inhibitor treatment completely blocked the effect of AT2R knockdown. These data indicate that AT2R signaling positively regulates myoblast differentiation and potentiates skeletal muscle regenerative potential, providing a new therapeutic target in wasting disorders such as CHF and CKD.

INTRODUCTION

Angiotensin II (Ang II), the main effector molecule of the renin-angiotensin system (RAS), has multiple physiological actions including regulation of blood pressure and salt/water balance through a variety of effects on the central nervous system, the adrenal gland, the vasculature and the kidney. Furthermore, the RAS plays an important role in the pathogenesis of cardiovascular disease, ranging from early endothelial dysfunction to congestive heart failure (CHF), renal or cerebrovascular disease (1). While most physiological effects of Ang II are mediated via the AT1 receptor, Ang II also signals via the AT2 receptor, whose function is incompletely understood.

There is increasing interest in potential effects of Ang II on skeletal muscle, not previously considered a major target organ for Ang II effects.
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Thus our group (2-9) and others (10-12) have shown that Ang II causes skeletal muscle wasting via increased protein breakdown and apoptosis and decreased protein synthesis. These findings have important clinical relevance since patients with chronic diseases such as advanced CHF or chronic kidney disease (CKD) often have increased Ang II levels (13-15) and cachexia, which independently worsens outcome (16). Indeed angiotensin-converting enzyme (ACE) inhibitor treatment improves weight loss in CHF patients (14) and Ang II type 1 receptor (AT1R) blockade prevents skeletal muscle atrophy in a rat model of CHF (17), suggesting that Ang II could play an important role in the development of cachexia and that the RAS could represent a common mechanism regulating skeletal muscle function in chronic diseases.

We have shown that most of the atrophic effects of Ang II on skeletal muscle are indirect, i.e., mediated by intermediate molecules including cytokines and glucocorticoids (4, 18), which is consistent with the fact that adult muscle fibers express little to no Ang II receptors (18, 19). However we have recently demonstrated that Ang II also acts directly on skeletal muscle via its effects on satellite cells (19). These mitotically quiescent mononuclear muscle stem cells are the key orchestrators of the regenerative response to injury. They become activated, proliferate, form myoblasts, and then terminally differentiate and fuse to form multinucleated myotubes, thereby repairing damaged myofibers (20). We found that satellite cells express high levels of AT1R, and that Ang II inhibits satellite cell proliferation via AT1R signaling, leading to depletion of the satellite cell pool and reduced muscle regenerative capacity in a cardiotoxin-injury induced model (19). We also found that differentiated satellite cells expressed the AT2R. Our findings were consistent with reports that AT1R blockade or AT1R deficiency was beneficial in mouse models of myopathy and injury (21, 22), although there are contradictory reports that an ACE inhibitor or AT1R ablation significantly impaired skeletal muscle regeneration (23, 24).

The focus of the current study was to determine the potential functional role of the AT2R in skeletal muscle. Our findings indicate that AT2R expression is dramatically increased in satellite cells during differentiation and that the AT2R is a critical positive regulator of myoblast differentiation and skeletal muscle regenerative processes.

EXPERIMENTAL PROCEDURES

Animals - Animal protocols were approved by the Institutional Animal Care and Use Committee at Tulane University. 8- to 10-week-old male C57BL/6 mice (Charles River) were used in this study. Ang II (Phoenix Pharmaceuticals, 1.5 µg/kg/min), PD123319 (Pfizer, 30 mg/kg/d) and CGP42112 (Sigma, 10 µg/kg/min) were administered via osmotic minipump (Alzet model 1007D). CTX was injected as previously described (25).

Antibodies and reagents - Antibodies used for immunoblotting included: anti-AT2R (Millipore, 1:500); anti-myogenin (abcam, 1:500); anti-desmin (Cell Signaling, 1:1000); anti-ERK1/2 (Cell Signaling, 1:1000); anti-phospho-ERK1/2 (Cell Signaling 1:2000); anti-p38MAPK (Cell Signaling, 1:1000); anti-phospho-p38MAPK (Cell Signaling, 1:1000); anti-MEK1/2 (SantaCruz, 1:200); anti-phospho-MEK1/2 (SantaCruz, 1:200); anti-MKP-1 (SantaCruz, 1:100); anti-JNK (Cell Signaling, 1:1000); anti-phospho-JNK (Cell Signaling, 1:2000) and anti-mouse β-actin (Sigma, 1:10000). Antibodies used for immunohistochemical staining included: anti-eMyHC (Developmental Studies Hybridoma Bank, 1:100); anti-fast skeletal myosin (Sigma, 1:100); anti-slow skeletal myosin (Sigma, 1:2000); anti-laminin (Sigma, 1:50); anti-mouse IgG-Alexa488 (Invitrogen, 1:500) and anti-rabbit IgG-Alexa594 (Invitrogen, 1:500). ERK inhibitor U0126 and PD98059 were purchased from Cell Signaling and used at the final concentration of 10 µM and 25 µM, respectively.

Immunohistochemical staining - Skeletal muscle slow and fast myosin were stained on paraffin (6 µm) sections using Mouse on Mouse (M.O.M) immunodetection kit (Vector laboratories) according to the manufacturer’s instruction. Sections were co-stained with laminin to identify each myofiber.

Primary satellite cell and C2C12 cell culture - Satellite cells were collected as previously described (26, 27). Briefly, hindlimb muscles (gastrocnemius, soleus, tibialis anterior and extensor digitorum longus) were incubated with 0.2% (w/v) collagenase type II in DMEM and
10% horse serum for 2 h at 37°C. Digested muscles were dissociated by triturating with Pasteur pipette, washed and further digested with 1 U/ml dispase for 1 h at 37°C. Cells were filtered, washed and incubated in proliferation medium (DMEM supplemented with 20% FBS (Mediatech), 10% HS, 2% chicken embryo extract (US Biological), 1 mM sodium pyruvate, 2 mM L-glutamine and penicillin/streptomycin) for 40 min to let the fibroblasts attach onto the plate and unattached cells were plated onto Matrigel-coated plates in the proliferation medium at the density of 1x10^4 cells/cm^2. C2C12 myoblasts (ATCC) were cultured in C2C12 growth medium (DMEM supplemented with 20% FBS, 1 mM sodium pyruvate and 2 mM L-glutamine). After satellite cells and C2C12 cells reached confluence, cells were switched to the differentiation medium (DMEM supplemented with 2% horse serum, 1 mM sodium pyruvate and 2 mM L-glutamine) to induce differentiation. Myoblast fusion index was calculated as the percentage of nuclei in MyHC-positive myotubes divided by total number of nuclei.

**Quantitative RT-PCR** - Quantitative RT-PCR was performed as previously described (5) and PCR primers used in this study were obtained commercially (SABiosciences, PPM04482A for myogenin, PPM41610F for eMyHC, PPM04811A for AT2R and PPM03559F for Hprt).

Measurement of cell proliferation, viability and apoptosis - Cells were plated on 96 well plates in the proliferation medium and cell proliferation, cell viability and caspase-3/7 activity were measured when cells reached confluence (day 0) and 24hrs after cells were switched to differentiation medium (differentiation-day 1). For cell proliferation analysis, anti-Notch antibody (Millipore, 1:300) was added in culture together with BrdU (10 µM) and incubated for 24hr. BrdU incorporation was measured using a BrdU cell proliferation ELISA kit (Roche) according to the manufacturer’s instruction. Cell viability and caspase-3/7 were measured using a CellTiter-Glo luminescent cell viability assay (Promega) and Caspase-Glo 3/7 assay (Promega), respectively, according to the manufacturer’s instructions. For apoptosis and cell viability assay, staurosporine (1 mM, Cell Signaling) and mitomycin C (0.4 mg/ml, Sigma), respectively, were added as a positive control.

siRNA electroporation in vivo and transfection in vitro - siRNAs used in this study were obtained from Life Technologies (Silencer Select siRNA). AT2R siRNA-a, s201014; AT2R siRNA-b, s62151; control siRNA, 4390843. For *in vivo* siRNA electroporation, mice were anesthetized by ketamine/xylazine, hindlimbs were shaved and 35 µl of siRNA (20 µM) was injected into 5-6 different locations of gastrocnemius muscle with a 22-gauge needle syringe (Hamilton model 705). Transcutaneous pulses were applied by two stainless steel plate electrodes (Caliper Electrode model 384, BTX) with a distance between the two plates of 0.5 cm, and conductive gel was used to ensure the electrical contact. Electric pulses with a standard square wave were delivered by an electroporator (ECM830 Electro Square Porator, BTX). Eight pulses of 100 V/cm were administered to the muscle with a delivery rate of 1 pulse/s with each pulse 20 ms in duration. *In vitro* siRNA transfection was performed using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instruction. Cells were transfected when they reached ~40% confluence, and the second transfection was performed on the following day to ensure a high efficiency of transfection. C2C12 myoblasts and primary satellite cells were induced to differentiate two days after the initial transfection.

**RESULTS**

**AT2R expression increases during muscle regeneration** - Consistent with previous reports (25), a number of myogenic transcription factors were upregulated in the hindlimb muscle after CTX injury. Myogenin mRNA expression reached a maximal level on d3, which preceded maximal induction of embryonic myosin heavy chain (eMyHC) expression, a marker of differentiating myotubes. Whereas there was no detectable AT2R expression in skeletal muscle before the CTX injury, AT2R mRNA level was robustly increased during regeneration and reached a maximal level on d5, coinciding with the expression of eMyHC (Fig. 1A). These data suggest that AT2R expression is induced during the process of satellite cell differentiation. Consistent with these data, AT2R mRNA and protein expression was highly upregulated in primary satellite cells and C2C12 myoblasts after cells were induced to differentiate (Fig. 1B, C). These data strongly
suggest that upregulation of AT2R expression during satellite cell differentiation may play an important role in muscle regenerative processes.

**AT2R antagonist reduces and AT2R agonist potentiates muscle regeneration in vivo** - The robust increase of AT2R expression during muscle regeneration in vivo and during satellite cell/C2C12 myoblast differentiation in vitro (Fig. 1) suggested that the AT2R plays an important role in regulation of satellite cell differentiation and muscle regeneration. To investigate the role of AT2R during muscle regeneration in vivo, we infused mice with the AT2R antagonist PD123319 (28) in the setting of CTX-induced injury (Fig. 2A). We found that PD123319 infusion significantly reduced the size of regenerating myofibers, whereas the number of regenerating myofibers was not altered (Fig. 2B, C). To gain more insight into the AT2R-mediated regulation of regenerating myofiber number and size, we analyzed the slow and fast fiber sizes in regenerating areas. As shown in Fig. 2B, the sizes of regenerating myofibers positive for slow and fast myosin were both reduced to the same extent, suggesting the effect of AT2R suppression is not fiber type specific. We also analyzed the number of regenerating myofibers based on the number of centralized nuclei in each myofiber (Fig. 2E). There was a significant reduction in the number of regenerating myofibers with two or more centralized nuclei, suggesting that AT2R suppression prevents myoblast fusion. Furthermore, these alterations of skeletal muscle regeneration were associated with a decline in the expression of myogenin and eMyHC (Fig. 2F, G). Next we infused the AT2R agonist CGP42112 in C57BL/6 mice after CTX injury to determine the effect of AT2R activation on muscle regeneration (Fig. 3A). In contrast to the effects of AT2R antagonism and AT2R knockdown (Fig. 2), the infusion of the AT2R agonist in the setting of CTX-induced injury increased the size of regenerating myofibers, without altering the total number of regenerating myofibers (Fig. 3B, C). The increase of regenerating myofiber size was not fiber-type specific (Fig. 3D, E) as in the case of AT2R antagonist infusion. We also found that there was an increase in the number of centralized nuclei in regenerating myofibers. These changes in regenerating myofibers after AT2R agonist infusion were associated with increased expression of myogenin (Fig. 3D) and eMyHC (Fig. 3E). These data strongly suggest that AT2R activation positively regulates satellite cell differentiation and potentiates muscle regeneration.

**AT2R regulates myoblast differentiation via ERK1/2 signaling** - To further investigate mechanisms whereby the AT2R regulates skeletal muscle regeneration the AT2R was knocked down both in vivo and in vitro using siRNA. AT2R siRNA was delivered to mouse gastrocnemius muscle via electroporation-mediated siRNA transfer (29). In this experimental condition, the electric pulse itself damages the skeletal muscle (5), which results in induction of myogenin, eMyHC and AT2R in a manner similar to that of CTX-induced injury (Fig. 4A-D). AT2R siRNA markedly blunted the injury-induced increase in AT2R expression (Fig. 4B), and significantly suppressed the increase of myogenin and eMyHC (Fig. 4C, D), consistent with our AT2R antagonist infusion experiment (Fig. 2). These data are consistent with our hypothesis that the AT2R regulates satellite cell differentiation and muscle regeneration. To obtain further insights we transfected AT2R siRNA to C2C12 cells to analyze the effect of AT2R knockdown on myoblast differentiation. As shown in Fig. 5A, AT2R siRNA (we used two independent AT2R siRNA; see methods for detailed information) blocked the induction of AT2R expression that occurred during normal C2C12 myoblast differentiation. AT2R knockdown did not alter apoptosis, viability or proliferation of C2C12 cells, either before or after induction of differentiation (Fig. 5C). Importantly, AT2R knockdown markedly reduced the induction of myogenin and desmin, consistent with our in vivo observations (Fig. 5A). It has been shown that MAPK signaling plays an important role in C2C12 myoblast differentiation (30). Also, it has been suggested that AT2R signaling attenuates ERK1/2 activity in vascular smooth muscle cells (31), whereas nothing is known about AT2R downstream signaling in satellite cells. We analyzed MAPK signaling pathways in C2C12 myoblasts and myotubes after AT2R siRNA transfection. There was no detectable difference in phosphorylation of p38MAPK and JNK in response to AT2R knockdown (Fig. 5A, B). The phosphorylation of ERK1/2 was downregulated at the initial stage of C2C12 cell differentiation, followed by a
progressive increase up to day 3 of differentiation, consistent with a previous report (30). Importantly, we found that phosphorylation of ERK1/2 was increased after AT2R knockdown throughout the process of myoblast differentiation (Fig. 5A, B). This increase of p-ERK1/2 was associated with increased phosphorylation of MEK, especially at the initial stages of differentiation, suggesting that ERK1/2 phosphorylation was regulated by MEK. To further explore the involvement of ERK1/2 in AT2R downstream signaling, ERK1/2 was inhibited by the ERK inhibitors U0126 and PD98059, together with AT2R knockdown (Fig. 6). As expected, AT2R knockdown markedly reduced myogenin and desmin expression in differentiating C2C12 cells. However inhibition of ERK1/2 phosphorylation by U0126 and PD98059 largely restored the induction of myogenin and desmin expression in the setting of AT2R knockdown, (Fig. 6A). The ability of ERK inhibition to rescue the AT2R siRNA-mediated reduction in C2C12 differentiation was further confirmed by immunohistochemical staining of eMyHC in differentiating myotubes (Fig. 6B, C). The fusion index (calculated as the percentage of nuclei in eMyHC-positive myotubes divided by total number of nuclei) was markedly decreased by AT2R knockdown and this decrease was completely abrogated by ERK inhibition. These data indicate that the AT2R positively regulates myoblast differentiation via downregulation of ERK1/2 signaling.

DISCUSSION

Although RAS components, such as angiotensinogen and angiotensin-converting enzyme (ACE) are expressed in the skeletal muscle microcirculation (32), and in myoblasts (33), there has been limited data on biological effects of Ang II or other components of the RAS on skeletal muscle. Ang (1-7), mainly produced from Ang II by ACE2 and signaling through the Mas receptor, has been reported to regulate skeletal muscle insulin sensitivity (34, 35), decrease fibrosis and improve muscle function in a mouse model of muscle dystrophy (36). It has been shown by our group (2-9) and others (10-12) that Ang II causes skeletal muscle wasting via increased protein breakdown and apoptosis and decreased protein synthesis. Most of these effects of Ang II are indirect, i.e., mediated by intermediate molecules including cytokines and glucocorticoids (4, 18), which is consistent with the fact that adult muscle fibers express little to no Ang II receptors (18, 19). However recently we discovered that satellite cells express the AT1R and that Ang II acts via these receptors to prevent satellite cell proliferation and suppress muscle regeneration (19). This mechanism could be an important contributory factor to the loss of skeletal muscle that occurs in many clinical conditions in which Ang II levels are elevated. A critical unanswered question is the potential role of the AT2R in skeletal muscle regeneration. Here we show that there is coordinated temporal expression of AT2R in differentiating muscle stem cells. AT2R expression is not detectable in primary satellite cells and in C2C12 myoblasts basally but is strongly induced during differentiation (Fig. 1). Inhibition of AT2R by the AT2R antagonist PD123319 (Fig. 2) and by AT2R siRNA (Fig. 4-6) markedly inhibited myoblast differentiation and skeletal muscle regeneration, whereas the AT2R agonist CGP42112 potentiated muscle regeneration (Fig. 3). We found that the number of centralized nuclei in regenerating myofibers was reduced and increased by PD123319 and CGP42112, respectively, suggesting that AT2R regulates myoblast fusion processes (Fig. 2E and 3E). We also found that the effect of AT2R activation/inactivation on muscle regeneration is not fiber type specific (Fig. 2D and 3D). During differentiation of myoblasts, phosphorylation of ERK1/2 was initially downregulated, followed by an increase during myotube formation (Fig. 5). AT2R knockdown increased p-ERK1/2 in all the stages of differentiation, indicating that the AT2R negatively regulated ERK1/2 signaling. Indeed, blockade of ERK1/2 signaling by the ERK inhibitors U0126 or PD98059 restored myoblast differentiation in the setting of AT2R knockdown (Fig. 6).

To our knowledge this is the first report establishing a functional role of the AT2R in skeletal muscle regeneration. In fact very little is known about the potential role of the AT2R in skeletal muscle biology, although a recent report has suggested that the AT2R may be involved in fibrotic responses in dystrophic skeletal muscle (37). While AT2R expression is very low in rodent adult tissues, AT2R is reported to be the dominant Ang II receptor expressed in the fetus. In many
tissues such as the adrenal gland, kidney, heart, aorta, skin and skeletal muscle, AT2R expression appears on embryonic days 11 to 13 (E11-E13) and reaches a maximal level on E19 (38). AT2R expression then rapidly declines in newborn animals to low or undetectable levels. Interestingly, AT2R expression in the adult reappears in pathophysiological conditions such as vascular injury and cardiac remodeling (39, 40). In some tissues such as vascular smooth muscle the AT2R is reported to mediate effects counterbalancing those mediated by the AT1R, e.g., vasodilatation, NO release and inhibition of proliferation and growth (38, 41) . Furthermore, it has been shown that the AT2R regulates neuronal cell differentiation and nerve regeneration following crush injury of rat sciatic nerve (42). Thus the AT2R appears to play an important role in multiple organ development and in neural regeneration. However, the molecular mechanisms whereby the AT2R may regulate tissue regeneration are poorly understood. Our data clearly show that the AT2R positively regulates satellite cell differentiation and skeletal muscle regeneration via downregulation of ERK1/2 signaling. Considering the strong expression of AT2R during embryonic development and during neuronal differentiation, one can speculate that a similar signaling mechanism is important for AT2R-mediated effects on cellular differentiation in other tissues.

Both AT1R and AT2R belong to the seven-transmembrane domain receptor superfamily, they have 31% amino acid identity and bind to Ang II with nanomolar affinity, but downstream signaling pathways are vastly different (38, 43). AT1R is a classical G protein-coupled receptor and signals through Gq subunit and activates MAPKs. On the other hand, although the AT2R can couple to G proteins, in many cases it activates signaling cascades independent of G proteins. The major AT2R signaling transduction pathways include activation of protein phosphatases such as MAP kinase phosphatase-1 (MKP-1), activation of the NO/cGMP system and stimulation of phospholipase A2 with subsequent release of arachidonic acid (38, 44). Activation of protein phosphatases is of particular interest, because it has been shown that MAPK signaling may play an important role in satellite cell proliferation and differentiation (30, 45-47), although some studies are conflicting. Bennett et al. showed that MKP-1 regulates ERK2 activity differently depending on the myoblast differentiation stages (30). ERK2 is inactivated upon mitogen withdrawal (differentiation cue), concomitant with upregulation of muscle specific genes, and this ERK2 inactivation is regulated by MKP-1. On the other hand, endogenous MKP-1 expression is decreased at later stage of differentiation, and MKP-1 overexpression during differentiation prevented myotube formation. Contrary to these data, Jones et al. reported that inactivation of the ERK1/2 pathway did not affect myoblast differentiation, whereas it prevented myoblast proliferation (46). Also, Jones et al. showed that inhibition of p38MAPK promotes satellite cell quiescence and prevents differentiation (47). Whereas the basal activity of ERK1/2 changes dramatically during satellite cell differentiation, our data clearly show that the AT2R negatively regulates ERK1/2 signaling at all the stages of differentiation (Fig. 5A, B), and that ERK inhibitors prevented the reduction in regeneration caused by AT2R knockdown (Fig. 5, 6). Mechanism whereby ERK1/2 may differentially regulate satellite cell differentiation depending on the temporal stages of differentiation remains to be determined. In our experimental setting AT2R and ERK1/2 were inhibited throughout the differentiation processes, and future studies are required to precisely understand the AT2R’s function in satellite cells, especially in different stages of differentiation. In our experimental setting we did not observe changes in p38MAPK and JNK signaling during differentiation, and AT2R knockdown did not affect these pathways. Although AT2R knockdown led to an increase in phospho-MEK and phospho-ERK1/2, we did not detect changes in MKP-1 levels during differentiation and after AT2R siRNA transfection, suggesting that alteration of ERK1/2 activity was regulated independently of MKP-1. Interestingly, In ERK inhibitor treated cells, AT2R expression was increased both basally (1.87±0.20 fold increase in control siRNA+U0126 vs. control siRNA, N=3, p<0.05) and in the presence of AT2R siRNA (3.87±0.87 fold increase in AT2R siRNA+U0126 vs. AT2R siRNA, N=3, p=0.05), and AT2R and myogenin/desmin expression were highly correlated, suggesting that there is a positive
feedback loop whereby the AT2R and ERK1/2 interact to regulate myoblast differentiation. Namely, the AT2R inhibits ERK1/2 activity, which promotes differentiation, and the reduction in ERK1/2 activity increases AT2R expression, further sustaining myoblast differentiation (Fig. 7).

An important question raised by our findings is the mechanisms regulating AT2R expression during myoblast differentiation. Our data indicate that the AT2R is strongly induced during myoblast differentiation both transcriptionally and translationally. Little is known about AT2R transcriptional regulation. Ichiki et al. showed that the DNA segment between -1497 and -874 upstream of the AT2R transcription start site was important for basal AT2R expression in mouse fibroblast R3T3 cells (48), whereas it was suppressive to basal AT2R expression in rat pheochromocytoma PC12W cells (49). These data suggest that there are different mechanisms regulating AT2R expression in different cell types, but to date no transcription factors have been identified that directly regulate AT2R expression in any cells/tissues. Future studies identifying cis and transacting transcriptional pathways involved in regulation of AT2R expression will be critical to further determine mechanisms underlying AT2R-mediated satellite cell differentiation.

In summary, here we report for the first time that the AT2R plays a critical role in satellite cell differentiation and skeletal muscle regeneration. Expression of the AT2R is markedly induced in differentiating skeletal myoblasts, and the AT2R positively regulates myoblast differentiation via downregulation of ERK1/2 activity. Taken together with our recent findings that Ang II AT1R signaling depletes the satellite cell pool and inhibits muscle regeneration one can postulate that the balance between AT1R and AT2R signaling is critical for normal muscle regeneration. Since Ang II levels are elevated in chronic conditions, such as CHF and CKD, that are accompanied by myopathy, potential reductions in AT2R signaling could be particularly detrimental by resulting in preferential AT1R signaling. In fact, we have recently found that in a mouse model of myocardial infarction there is a markedly blunted increase in AT2R expression following injury, and that this relative reduction in AT2R expression correlates with markedly reduced muscle regeneration.

Skeletal muscle satellite cells could provide an excellent model system for the analysis of AT2R function in regulating tissue regeneration. Considering the AT2R’s function to potentiate myoblast differentiation, future studies assessing mechanisms of AT2R regulation (e.g. identification of upstream transcription factors) have the potential to lead to the development of novel interventions to potentiate skeletal muscle regenerative capacity, particularly in chronic disease conditions.
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Figure Legends

Figure 1. AT2R expression is increased during skeletal muscle regeneration and satellite cell differentiation

(A) The number of regenerating myofibers (myofibers with centralized nuclei) after CTX injection (top panel, with representative pictures). Gastrocnemius muscle was injected with CTX on d0 and muscles were harvested on d3, 5 and 7. Paraffin sections were prepared from the middle of the gastrocnemius muscles and regenerating myofiber number was quantified per section. For qRT-PCR analysis (bottom panel), RNA was extracted from the whole gastrocnemius muscles. (B) The expression of AT2R during primary satellite cell (SC) and C2C12 myoblast differentiation. Primary satellite cells were collected from C57BL/6 mouse hindlimb muscles and cultured in proliferation medium. After cells reached confluence (d0), the medium was switched to differentiation medium and cells were harvested after 1, 2 and 3 days. C2C12 myoblasts were cultured and harvested in the same condition as primary satellite cells. AT2R, myogenin and desmin expression was analyzed by immunoblotting. (C) qRT-PCR analysis of AT2R, myogenin and eMyHC in the same conditions as in (B). N=5, mean±SEM, *p<0.05, **p<0.01 compared to day 0.

Figure 2. AT2R antagonist inhibits skeletal muscle regeneration in vivo

(A) Experimental model. CTX was injected and AT2R antagonist PD123319 (PD) infusion (30 mg/kg/d) was started on day 0. Gastrocnemius muscles were collected on d3, 5 and 7. For H&E staining, paraffin sections were prepared from the middle of the gastrocnemius muscles. For qRT-PCR analysis, RNA was extracted from whole gastrocnemius muscle. (B) Number of regenerating myofibers (myofibers with centralized nuclei) after CTX injection and PD infusion on d5. The number of regenerating myofibers are shown per section. (C) CSA of regenerating myofibers after CTX injection and PD infusion on d5. (D) The same sections as in (C) were stained for skeletal muscle slow and fast myosin, and the seize of the regenerating myofibers was quantified. (E) The number of regenerating myofibers was analyzed on the same sections as in (B) and categorized based on the number of centralized nuclei in each myofiber. (F, G) qRT-PCR analysis of myogenin (D) and eMyHC (E) in the gastrocnemius muscle after CTX injection and PD infusion on d0, 3, 5 and 7. N=5-6. Mean±SEM. *p<0.05.

Figure 3. AT2R agonist potentiates skeletal muscle regeneration in vivo

(A) Experimental model. CTX was injected and AT2R antagonist CGP42112 (CGP) infusion (10 µg/kg/min) was started on day 0. Gastrocnemius muscles were collected on d3, 5 and 7. For H&E staining, paraffin sections were prepared from the middle of the gastrocnemius muscles. For qRT-PCR analysis, RNA was extracted from whole gastrocnemius muscle. (B) Number of regenerating myofibers (myofibers with centralized nuclei) after CTX injection and CGP infusion on d5. The number of regenerating myofibers are shown per section. (C) CSA of regenerating myofibers after CTX injection and CGP infusion on d5. (D) The same sections as in (C) were stained for skeletal muscle slow and fast myosin, and the seize of the regenerating myofibers was quantified. (E) The number of regenerating myofibers was analyzed on the same sections as in (B) and categorized based on the number of centralized nuclei in each myofiber. (F, G) qRT-PCR analysis of myogenin (D) and eMyHC (E) in the gastrocnemius muscle after CTX injection and CGP infusion on d0, 3, 5 and 7. N=5-6. Mean±SEM.
AT2 receptor potentiates skeletal muscle regeneration

*\( p<0.05 \).

**FIGURE 4. AT2R knockdown inhibits skeletal muscle regeneration in vivo**

(A) Experimental model. siRNA electroporation was performed on day 0. In this experimental condition, the electric pulse damages muscles and induces regeneration. Gastrocnemius muscles were collected on d3, 5 and 7, and RNA was extracted. (B-D) qRT-PCR analysis of AT2R (B), myogenin (C) and eMyHC (D) in the gastrocnemius muscle after siRNA electroporation on d0, 3, 5 and 7. N=5-6. Mean±SEM. *\( p<0.05 \)

**FIGURE 5. AT2R knockdown inhibits myoblast differentiation**

(A) Immunoblotting of myoblast differentiation markers and MAPK signaling components in C2C12 cells after AT2R knockdown. C2C12 myoblasts were cultured in proliferation medium and transfected with AT2R or control siRNA. Two independent AT2R siRNAs were used, termed AT2R-a (Aa) and AT2R-b (Ab). After cells reached confluence (d0), the medium was switched to differentiation medium and cells were harvested after 1, 2 and 3 days. (B) Quantification of p-ERK1/2, p-p38MAPK and p-JNK calculated from (A). Data are presented as the ratio to total level of corresponding protein. (C) Cell proliferation, apoptosis and viability in C2C12 cells. C2C12 myoblasts were transfected with control (C) or AT2R (A) siRNA and cell proliferation, apoptosis and viability were measured before and 1 day after induction of differentiation. Anti-Notch antibody (N), staurosporin (S) and mitomycin C (M) were used as positive control for cell proliferation, apoptosis and cell viability, respectively. (–) indicates non-transfected control. Mean±SEM. N=3 and 8 in (B) and (C), respectively. *\( p<0.05 \), **\( p<0.01 \).

**FIGURE 6. AT2R positively regulates myoblast differentiation via ERK1/2 signaling**

(A) Immunoblotting of myoblast differentiation markers of C2C12 cells. C2C12 myoblasts were cultured in proliferation medium, transfected with AT2R siRNA and medium switched to proliferation medium ±ERK1/2 inhibitors U0126 and PD98059 (PD). After cells reached confluence, the medium was switched to differentiation medium and cells were harvested after 24 hr. (B) Immunohistochemical staining of eMyHC in C2C12 myotubes. C2C12 myoblasts were cultured in the same condition as in (A), and myotube formation was analyzed 2 days after differentiation. (C) Fusion index (calculated as the percentage of nuclei in eMyHC-positive myotubes divided by total number of nuclei) of C2C12 myotubes calculated from (B). Mean±SEM, N=5, **\( p<0.01 \).

**FIGURE 7. Proposed model for AT2R-mediated regulation of satellite cell differentiation**

Initial cue(s) directing differentiation lead to rapid upregulation of AT2R expression. During satellite cell differentiation, ERK1/2 phosphorylation is suppressed at the initial stage of differentiation, followed by an increase in the later stage (shown in dotted line). AT2R inhibits ERK1/2 signaling at all the stages of differentiation, resulting in activation of myogenic gene expression (myogenin, desmin) and myoblast differentiation. ERK1/2 inhibition increases AT2R expression, suggesting a positive-feedback regulation. It is of note that it has been suggested that ERK1/2 positively regulates myotube formation in the later stage of differentiation, independently from the regulation of differentiation markers (not shown in the figure, see Discussion).
Figure 1

A

![Graph showing the number of regenerating myofibers (fibers/section) over time (days after CTX injury). The graph includes a bar chart illustrating fold change over time.](image)

B

|       | SC  | C2C12 |
|-------|-----|-------|
| d0    |     |       |
| d1    |     |       |
| d2    |     |       |
| d3    |     |       |

AT2R

myogenin

desmin

β-actin

C

![Graphs showing fold change over time for myogenin, eMyHC, and AT2R in SC and C2C12 cells.](image)
Figure 2

A. (**PD**123319) CTX

B. CTX injury

C. CTX harvest

D. CTX osmotic minipump

E. CTX qRT-PCR

F. CTX & CSA

G. CTX

*) *p < 0.05

**p < 0.01

ns = not significant

- Sham
- PD
- Sham+CTX
- PD+CTX
Figure 4

A diagram showing the timeline of siRNA treatment and harvest.

Graphs showing the fold change of AT2R and other myogenic markers (myogenin and eMyHC) over time with siRNA treatment compared to control siRNA.

- **AT2R**
  - Time (d): 0, 1, 2, 3, 4, 5, 6, 7
  - Fold change: 0, 50, 100, 150, 200
  - Legend: Control siRNA, AT2R siRNA

- **myogenin**
  - Time (d): 0, 1, 2, 3, 4, 5, 6, 7
  - Fold change: 0, 10, 20, 30, 40
  - Legend: Control siRNA, AT2R siRNA

- **eMyHC**
  - Time (d): 0, 1, 2, 3, 4, 5, 6, 7
  - Fold change: 0, 50, 100, 150, 250
  - Legend: Control siRNA, AT2R siRNA

Note: The graphs show significant changes marked by asterisks (** and *).
Figure 5

A) Differentiation day 0

| siRNA | d0 | d1 | d2 | d3 |
|-------|----|----|----|----|
| C     | C  | C  | C  |     |
| Aa    | Aa | Aa | Aa |     |
| Ab    | Ab | Ab | Ab |     |

- AT2R
- myogenin
- desmin
- p-ERK1/2
- ERK1/2
- p-MEK
- MEK
- MKP-1
- p-p38 MAPK
- p38 MAPK
- p-JNK
- JNK
- β-actin

B) Graphs showing the expression of p-ERK/ERK, p-p38MAPK/p38MAPK, and p-JNK/JNK over time (day).

C) Differentiation day 0

- **Cell proliferation**
- **Apoptosis**
- **Cell viability**
Figure 6

A

| siRNA | DMSO | U0126 | PD |
|-------|------|-------|----|
| C     | A    | C     | C  |
| AT2R  |      |       |    |
| myogenin |     |       |    |
| desmin |      |       |    |
| p-ERK1/2 |    |       |    |
| ERK1/2 |      |       |    |

B

Control siRNA

AT2R siRNA

C

Fusion index (%)

**ns ns ns**

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Figure 7

AT2R

Ang II

MEK

ERK1/2

myoblast differentiation

myogenin etc.

Differentiation Cue

AT2R

ERK1/2

MEK

Ang II
Angiotensin Type 2 Receptor Signaling In Satellite Cells Potentiates Skeletal Muscle Regeneration
Tadashi Yoshida, Tashfin S. Huq and Patrice Delafontaine

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