TNF induction of atrogin-1/MAFbx mRNA depends on Foxo4 expression but not AKT-Foxo1/3 signaling

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Moylan JS, Smith JD, Chambers MA, McLoughlin TJ, Reid MB. TNF induction of atrogin-1/MAFbx mRNA depends on Foxo4 expression but not AKT-Foxo1/3 signaling. Am J Physiol Cell Physiol 295: C986–C993, 2008. First published August 13, 2008; doi:10.1152/ajpcell.00041.2008.—Murine models of starvation-induced muscle atrophy demonstrate that reduced protein kinase B (AKT) function upregulates the atrophy-related gene atrogin-1/MAFbx (atrogin). The mechanism involves release of inhibition of Forkhead transcription factors, namely Foxo1 and Foxo3. Elevated atrogin mRNA also corresponds with elevated TNF in inflammatory catabolic states, including cancer and chronic heart failure. Exogenous tumor necrosis factor (TNF) increases atrogin mRNA in vivo and in vitro. We used TNF-treated C2C12 myotubes to test the hypothesis that AKT-Foxo1/3 signaling mediates TNF regulation of atrogin mRNA. Here we confirm that exposure to TNF increases atrogin mRNA (+125%). We also confirm that canonical AKT-mediated regulation of atrogin is active in C2C12 myotubes. Inhibition of phosphoinositol-3 kinase (PI3K)/AKT signaling with wortmannin reduces AKT phosphorylation (~87%) and increases atrogin mRNA (+340%). Activation with insulin-like growth factor (IGF) increases AKT phosphorylation (+126%) and reduces atrogin mRNA (~15%). Although AKT regulation is intact, our data suggest it does not mediate TNF effects on atrogin. TNF increases AKT phosphorylation (+50%) and stimulation of AKT with IGF does not prevent TNF induction of atrogin mRNA. Nor does TNF appear to signal through Foxo1/3 proteins. TNF has no effect on Foxo1/3 mRNA or Foxo1/3 nuclear localization. Instead, TNF increases nuclear Foxo4 protein (+55%). Small interfering RNA oligos targeted to two distinct regions of Foxo4 mRNA reduce the TNF-induced increase in atrogin mRNA (~34% and ~32%). We conclude that TNF increases atrogin mRNA independent of AKT via Foxo4. These results suggest a mechanism by which inflammatory catabolic states may persist in the presence of adequate growth factors and nutrition.

skeletal muscle; cachexia; atrophy; ubiquitin; cytokines

THE BULK OF MUSCLE PROTEIN lost during muscle atrophy is degraded by the ubiquitin-proteasome system (19, 25, 35). The specificity of this system is regulated by ubiquitin ligases (E3 proteins). Atrogin-1/MAFbx (atrogin) is a muscle-specific E3 protein that is upregulated in catabolic states that include humans under voluntary bed rest (37), or with spinal cord injury (46), amyotrophic lateral sclerosis (27), or chronic obstructive pulmonary disease (COPD) (9); and in animal models of cancer (4), sepsis (12, 49), aging (3), chronic heart failure (29), chronic kidney disease (10), diabetes (7, 24), starvation (20, 24), denervation (39), and unloading (17). The role of atrogin as a mediator of muscle atrophy is supported by studies that show overexpression in cell culture myotubes results in a fivefold reduction in myotube diameter (2). In addition, the absence of atrogin results in a 56% reduction in muscle atrophy after denervation in knockout mice (2).

Atrogin gene expression is under the transcriptional control of Forkhead box O transcription factors (Foxo). Foxo1, Foxo3, and Foxo4 are related Foxo proteins thought to regulate atrogin under a variety of conditions. Foxo1 and Foxo3 mRNA are upregulated with starvation (21, 24), diabetes (26), cachexia, and aging (13, 22, 24). Foxo4 is less well studied but is modulated in heart failure models of atrophy (42).

Foxo1, Foxo3, and Foxo4 activity is regulated by phosphoinositol-3 kinase (PI3K)-protein kinase B (AKT) signaling (41, 42, 45). Basal AKT function maintains Foxo in a phosphorylated state that favors cytoplasmic retention and repression of transcriptional activity. Overexpression of IGF can further suppress Foxo activity through stimulation of AKT (15, 16, 42). Wortmannin, a PI3K inhibitor, depresses AKT activity and Foxo phosphorylation (28, 40, 44). With starvation or dexamethasone treatment, AKT activity and Foxo1 and Foxo3 phosphorylation are decreased. Foxo1/3 subsequently translocate to the nucleus to drive atrogin expression (41, 45). The activity of Foxo4 under these conditions has not been established.

Atrogin mRNA has been shown to increase with tumor necrosis factor (TNF) exposure in muscles of rats (12) and mice (31) and in primary and C2C12 myotubes (30, 31). The goal of our current study was to define components of the signaling pathway that mediate TNF-induced atrogin expression. We postulated that a similar AKT-Foxo1/3-dependent mechanism mediates TNF effects on atrogin expression in differentiated muscle cells. This model (Fig. 1A) was evaluated by using mature C2C12 myotubes to test two hypotheses: 1) TNF induces atrogin expression through inhibition of AKT. To assess the role of AKT, we measured changes in atrogin expression and AKT phosphorylation after a time course of TNF exposure. We also measured the inhibitory potential of AKT activation on TNF-induced atrogin expression. 2) TNF induces atrogin expression through activation of Foxo1/3 transcription factors. To assess the roles of Foxo1, Foxo3, and Foxo4, we measured changes in Foxo mRNA, protein, and nuclear translocation after TNF exposure.

Here we confirm that elements of the canonical AKT-Foxo1/3-atrogin pathway are active in C2C12 myotubes. Inhibition of

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Akt or overexpression of Foxo1 causes an increase in atrogin mRNA. Paradoxically, we demonstrate that TNF increases atrogin mRNA despite simultaneous activation of AKT. In addition, TNF has no apparent effect on endogenous Foxo1 or Foxo3 activity. Instead, it induces nuclear translocation of Foxo4 protein. Finally, myotubes treated with Foxo4 small interfering RNA (siRNA) have a diminished atrogin response to TNF. Thus we propose a revised model where TNF bypasses AKT inhibitory signaling to activate Foxo4-mediated atrogin expression (Fig. 1B).

EXPERIMENTAL PROCEDURES

Cell culture. C2C12 myoblasts (American Type Culture Collection, Rockville, MD) were seeded at a density of 8,000–10,000 cells/cm² and allowed to proliferate in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 25 mg/ml gentamicin (GIBCO-Invitrogen, Carlsbad, CA) in 5% CO₂ at 37°C. After 2 days the myoblasts were shifted to differentiation medium (Dulbecco’s modified Eagle’s medium-2% horse serum-25 mg/ml gentamicin). After 4–6 days of differentiation, myotubes were treated with the following reagents: 6 ng/ml recombinant mouse TNF (Pierce, Rockford, IL), 10 ng/ml recombinant human IGF-1 (Invitrogen), 100 nM wortmannin (Sigma-Aldrich, St. Louis, MO), or 1 μM 4-hydroxytamoxifen (Sigma-Aldrich).

Stable Foxo1/TSS-ER C2C12 cell lines were generated according to Bastie et al. (1). Briefly, the AKT phosphorylation sites in Foxo1 (Thr24, Ser256, and Ser319) were mutated to alanine. The COOH-terminus was fused to a modified ligand-binding domain of the estrogen receptor. This ligand-binding domain is transcriptionally inactive and has been mutated such that it specifically responds to tamoxifen but not to endogenous estrogens (33). The construct was cloned into the pBABE retroviral vector containing the puromycin selection marker and stably transfected cells were isolated.

Relative quantification real time PCR (qPCR). Reverse transcription was performed with Murine-Malone myeloma virus reverse transcriptase and random hexamers (Promega, Madison, WI) plus 2 μg total RNA isolated with TRIzol reagent (Invitrogen). The mouse cDNA sequences for atrogin-1/MAFbx (NM-026346), Foxo1 (NM-019739.2), Foxo3 (NM-019740.2), Foxo4 (Mllt7, NM-018789.1), and β-actin (NM-007393.1) were obtained from GenBank. PCR primers were designed from the cDNA sequences using Primer Express 1.5 software (Applied Biosystems, Foster City, CA). Primer sequences are as follows: atrogin forward 5’-ATGCA-CACGGTGGCACAGAGG-3’; reverse 5’-GTGAAGACACATCATGCTCA-3’; Foxo1 forward 5’-GTGAACACAGAATCTGCTCA-3’; reverse 5’-CACAGTCCAAGGCTCATA-3’; Foxo3 forward 5’-AGGCGTG-TACTGAGGACCT-3’; reverse 5’-CTCTGCCGATATGGAAG-3’; Foxo4 forward 5’-CAAGAAGAAGCCTGCTGTC-3’; reverse 5’-CTGACGCGTCTAGACCTATTA-3’; β-actin forward 5’-AGGCCAGGCAAGAGAGGTGA-3’; reverse 5’-CCATGTGGTGCCACTCTTGTGAA-3’.

Synthesized primers were purchased from Invitrogen. PCR was performed using Applied Biosystems 7500 Real Time PCR system. Targets were amplified from 50 ng of cDNA using SYBR Green Master Mix reagent (stage 1, 1 cycle, 50°C, 2 min; stage 2, 1 cycle, 95°C, 10 min; stage 3, 40 cycles, 95°C, 15 s, 60°C, 1 min; Applied Biosystems). Reactions were performed in duplicate or triplicate for each cDNA sample. The abundance of target mRNA relative to β-actin mRNA was determined using the comparative cycle threshold method (14, 34).

Western blot analysis. Cells were washed once with phosphate-buffered saline (PBS) then resuspended and briefly sonicated in 2x sample loading buffer (120 mM Tris, pH 7.5, 200 mM DTT, 20% glycerol, 4% SDS, and 0.002% bromphenol blue). Proteins were fractionated on 4–15% SDS-polyacrylamide gels (Criterion precast gels, Bio-Rad, Hercules, CA). Fractionated proteins were transferred to reduced-fluorescence polyvinylidifluoride membrane (Immobilon-FL, Millipore, Bedford, MA). Membranes with transferred proteins were blocked for 1 h at room temperature in Odyssey Blocking Buffer (LI-COR, Lincoln, NE). Primary antibodies were incubated overnight at room temperature in Odyssey Blocking Buffer mixed 1:1 with PBS plus 0.2% Tween. Secondary antibodies were incubated for 30 min in Odyssey-PBS-0.2% Tween plus 0.01% SDS. Total AKT antibody and atrogin antibody were purchased from ECM Biosciences (Versailles, KY). Foxo1, Foxo4, and phospho-specific AKT (S473) antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Foxo3 antibody was purchased from Upstate (Lake Placid, NY). Myosin antibody was from Sigma. Fluorescent secondary antibodies were used for detection (goat anti-mouse Alexa-680, Molecular Probes-Invitrogen; goat anti-rabbit IRD800, Rockland Immunochemicals, Gilbertsville, PA). Fluorescence was imaged and results were quantified using the Odyssey Infrared Imaging System (LI-COR).

Immunocytochemistry. Foxo1/TSS overexpressing C2C12 skeletal myotubes were grown on glass coverslips coated in ECL cell attachment matrix (Upstate, Charlotteville, VA) for 4 days in DMEM supplemented with 2% horse serum (Invitrogen). Foxo1/TSS myotubes were treated with either vehicle (0.1% vol/vol EtOH final concentration) or 1 μM 4-hydroxytamoxifen (4-HT) for 2 h. Myotubes were then washed in PBS, fixed in 4% formaldehyde in PBS, permeablized (0.5% Triton X-100 in PBS), blocked (1 M Tris-HCl, pH 7.6, supplemented with 3% BSA, 0.2% gelatin, 0.05% Tween-20), and incubated overnight at 4°C with primary antibody directed against the Foxo1 protein (Cell Signaling). Myotubes were then washed in PBS, incubated with a TRITC-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA), and mounted in Vectashield mounting medium supplemented with 4’,6-diamidino-2-phenylindole (Vector Labs, Burlingame, CA). Myotubes were assessed for Foxo1 localization using a Zeiss LSM 510 confocal microscope (Carl Zeiss Microimaging, Thornwood, NY).

siRNA. Two Silencer predesigned siRNAs for Foxo4 (siRNA IDs: no. 184930 and no. 184932) and Negative Control no. 2 siRNA were purchased from Ambion (Austin, TX). siRNA (100 μM) was transfected
into day 4 myotubes using oligofectamine diluted 1:5 with DMEM according to the manufacturer’s instructions (Invitrogen). Changes in atrogin mRNA and protein were assayed 72 h posttransfection.

Statistical analysis. Data were normally distributed and are expressed as means ± SE. Student’s t-test was used for the statistical comparison of two means; ANOVA was used for the comparison of multiple means. When ANOVA revealed significant differences, Tukey’s post hoc test for multiple comparisons was performed. *P values < 0.05 were considered significant.

RESULTS

TNF modulation of atrogin. TNF regulation of atrogin mRNA and the effect on myosin levels was measured by real-time PCR and Western blot analysis. Atrogin mRNA increases early and peaks at 2 h. A decrease in myosin protein is detected after 72 h of daily TNF treatments (Fig. 2A). These data confirm earlier reports that show TNF induces atrogin expression (30) and myosin loss (8, 32). In addition, our data are the first to show that TNF causes an elevation in atrogin protein that begins to accumulate by 6 h (Fig. 2A) and remains elevated with chronic, repeated exposure to TNF (Fig. 2B).

AKT/Foxo pathway regulation of atrogin. The contribution of AKT/Foxo signaling to control of atrogin expression was confirmed by exposing myotubes to wortmannin, an inhibitor of PI3K that reduces AKT activity (28, 40, 44), IGF, a stimulator of PI3K/AKT signaling that increases AKT activity (15, 16, 42), or overexpression of a Foxo1 mutant (Foxo1/TSS) resistant to inhibition by AKT and activated by 4-HT (1, 33). Inhibition or activation of AKT was evaluated by Western blot analysis with phospho-specific and total AKT antibodies. Treatment of myotubes with wortmannin for 2 h produced the expected decrease in AKT phosphorylation and increase in atrogin mRNA (Fig. 3A). IGF effects were reversed; as phospho-AKT increased, atrogin mRNA decreased (Fig. 3B). Foxo1/TSS nuclear translocation, as assessed by immunofluo-
rescent staining, caused a corresponding rise in atrogin mRNA (Fig. 3C). These data suggest that basal atrogin mRNA levels are held in check by AKT. This suppression is augmented by IGF, whereas AKT inhibition or Foxo1/TSS nuclear translocation allows atrogin mRNA to accumulate.

**TNF modulation of AKT.** The control AKT signaling exerts on atrogin is demonstrated by experiments described in Fig. 3, thus is seemed likely that TNF would modulate this pathway to promote atrogin expression. Paradoxically, TNF acted opposite of our expectations and induced an increase AKT phosphorylation (Fig. 4A). Phosphorylation of AKT begins early and peaks at 2 h, following a time course similar to atrogin induction (Fig. 2A). Thus TNF promotes atrogin expression not because of suppression of AKT signaling, but despite an increase in AKT signaling. Further evidence to support this conclusion is shown in Fig. 4B. IGF, through activation of AKT, is known to suppress glucocorticoid-induced atrogin expression (45). Therefore, we treated myotubes with IGF 30 min before the addition of TNF. Two hours post-TNF we measured AKT phosphorylation and atrogin expression. IGF pretreatment increased AKT phosphorylation eightfold over TNF alone, but TNF-induced atrogin expression was unaffected. Thus the mechanism of TNF activation of atrogin appears to be independent of the canonical AKT pathway.

**Foxo isoforms in C2C12 myotubes.** Foxo proteins are also a component of our model (Fig. 1). But before evaluating TNF effects on Foxo proteins, we wanted to know more about basal expression of the different isoforms in C2C12 myotubes. Figure 5A shows the relative abundance of Foxo isoform mRNAs. Foxo1 is most abundant and is expressed as 100%. Foxo3 and Foxo4 are 65% and 15% of Foxo1 levels, respectively. Since Foxo proteins are in part regulated by control of nuclear localization, we measured the nuclear and cytoplasmic distribution of Foxo isoforms (Fig. 5B). Both Foxo1 and Foxo3 have a greater presence in the nuclear fraction with nuclear-to-cytoplasmic ratios of 1.4 and 0.74, respectively. In contrast, Foxo4 is predominantly cytoplasmic with a nuclear-to-cytoplasmic ratio of 0.05.

**Foxo isoform responses to TNF.** To assess whether TNF modulates Foxo isoform activity, we treated myotubes with TNF and measured changes in mRNA. We also assessed changes in nuclear localization by cell fractionation and Western blot analysis. TNF has no affect on Foxo isoform mRNA (Fig. 6A) nor does
it affect Foxo1 or Foxo3 nuclear localization. TNF does increase Foxo4 nuclear localization (Fig. 6B). These data suggest that TNF modulates Foxo, and the response is isoform specific with Foxo4 being most sensitive.

**Foxo4 knockdown and reduction of the atrogin response to TNF.** To further evaluate Foxo4 as a TNF-sensitive factor, we used siRNA to selectively depress Foxo4 mRNA and protein. Figure 7A shows that siRNAs targeted to two different regions of the Foxo4 mRNA and a nonspecific siRNA control (siFoxo4-0, siFoxo4-2, and NS) were transfected into myotubes 96 h postdifferentiation. Seventy-two hours posttransfection, myotubes were treated for 2 h with 6 ng/ml TNF. A: Foxo4 mRNA, as assayed by real time PCR, and protein, as assayed by Western blot analysis, were reduced by both siFoxo4-0 and siFoxo4-2 relative to the nonspecific siRNA control (*P < 0.05, n = 3). B: TNF-induced increase in atrogin mRNA, as measured by real time PCR, was reduced by siFoxo4-0 and siFoxo4-2 relative to the nonspecific siRNA control (34% average reduction, *P < 0.05, n = 3). C: linear regression showing the relationship between changes in Foxo4 protein and changes in atrogin mRNA.

Fig. 6. Foxo isoform response to TNF. A: C2C12 myotubes were treated with 6 ng/ml TNF for 2 h; total RNA was isolated and assayed by real time PCR for changes in Foxo1, Foxo3, and Foxo4 mRNA. Foxo isoform mRNAs did not change with TNF. B: nuclear and cytoplasmic extracts were prepared from TNF-treated C2C12 myotubes, and Foxo isoform localization was measured by Western blot analysis with antibodies specific for Foxo1, Foxo3, and Foxo4. Only Foxo4 translocates to the nucleus in response to TNF (*P < 0.05).

Fig. 7. Downregulation of Foxo4 diminishes the induction of atrogin mRNA in response to TNF. Small interfering RNAs (siRNAs) 100 μM specific to 2 regions of the Foxo4 mRNA and a nonspecific siRNA control (siFoxo4-0, siFoxo4-2, and NS) were transfected into myotubes 96 h postdifferentiation. Seventy-two hours posttransfection, myotubes were treated for 2 h with 6 ng/ml TNF. A: Foxo4 mRNA, as assayed by real time PCR, and protein, as assayed by Western blot analysis, were reduced by both siFoxo4-0 and siFoxo4-2 relative to the nonspecific siRNA control (*P < 0.05, n = 3). B: TNF-induced increase in atrogin mRNA, as measured by real time PCR, was reduced by siFoxo4-0 and siFoxo4-2 relative to the nonspecific siRNA control (34% average reduction, *P < 0.05, n = 3). C: linear regression showing the relationship between changes in Foxo4 protein and changes in atrogin mRNA.
of Foxo4 reduced Foxo4 mRNA by 32% and 56%, and Foxo4 protein by 19% and 25%, relative to a nonspecific siRNA control. Foxo4 siRNA had a modest effect on basal atrogin levels (reduced 8%, \( P < 0.2 \) and 17%, \( P < 0.05 \)) and diminished the response to TNF by an average of 34% relative to the nonspecific control (Fig. 7B). Figure 7C shows that basal and TNF-induced atrogin mRNA levels were proportional to Foxo4 protein content; as siRNA depressed Foxo4, atrogin mRNA fell. Thus Foxo4 appears to modulate atrogin mRNA.

**IGF regulation of Foxo4.** Since Foxo4 appears to mediate TNF-induced atrogin mRNA, and IGF fails to inhibit this response, we hypothesized Foxo4 might resist IGF action. We measured phosphorylation of the AKT inhibitory sites on Foxo4 after IGF or IGF+TNF treatment. Figure 8A shows that phospho-Foxo4 levels increase with IGF and this is not affected by pretreatment with TNF. We also measured Foxo4 nuclear translocation (Fig. 8B). In contrast to phosphorylation, 2 h of IGF exposure did not drive Foxo4 from the nucleus nor did pretreatment prevent TNF-induced nuclear translocation. These data suggest an alternate activation mechanism overrides inhibitory phosphorylation to promote nuclear translocation of Foxo4.

**DISCUSSION**

The goal of our current study was to define components of the signaling pathway that mediate TNF-induced atrogin expression. We proposed that TNF would function similar to starvation where atrogin mRNA is elevated through AKT inhibition and Foxo1 and/or Foxo3 activation. Instead, our results suggest that induction of atrogin by TNF bypasses AKT-Foxo1/3 signaling and triggers an alternate pathway. Whereas starvation or glucocorticoids reduce AKT phosphorylation, TNF increases it. IGF inhibits starvation- and glucocorticoid-induced atrogin expression but has no influence on TNF regulation. These observations contribute to a gathering body of literature that suggest inflammation-related atrophy is not controlled by AKT-Foxo1/3 signaling. For example, COPD patients with limb muscle atrophy have elevated atrogin and MuRF1 mRNA; however, phosphorylated AKT is also increased (9). In addition, patients with amyotrophic lateral sclerosis (ALS) exhibit skeletal muscle atrophy and increased atrogin mRNA and protein. However, neither Foxo1 nor Foxo3 protein levels differ from healthy control individuals (27). Finally, Dehoux et al. (6) show IGF does not reverse C2C12 myotube atrophy caused by an inflammatory cocktail of TNF and IFN-\( \gamma \). In contrast to our data, however, they show IGF reverses the cytokine-induced increase in atrogin mRNA. The apparent discrepancy may be due to differences in experimental design and cytokine stimuli. Our experiments measured early responses 2 h after treatment with TNF. We tested the effects of IGF pretreatment on these early responses and found atrogin expression was unaffected. Dehoux et al. (6) measured a longer-term response 24 h after treatment with both TNF and IFN\( \gamma \). They tested the effects of IGF posttreatment on the longer-term response and found atrogin mRNA levels were blunted. Combined, these data suggest there may be two phases to cytokine regulation of atrogin: an early phase that is independent of AKT and a prolonged phase that is sensitive to AKT regulation. This second phase could involve IFN\( \gamma \), an idea consistent with a report by Smith et al. (43) that shows IFN\( \gamma \) depresses AKT phosphorylation. Both studies measured the effects of IGF on atrogin mRNA. IGF-cytokine combination studies that also measure atrogin protein would further clarify these issues.

Our results suggest that neither Foxo1 nor Foxo3 respond to TNF. Instead, Foxo4 appears to be the TNF-responsive isoform. Under basal conditions Foxo4 protein is mostly restricted to the cytoplasm and TNF does not reverse IGF-induced phosphorylation. Knockdown of Foxo4 protein by siRNA represses TNF regulation of atrogin. These data prompted us to refine our model and propose that Foxo4 mediates TNF-induced atrogin expression. Our results are reinforced by data from mouse models of heart failure, a pathophysiological process accompanied by chronic inflammation. Twelve weeks after induction of heart failure, animals exhibit skeletal muscle atrophy with...
increased active Foxo4 protein and elevated atrogin mRNA (42). Further experiments with Foxo4-null mice or cells stably transfected with Foxo4 shRNA will be important to better define the role Foxo4 plays in atrophy associated with chronic inflammation.

There are several potential mechanisms by which TNF might activate Foxo4. Among these, response to changes in intracellular oxidant activity is a common regulatory theme. Skeletal muscle oxidant activity is elevated in chronic inflammatory states or with exposure to TNF (18, 36). These increases in cellular oxidant activity are thought to be pro-catastolic (38, 36) and can activate Foxo4 by at least four mechanisms. First, Foxo factors are subject to regulation by redox-sensitive reversible acetylation (23). Acetylation, which inhibits Foxo transcriptional activity, is catalyzed by histone acetyltransferase cAMP-response element-binding protein-binding protein (5). Oxidative stress releases this inhibition by deacetylation via NAD-dependent deacetylase hSirt2 (SIRT1) (23, 48). Second, oxidative stress can stimulate rapid mono-ubiquitination of Foxo4, causing nuclear translocation and increased transcriptional activity in human embryonic kidney cells (47). Third, H2O2 or TNF treatment of fibroblasts increases activation of Foxo4 via jun NH2-terminal kinase (JNK)-dependent phosphorylation of threonines 447 and 451 (11). JNK is also activated by H2O2 or TNF in cultured myotubes (30), suggesting JNK could mediate TNF/Foxo4 signaling in muscle. Finally, Li et al. (30) demonstrated that TNF-induced atrogin expression is dependent on p38 mitogen-signaling in muscle. Finally, Li et al. (30) demonstrated that TNF-induced atrogin expression is dependent on p38 mitogen-activated protein kinase (p38 MAPK). Like JNK, p38 MAPK activity is increased by H2O2 or TNF in skeletal muscle myotubes, and suppression of this activity prevents the induction of atrogin (30).

The signaling mechanisms that regulate atrogin expression appear to differ depending on catabolic stimulus. AKT regulation of atrogin is modulated by low nutrients, dexamethasone, or pharmacological inhibition. All of these reduce AKT activity, relieving Foxo1, Foxo3 (41, 45), and presumably Foxo4 inhibition. TNF and/or potentially inflammation in general appear to act via a second pathway. Our current findings indicate TNF increases Foxo4 activity through an AKT-independent, growth factor-insensitive process. Thus the TNF/Foxo4 pathway is a mechanism by which inflammation might upregulate atrogin and promote muscle catabolism despite high levels of circulating nutrients or growth factors. The intermediate steps in this pathway and the mechanism by which TNF bypasses AKT regulation of Foxo4 are yet to be defined.

GRANTS

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