IGF-1 prevents ANG II-induced skeletal muscle atrophy via Akt- and Foxo-dependent inhibition of the ubiquitin ligase atrogin-1 expression

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Yoshida T, Semprun-Prieto L, Sukhanov S, Delafontaine P. IGF-1 prevents ANG II-induced skeletal muscle atrophy via Akt- and Foxo-dependent inhibition of the ubiquitin ligase atrogin-1 expression. Am J Physiol Heart Circ Physiol 298: H1565–H1570, 2010. First published March 12, 2010; doi:10.1152/ajpheart.00146.2010.—Congestive heart failure is associated with activation of the renin-angiotensin system and skeletal muscle wasting. Angiotensin II (ANG II) has been shown to increase muscle proteolysis and decrease circulating and skeletal muscle IGF-1. We have shown previously that skeletal muscle-specific overexpression of IGF-1 prevents proteolysis and apoptosis induced by ANG II. These findings indicated that downregulation of IGF-1 signaling in skeletal muscle played an important role in the wasting effect of ANG II. However, the signaling pathways and mechanisms whereby IGF-1 prevents ANG II-induced skeletal muscle atrophy are unknown. Here we show ANG II-induced transcriptional regulation of two ubiquitin ligases atrogin-1 and muscle ring finger-1 (MuRF-1) that precedes the reduction of skeletal muscle IGF-1 expression, suggesting that activation of atrogin-1 and MuRF-1 is an initial mechanism leading to skeletal muscle atrophy in response to ANG II. IGF-1 overexpression in skeletal muscle prevents ANG II-induced skeletal muscle wasting and the expression of atrogin-1, but not MuRF-1. Dominant-negative Akt and constitutively active Foxo-1 blocked the ability of IGF-1 to prevent ANG II-mediated upregulation of atrogin-1 and skeletal muscle wasting. Our findings demonstrate that the ability of IGF-1 to prevent ANG II-induced skeletal muscle wasting is mediated via an Akt- and Foxo-1-dependent signaling pathway that results in inhibition of atrogin-1 but not MuRF-1 expression. These data suggest strongly that atrogin-1 plays a critical role in mechanisms of ANG II-induced wasting in vivo.

angiotensin II; insulin-like growth factor-1

CONGESTIVE HEART FAILURE (CHF) is a leading cause of cardiovascular mortality and morbidity (8). CHF is associated with elevated circulating levels of angiotensin II (ANG II) (10) and muscle wasting, which is an important predictor of poor outcome in patients with this disease (2, 22). We have demonstrated that ANG II infusion in mice stimulates muscle protein wasting by activating proteolytic pathways involving caspase-3 and the ubiquitin-proteasome system, accompanied by depression of circulating and skeletal muscle insulin-like growth factor-1 (IGF-1). These findings suggested that downregulation of IGF-1 signaling in skeletal muscle played an important role in the wasting effect of ANG II. Studies using in vitro models of muscle atrophy such as dexamethasone-induced proteolysis have indicated that IGF-1 acts through Akt and Foxo transcription factors to suppress expression of the two E3 ubiquitin ligase genes atrogin-1 and muscle ring finger-1 (MuRF-1) (16). Atrogin-1 and MuRF-1 are highly induced in various muscle atrophy models (7). We have shown that in transgenic mice overexpressing IGF-1 specifically in skeletal muscle ANG II-induced caspase-3 activation, protein ubiquitination and muscle atrophy are inhibited (20). In the present study we used electroporation of plasmid constructs in vivo to determine signaling mechanisms whereby IGF-1 inhibits ANG II-induced atrophy. Our data demonstrate that IGF-1 prevents ANG II-induced skeletal muscle wasting through an Akt/Foxo-dependent pathway in vivo and suggest an important role for atrogin-1 in ANG II effects on skeletal muscle.

MATERIALS AND METHODS

Materials. Recombinant human IGF-1 was from Tercica. Antibodies against phospho-Akt (Ser473), phospho-Foxo-1 (Ser256), total Akt, and total Foxo-1 were purchased from Cell Signaling Technology. Human IGF-1 expression plasmid is a generous gift from Dr. Thissen (17). dnAkt and caFoxo-1 expression plasmids were constructed from adenoviral expression vectors described previously (7, 19).

Animal studies. Animal protocols were approved by the Institutional Animal Care and Use Committee at Tulane University. Nine- to ten-week-old male FVB (Charles River), C57BL/6 (Charles River), and IGF-1-transgenic [myosin light chain (MLC)/migf-1] mice were used in this study. Osmotic minipumps (Alzet model 1007D; Durect) were implanted to infuse ANG II at a rate of 1 mg·kg⁻¹·min⁻¹ for FVB and MLC/migf-1 mice and 0.5 mg·kg⁻¹·min⁻¹ for C57BL/6, respectively. The body weight and food intake of each ANG II-infused mouse were measured daily, and a corresponding vehicle-infused control mouse was given the same amount of food as that eaten by the ANG II-infused mouse on the previous day (i.e., mice were pair fed). Blood pressures were measured noninvasively using the Visitech BP-2000 (Visitech Systems) as previously described (4). One or seven days after implantation, mice were anesthetized and tissues were removed, weighed, and snap-frozen in liquid nitrogen and stored at −80°C until processed.

Skeletal muscle electroporation. Two weeks before ANG II minipump implantation, each mouse was anesthetized with a mixture of 75 mg/kg ketamine (Ketalar; Pfizer, Oslo, Norway) and 15 mg/kg xylazine hydrochloride (Rompun; Bayer, Fernwald, Germany) administered by injection intraperitoneally. Mice hindlimbs were shaved, and plasmids (1 µg/µl; dissolved in saline) were injected into five different sites (total volume per muscle = 50 µl) in each 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.

Quantitative real-time RT-PCR. Gastrocnemius muscles were homogenized in Tripure isolation reagent (Roche), and total RNA was isolated following manufacturer’s instructions. cDNA synthesis was performed using the RT2 first-strand kit (SABiosciences, Frederick, MD). Real-time PCR was performed using a 40-cycle two-step PCR.
protocol in the iCycler IQ real-time detection system (Bio-Rad, Hercules, CA). Hprt1 gene expression was used as an internal control (14). PCR primers used in this study were obtained commercially [SABiosciences; PPM38061A for atrogin-1, PPM61045A for MuRF-1, PPM03387E for IGF-1, PPM04714E for IGF-1 receptor (IGF-1R), and PPM03559E for Hprt1].

**Immunoblotting.** Samples were solubilized in radioimmunoprecipitation assay buffer, and proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels. Proteins were blotted onto polyvinylidene difluoride membranes and, after being blocked with 5% BSA/0.1% Tween 20, incubated with primary antibodies in the same solution. Bound antibodies were detected by anti-rabbit or -mouse IgG conjugated with peroxidase and subsequently detected by chemiluminescence.

**Luciferase reporter gene assay.** 5 kbp and 1 kbp upstream promoter regions of atrogin-1 and MuRF-1 were cloned from C57BL/6 mouse genomic DNA by PCR using the following primers: 5'-GCGGTCGACGGAATATAGACTTGCAGATC-3' and 5'-GCGGTCGACGCACGCTGTACGACGCGAC-3' for atrogin-1 5kbp; 5'-GGCGGTACCGCTCCAGAGCTGTCAGGCTATC-3' and 5'-GGCGGTACGGTACAGAGCGCGGACGCGAC-3' for atrogin-1 1kbp; 5'-GGGTGTCGACCTCAGGACGGACTGTC-3' and 5'-GGCGGTACGGTACAGAGCGCGGACGCGAC-3' for MuRF-1 5kbp; 5'-GGCGGTACCGCTCCAGAGCTGTCAGGCTATC-3' and 5'-GGCGGTACGGTACAGAGCGCGGACGCGAC-3' for MuRF-1 1kbp. These DNA fragments were subcloned into pGL4.12[luc2CP] (Promega). Twenty micrograms of plasmids containing atrogin-1 or MuRF-1 promoter sequences were electroporated into gastrocnemius muscle together with 2 μg of pGL4.74[hRluc/TK] vector (Promega), followed by ANG II minipump implantation 2 wk after electroporation. Gastrocnemius muscles were collected at day 1 of the infusion. Skeletal muscle lysates were prepared, and luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instruction.

**Statistical analysis.** All data represent means ± SE of at least five animals in each group, and results were analyzed using Student’s t-test when data from two experimental groups were compared or ANOVA followed by Bonferroni’s multiple comparison test when data from three groups were studied.

**RESULTS**

**ANG II-induced upregulation of ubiquitin ligase expression precedes IGF-1 downregulation; differential regulation of ubiquitin ligases in MLC/mIgf-1 mice.** We previously reported that after 7 days of ANG II infusion in mice, IGF-1 mRNA levels were reduced but mRNA levels of the ubiquitin ligases atrogin-1 and MuRF-1 were increased in skeletal muscle (20, 23). Since IGF-1 has been reported to suppress expression of atrogin-1 and MuRF-1 (21), our findings suggested that downregulation of IGF-1 could be involved in upregulation of ubiquitin ligase expression. We determined the time course-dependent expression of these genes in the skeletal muscle of ANG II-infused mice using quantitative PCR. Atrogin-1 and MuRF-1 expression was rapidly upregulated within 1 day after ANG II infusion, followed by a gradual reduction over 7 days (Fig. 1, A and B). On the other hand, reduced IGF-1 expression was only seen 7 days after infusion (Fig. 1C), indicating that upregulation of ubiquitin ligase expression was unlikely to result from reduced IGF-1 signaling. IGF-1R expression increased 1 and 4 days after infusion and returned to basal levels after 7 days (Fig. 1D). MLC/mIgf-1 mice overexpress a skeletal muscle-specific isoform of IGF-1 preferentially in fast fiber muscles under the control of the myosin light chain promoter (13) and are resistant to ANG II-induced skeletal muscle atrophy (20). In gastrocnemius muscle of MLC/mIgf-1 mice, basal expression of atrogin-1 was significantly repressed, and ANG II infusion failed to increase its expression (Fig. 1F). On the other hand, neither basal nor ANG II-induced MuRF-1 expression was affected in skeletal muscle of MLC/mIgf-1 mice (Fig. 1G). Consistent with our previous data, the ANG II-induced reduction in gastrocnemius weight was inhibited in MLC/mIgf-1 mice (Fig. 1E).

**IGF-1 plasmid electroporation blocks atrogin-1 but not MuRF-1 upregulation in ANG II-infused mice.** Electroporation of plasmid constructs has been reported to be a useful technolog-
ology for the analysis of signaling pathways involved in skeletal muscle hypertrophy and atrophy (17, 18). We determined the efficiency of gene expression by using enhanced green fluorescent protein-encoding plasmid electroporated to gastrocnemius muscle and observed highly efficient and long-lasting expression as reported previously (Fig. 2, A and B) (1, 12, 17, 18). However, we found that the electric pulse associated with plasmid electroporation to gastrocnemius muscle significantly increased atrogin-1 and MuRF-1 mRNA levels, whereas plasmid injection alone had no effect on gene expression (Fig. 2, E and F). Furthermore, electroporation was associated with a marked but transient increase in β-actin expression (Fig. 2B). The increase of ubiquitin ligase gene expression reached the maximal level 1 day after electroporation, followed by a gradual decrease and return to basal levels 1 to 2 wk after electroporation (Fig. 2, G and H). These data indicate that a recovery period is required after plasmid electroporation to analyze the signaling pathways associated with skeletal muscle atrophy. Since the elevation of atrogin-1 and MuRF-1 completely returned to basal levels after 2 wk of electroporation, we chose a 2-wk recovery period after plasmid electroporation to analyze minipump implantation. Human IGF-1 (hIGF-1) mRNA levels were highly upregulated in hIGF-1 plasmid electroporated gastrocnemius muscles (Fig. 2C), without significant detection of plasma hIGF-1 (Fig. 2D).

Following electroporation of hIGF-1 or control empty vector into mouse gastrocnemius muscle and the 2-wk recovery period, mice were implanted with ANG II or saline minipumps and skeletal muscle weight and ubiquitin ligase expression were measured 1 and 7 days after infusion. As reported previously, ANG II infusion caused a loss of body weight in pair-fed mice (P < 0.01 by ANOVA compared with sham; Fig. 3A) and reduced food intake (Fig. 3B). The body weight change in the sham group before and after minipump implantation was not significant. Local hIGF-1 expression prevented the loss of skeletal muscle weight induced by ANG II (Fig. 3C) and markedly suppressed ANG II-induced increase of atrogin-1 expression (Fig. 3D). On the other hand, MuRF-1 expression caused by ANG II was not affected by local hIGF-1 overexpression (Fig. 3E).

To confirm these findings and to obtain initial insights into mechanisms whereby ANG II increases ubiquitin ligase mRNA levels, we generated luciferase reporter gene constructs that contain 5 kbp and 1 kbp of atrogin-1 and MuRF-1 upstream promoter regions and analyzed these promoter activities in gastrocnemius muscle (Fig. 3F). Our data showed that ANG II induction of atrogin-1 and MuRF-1 transcriptional activity was mediated by 1 kbp and 5 kbp upstream regions of atrogin-1 and MuRF-1, respectively. Electroporation of IGF-1 expression plasmids together with these luciferase vectors showed that IGF-1 suppressed ANG II-induced atrogin-1 promoter activity, and this was the case using both 5 kbp and 1 kbp constructs. However, IGF-1 coexpression failed to suppress ANG II-induced MuRF-1 promoter activity.

IGF-1 prevents ANG II-induced skeletal muscle atrophy via Akt-Foxo signaling. It has been previously demonstrated that induction of myotube hypertrophy by IGF-1 depends on phosphatidylinositol 3-kinase-mediated activation of Akt (15). Akt phosphorylates the Forkhead box (Foxo) class of transcription factors, resulting in their nuclear exclusion, thereby inhibiting their transcriptional activity. Activation of the IGF-1-PI3K-protein kinase B (Akt) signaling cascade (19) is essential for the IGF-1-induced death of myotubes in vitro (28) as well as the myotube hypertrophy (29) and the increase in mTor activity (30). We found that IGF-1 also suppressed the expression of MuRF-1 and atrogin-1 in a luciferase reporter assay (Fig. 3G).

To analyze the signaling pathways involved in skeletal muscle atrophy, we used a retrovirus plasmid electroporation approach to deliver IGF-1 or control plasmids into gastrocnemius muscle and observed highly efficient and long-lasting expression by Northern blotting 5 days after electroporation. Endogenous and plasmid-derived IGF-1 transcripts are indicated by white and black arrow, respectively. I, IGF-1 plasmid; C, control plasmid. hIGF-1 mRNA expression was analyzed by Northern blotting 5 days after electroporation. Endogenous and plasmid-derived IGF-1 transcripts are indicated by white and black arrow, respectively. I, IGF-1 plasmid; C, control plasmid. 

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Akt-Foxo pathway inhibits skeletal muscle atrophy induced by starvation, denervation, and glucocorticoids (16–18, 21). To determine the downstream signaling mechanisms mediating IGF-1 prevention of ANG II-induced skeletal muscle atrophy, we used dominant-negative and constitutively active Akt and Foxo-1 variants.

The dominant-negative form of Akt (dnAkt) has alternative residues substituted for lysine at position 179, threonine at position 308, and serine at position 473 (7, 19) and when electroporated to gastrocnemius muscle markedly reduced levels of phosphorylated Akt (Fig. 4A). Electroporation of dnAkt, but not of wild-type Akt, along with IGF-1 blocked the ability of IGF-1 to prevent ANG II-induced loss of skeletal muscle weight (Fig. 4B). Furthermore, IGF-1 failed to inhibit ANG II-induced atrogin-1 gene expression in the presence of dnAkt (Fig. 4C). On the other hand, ANG II-induced MuRF-1 expression was not affected by dnAkt expression (Fig. 4D).

The constitutively active form of Foxo-1 (caFoxo-1) has alanine residues substituted for those serine/threonine residues that are phosphorylated by Akt, which prevents its inactivation by phosphorylation. Similarly to the results obtained using dnAkt, when caFoxo-1 was electroporated to gastrocnemius muscle, IGF-1 could not counteract the ANG II-induced loss of skeletal muscle weight (Fig. 5B). Furthermore, caFoxo-1 upregulated basal levels of atrogin-1 mRNA in sham-infused animals and prevented the IGF-1-mediated repression of atrogin-1 gene expression in ANG II-infused mouse skeletal muscle (Fig. 5C). However, neither basal nor ANG II-induced MuRF-1 expression was affected by caFoxo-1 expression (Fig. 5D).

**DISCUSSION**

We have previously shown that ANG II induces weight loss in part via a catabolic effect on skeletal muscle that is characterized by downregulation of IGF-1 expression, reduced pAkt and pFoxo expression, and upregulation of ubiquitin ligase expression (20). In MLC/mIgf-1 mice the ANG II induction of weight loss was blocked, and this effect was accompanied by maintenance of pAkt levels, suggesting potential involvement of the Akt-Foxo pathway in the ability of IGF-1 to prevent
ANG II-induced atrophy. Our current study establishes that Akt-Foxo-dependent signaling is required for the rescue effect of IGF-1. Furthermore, the rapid induction of ubiquitin ligase gene expression by ANG II and the ability of IGF-1 electroporation to block ANG II upregulation of atrogin-1 expression suggest strongly that atrogin-1 plays a critical role in the catabolic effect of ANG II.

Because IGF-1 has been shown to repress atrogin-1 expression in vivo in other catabolic states (13, 21) we hypothesized that the reduction in IGF-1 expression induced by ANG II could have resulted in upregulation of ubiquitin ligase expression. However, time course analysis (Fig. 1) clearly showed that the rapid upregulation of atrogin-1 and MuRF-1 in response to ANG II occurred within 24 h and preceded downregulation of IGF-1. Thus upregulation of ubiquitin ligase expression is likely to play an important role in early mechanisms leading to loss of muscle in response to ANG II and is followed by reduced IGF-1 expression and signaling, which likely contributes to ongoing loss of muscle.

To determine the role of the Akt and Foxo pathways in the ability of IGF-1 to prevent ANG II muscle loss, we electroporated IGF-1 plasmid together with dnAkt and caFoxo-1 plasmids. The blocking effect of dnAkt on IGF-1 inhibition of ANG II-induced atrogin-1 expression and muscle wasting (Fig. 4) demonstrates that Akt is required for the rescue effect of IGF-1. Akt is a serine/threonine kinase with multiple downstream targets including Foxo, BCL2-antagonist of death, caspase-9, glycogen synthase kinase-3, and tuberous sclerosis 2 (6). The Foxo family consists of four members in mammalian cells: Foxo-1, Foxo-3a, Foxo-4, and Foxo-6. Foxo-1 and Foxo-3 have received the most attention with regard to skeletal muscle atrophy because they are upregulated in various models of atrophy (9). The ability of caFoxo to inhibit the rescue effect of IGF-1 (Fig. 5) is consistent with a model in which IGF-1-induced phosphorylation and inactivation of Foxo-1 protein via an Akt signaling pathway plays a critical role in the ability of IGF-1 to inhibit ANG II-induced ubiquitin ligase expression and muscle wasting.

IGF-1 inhibition of muscle atrophy in other catabolic conditions (5, 17) has been suggested to be due to its ability to repress expression of atrogin-1 and MuRF-1. Our findings using MLC/mIgf-1 mice and electroporation of IGF-1 plasmid clearly showed that overexpression of IGF-1 in skeletal muscle blocks ANG II upregulation of atrogin-1, but not MuRF-1. This finding is surprising since in most other catabolic conditions these ubiquitin ligases are coordinately regulated (3, 11). For instance, in dexamethasone-induced atrophy intramuscular injection of IGF-1 prevented Akt/Foxo signaling, resulting in repression of atrogin-1 and MuRF-1 expression (17). However, it is important to note that in NF-κB-mediated muscle atrophy there is an increase in MuRF-1 expression but no change in atrogin-1 (5). To confirm our findings and to obtain initial insights into mechanisms whereby ANG II upregulates ubiquitin ligase expression, we cloned upstream promoter sequences from both atrogin-1 and MuRF-1 mouse genomic DNA and generated promoter reporter gene constructs. Our data clearly demonstrate marked upregulation of reporter gene expression in the skeletal muscles of ANG II-infused animals and abrogation of atrogin-1 but not MuRF-1 luciferase activity following electroporation of IGF-1. These findings unequivocally demonstrate that ANG II transcriptionally regulates atrogin-1/MuRF-1 expression but that the rescue effect of IGF-1 is characterized by inhibition of atrogin-1 but not MuRF-1, strongly suggesting specific involvement of atrogin-1 but not MuRF-1 in ANG II-induced wasting. This is schematically represented in Fig. 6.

To our knowledge this is the first report of a catabolic condition in which IGF-1 differentially represses atrogin-1...
expression without altering MuRF-1 expression. It is of note that little is known about transcriptional control of atrogin-1 and MuRF-1 expression, although Foxo-3 has been shown to stimulate transcription of the atrogin-1 promoter (16). Our findings demonstrate that ANG II infusion in mice strongly increases skeletal muscle atrogin-1 and MuRF-1 mRNA levels, likely in large part via a transcriptional effect. However, this effect of ANG II is unlikely to be a direct effect since we have found that adult rodent skeletal muscles express little to no ANG II receptors (23). Thus it is more likely that the ANG II upregulation of ubiquitin ligase expression is mediated via other molecules such as glucocorticoids, IL-6, and serum amyloid A that we have shown to be involved in mediating the catabolic effects of ANG II (20, 23).

A novel aspect of our study is the discovery that the electric pulse application associated with plasmid electroporation into skeletal muscle is sufficient to increase ubiquitin ligase expression for up to 1 wk (Fig. 2, G and H). This effect likely results from an injury response since β-actin expression was also transiently increased in electroporated muscle (Fig. 2B). Because electroporation is increasingly applied for the study of skeletal muscle signaling pathways (1, 13, 17, 18), our findings have important implications for the application of this technology and indicate that an appropriate recovery period is critical, particularly for the study of skeletal muscle atrophy-associated signaling.

In summary, we have shown that the upregulation of atrogin-1 and MuRF-1 expression in skeletal muscles of ANG II-infused mice is mediated via a transcriptional effect that precedes ANG II-induced downregulation of IGF-1 expression. The ability of IGF-1 to prevent ANG II-induced skeletal muscle wasting is mediated via an Akt- and Foxo-1-dependent signaling pathway that results in inhibition of atrogin-1 and not MuRF-1 expression. These data suggest strongly that atrogin-1 plays a critical role in mechanisms of ANG II-induced wasting in vivo.

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DISCLOSURES

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

REFERENCES