

## IL-6 and Serum Amyloid A Synergy Mediates Angiotensin II–Induced Muscle Wasting

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### ABSTRACT

Animal studies suggest that increased levels of circulating angiotensin II (AngII) could contribute to the loss of lean body mass in chronic kidney disease, but the mechanism by which this occurs is unclear. Here, AngII infusion increased circulating IL-6 and its hepatic production in wild-type mice, suggesting that AngII-induced inflammation may trigger muscle loss. AngII infusion also stimulated the suppressor of cytokine signaling (SOCS3) in muscle, which led to loss of insulin receptor substrate 1 (IRS-1), thereby impairing insulin/IGF-1 signaling and enhancing protein degradation. All of these responses to AngII were suppressed in IL-6–deficient mice. Recombinant human IL-6 (rhIL-6) treatment of cultured myotubes only minimally increased SOCS3, however, suggesting the contribution of other mediators. Because AngII increases hepatic serum amyloid A (SAA) expression in an IL-6–dependent manner, we treated wild-type mice with rhIL-6 and an SAA1-overexpressing adenovirus; the combination led to a significantly greater increase in SOCS3 and decrease in IRS-1 compared with either rhIL-6 or SAA1 alone. We observed similar effects on SOCS3 and IRS-1 when we treated cultured muscle myotubes with rhIL-6 and SAA1. Taken together, these results suggest an interorgan response to high levels of AngII: Hepatic production of IL-6 and SAA increases, and these mediators act synergistically to impair insulin/IGF-1 signaling, which promotes muscle proteolysis. Targeting the high levels of IL-6 and SAA in catabolic disorders might be a therapeutic approach to prevent muscle wasting.

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Increased circulating angiotensin II (AngII) occurs in several conditions that are characterized by muscle wasting (e.g., chronic kidney disease [CKD], congestive heart failure [CHF]).<sup>1,2</sup> Experimentally, long-term AngII infusion into mice stimulates muscle protein wasting by activating two proteolytic mechanisms: Caspase-3 and the ubiquitin-proteasome system (UPS).<sup>3</sup> Identifying mechanisms linking AngII with muscle wasting is of interest because blocking AngII in patients with CHF can ameliorate their loss of muscle mass.<sup>2</sup>

The mechanisms underlying AngII-induced loss of lean body mass could involve a direct effect of AngII on muscle, but there is evidence that AngII type 1 receptors (AT<sub>1</sub>) are not expressed in skeletal muscle.<sup>4</sup> Alternatively, AngII could trigger muscle protein losses indirectly, possibly through inflammatory

mechanisms, because AngII stimulates inflammation.<sup>5</sup> There is abundant evidence that CKD and cardiovascular diseases are complicated by inflammation,<sup>2,6</sup> and inflammation has been associated with activation of E3 ubiquitin ligase MuRF1 in muscle and, therefore, could stimulate protein degradation in the UPS.<sup>7</sup> Inflammation also causes insulin resistance: In one mechanism, TNF- $\alpha$  or fatty acids stimulate

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phosphorylation of IRS-1ser<sup>307</sup>, leading to suppression of insulin/IGF-1 signaling.<sup>8–10</sup> This is relevant because impaired insulin/IGF-1 signaling is closely linked to activation of muscle protein degradation.<sup>3,11,12</sup> Indeed, overexpression of IGF-1 in muscle prevents AngII-induced muscle wasting.<sup>3</sup> Additional evidence for impaired insulin/IGF-1 signaling is that glucocorticoids exert a permissive effect in mediating muscle proteolysis in conditions associated with impaired insulin/IGF-1 signaling, such as acidosis, insulin deficiency/resistance, starvation, and sepsis.<sup>3,12–16</sup> Moreover, administration of a steroid receptor antagonist substantially reduces muscle wasting in AngII-infused mice. Because impaired insulin/IGF-1 signaling occurs in inflammation and several conditions causing muscle catabolism, identifying how AngII impairs insulin/IGF-1 signaling could uncover mechanisms in catabolic conditions.

In examining whether inflammation contributes to AngII-induced muscle wasting, we found that AngII infusion raises circulating IL-6. The increase in IL-6 is essential for AngII-induced muscle wasting, and it interacts synergistically with serum amyloid A (SAA) to increase muscle suppressor of cytokine signaling (SOCS3) expression, leading to reduced IRS-1 levels in muscle. These results uncover a new role for an acute-phase reactant protein.

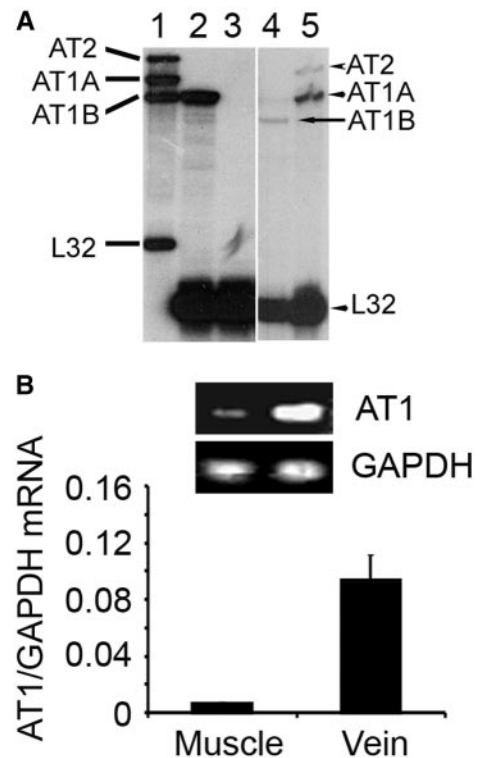
## RESULTS

### Skeletal Muscle and Muscle-Derived Cells Do not Express AngII Receptors

To seek functional AngII receptors, we examined AngII receptor expression in C2C12 cells derived from mice, L6 cells from rats, and mouse skeletal muscle. In C2C12 myotubes, treatment with 1  $\mu$ M AngII did not cause an intracellular calcium spike or phosphorylation of extracellular signal-regulated kinase (ERK; a downstream target of AngII), but in C2C12 infected with an adenovirus encoding AT<sub>1</sub>,<sup>17</sup> AngII stimulated a calcium spike and ERK phosphorylation (Supplemental Figure 1). In L6 cells, we found no expression of AT<sub>2</sub>, AT<sub>1A</sub>, or AT<sub>1B</sub> receptors by ribonuclease protection assay<sup>18,19</sup> (Figure 1A). By real-time PCR (RT-PCR), we found no detectable AT<sub>1</sub> receptor mRNA in C2C12 cells, a small amount in gastrocnemius muscle (possibly due to vessels within the muscle) and abundant AT<sub>1</sub> receptor mRNA in mouse vein (Figure 1B). These results are consistent with immunostaining results which revealed AT<sub>1</sub> receptors in blood vessels of muscle but not in muscle itself.<sup>4</sup>

### AngII Induces IL-6 Expression in Mice

The absence of AngII receptors indicates that it acts through mediators to cause muscle wasting. Because AngII can cause inflammation,<sup>5</sup> we measured inflammatory mediators induced by AngII. Male pair-fed, wild-type (WT), C57/BL6 mice were infused with PBS or AngII. One week later, we found increased plasma levels of chemokines and cytokines in AngII-infused mice; the largest increase was IL-6 (Table 1). There was an associated 10-



**Figure 1.** Skeletal muscle and cells derived from it do not express AngII receptors. (A) AngII receptors analyzed by ribonuclease protection assay (lane 1, probes for AT<sub>2</sub>, AT<sub>1A</sub>, and AT<sub>1B</sub>; lane 2, rat aorta smooth muscle cells as a positive control for AT<sub>1B</sub>; lane 3, L6 cells; lane 4, positive control for AT<sub>1B</sub>; lane 5, positive control for AT<sub>2</sub> [upper band] and AT<sub>1A</sub> [lower band]). The rat ribosomal L-32 gene was used as the housekeeping gene; probes containing polylinker regions are longer than the protected bands. (B) AngII receptors in mouse muscle, mouse vein, or C2C12 cells analyzed by RT-PCR.

fold increase in IL-6 mRNA in liver plus much smaller increases in kidney and muscle (Supplemental Figure 2).

### AngII-Induced Muscle Wasting Depends on IL-6

To examine whether IL-6 plays a role in AngII-induced muscle wasting, we compared IL-6 knockout (KO) and WT mice infused with AngII or PBS. After 7 d of AngII infusion, the weights of mixed-fiber gastrocnemius and tibialis anterior (TA) muscles and the red fiber, soleus muscles of WT mice were significantly reduced. The AngII-induced loss of muscle mass was significantly blocked in IL-6 KO mice (Table 2). Likewise, the frequency distribution of cross-sectional areas (CSA) of fibers in TA muscles of WT mice infused with AngII was shifted toward smaller sizes compared with results of PBS infusion (Figure 2, A and C). Again, the shift in CSAs as a result of AngII was blocked in TA muscles from IL-6 KO mice (Figure 2, B and D). The mean CSA in TA muscles of AngII-infused WT mice was lower than that in PBS-infused WT mice. This difference was not present in AngII-infused IL-6 KO mice (Figure 2E).

We also examined whether AngII affects fiber types I, IIA, or

**Table 1.** Changes in plasma cytokines induced by AngII<sup>a</sup>

Cytokine or Chemokine	PBS Infusion (pg/ml)	AngII Infusion (pg/ml)	Fold Change (AngII versus PBS)	P
KC	38.59 ± 6.25	76.50 ± 0.83	1.98	0.037
RANTES	7.19 ± 0.08	8.33 ± 0.33	1.16	0.019
IL-1 $\beta$	6.75 ± 1.35	7.13 ± 1.01	1.06	0.126
IL-1 $\alpha$	11.38 ± 3.03	17.73 ± 0.57	1.56	0.054
IL-6	3.27 ± 2.12	17.60 ± 0.87	5.38	0.005
IL-10	35.52 ± 5.93	1.00 ± 0.05	0.03	0.003
IL-12p(70)	52.42 ± 13.78	13.96 ± 11.00	0.27	0.024
TNF- $\alpha$	4.48 ± 0.78	6.02 ± 1.50	1.34	0.053
IFN- $\gamma$	32.78 ± 2.50	43.92 ± 1.40	1.34	0.052

<sup>a</sup>Circulating concentrations of cytokines and chemokines were measured in plasma samples from mice infused with PBS or AngII for 7 d (n = 4 mice in each group).

**Table 2.** Skeletal muscle and body weights of WT and IL-6 KO infused with AngII or PBS<sup>a</sup>

Parameter	Gastrocnemius (mg)	Soleus (mg)	TA (mg)	Body Weight (g)
WT				
PBS	131.30 ± 2.87	10.60 ± 0.59	53.18 ± 2.28	23.50 ± 0.39
AngII	116.40 ± 3.61 <sup>b</sup>	8.60 ± 0.12 <sup>b</sup>	46.40 ± 1.54 <sup>b</sup>	21.90 ± 0.44
IL-6 KO,				
PBS	128.60 ± 6.50	10.90 ± 1.20	51.60 ± 1.47	23.80 ± 0.59
AngII	123.70 ± 3.97	10.10 ± 1.11	50.10 ± 1.75	22.20 ± 0.69

<sup>a</sup>Data are means ± SEM; n = 12 mice in each group.

<sup>b</sup>P < 0.05, AngII versus PBS infused.

IIB, using immunostaining of TA muscles from three mice of each group. AngII infusion shifted the CSA frequency distribution toward smaller size in type IIB fibers, and the mean CSA of type IIB fibers was smaller compared with results from PBS-infused WT mice. This difference was not observed in type I or IIA fibers or in TA muscles from IL-6 KO mice (Supplemental Figure 3). Thus, type IIB fibers may be more susceptible to AngII-induced proteolysis.

### Proteolytic Pathways Activated by AngII Infusion Depend on IL-6

To assess how AngII causes muscle wasting, we examined evidence for caspase-3 and the UPS proteolytic activities. The 14-kD actin fragment “footprint” of caspase-3 activity<sup>3,11,12,20–22</sup> was increased by AngII (Figure 3A). Immunohistochemistry with an antibody recognizing active caspase-3 confirmed the increase in caspase-3 activity induced by AngII<sup>11</sup> (Figure 3, C and D). There also was an increase in the mRNAs for the E3 ubiquitin ligases Atrogin-1/MAFbx and MuRF-1, consistent with activation of UPS proteolysis<sup>23–25</sup> (Figure 3B). Notably, these responses to AngII infusion were significantly suppressed in muscle of IL-6 KO mice.

### Decreased Muscle Levels of IRS-1 and Phosphorylated Akt Induced by AngII Depend on IL-6

Defective insulin/IGF-1 signaling stimulates muscle protein degradation by caspase-3 and the UPS.<sup>11,12,20</sup> To determine whether similar events contribute to AngII-induced muscle wasting, we measured IRS-1 and phosphorylated Akt (p-Akt) in muscle. AngII significantly reduced IRS-1 and p-Akt in muscle of WT mice.

Both responses were blocked in AngII-infused IL-6 KO mice (Figure 4).

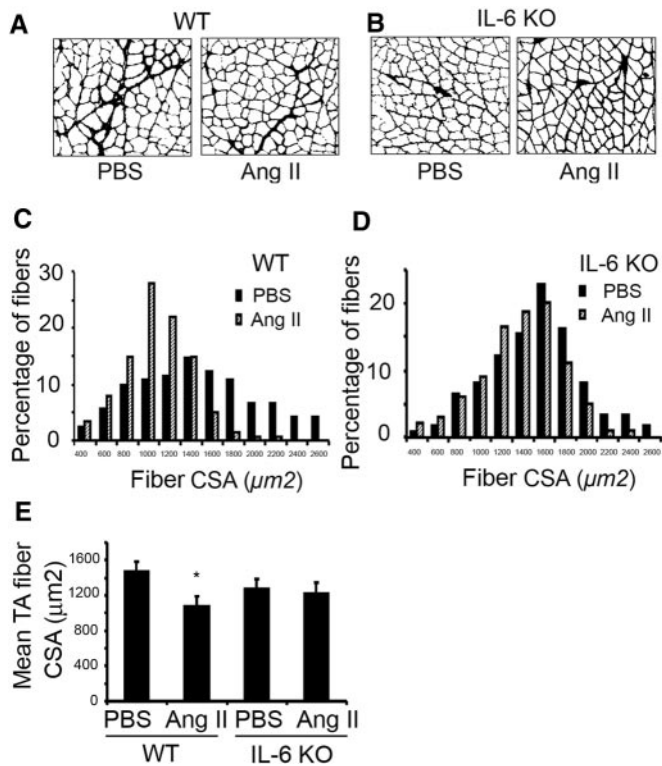
### AngII-Induced SOCS3 Expression in Muscle Depends on IL-6

To understand how AngII downregulates IRS-1, we examined SOCS3, which is stimulated by cytokines or growth factors and can increase IRS-1 degradation by the UPS.<sup>26</sup> In AngII-infused WT mice, SOCS3 mRNA and protein levels in muscle were sharply increased (Figure 5). In contrast, SOCS3 mRNA and protein in muscle of AngII-infused, IL-6 KO mice did not significantly change.

### IL-6 and SAA Act Synergistically to Increase SOCS3 Expression and Decrease IRS-1 Levels

Because IL-6 does not increase protein degradation in isolated muscles,<sup>27</sup> we examined other AngII-induced circulating factors. There was a sharp increase in circulating SAA, an acute-phase protein, and this increase was eliminated in AngII-infused, IL-6 KO mice (Supplemental Figure 4A). Again, the liver was the major source of SAA, although there was some SAA1 mRNA expression in muscle and kidney (Supplemental Figure 4B).

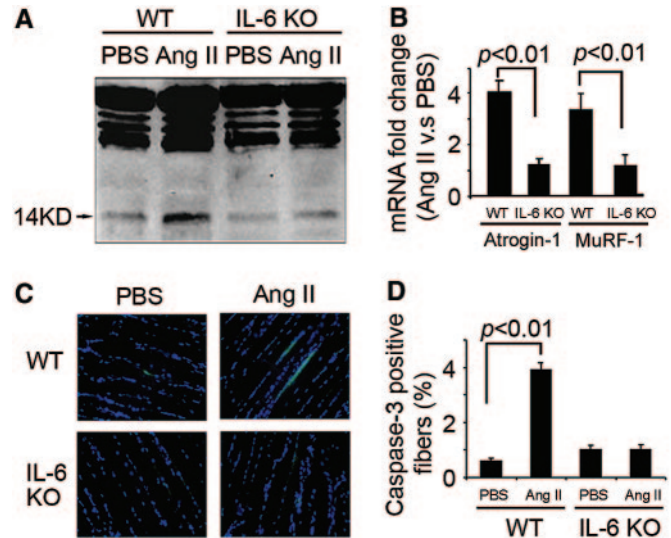
To assess whether SAA1 and IL-6 change muscle metabolism synergistically *in vivo*, we injected tail veins of WT mice with adenoviruses that overexpress either SAA1 or green fluorescence protein.<sup>28</sup> Overexpression of SAA1 alone increased SOCS3 mRNA or protein by 51.5 and 36.5%, respectively, whereas rhIL-6 injection increased SOCS3 mRNA and protein by 56.7 and 38.7%, respectively (Figure 6, A and B). When



**Figure 2.** The muscle atrophy induced by AngII infusion is significantly suppressed in IL-6 KO mice. PBS-infused mice were paired with AngII-infused mice, and the experiments were repeated five times with three animals in each group. (A) TA muscles of WT mice were stained for laminin after 7 d of PBS or AngII infusion. (B) TA muscles of IL-6 KO mice were also stained for laminin. (C) Frequency distribution of fiber sizes (CSA) in TA muscles of AngII- or PBS-infused WT mice. (D) Frequency distribution of CSA in fibers of TA muscles from AngII- or PBS-infused IL-6 KO mice. (E) Mean  $\pm$  SEM of CSA in TA fibers from each group of mice (\* $P < 0.05$  for AngII-infused versus PBS-infused WT mice).

SAA1 overexpression and rhIL-6 injection were combined, SOCS3 mRNA and protein increased 150 and 80.3%, respectively. The latter responses were accompanied by a reciprocal and significant decrease in muscle IRS-1 and p-Akt.

Interpreting results of injecting rhIL-6 and/or the SAA1 adenovirus is complicated because green fluorescence protein or SAA adenoviruses caused an increase in circulating IL-6, whereas rhIL-6 injection raised circulating SAA (Supplemental Figure 5, A and B). For this reason, we studied C2C12 myotubes: Treatment with a high concentration of rhIL-6 produced a minimal decrease in IRS-1 (9.4%) and no detectable change in p-IRS-1ser<sup>307</sup> (Figure 7A). Treatment with SAA1 alone yielded similar results; however, treatment of C2C12 myotubes with rhIL-6 + SAA1 reduced IRS-1 60% ( $P < 0.01$ ). This decrease was accompanied by increases in SOCS3 mRNA (150%) and protein (seven-fold; Figure 7, B and C). Individually, rhIL-6 or SAA1 increased SOCS3 mRNA by approximately 45% (Figure 7C). These responses provide a mechanism for the decrease in IRS-1 because SOCS3 binds to IRS-1 and promotes its degradation by the UPS.<sup>26</sup>



**Figure 3.** Loss of IL-6 prevents AngII-induced activation of proteolytic pathways in muscle. (A) Representative blots of the 14-kD actin fragment resulting from caspase-3 action in gastrocnemius muscle.<sup>20</sup> AngII increased the 14-kD fragment in muscle of WT mice but not IL-6 KO mice. (B) Similar results occurred with RT-PCR measurements of the mRNAs (corrected for GAPDH mRNA) of E3 ubiquitin-conjugating enzymes Atrogin-1/MuRF1. AngII increased Atrogin-1/MuRF1 in muscle of WT but not IL-6 KO mice. (C) Active caspase-3 in TA muscle was detected by immunostaining (green color; nuclei, blue color). (D) The percentage of cells staining positively for activated caspase-3 was calculated by counting the number of cells with activated caspase-3 present in 500 fibers ( $n = 5$  mice in each group).

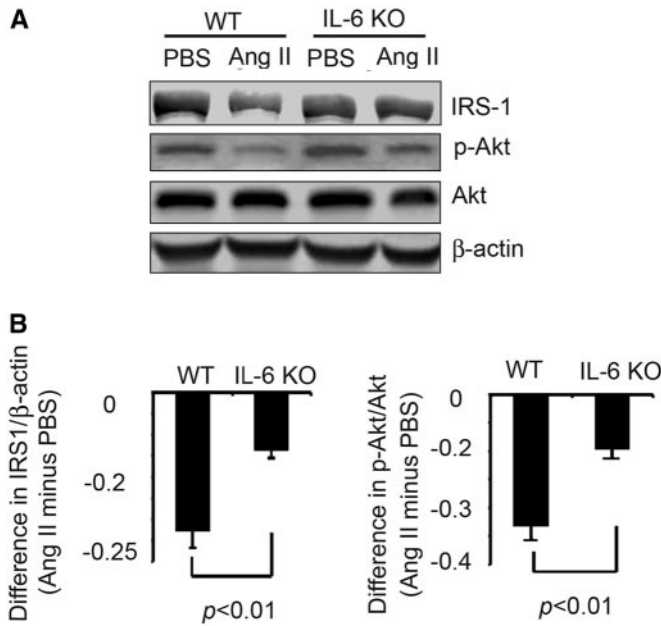
We also evaluated STAT3, an IL-6-activated transcription factor that stimulates SOCS3 expression.<sup>29</sup> Treatment with rhIL-6 alone led to STAT3 phosphorylation, but the addition of SAA1 and rhIL-6 led to a stronger ( $P < 0.05$ ) STAT3 phosphorylation and a major increase in SOCS3 expression. Notably, the increase in p-STAT3 preceded SOCS3 expression (Figure 7B). We also found that the combination increased p-IRS-1ser<sup>307</sup> two-fold ( $P < 0.05$ ), providing another mechanism for IRS-1 degradation.<sup>11</sup> A decrease in muscle IRS-1 could suppress insulin/IGF-1 signaling, triggering protein degradation.<sup>11,12</sup>

To confirm stimulation of proteolysis by IL-6 and SAA1, we studied L6 myotubes. Treatment with high concentrations of either SAA1 or rhIL-6 did not significantly change the rate of protein degradation; the myotube size; the level of the 14-kD actin fragment; or mRNA levels of Atrogin-1, MuRF1, or ubiquitin. In contrast, treatment with rhIL-6 + SAA1 increased protein degradation, the 14-kD actin fragment, and the mRNAs of Atrogin-1 and ubiquitin but not MuRF1; myotube size was reduced (Supplemental Figure 6).

## DISCUSSION

Several complications of CKD, including CHF, hypertension, and progression of renal insufficiency, as well as loss of lean body mass

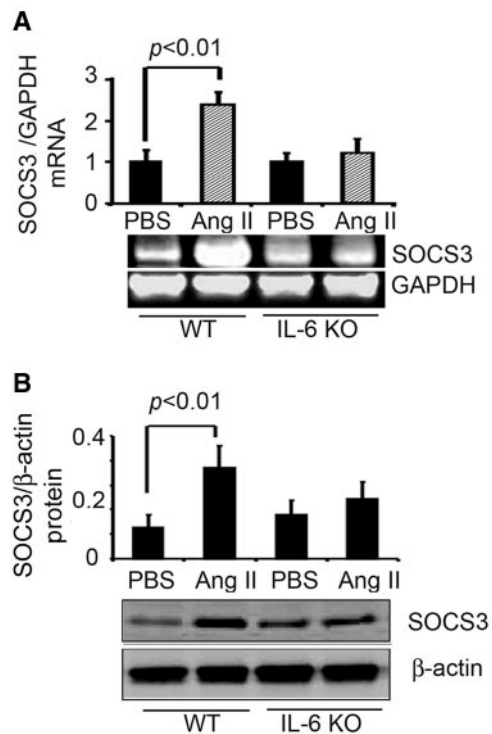




**Figure 4.** IL-6 KO prevents the reduction in IRS-1 and Akt phosphorylation which is induced by AngII infusion. (A) The levels of IRS-1 and p-Akt in gastrocnemius muscles of WT and IL-6 KO mice after 7 d of PBS or AngII infusion were detected by Western blot. (B) Differences in the densities of IRS-1 (corrected for  $\beta$ -actin; left), p-Akt (corrected for total Akt; right) from A are shown. Values from mice infused with AngII minus value in PBS-infused mice were calculated from five mice in each group.

are linked to increased AngII.<sup>3,30,31</sup> To identify how AngII affects muscle protein metabolism, we initially sought to determine whether functional AngII receptors are present in muscle or muscle cells<sup>4</sup> (Figure 1). The absence of AT<sub>1</sub> receptors indicates that AngII works through other factors to cause muscle wasting. In AngII-infused normal mice, we found high circulating levels of IL-6 and SAA; their major source was the liver, indicating contributions of other organs to muscle wasting (Supplemental Figures 2 and 4). Additional studies will be needed to determine the contribution of muscle production of IL-6 and SAA to the proteolytic response we measured in muscle. Thus, muscle proteolytic responses to AngII depend on contributions of other organs. We also found that IL-6 + SAA acted synergistically to increase SOCS3 expression in muscle. The increase in SOCS3 can account for downregulation of IRS-1 and impaired insulin/IGF-1 signaling (documented as decreased Akt phosphorylation [Figure 4]). This pathway explains the loss of muscle mass because we found that AngII infusion into mice overexpressing IGF-1 in muscle do not experience accelerated muscle protein degradation.<sup>3</sup> We conclude that AngII-induced muscle wasting is due to defects in insulin/IGF-1 signaling with activation of caspase-3 and the UPS to degrade muscle protein.<sup>11,12,21,25,32</sup> Indeed, AngII or rhIL-6 + SAA1 did stimulate proteolytic pathways in muscle (Figure 3, Supplemental Figure 6).

We extensively examined muscle and muscle-derived cells for AngII receptors using ribonuclease protection assay and

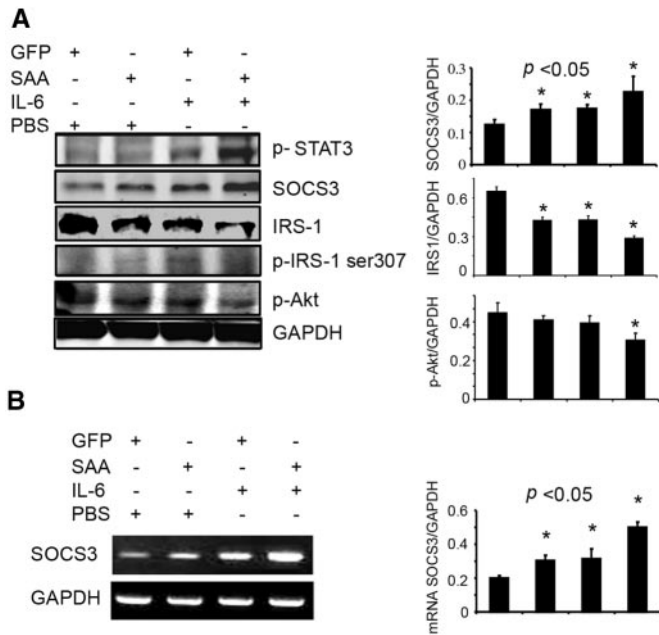


**Figure 5.** AngII infusion stimulates IL-6–dependent expression of SOCS3 in muscle. (A) SOCS3 mRNA in gastrocnemius muscles of AngII- or PBS-infused mice was detected by RT-PCR (corrected for GAPDH mRNA). (B) SOCS3 protein in gastrocnemius muscles of AngII- or PBS-infused mice was detected by Western blot (corrected for  $\beta$ -actin). In each figure, a representative blot is shown in the bottom panel with quantitative results in the top panel. Results from five mice in each group were quantified. The significant increase in SOCS3 induced by AngII was not present in IL-6 KO mice.

RT-PCR (Figure 1). Assessment of an AngII-induced calcium spike or phosphorylation of ERK, an AngII target protein, did not reveal the presence of functional AngII receptors in muscle cells (Supplemental Figure 1). Consequently, we cannot explain reports concluding that AngII causes signaling responses in C2C12 myotubes or that the absence of AngII stimulates muscle cell development.<sup>33,34</sup> In some cases, nonspecific events can occur with high (10  $\mu$ M) AngII concentrations.

Because we did not find AngII receptors in mouse muscle or C2C12 or L6 muscle cells, AngII must act by circulating factors that stimulate muscle proteolysis. The AngII-induced increase in circulating IL-6 and SAA (Table 1, Supplemental Figures 2 and 4) led to stimulation of proteolytic pathways and smaller muscles. Notably, in IL-6 KO mice, AngII did not stimulate pathways of muscle protein breakdown or muscle wasting (Figure 3). We note that the absence of IL-6 did not completely block the loss of muscle mass. This could be related to food deprivation, because high levels of AngII suppress appetite and starvation can impair muscle protein metabolism.<sup>35,36</sup>

How does the absence of IL-6 prevent activation of proteolytic pathways in muscle despite AngII infusion? We found that AngII decreased Akt phosphorylation, a response that triggers

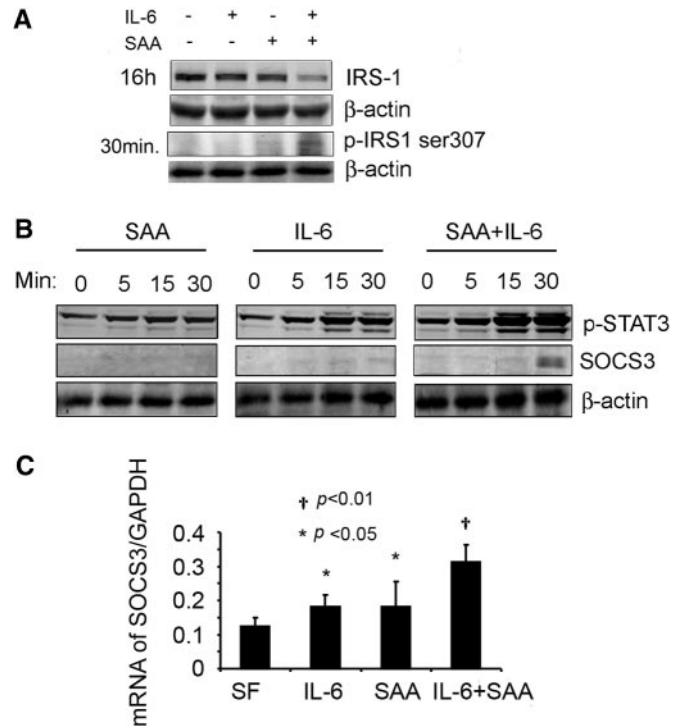


**Figure 6.** Mice treated to overexpress SAA1 and infused with rhIL-6 exhibit increased SOCS3 expression and decreased IRS-1 in gastrocnemius muscles. (A) Levels of p-STAT3, p-IRS-1<sup>ser307</sup>, p-Akt, SOCS3, and IRS-1 in gastrocnemius muscles of mice injected with a recombinant adenovirus expressing SAA1 and/or injected with recombinant IL-6 were detected by Western blot (corrected for GAPDH). (Left) Representative blot and the right panel is a quantification of band densities. (B) SOCS3 mRNA in gastrocnemius muscles of mice described for A were detected by RT-PCR (corrected for GAPDH). Nine mice in each group were studied.

activation of caspase-3 and the UPS in muscle,<sup>3,11,25,32</sup> but when AngII was infused into IL-6 KO mice, there was no decrease in muscle p-Akt (Figure 4). This is because of the finding that AngII did not decrease IRS-1 in these animals, and, hence, insulin/IGF-1 signaling would not be impaired (Figure 4).

Why is IL-6 necessary for the decrease in muscle IRS-1? Cytokines, including IL-6, stimulate the expression of SOCSs, which in turn stimulates UPS-mediated degradation of IRS-1.<sup>26</sup> In AngII-infused mice, we found an increase in muscle SOCS3 levels in WT but not in IL-6 KO mice (Figure 5). We conclude that IL-6 is necessary because it stimulates IRS-1 degradation by upregulating SOCS3 in muscle.

Circulating IL-6 is linked to muscle wasting because injection of IL-6 into rodents produces muscle catabolism; alternatively, treatment with an IL-6 receptor antibody prevents muscle atrophy in mice that overexpress IL-6.<sup>27,37,38</sup> IL-6 actions are complex, however, because IL-6 alone does not stimulate protein degradation in isolated muscles.<sup>27</sup> Indeed, we found that rhIL-6 alone minimally decreased IRS-1 in C2C12 myotubes (Figure 7A), prompting a search for another mediator. In AngII-infused mice, we found a 100-fold increase in SAAs in an IL-6–dependent manner (Supplemental Figure 4A). Moreover, C2C12 myotubes treated with rhIL-6 + SAA1 exhibited a significant increase in SOCS3 and a significant decrease in



**Figure 7.** In C2C12 muscle cells, SAA1 enhances the rhIL-6–induced increase in SOCS3 expression while reducing IRS-1. (A) C2C12 myotubes were treated with rhIL-6, SAA1, or rhIL-6 + SAA1 for 16 h, and IRS-1 was measured by Western blotting (corrected by β-actin). In addition, p-IRS-1<sup>ser307</sup> was measured after 30 min of treatment. (B) C2C12 myotubes were treated with SAA1 and/or rhIL-6 for the indicated times, and p-STAT3 and SOCS3 were measured. (C) SOCS3 mRNA expression was analyzed by RT-PCR (corrected for GAPDH). The experiments were repeated three times, and, in each case, the combination of SAA1 and rhIL-6 resulted in the greatest changes in IRS-1, SOCS3 (protein and mRNA), and p-IRS-1<sup>ser307</sup>.

IRS-1 (Figure 7). When we treated C2C12 myotubes with rhIL-6 or SAA1 alone, we found a small response of SOCS3, and, in this case, IRS-1 did not decrease.

The concentrations of IL-6 or SAA1 studied in C2C12 myotubes was much higher than circulating levels (Table 1, Figure 1, Supplemental Figure 6) but similar to those used by others.<sup>29,39</sup> Despite this difference, SOCS3 and IRS-1 changed minimally in C2C12 myotubes treated with rhIL-6 or SAA1 alone. Possibly, mediators other than SAA1 may be required to stimulate muscle protein degradation *in vivo*. For example, in several catabolic conditions, including AngII-stimulated muscle protein breakdown,<sup>3</sup> glucocorticoids are required to stimulate muscle proteolysis *in vivo*.

Cytokine-induced SOCS3 expression is activated by the transcription factor STAT3,<sup>29</sup> and we found that rhIL-6 + SAA1 significantly increased STAT3 phosphorylation (Figure 7B). This synergistic response was also found *in vivo*: In WT mice treated to express SAA1 and injected with rhIL-6, STAT3 phosphorylation increased as did SOCS3 mRNA and protein levels; IRS-1 protein was decreased (Figure 6).

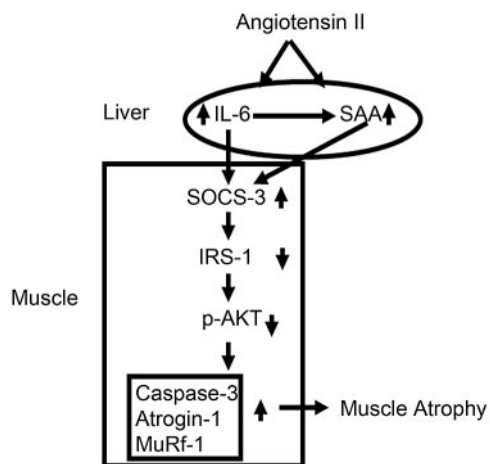
The mechanism by which SAA synergizes with IL-6 to increase SOCS3 transcription is unknown. SAA alone could act through a receptor or, alternatively, might stimulate other transcription factors (e.g., NF- $\kappa$ B) *via* the receptor for advanced glycosylation end products.<sup>28,40,41</sup> In our *in vivo* experiments, SAA1 overexpression or rhIL-6 administration individually was associated with an increase in SOCS3 and decrease in IRS-1, but the combination of SAA1 + rhIL-6 reduced IRS-1 significantly more ( $P < 0.01$ ). The apparent decrease in IRS-1 induced by SAA1 or rhIL-6 alone can be attributed to the *in vivo* interaction between SAA and IL-6; SAA1 overexpression raised circulating IL-6, and rhIL-6 treatment raised SAA (Supplemental Figure 5). Taken together, our results indicate that AngII stimulates IL-6–dependent expression of SAA, raising its circulating level. SAA interacts with IL-6 to increase SOCS3 and decrease IRS-1. When SAA + IL-6 act together, there is a 60% decrease in IRS-1 impairing insulin/IGF-1 signaling (*i.e.*, decreased p-Akt). The result is activation of proteolytic pathways in muscle (Figure 8). To examine the interaction of IL-6 with SAA more completely, it would be of interest to infuse AngII into a SAA KO mouse, but this mouse is not available.

In conclusion, interactions among organs can influence muscle protein metabolism. Specifically, AngII stimulates an interaction between the liver and skeletal muscle because the liver becomes the major source of IL-6 and SAA. The result is stimulation of protein breakdown in muscle. This interaction is of special interest because circulating IL-6 rises in a number of catabolic conditions, including CKD.<sup>6</sup> These results also suggest there could be new roles for acute-phase proteins.

## CONCISE METHODS

### Reagents

rhIL-6 and SAA1 were obtained from Bender Medsystems (Burlingame, CA) and PeproTech (Rocky Hill, NJ), respectively. The SAA produced in



**Figure 8.** A scheme for the interaction of IL-6 and SAA in stimulating SOCS3 to reduce IRS-1 and impair insulin/IGF-1 signaling (signified by a decrease in p-Akt). Impaired insulin/IGF-1 signaling activates proteolytic pathways, caspase-3, and the UPS, leading to muscle wasting.

the liver was identified by RT-PCR as SAA1; the ELISA method (see the ELISA and Multiplex Assay section) for measuring plasma SAA recognized SAA1 and SAA2. Antibodies against phospho-Akt (Ser473), phospho-STAT3 (Tyr 705), and phospho-ERK (42/44) were purchased from Cell Signaling Technology (Beverly, MA). The C-terminus–specific anti-actin, anti-laminin, and anti- $\beta$ -actin antibodies and AngII and horse serum were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against IRS-1 and p-IRS-1ser<sup>307</sup> were purchased from Upstate Biotechnology (Waltham, MA). Antibodies against ERK or SOCS3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Chemicon (Temecula, CA). DMEM and FBS were from Cellgro Mediatech (Manassas, VA). Rat L6 and C2C12 mouse myoblasts from American Type Culture Collection (Manassas, VA) were grown in DMEM to 80% confluence before incubation in DMEM with 2% horse serum for 4 d, stimulating differentiation into myotubes. Myotubes were used in all cell experiments and were treated with 2  $\mu$ g/ml SAA1 and/or 10 ng/ml rhIL-6.<sup>29,39</sup>

### Intracellular Calcium Measurements

Fura-2/AM was purchased from Molecular Probes (Eugene, OR). C2C12 myoblasts infected with a control Ad-TRACK or Ad-TRACK-AT<sub>1</sub> receptor adenoviruses.<sup>5</sup> These were plated on 25-mm coverslips and differentiated into myotubes by incubating in 2% horse serum. The myotubes were washed in calcium-free PBS and loaded with 2  $\mu$ M Fura-2/AM diluted in PBS at room temperature for 1 h. After two washings, the myotubes were equilibrated in PBS for 20 min at room temperature before imaging using an EPI200 Nikon microscope equipped with a TE200-IUC Quantitative Fluorescence Live-Cell and Multidimensional Imaging System and a digital monochrome cooled CCD Roper Coolsnap HQ camera (Roper Scientific, Tucson, AZ). Ca<sup>2+</sup> from single myotubes was recorded every 5 s and quantified by calculating the change in fluorescence ratio expressed as a percentage of the AngII-induced value divided by the basal fluorescence value.

### ELISA and Multiplex Assay

Cytokines and chemokines (mKC, mRANTES, mINF $\gamma$ , mIL-1 $\beta$ , mIL-1 $\alpha$ , mIL-6, mIL-10, mIL-12[p70], and mTNF- $\alpha$ ) in mouse plasma were quantified by the Lincplex assay kit (Linco Research, St. Charles, MO) and Luminex 100 platform (Bio-Rad, Hercules, CA) according to the manufacturers' protocol. The concentration of SAA1 and SAA2 in mouse plasma was measured by ELISA kit from Biosource (Camarillo, CA), and, as with cytokines/chemokines, duplicates were measured.

### AngII Infusion Model

All animal experiments were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. IL-6 KO and WT mice (both of C57/BL6 background) were purchased from Jackson Laboratory (Bar Harbor, ME). Osmotic minipumps (ALZET model 2004; ALZA Corp, Cupertino, CA) were subcutaneously implanted to infuse AngII at 1.5  $\mu$ g/kg per min. AngII increased the BP of WT mice as demonstrated previously.<sup>5</sup> Note that mice in this study were 6 to 10 wk old and, hence, younger than mice

studied previously to avoid the known metabolic changes that might occur in older IL-6 KO mice.<sup>5</sup> Body weight and food intake of each mouse were measured daily. For pair feeding, PBS-infused WT mouse was given the same amount of food as eaten by the AngII-infused mouse on the previous day. After 7 d, mice were anesthetized and tissues were removed, weighed, and snap-frozen in liquid nitrogen before storing at  $-80^{\circ}\text{C}$ .

### Protein Degradation

The rate of protein degradation in cultured L6 myotubes was measured from the release of L-[ $^{14}\text{C}$ ]phenylalanine from prelabeled cells as described previously.<sup>42</sup> The experiment was repeated six times.

### RT-PCR

Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA), cDNA was synthesized using the first-strand cDNA synthesis kit with oligo dT (12 through 18) primers (Invitrogen, Carlsbad, CA), and RT-PCR was performed (Opticon real-time PCR; MJ Research, Waltham, MA). The RT-PCR conditions were as follows:  $42^{\circ}\text{C}$  for 20 min and then 40 cycles at  $95^{\circ}\text{C}$  for 5 min,  $94^{\circ}\text{C}$  for 20 s, and  $72^{\circ}\text{C}$  for 42 s. The melting curve was run at 65 to  $95^{\circ}\text{C}$ . Relative expression was calculated from cycle threshold values corrected for GAPDH [Ct; relative expression =  $2^{(\text{sample Ct} - \text{GAPDH Ct})}$ ]. The specificity of RT-PCR was confirmed using agarose gel electrophoresis. The primers were as follows: Mouse SOCS3 5'-CACAGCAAGTTTCCCGCCGCC-3', 5'-GTGCACCAGCTTGAGTACACA-3'; mouse Atrogin-1 5'-GAGGCAGATTCGCAAGCGTTTGAT-3', 5'-TCCAGGAGAGAATGTGGCAGTGTT-3'; mouse MuRF-1 5'-AGTGTCCATGTCTGGAGGTCGTTT-3', 5'-ACTGGAGCACTCCTGCTGTAGAT-3'; mouse IL-6 5'-GAGGATACTCCCAACAGACC-3', 5'-AAGTGCATCATCGTTGTTTCATACA-3'; mouse SAA1 5'-ACATGAAGGAAGCTGGCTGGAA-3', 5'-ATGTCTGTTGGCTTCCTGGTCA-3'; and mouse AT<sub>1</sub> receptor 5'-AGCCGTCATCCACCGAAATGTGTA-3', 5'-TTAGGAAAGGGAACAGGAAGCCCA-3'.

### mRNA Expression of AT<sub>2</sub>, AT<sub>1A</sub>, and AT<sub>1B</sub> Receptors

A rat AT<sub>2</sub> probe was generated (NM\_012494, nucleotides 745 through 1177) from fetal kidney tissue using RT-PCR. A rat AT<sub>1A</sub> probe (NM\_030985) and AT<sub>1B</sub> probe (NM\_031009) were designed from the 3' untranslated regions, which have low homology between the two receptors. AT<sub>1A</sub> (323 bp) and AT<sub>1B</sub> (266 bp) probes were generated from fetal lung and pituitary gland tissues, respectively, by RT-PCR. Three micrograms of RNA from rat aorta smooth muscle cells and L6 myotubes were used in an RNase protection assay using the Torrey Pines Biolabs kit (Houston, TX) as described previously.<sup>18,19</sup>

### Muscle Fiber Size

Mouse TA muscles were flash-frozen in embedding medium. Cross-sections were fixed in 4% paraformaldehyde at room temperature. After blocking and washing, the slides were incubated for 16 h at  $4^{\circ}\text{C}$  with anti-laminin antibody (Sigma-Aldrich). Alternatively, slides were incubated with type I (Sigma-Aldrich) or type IIA or IIB antibodies (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); sections were subsequently incubated with the Alexa Fluor secondary antibody (1:400; Molecular Probes) for 30 min at

room temperature. After extensive washing with 0.1% TTBS, CSAs of type I, IIA, or IIB fibers were determined using Sigma Pro v5. In each section, at least 500 fibers were examined.

### Statistical Analysis

Values are presented as means  $\pm$  SEM. Results were analyzed using the *t* test when results from two experimental groups were compared or using ANOVA when data from three groups were studied. For data analyzed by ANOVA, pair-wise comparisons were made by the Student-Newman-Keuls test.  $P \leq 0.05$  was considered statistically significant.

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### DISCLOSURES

None.

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