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Losartan Restores Skeletal Muscle Remodeling and Protects Against Disuse Atrophy in Sarcopenia

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Abstract

Sarcopenia, a critical loss of muscle mass and function because of the physiological process of aging, contributes to disability and mortality in older adults. It increases the incidence of pathologic fractures, causing prolonged periods of hospitalization and rehabilitation. The molecular mechanisms underlying sarcopenia are poorly understood, but recent evidence suggests that increased transforming growth factor–β (TGF-β) signaling contributes to impaired satellite cell function and muscle repair in aged skeletal muscle. We therefore evaluated whether antagonism of TGF-β signaling via losartan, an angiotensin II receptor antagonist commonly used to treat high blood pressure, had a beneficial impact on the muscle remodeling process of sarcopenic mice. We demonstrated that mice treated with losartan developed significantly less fibrosis and exhibited improved in vivo muscle function after cardiotoxin-induced injury. We found that losartan not only blunted the canonical TGF-β signaling cascade but also modulated the noncanonical TGF-β mitogen-activated protein kinase pathway. We next assessed whether losartan was able to combat disuse atrophy in aged mice that were subjected to hindlimb immobilization. We showed that immobilized mice treated with losartan were protected against loss of muscle mass. Unexpectedly, this protective mechanism was not mediated by TGF-β signaling but was due to an increased activation of the insulin-like growth factor 1 (IGF-1)/Akt/mammalian target of rapamycin (mTOR) pathway. Thus, blockade of the AT1 (angiotensin II type I) receptor improved muscle remodeling and protected against disuse atrophy by differentially regulating the TGF-β and IGF-1/Akt/mTOR signaling cascades, two pathways critical for skeletal muscle homeostasis. Thus, losartan, a Food and Drug Administration–approved drug, may prove to have clinical benefits to combat injury-related muscle remodeling and provide protection against disuse atrophy in humans with sarcopenia.

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INTRODUCTION

Sarcopenia refers to the physiological loss of skeletal muscle mass and function during aging (1). Several age-related changes occur in skeletal muscle including a decrease in myofiber size and number and a diminished ability of satellite cells to activate and proliferate upon injury, leading to impaired muscle remodeling (2, 3). The progressive loss of muscle mass poses health risks for older adults that lead to a decrease in physical activity and a rise in the incidence of falls and related fractures. Rehabilitation time is often prolonged after injury, which in turn extends the duration of bed rest leading to disuse atrophy, an additional variable interfering with successful recovery (4).

Sarcopenia is a major public health problem affecting about 25% of people younger than 70 years and 40% of people aged 80 years and older (5). In 2000, sarcopenia-related healthcare expenses totaled about $18.5 billion in the United States (6). Considering the impact of sarcopenia on the well-being of older adults and the healthcare system in general, it is critical to identify therapeutic strategies to maintain skeletal muscle homeostasis and repair.

The molecular mechanisms underlying sarcopenia are largely unknown. One theory attributes the loss of muscle mass to an age-related increase in transforming growth factor–β (TGF-β) signaling (3). Increased TGF-β activity inhibits satellite cell activation (3, 7), impairs myocyte differentiation (7, 8), and leads to the formation of fibrotic tissue in response to skeletal muscle injury (9). TGF-β is known to signal through its canonical (Smad-dependent) and noncanonical (Smad-independent) pathways. The Smad-dependent pathway leads to phosphorylation of Smad2, Smad3, or both, which then binds to Smad4, and this complex translocates into the nucleus where it activates and represses transcription (10). The noncanonical TGF-β cascade signals through the mitogen-activated protein (MAP) kinase pathway, which includes the extracellular signal–regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinases, and p38 (11). Changes in the canonical and noncanonical TGF-β signaling pathways contribute to different aspects of impaired muscle regeneration and sarcopenia. In particular, they have been implicated in the inhibition of various myogenic regulatory factors (MRFs), leading to insufficient regeneration and the formation of tissue fibrosis (3, 12–14).

Observations regarding the expression profile of the canonical TGF-β signaling pathway in disuse atrophy are controversial (15, 16). In contrast, it is well documented that loss of muscle mass during disuse in young and aged skeletal muscle is associated with an increase of the noncanonical TGF-β (MAP kinase) pathway (17–19). Notably, sarcopenic muscle lacks the ability to sufficiently recover from disuse-induced atrophy as compared to young muscle (19).

Previous studies have shown that the administration of losartan, an angiotensin II type 1 (AT1) receptor blocker, inhibits canonical TGF-β signaling activity and promotes muscle remodeling in mouse models of Marfan syndrome (MFS) and dystrophin-deficient Duchenne muscular dystrophy (DMD) (20). Furthermore, treatment with losartan after infliction of muscle injury also improved regeneration in normal adult murine skeletal muscle by reducing fibrotic tissue formation (21).

Considering the proven benefits of losartan on muscle physiology, we evaluated whether administration of losartan would have an impact on two common ailments affecting skeletal muscle of sarcopenic individuals, impaired muscle remodeling after injury and disuse atrophy, using an aging mouse model. Our data demonstrate that losartan facilitated the remodeling of sarcopenic skeletal muscle after injury and protected it from disuse atrophy during immobilization. Our findings indicate that losartan exerted its effects by modulating multiple pathways critical for skeletal muscle homeostasis.

RESULTS

Losartan improves muscle remodeling and in vivo function in sarcopenic mice

Sarcopenia is characterized by impaired regeneration that results in the replacement of skeletal muscle with fibrotic tissue upon injury. To determine whether losartan modulates muscle remodeling in sarcopenia, we treated 21-month-old mice with either losartan or placebo and subsequently injected them with cardiotoxin (CT) in the tibialis anterior (TA) muscle. Aged mice that were neither injected with CT nor treated with losartan or placebo were used as a control (fig. S1). At 4 days after CT-induced injury, both losartan- and placebo-treated muscles showed signs of muscle injury and early indications of regeneration (Fig. 1A). The number of muscle fibers expressing developmental myosin, a marker for regenerating muscle cells, was similar between the losartan- and the placebo-treated groups (Fig. 1B and fig. S1C). By 19 days after CT injury, placebo-treated animals exhibited impaired muscle remodeling with large areas of fibrosis (Fig. 1, C and D). In contrast, losartan-treated mice displayed significantly less fibrotic tissue and overall improved muscle architecture in response to muscle injury (Fig. 1, C and D).

To ascertain the function of the muscle after regeneration, we tested the in vivo functional performance of the ankle dorsiflexor muscle (TA) as previously described (22, 23). The ratio of tetanic to twitch tension, a sensitive measure of muscle function (24–26), was used to evaluate the effect of losartan and placebo on functional recovery. Animals treated with losartan had a significant increase in the tetanic/twitch ratio compared to the placebo-treated animals, indicating that losartan enhanced functional recovery 19 days after CT injury (Fig. 1E). Thus, losartan treatment significantly improved muscle remodeling and functional recovery in sarcopenic mice.

AT1 receptor blockade modulates canonical and noncanonical TGF-β signaling cascades

Impaired regeneration of aged muscle is, at least in part, caused by an age-related increase in canonical TGF-β signaling that results in insufficient satellite cell activation in response to injury (3). Evidence suggests that alterations of the noncanonical TGF-β signaling cascade also contribute to the pathogenesis of sarcopenia (14). Because losartan has been shown to mediate the canonical and noncanonical TGF-β cascades (fig. S2) (20, 27–30), we assessed the expression pattern of downstream targets of both pathways in our mice. At 4 days after CT injury, there was an injury-related increase in phospho-Smad2 (pSmad2) and phospho-ERK (pERK) protein levels in the placebo- and losartan-treated mice (Fig. 2A). The levels of pSmad2 and pERK remained elevated 19 days after CT injury in the placebo group but were significantly reduced in the losartan-treated group (Fig. 2, B and D to F). Furthermore, we observed a decrease in the expression of phospho-p38 (p-p38) in the placebo-treated group at 4 days after CT when compared to the noninjected control and losartan-treated animals (Fig. 2, A and C). Thus, losartan-mediated modulation of canonical and noncanonical TGF-β signaling during later stages of muscle remodeling reduced fibrotic tissue formation and improved muscle function after infliction of muscle injury.

Modulation of canonical and noncanonical TGF-β signaling affects expression of MRFs

Canonical and noncanonical TGF-β signaling pathways play a role in muscle regeneration and repair by regulating the MRFs (31–33). Upon muscle injury, Pax7 is expressed in activated and proliferating satellite cells, whereas MyoD is mainly restricted to cycling myoblasts. In contrast, myogenin is critical for the differentiation and fusion of myocytes into myofibers (34, 35). Myoblast expression of p21, which permits cells to irreversibly withdraw from the cell cycle, is necessary for muscle differentiation (36). Therefore, the expression levels of Pax7, MyoD, myogenin, and p21 were analyzed.

Expression of p21 and myogenin was decreased in both the placebo-and the losartan-treated groups at 4 days after CT injury (Fig. 3A). This decrease was expected because these proteins are not necessary for the early muscle regeneration response, but are critical for late-stage muscle differentiation, which occurs after the initial satellite cell proliferation. In contrast, we observed significant differences in the expression of MRFs between placebo- and losartan-treated mice at later stages in the muscle remodeling process. Expression of Pax7 and MyoD remained elevated 19 days after CT injury in the placebo-treated animal but returned to baseline levels in the losartan-treated animal (Fig. 3B). Conversely, myogenin and p21 expression were significantly increased in the losartan-treated animals as compared to the placebo-treated animals at 19 days after CT (Fig. 3, B to D). Together, these data suggest that aged regenerating skeletal muscle was unable to transition from a proliferation stage of satellite cells into the differentiation process of muscle fibers. Modulation of the canonical and noncanonical TGF-β signaling cascades restored the necessary down-regulation of Pax7 and MyoD and up-regulation of p21 and myogenin at the muscle differentiation stage, which permitted successful remodeling of muscle injury.

Losartan prevents disuse atrophy in sarcopenic mice

Previous evidence suggests that skeletal muscle atrophy caused by disuse is exaggerated during aging (37). Furthermore, immobilization using different techniques is associated with transient alterations of the canonical (15) and noncanonical TGF-β signaling pathways (19, 38). We therefore evaluated whether losartan would have beneficial effects on skeletal muscle of 21-month-old mice subjected to immobilization of the TA muscle for 21 days using a surgical staple procedure (39).

Immobilization caused a significant 16% decrease in the wet TA weight of the placebo-treated group as compared to the contralateral TA that was not observed when comparing the immobilized and con-tralateral TA of the losartan-treated groups (Fig. 4C and fig. S3). His-tological analyses of immobilized muscles revealed small areas of fibrosis in the placebo-treated mice (Fig. 4A). Unexpectedly, detailed morphometric analyses of the minimum Feret's diameter (MFD) did not reveal a difference in muscle fiber size in either TA of the control, placebo-treated, or losartan-treated mice (Fig. 4, A and D, and figs. S4 and S5). This result suggests that actual loss of muscle fibers rather than simple atrophy, generally observed in young mice (39), may be responsible for the differences in wet muscle weight. Therefore, we assessed the cross-sectional area (CSA) of the intact TA and the total myofiber number in our animals. Both CSA and total myofiber number were significantly reduced in placebo-treated mice by 35 and 33%, respectively (Fig. 4, B, E, and F, and fig. S3). In contrast, losartan treatment prevented the decrease of CSA, and mice exhibited a total myo-fiber count similar to that of the nonimmobilized control group (Fig. 4, B, E, and F). In summary, losartan protected aged mice against disuse atrophy by preventing loss of muscle fibers rather than preventing atrophy of the individual myofibers.

AT1 receptor blockade mediates the IGF-1/Akt/mTOR signaling cascade in skeletal muscle

We next evaluated whether the protective effect of losartan on disuse atrophy was due to the modulation of the canonical and noncanonical TGF-β signaling pathways. There were no significant changes observed in the expression of pSmad2 or pERK protein levels in the immobilized TA of the placebo- and losartan-treated mice (Fig. 5A). There was, however, a significant decrease of p-p38 in the TA of the losartan-treated mice as compared to the nonimmobilized control and placebo-treated mice (Fig. 5, A and B).

Because losartan did not seem to affect the regulation of the canonical and noncanonical TGF-β signaling cascades, we next analyzed the expression of the insulin-like growth factor 1 (IGF-1)/Akt/mammalian target of rapamycin (mTOR) signaling cascade. A previous study

indicated that losartan is able to mediate the activity of the IGF-1/Akt/mTOR pathway (40). Additionally, this pathway is known to play a pivotal role in regulating muscle mass (41) and is generally decreased in muscle atrophy induced by immobilization (19, 38). As expected, the IGF-1/Akt/mTOR pathway was down-regulated in the placebo-treated TA muscle subjected to immobilization (Fig. 6, A to E). In contrast, we observed a significant increase in the expression of phospho-Akt (pAkt), phospho-FoxO3a (pFoxO3a), phospho-mTOR (p-mTOR), and phospho–4E-BP1 (p4E-BP1) in the losartan-treated animals as compared to the placebo-treated animals (Fig. 6, A to E). Thus, increased expression of the IGF-1/Akt/mTOR pathway in the losartan-treated mice likely mediated protection against the loss of muscle mass during immobilization; this indicates that blockade of the AT1 receptor in skeletal muscle can affect multiple pathways critical for the maintenance of muscle mass and homeostasis.

DISCUSSION

Preservation of skeletal muscle mass is achieved by maintaining a homeostatic balance between muscle regeneration, protein synthesis, and protein degradation. This balance is significantly perturbed during the physiological process of aging, leading to a loss of muscle mass and a decline in function over time. The decrease in the ability to regenerate after injury and the exaggerated atrophic response to disuse of sarcopenic muscles are two major clinical scenarios that contribute to morbidity and mortality in the aging population (19, 42).

Here, we demonstrate that the ability to repair skeletal muscle after injury is restored upon treatment with the AT1 receptor blocker losartan in sarcopenic mice. Furthermore, losartan treatment can prevent loss of muscle mass induced by hindlimb immobilization. Our data provide evidence that blockade of the AT1 receptor modulates multiple critical pathways associated with skeletal muscle homeostasis including the canonical and noncanonical TGF-β signaling cascades as well as the IGF-1/Akt/mTOR pathway.

TGF-β signaling, a known inhibitor of skeletal muscle regeneration and remodeling, promotes the formation of fibrotic tissue (7–9). Previous studies showed that losartan inhibited canonical TGF-β signaling, thereby improving muscle regeneration and function in mouse models of MFS and DMD (20). Indeed, administration of losartan after the induction of muscle laceration injuries in adult mice significantly decreased the formation of fibrosis (21). Our results presented here shed further light into the mechanism of action of losartan in skeletal muscle. We demonstrate that blockade of the AT1 receptor during regeneration not only inhibits the canonical but also modulates the noncanonical TGF-β signaling cascade.

The TGF-β signaling pathway is one of the many pathways altered in skeletal muscle during the physiological process of aging. Specifically, increases in the canonical and noncanonical TGF-β pathways as well as alterations of Notch and WNT signaling pathways have been associated with an inability to activate satellite cells and repair injured muscle (3, 43, 44). In the context of alterations of the canonical TGF-β signaling cascade, it has been suggested that an imbalance between TGF-β and Notch signaling increases the production of the cyclin-dependent kinase inhibitor p21 (3). However, our data show an exaggerated increase of TGF-β signaling without an impaired muscle regeneration response or increase in p21 expression at early stages of the muscle repair process. In contrast, our results demonstrate that canonical TGF-β signaling remains increased during later stages of regeneration associated with a decrease of p21 expression. It is certainly possible that the different observations at early stages of muscle repair might be due to in vitro–performed experiments versus our in vivo mouse studies. However, our observations agree with previous evidence that an increase of canonical TGF-β signaling inhibits expression of p21 in the C2C12

murine muscle cell line (45) and that p21 is indeed necessary for skeletal muscle differentiation and remodeling in response to injury in vivo (36).

Our finding that losartan also modulates the noncanonical TGF-β signaling cascade is of particular interest because this pathway has previously been implicated in various stages of the muscle repair response. During the early stages of regeneration, we show injury-related expression of these proteins believed to be necessary for efficient regeneration. ERK1/2 is postulated to enhance myoblast proliferation during the acute stage of muscle repair; however, evidence suggests that its sustained expression may repress muscle-specific gene expression and myoblast differentiation (12). Our data demonstrating an up-regulation of ERK1/2 in placebo-treated mice during the acute and later stages of regeneration further support this hypothesis. However, it is important to emphasize that the decrease in ERK signaling could be directly related to blockade of the AT1 receptor independent of TGF-β signaling (27, 30) (fig. S1). In contrast to the biphasic expression of ERK, the expression of p38α is required throughout the process of muscle remodeling; it is critical for the exit of myoblasts from the cell cycle and the induction of muscle-specific genes necessary for myofiber recruitment and formation (13). Our results show a delayed up-regulation of phosphorylated p38 in the placebo-treated mice at 19 days after CT as compared to the losartan-treated mice that have an increase at 4 days. Thus, we suggest that this delay in the expression of p38 contributes to the impaired muscle remodeling process observed in the placebo-treated mice.

Evidence suggests that the canonical and noncanonical TGF-β pathway regulates members of the MRF family (46). These factors include MyoD, Myf5, myogenin, and MRF4. Additional key players during myogenesis are Pax7, which is expressed during satellite cell activation, and p21, which permits irreversible withdrawal of satellite cells from the cell cycle, a critical and necessary step for the differentiation and maturation of muscle fibers (36). Our observations of an increase in Pax7 and MyoD at 4 and 19 days after CT injection in placebo-treated animals suggest that aged mice fail to transition from a state of satellite cell proliferation toward muscle differentiation and fusion (47). It is likely that losartan-induced blunting of the canonical and noncanonical TGF-β signaling pathways permits muscle remodeling by improving the physiological environment of satellite cells, which is critical for satellite cell function and their ability to regenerate and repopulate myofibers (33).

Additionally, we investigated disuse atrophy, which poses a frequent problem for individuals of all ages, but is particularly challenging for older adults. When skeletal muscle is subjected to immobilization for a period of time, muscle atrophy occurs (1). This atrophic response is a completely reversible process in the younger population (1); however, as a result of the physiological process of aging, animal models and humans are known to exhibit an exaggerated atrophy in response to disuse and an inability to rebuild muscle mass after immobilization (19, 42). Studies performed in human subjects reported a 30% loss of skeletal muscle mass after only 2 weeks of immobilization in older men as compared to a loss of less than 2% in young men, and only 2.5% of the loss muscle repopulated (43). Our data suggest that the decrease in muscle mass of aged rodents and humans subjected to immobilization is in fact due to a loss of muscle fibers rather than actual atrophy of myofibers generally observed in the young (39, 48).This provides a mechanistic explanation for the exaggerated response to disuse and the inability to recover with aging. Furthermore, the ability to prevent this loss of muscle fibers with losartan provides a rationale to explore this drug as a potential therapeutic option for disuse atrophy in older adults.

We did not observe significant alterations in the canonical or non-canonical TGF-β signaling pathways in our placebo- or losartan-treated immobilized animals with the exception of p38.

Previous studies have shown immobilization-induced alterations in these pathways. Specifically, an increase in the MAP kinase pathway has been suggested to contribute to the loss of muscle mass during disuse atrophy (17, 18, 49). The levels of p38 expression in the losartan-treated immobilized TA were significantly reduced, supporting the notion that when p38 is up-regulated during immobilization, it induces atrophy (39, 49). Because our analyses were performed after 21 days of immobilization, it is possible that transient alterations of these pathways may have occurred at an earlier time point. Because altered TGF-β signaling did not appear to play a major role in conferring protection against disuse atrophy in this immobilization model, we performed analyses of the IGF-1/Akt/mTOR pathway, which is a critical mediator of skeletal muscle proteolysis and synthesis and has been shown to be modulated by losartan treatment in skeletal muscle (40, 41). Phosphorylated Akt phosphorylates and activates mTOR signaling, thereby causing an increase of protein synthesis. In addition, Akt phosphorylates and inactivates the transcription factor FoxO3a, preventing muscle protein degradation. The IGF-1/Akt/mTOR pathway and the inactivated form of FoxO3a are down-regulated during various challenges, causing muscle atrophy (17). Our analyses of placebo-treated, immobilized TA muscle of aged mice revealed the expected decrease of members of the IGF-1/Akt/mTOR signaling cascade pathway. In contrast, losartan treatment prevented down-regulation of the expression profile of this pathway and resulted in an up-regulation of mTOR activation, suggesting that increased protein synthesis and inhibition of protein degradation may contribute to protection against disuse atrophy in sarcopenia.

Our results indicate that the blockade of the AT1 receptor has beneficial effects on skeletal muscle remodeling in response to injury and conferring protection against disuse atrophy in sarcopenia by modulating the TGF-β and IGF-1/Akt/mTOR signaling cascades. Previous studies in young rats have shown that angiotensin II is necessary for a hypertrophic response elicited by muscle overload and that the effect may be partly mediated by the AT1 receptor (50). Together, these results suggest that there are age-related differences in response to AT1 receptor blockers in skeletal muscle.

With the number of individuals older than 60 years doubling over the next 40 years, sarcopenia is a major public health problem (51). Additionally, normal muscle mass and strength are required to perform daily activities. Skeletal muscle injuries and disuse atrophy are clinical scenarios that increase morbidity and rehabilitation time of the aging population and represent additional challenges for geriatric healthcare providers. Our observations show that losartan can effectively improve skeletal muscle regeneration and preserve mass in physiological challenging conditions using a sarcopenic mouse model. Notably, losartan is a Food and Drug Administration–approved drug that is well tolerated in all age groups, with rare events of low blood pressure reported as a side effect in the elderly population (52). In our studies, losartan was administered before the induction of either injury or immobilization; thus, future clinical trials should consider administering losartan during the early stages of muscle injury and/or immobilization. In summary, these preclinical studies provide the basis for new therapeutic strategies in patients with sarcopenia.

MATERIALS AND METHODS

Animals

All mouse protocols were approved by the Animal Care and Use Committee of Johns Hopkins University School of Medicine. Male C57BL/6 mice (21 months old) were obtained from the National Institute on Aging. A subset of the mice was subjected to losartan ad libitum in their water (0.9 g/liter, Cozaar, Merck) for 1 week before the induction of injury or immobilization, and the losartan treatment continued until cessation of the experiment. Daily water intake was monitored to be 3 to 3.3 ml per mouse per day. The

rationale for using this concentration was derived from detailed studies titrating losartan doses in mice to achieve a hemodynamic effect of a 10 to 20% decrease in blood pressure and heart rate comparable to the desired response in humans. This dose is slightly higher than what is used in humans, which is not surprising, considering differences of body surface area and drug metabolism between mice and humans (for more details, see the Supplementary Material). For injury-regeneration experiments, losartan-treated and untreated (placebo) mice were injected with 100 μl of CT (10 μM *Naja nigricollis,* Calbiochem) into their TA. The mice were sacrificed at 4 and 19 days after CT injection after inhalation of isoflurane (IsoFlo, Abbott). The TA muscles were excised and prepared for subsequent experiments. For immobilization experiments, the mice were anesthetized before the procedure. The right hindlimb was immobilized by stapling the foot to the limb using a surgical stapler (Autosuture Royal 35W stapler) (39). The mice were dissected after 21 days of immobilization. Both TAs were excised, weighed, and used for subsequent experiments. Control mice were subjected to anesthesia only.

Histology/immunofluorescence

Muscle samples were embedded in optimal cutting temperature (OCT) compound (Electron Microscopy Sciences) and sectioned at 10 μm using a cryostat (Microm HM 550). Subsequently, the sections were then stained with hematoxylin and eosin or immunofluorescence. For immunofluorescence, the sections were blocked with 5% bovine serum albumin at room temperature and incubated with the primary antibodies overnight at 4°C and with secondary antibodies at room temperature for 1 hour. Primary antibodies include developmental myosin (Novocastra) and laminin γ1 (Chemicon). Secondary antibodies include Alexa Fluor 488 and 594 (Invitrogen). All images were taken with an Eclipse i80 microscope (Nikon).

Morphometry

For injury-regeneration experiments, the percentage of fibrosis was calculated by dividing the total damaged area by the area of fibrosis using Nikon NS elements 2.0 software. For immobilization experiments, the MFD of the myocytes (20, 53), total cell number, and CSA were determined using Nikon NS elements 2.0 software. About 1000 myocytes were analyzed per muscle for the MFD.

Protein extraction/Western blot analysis

Protein was extracted from flash-frozen TA muscles using T-PER (Thermo Scientific) with the addition of protease (Complete Mini, Roche) and phosphatase (PhosSTOP, Roche) inhibitors. Equal concentrations of protein were electrophoresed using a bis-tris gel (Invitrogen) and transferred onto a nitrocellulose membrane. Membranes were incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: pSmad2 ($Ser^{465/467}$), total Smad2, pERK1/2 (Thr^{202}/Tyr^{204}), total ERK1/2, p-p38 (Thr^{180}/Tyr^{182}), total p38, pAkt (Ser^{473}), total Akt, total FoxO3a, p-mTOR (Ser^{2448}), total mTOR, p4E-BP1 (Ser^{65}), total 4E-BP1 (Cell Signaling), myogenin, p21 (Santa Cruz), MyoD (BD Pharmingen), Pax7 (R&D Systems), pFoxO3a (Ser^{253}) (Millipore), and total actin (Sigma). Horseradish peroxidase–conjugated secondary antibodies were used to detect bands (Amersham). Quantitative Western blot analyses were performed with ImageJ (National Institutes of Health).

In vivo muscle function

Functional performance of the ankle dorsiflexor muscle (TA) was assessed in vivo as previously described (22). In the deeply anesthetized mouse, the knee was immobilized with a clamp and the foot was secured (90° to the tibia) in a custom footplate on a 300B-LR

servomotor (Aurora Scientific). Single twitch (0.1-ms square wave pulse at >10% threshold voltage needed to elicit maximal contraction) and tetanic contractions (250-ms train of pulses at 80 Hz) were assessed by percutaneous stimulation of the common peroneal nerve. A sequence of three successive twitch and tetanic pulses (30-s rest interval) was evaluated, and the peak response in each was used for analysis.

Statistical analysis

All values are expressed as means ± SEM. Significance was determined by either unpaired Student's *t* tests or one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls method. Significance was set at $P \leq 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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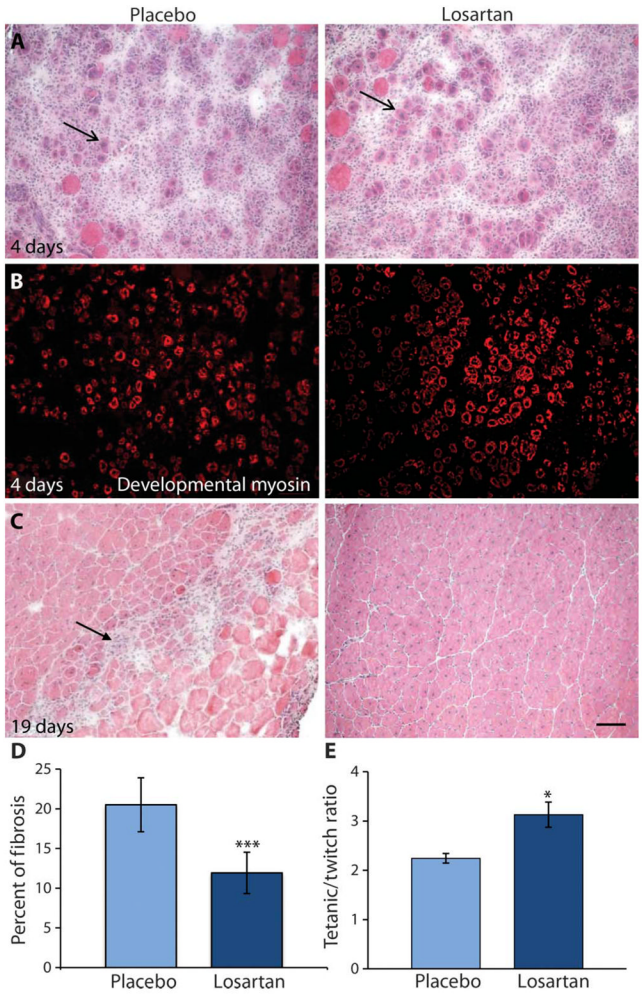

Fig. 1.

Losartan improves muscle remodeling and in vivo function in sarcopenic mice. (**A** to **C**) Histological analyses of the tibialis anterior (TA) muscle after cardiotoxin (CT) injection of placebo-treated (left) and losartan-treated (right) 21-month-old C57BL/6 male mice. Hematoxylin-eosin (H&E) (A) shows evidence of early signs of regeneration indicated by open arrows and no phenotypic differences between the treatment groups. Developmental myosin immunofluorescence (B) confirms similar amounts of newly regenerating cells. H&E staining (C) at 19 days after CT reveals impaired regeneration in the placebo-treated animal evident by fibrosis (closed arrow). Scale bar, 200 μm. (**D**) The amount of fibrosis was quantified and expressed as a percentage of the damaged area. (**E**) In vivo function of the TA was assessed using the titanic/twitch ratio 19 days after injury. Data are means ± SEM (n = 4 to 7 animals). *P < 0.05; ***P < 0.001, unpaired t test.

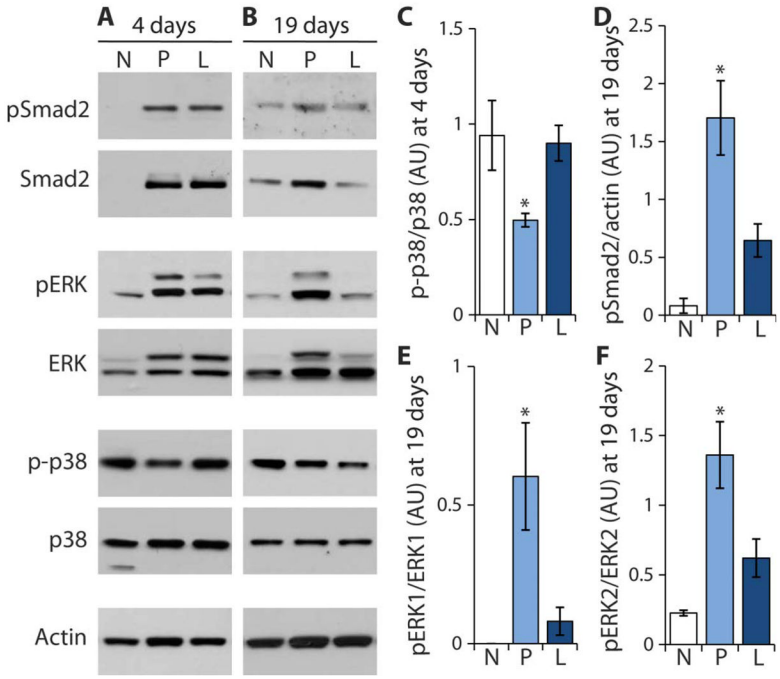

Fig. 2.

AT1 blockade modulates the canonical and noncanonical TGF-β pathways during muscle remodeling. (**A** and **B**) Western blot analyses of the TA using antibodies against the proteins indicated show the levels of the canonical and noncanonical TGF-β pathways in noninjected controls (N) and in the placebo (P) and losartan (L) groups at 4 days (A) and 19 days (B) after CT. Actin was used as a loading control. (**C** to **F**) Levels of p-p38 (C), pSmad2 (D), and pERK (E and F) were quantified using relative expression in arbitrary units (AUs). Data are means ± SEM (n = 4 animals). *P < 0.05, one-way ANOVA with Student-Newman-Keuls method.

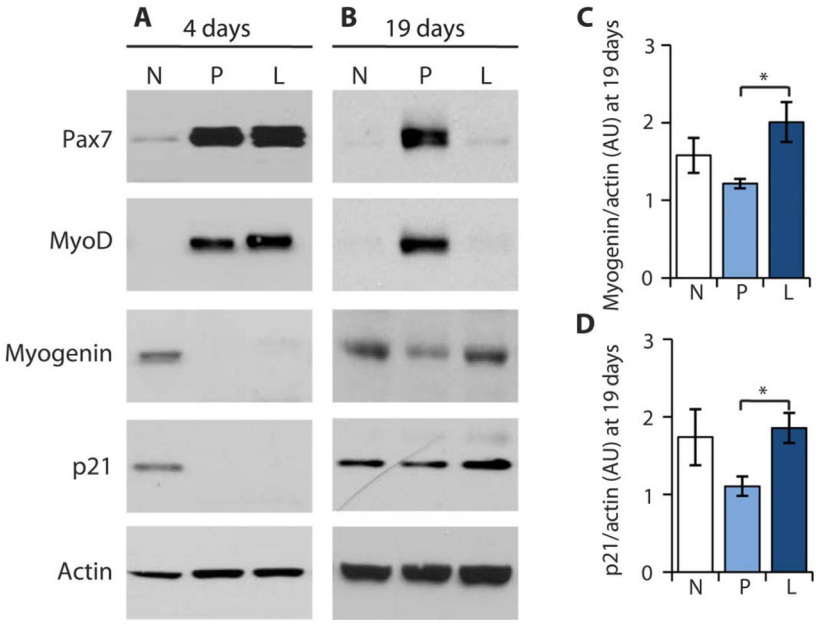

Fig. 3.

Modulation of TGF-β pathways affects expression of the myogenic regulatory pathway. (**A** and **B**) Western blot analyses of the myogenic regulatory pathway at 4 days (A) and 19 days (B) after CT in the TA of non-injected control (N), placebo-treated (P), and losartan-treated (L) mice. Actin was used as a loading control. (**C** and **D**) Relative expression was calculated for myogenin (C) and p21 (D) in arbitrary units (AUs). Data are means ± SEM ($n = 4$ animals). *$P < 0.05$, one-way ANOVA with Student-Newman-Keuls method.

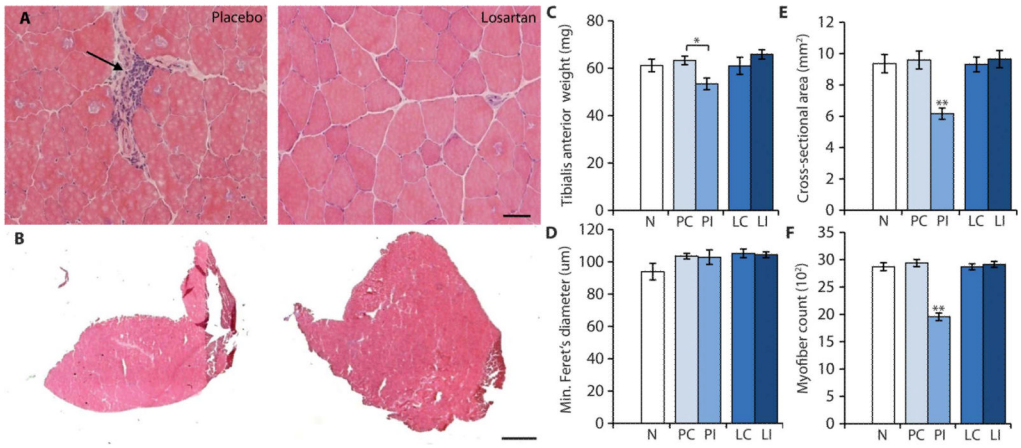

Fig. 4.

Losartan prevents disuse atrophy in sarcopenic mice. (**A** and **B**) H&E staining of the immobilized TA muscle of placebo-treated (left) and losartan-treated (right) animals does not show differences in the size of the individual myofibers (A) (scale bar, 100 μm) but reveal differences in the cross-sectional area (CSA) of the intact immobilized TA of the placebo group (B) (scale bar, 500 μm). There were also areas of fibrosis present (arrow) after immobilization only with placebo treatment (A). (**C** to **F**) Quantitative analyses were used to confirm histological findings by comparing the TAs from the nonimmobilized control (N), contralateral placebo (PC), immobilized placebo (PI), contralateral losartan (LC), and immobilized losartan (LI) limbs. (C) Comparisons were made between the TA weights of the contralateral and immobilized legs within the treatment groups (n = 5 to 6 animals). *P < 0.05, unpaired t test. (D to F) The evaluation of minimum Feret's diameter (MFD), CSA, and myofiber count was made between the subgroups (n = 5 to 6 animals). **P < 0.01, one-way ANOVA with Student-Newman-Keuls method.

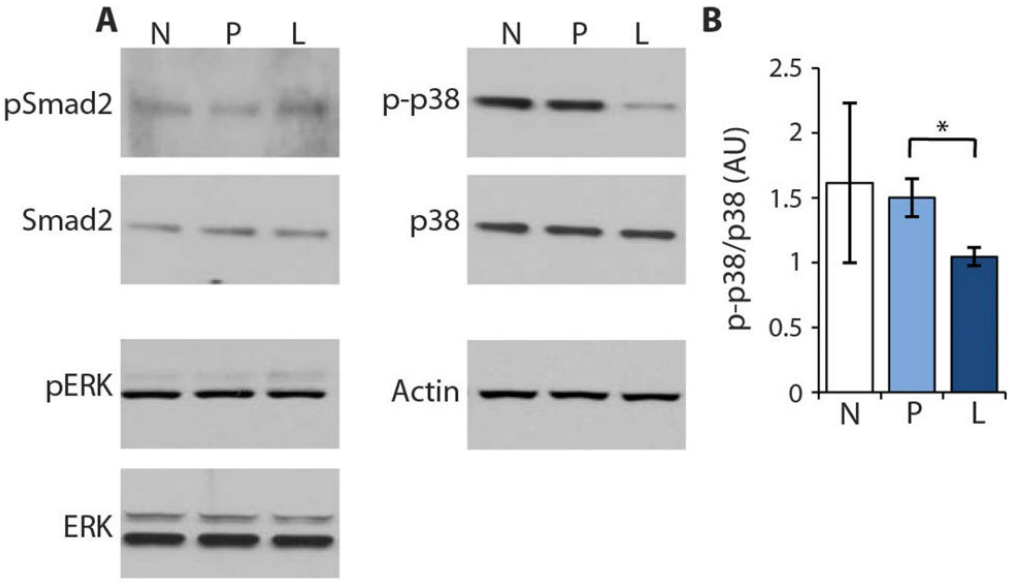

Fig. 5.

Losartan does not inhibit canonical and noncanonical TGF-β pathways in immobilized aged mice. (**A**) Western blot analyses of the canonical and noncanonical TGF-β pathway in the nonimmobilized control (N) and immobilized placebo (P) and losartan (L) TAs. Actin was used as a loading control. (**B**) The levels of p-p38 were quantified using relative arbitrary units (AUs). Data are means ± SEM ($n = 4$ animals). *$P < 0.05$, one-way ANOVA with Student-Newman-Keuls method.

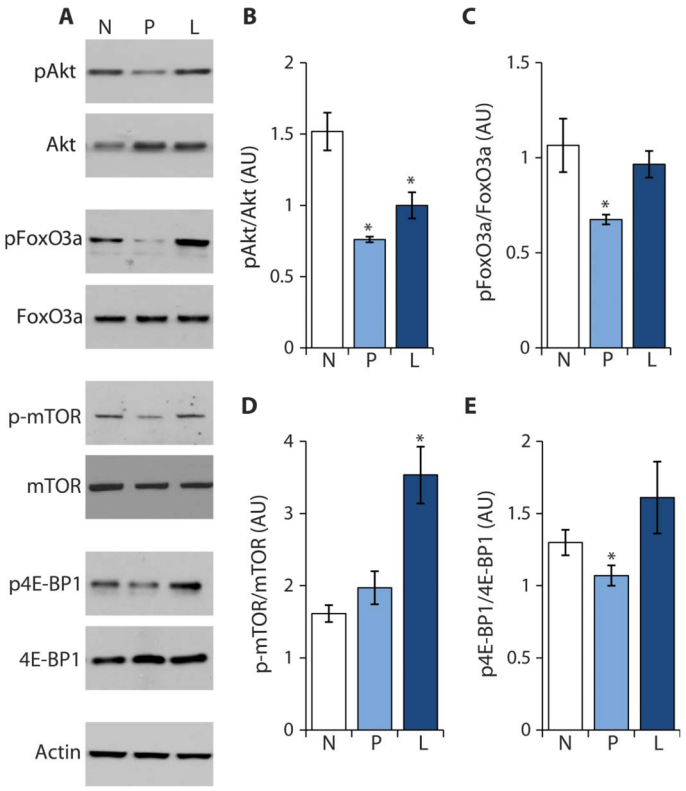

Fig. 6.

AT1 blockade up-regulates the IGF-1/Akt/mTOR pathway during immobilization in sarcopenic muscle. (**A** to **E**) Analyses of the indicated proteins of the IGF-1/Akt/mTOR pathway (A) illustrate the effects of immobilization on the expression of pAkt (B), pFoxO3a (C), p-mTOR (D), and p4E-BP1 (E) in the TA of the nonimmobilized control (N) and immobilized placebo (P) and losartan (L) groups, expressed in relative arbitrary units (AUs). Actin was used as a loading control. Data are means ± SEM (n = 4 animals). *P < 0.05, one-way ANOVA with Student-Newman-Keuls method.