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SEPSIS INCREASES THE EXPRESSION AND ACTIVITY OF THE TRANSCRIPTION FACTOR FORKHEAD BOX O 1 (FOXO1) IN SKELETAL MUSCLE BY A GLUCOCORTICOID-DEPENDENT MECHANISM

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Abstract

Sepsis-induced muscle wasting has severe clinical consequences, including muscle weakness, need for prolonged ventilatory support and stay in the intensive care unit, and delayed ambulation with risk for pulmonary and thromboembolic complications. Understanding molecular mechanisms regulating loss of muscle mass in septic patients therefore has significant clinical implications. FOXO transcription factors have been implicated in muscle wasting, partly reflecting upregulation of the ubiquitin ligases atrogin-1 and MuRF1. The influence of sepsis on FOXO transcription factors in skeletal muscle is poorly understood. We tested the hypothesis that sepsis upregulates expression and activity of FOXO transcription factors in skeletal muscle by a glucocorticoid-dependent mechanism. Sepsis in rats increased muscle FOXO1 and 3a mRNA and protein levels but did not influence FOXO4 expression. Nuclear FOXO1 levels and DNA binding activity were increased in septic muscle whereas FOXO3a nuclear levels were not increased during sepsis. Sepsis-induced expression of FOXO1 was reduced by the glucocorticoid receptor antagonist RU38486 and treatment of rats with dexamethasone increased FOXO1 mRNA levels suggesting that the expression of FOXO1 is regulated by glucocorticoids. Reducing FOXO1, but not FOXO3a, expression by siRNA in cultured L6 myotubes inhibited dexamethasone-induced atrogin-1 and MuRF1 expression, further supporting a role of FOXO1 in glucocorticoid-regulated muscle wasting. Results suggest that sepsis increases FOXO1 expression and activity in skeletal muscle by a glucocorticoid-dependent mechanism and that glucocorticoid-dependent upregulation of atrogin-1 and MuRF1 in skeletal muscle is regulated by FOXO1. The study is significant because it provides novel information about molecular mechanisms involved in sepsis-induced muscle wasting.

Keywords

FOXO transcription factors; muscle wasting; ubiquitin ligases; atrogin-1; MuRF1

INTRODUCTION

Because muscle wasting in various catabolic conditions at least in part reflects upregulated transcription of genes in the ubiquitin-proteasome proteolytic pathway, in particular the

ubiquitin ligases atrogin-1 and MuRF1 (Bodine et al, 2001; Clarke et al, 2007; Gomes et al, 2001; Wray et al, 2003), and of autophagy-related genes (Mammucari et al, 2007; Zhao et al, 2007), the potential role of transcription factors that may regulate gene activation in atrophying muscle has been the focus of several recent reports (Guttridge, 2004; Hasselgren, 2007a). For example, studies from our and other laboratories suggest that NF- κ B (Cai et al, 2004; Penner et al, 2001; Van Gammeren et al, 2009), C/EBP β and δ (Penner et al, 2002; Yang et al, 2005), AP-1 (Moore-Carrasco et al, 2006; Penner et al, 2001), and the glucocorticoid receptor (Hall-Angeras et al, 1991; Tiao et al, 1996; van Raalte et al, 2009) may participate in the regulation of gene activation during muscle wasting.

An additional group of transcription factors that may be involved in muscle wasting are the Forkhead box O (FOXO) transcription factors FOXO1, FOXO3a, and FOXO4. FOXO-dependent gene activation can be regulated by increased overall expression and by posttranslational modifications of the transcription factors (Brunet et al, 2004; Hasselgren, 2007b; Katoh, 2004). Phosphorylation and dephosphorylation, resulting in inactivation and activation, respectively, are the most studied posttranslational modifications of FOXO transcription factors, at least in the context of muscle wasting (Hasselgren, 2007b; Mammucari et al, 2007; Sandri et al, 2004; Stitt et al, 2004), and influence their activity mainly by regulating the trafficking of FOXOs between the cytoplasm and nucleus (Van der Heide et al, 2004).

Initial evidence for a role of FOXO transcription factors in the regulation of muscle mass was reported by Furuyama et al (2002, 2003) and Kamei et al (2003). In their studies, FOXO1 and 3a expression was increased in skeletal muscle during aging, starvation, diabetes, and glucocorticoid treatment, conditions that are associated with pronounced muscle wasting. In subsequent experiments, Kamei et al (2004) created transgenic mice specifically overexpressing FOXO1 in skeletal muscle and found that these mice developed muscle atrophy and increased expression of lysosomal cathepsin L. In a more recent study by Liu et al (2007), downregulation of FOXO1 in cultured muscle cells in vitro or in mouse skeletal muscles in vivo resulted in reduced expression of myostatin and increased expression of MyoD, providing additional support for the concept that FOXO1 may be involved in the regulation of muscle mass.

Further evidence for a role of FOXO transcription factors in the regulation of muscle homeostasis was reported by Sandri et al (2004) and Stitt et al (2004). In one of those studies (Sandri et al, 2004), depriving cultured myotubes of growth factors ("starvation") or treating them with dexamethasone resulted in myotube atrophy, reduced amounts of phosphorylated FOXO1, 3a, and 4, nuclear translocation of FOXO factors, increased FOXO DNA binding and activation of the atrogin-1 gene. When constitutively active FOXO3a was transfected into mouse tibialis anterior muscle by electroporation, muscle fiber diameter decreased by approximately 60%. In another study (Stitt et al, 2004), treatment of cultured myotubes with IGF-1 resulted in Akt-mediated phosphorylation and inactivation of FOXO transcription factors, downregulated expression of atrogin-1 and MuRF1, and myotube hypertrophy. In addition to regulating the transcription of atrogin-1 and MuRF1, recent studies suggest that FOXO transcription factors also regulate the expression of autophagy-related genes, resulting in stimulated autophagy and lysosomal proteolysis in atrophying muscle (Mammucari et al, 2007; Zhao et al, 2007).

Although previous reports suggest that various conditions associated with loss of muscle mass are associated with activation of FOXO1 and 3a (Furuyama et al, 2002, 2003; Kamei et al, 2004), the influence of sepsis on the expression and activity of FOXO transcription factors is poorly understood. In a recent report by Nystrom and Lang (2008), sepsis induced by cecal ligation and puncture (CLP) in mice resulted in a 2.5- to 3-fold increase in FOXO1

and 3a mRNA levels in gastrocnemius muscle but the influence of sepsis on total and phosphorylated FOXO protein levels and FOXO activity is not known from that study. In addition, the mechanism of sepsis-induced FOXO1 and 3a expression and activity is unknown. In particular, the role of glucocorticoids in the regulation of FOXO transcription factors in skeletal muscle during sepsis has not been reported. This is significant because glucocorticoids play an important role in sepsis-induced muscle wasting (Hasselgren, 1999; Shakman et al, 2008) and were found in previous reports to regulate the expression of genes in the ubiquitin-proteasome pathway in septic muscle (Sun et al, 2008; Tiao et al, 1996).

In the present study, we determined the influence of sepsis, induced by cecal ligation and puncture (CLP) in rats, on the expression and activity of the FOXO1, 3a, and 4 transcription factors in skeletal muscle and tested the hypothesis that glucocorticoids are involved in sepsis-induced activation of FOXO1.

MATERIALS AND METHODS

Experimental animals

Sepsis was induced in male Sprague-Dawley rats (50–60 g body weight) by cecal ligation and puncture (CLP) as described in detail previously (Hall-Angeras et al, 1991; Penner et al, 2002; Tiao et al, 1996; Wray et al, 2003). Control rats underwent sham-operation, i.e., laparotomy and manipulation, but no ligation or puncture, of the cecum. Rats were resuscitated with 10 ml/100 g body weight of saline administered subcutaneously on the back at the time of sham-operation or CLP to prevent hypovolemia and septic shock. The animals had free access to water but food was withheld after the surgical procedures to avoid the influence of differences in food intake between septic and sham-operated rats on metabolic changes in muscle. In several previous experiments in which we studied sepsis-induced muscle wasting, rats weighing 50–60 g were used because rats of this size have lower extremity muscles that are thin enough to allow for in vitro incubation and measurement of protein degradation rates under physiological conditions (Fareed et al, 2006; Hall-Angeras et al, 1991; Penner et al, 2001, 2002; Tiao et al, 1994, 1996; Wray et al, 2003). Rats of the same size were used here in order to make it possible to compare the present observations with previous studies. At different time points (4, 8, and 16 h) after sham-operation or CLP, extensor digitorum longus (EDL) muscles were harvested, immediately frozen in liquid nitrogen, and stored at -80°C until used for determination of atrogin-1 and MuRF1 mRNA levels, FOXO mRNA and protein levels, and FOXO1 DNA binding activity as described below. EDL muscles were studied here because in previous studies we found that white, fast-twitch skeletal muscles are particularly sensitive to the effects of sepsis (Tiao et al, 1994; 1997).

To test the role of glucocorticoids in FOXO activation, two series of experiments were performed. First, sham-operated and septic rats were treated with the glucocorticoid receptor antagonist RU38486 (10 mg/kg) administered intraperitoneally 2 h before sham-operation or CLP. Other rats received a corresponding volume (0.3 ml) of vehicle (50% ethanol in distilled water). EDL muscles were removed 16 h after sham-operation or CLP for determination of FOXO1 mRNA levels or measurement of protein breakdown rates performed as described below. We found in previous studies that treatment of rats with RU38486 prevented sepsis-induced muscle proteolysis and activation of the ubiquitin-proteasome pathway (Hall-Angeras et al, 1991; Tiao et al, 1996; Wray et al, 2003) but the effect of RU38486 on sepsis-induced upregulation of FOXO transcription factors is not known. In a second series of experiments, rats were treated with dexamethasone (10 mg/kg) or corresponding volume of vehicle administered intraperitoneally as described previously (Tiao et al, 1996; Yang et al, 2005a). Rats had free access to water but food was withheld after the injections. EDL muscles were harvested 16 h after administration of

dexamethasone or vehicle for determination of FOXO1, atrogin-1, and MuRF1 mRNA levels.

The animal experiments were approved by the Institutional Animal Care and Use Committee at the Beth Israel Deaconess Medical Center (Boston, MA).

Muscle incubations

Protein breakdown rates were determined in incubated EDL muscles as described in detail previously (Fareed et al, 2006; Tiao et al, 1994). In short, intact EDL muscles were incubated at resting length under physiological conditions for 2 h. Protein breakdown rates were measured as net release of free tyrosine into the incubation medium during the 2 h incubation. The incubation medium contained cycloheximide (0.5 mM) to prevent re-incorporation into protein of tyrosine released by proteolysis. Tyrosine was determined as described by Waalkes and Udenfriend (1957). The results were expressed as nmol/g ww $\times$ 2h.

Cell culture

L6 rat skeletal muscle cells (American Type Culture Collection, Manassas, VA) were maintained and cultured as described in detail recently (Menconi et al, 2008; Wei et al, 2005; Yang et al, 2005a;). In short, L6 muscle cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin in 10 % CO₂ atmosphere at 37°C. When the cells reached approximately 80% confluence, they were removed by trypsinization (0.25% trypsin in phosphate buffered saline, PBS) and seeded in 10-cm culture dishes in the presence of 10% FBS until they reached approximately 80% confluence, at which time the medium was replaced with DMEM containing 2% FBS for induction of differentiation into myotubes. After 5–7 days, when formation of myotubes was observed, cytosine arabinoside (10 μ M) was added to the culture medium for 48 h in order to remove any remaining dividing myoblasts.

In order to test the role of FOXO1 and 3a in the regulation of atrogin-1 and MuRF1 expression, differentiated L6 myotubes were transfected with FOXO1 or FOXO3a siRNA or corresponding concentrations of non-targeting (scrambled) RNA (Dharmacon Technologies, Lake Placid, NY). The RNA constructs were added to the culture medium at a concentration of 165 nM together with the transfection reagent Lipofectamine RNAiMAX (Invitrogen, Grand Island, NY). After 5 h, the medium was changed and the myotubes were cultured for an additional 48 h in DMEM with 2% FBS in order to allow for continued silencing of the appropriate gene. Myotubes were then treated for 24 h with 1 μ M dexamethasone dissolved in ethanol (final concentration 0.1% in the culture medium) or corresponding concentration of vehicle whereafter they were harvested for determination of mRNA levels for FOXO1, FOXO3a, atrogin-1 and MuRF1 by real-time PCR and for determination of FOXO1 protein levels by Western blot analysis as described below. In a separate experiment, the effect of FOXO1 siRNA on protein degradation rates was determined. In that experiment, myotube proteins were labeled with 3H-tyrosine for 48 h after the transfection with FOXO1 siRNA and protein degradation was assessed by measuring the release of trichloroacetic acid-soluble radioactivity during 24 h as described in detail previously (Hong and Forsberg, 1995; Menconi et al, 2008; Wang et al, 1998). The results were expressed as percentage of protein degraded over 24 h (%/24 h).

Real-time PCR

mRNA levels for FOXO 1, 3a, and 4 and atrogin-1 and MuRF1 were determined by real-time PCR. Total RNA was extracted from EDL muscles and cultured L6 myotubes and real-

time PCR was performed as described in detail recently (Fareed et al, 2006; Wei et al, 2005; Yang et al, 2005a). Multiplex RT-PCR with amplification of 18S RNA as endogenous control, TaqMan analysis and subsequent calculations were performed with an ABI Prism 7700 Sequence Detection System (Perkin-Elmer, Foster City, CA). The sequences of the forward, reverse, and double-labeled oligonucleotides for atrogin-1 and MuRF1 used here were reported recently (Fareed et al, 2006; Menconi et al, 2008). The corresponding sequences for FOXO1 were forward, 5'-ACC CCA TGC AGA TGA GTG C-3', reverse, 5'-TCC TAC CAT AGC CAT TGC AGC-3', and double-labeled TaqMan oligonucleotide probe, 5'-CTG GGC AGC TAC TCC TCG GTG AGC-3'. The corresponding sequences for FOXO4 were forward, 5'-TCA GCC AGG CCA TTG AAA G-3', reverse, TCC ATT CGT ATA TCT GGG CGA-3', and double labeled TaqMan oligonucleotide probe, 5'-CCC CGG AGA AGC GGC TGA CA-3'. FOXO3a mRNA levels were determined using the ABI TaqMan Gene Expression Assay (Assay ID: Rn01441087_m1). Amplification of 18S RNA was performed in the same reaction tubes as an internal standard with an alternatively labeled probe (VIC-labeled probe) to distinguish its product from those derived from atrogin-1, MuRF1, FOXO1, FOXO3a, and FOXO4 RNA. Atrogin-1, MuRF1, FOXO1, 3a, and 4 mRNA concentrations were normalized to the 18S mRNA levels and were expressed as arbitrary units (AU).

Western blotting

Western blotting was performed to determine protein expression of total and phosphorylated FOXO transcription factors. The protocol used for Western blotting was described in detail recently (Wei et al, 2005; Yang et al, 2005a). Total and phosphorylated FOXO1, 3a, and 4 protein levels were determined in total muscle and nuclear extracts. Total FOXO1 protein levels were determined in cultured L6 myotubes transfected with non-targeting RNA or FOXO1 siRNA as described above. Total muscle extracts were prepared by homogenizing muscles in ice-cold RIPA buffer (10 mM Tris-HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 1% Nonidet P-40) containing protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and phosphatase inhibitor cocktail (Sigma, St Louis, MO) followed by centrifugation at $10,000 \times g$ for 10 min at 4°C. The supernatant (total muscle extract) was stored at -80°C until analysis. In order to extract nuclear proteins, muscles were homogenized in 0.2 ml of ice-cold Buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 2.5 mM DTT, 0.1% Triton X-100) containing protease inhibitor cocktail (Roche Applied Science). The homogenate was centrifuged at $250 \times g$ for 5 min at 4°C. The supernatant was discarded and the nuclear pellet was resuspended in 100 µl ice-cold Buffer A and the suspension was incubated on ice for 15 min. After centrifugation ($250 \times g$ for 5 min at 4°C), the supernatant (nuclear extract) was stored at -80°C until analysis. When total FOXO1 protein levels were determined in myotubes, whole cell lysates were prepared as described in detail previously (Yang et al, 2005b). Because basal levels of FOXO1 protein were low, tissue from three 10-cm culture dishes was combined for each Western blot. Aliquots (50 µg protein) of total muscle or nuclear extracts or whole cell lysates (for myotube experiments) were used for Western blotting performed as described in detail recently (Wei et al, 2005; Yang et al, 2005a). In short, protein extracts were subjected to SDS-PAGE using 10% gels, followed by transfer to PVDF membranes. The membranes were blocked with 5% non-fat milk in TTBS buffer (50 mM Tris-HCl, 150 mM NaCl, and 1% Tween-20, pH 7.4) and incubated with the following primary antibodies and the appropriate secondary antibodies: a rabbit polyclonal anti-human FOXO1 antibody (1:1000, Cell Signaling Technology, Danver, MA); a rabbit polyclonal anti-rat phospho(Ser256)-FOXO1 antibody (1:1000, Cell Signaling Technology); a rabbit polyclonal anti-rat FOXO3a antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA); a rabbit polyclonal anti-rat phospho(Thr32)-FOXO3a antibody (1:1000, Upstate, Temecula, CA); a rabbit polyclonal anti-rat FOXO4 antibody (1:1000, Santa Cruz Biotechnology); a rabbit polyclonal anti-rat

phospho(Ser197)-FOXO4 antibody (1:1000, Santa Cruz Biotechnology). The phosphorylation sites of the FOXO factors examined here (Ser256 for FOXO1, Thr32 for FOXO3a, and Ser197 for FOXO4) were based on previous studies in which they were implicated in activation of the transcription factors (Matsuzaki et al, 2005; Sandri et al, 2004). A mouse monoclonal anti-rat α tubulin antibody (1:2000, Sigma Aldrich, St Louis, MO) was used for loading control when total muscle extracts were immunoblotted. When nuclear extracts were analyzed, an anti-rat lamin A/C antibody (1:2000, Cell Signaling Technology) was used for loading control. To validate the nuclear extracts, a goat polyclonal anti-rat superoxide dismutase (SOD, a cytosolic protein) antibody was used (1:1000, Cell Signaling Technology). Immunoreactive protein bands were detected by using the Western Lightning kit for enhanced chemiluminescence detection (Perkin-Elmer Life Sciences) and analyzed using the public domain Image J program (<http://rsb.info.nih.gov/ij/index.html>). The bands were quantified by densitometry and normalized to the appropriate loading controls.

FOXO1 DNA binding activity

FOXO1 DNA binding activity was determined in nuclear muscle extracts using the FOXO1 EZ-Transcription Factor Assay Kit (Upstate, Temecula, CA). Nuclear extracts were prepared as described above and the DNA binding activity was measured by following the manufacturer's instructions. In this assay, a double stranded biotinylated oligonucleotide containing a consensus sequence for FOXO binding is mixed with the nuclear extract in a transcription factor assay buffer provided in wells on a Streptavidin coated plate. FOXO1 contained in the nuclear extract binds to the consensus sequence and the bound FOXO1 protein is detected with a specific anti-FOXO1 primary antibody. A horse radish peroxidase-conjugated secondary antibody is then used for colorimetric detection in a spectrophotometric plate reader. Also included in the kit are a positive control whole cell lysate, a non-specific double stranded oligonucleotide, and a specific double stranded competitor oligonucleotide.

Statistics

Results are reported as means $\pm$ SEM. Statistical analysis was performed by using Student's t-test or one-way ANOVA followed by Tukey's post hoc test as appropriate. $p < 0.05$ was considered statistically significant.

RESULTS

In initial experiments, we examined the expression of FOXO1, 3a, and 4 in EDL muscles at different time points after induction of sepsis by CLP in rats. FOXO1 mRNA levels were increased 8 and 16 h after CLP (Fig 1A). The increase in FOXO1 mRNA levels was accompanied by increased amounts of total and phosphorylated (Ser 256) FOXO1 protein (Fig 1B–D). Although phosphorylated FOXO1 (pFOXO1) (Ser 256) levels were increased, total FOXO1 protein levels were increased to an even greater extent, resulting in reduced pFOXO1 (Ser 256)/total FOXO1 ratio (Fig 1E). This result suggests that an increased amount of unphosphorylated FOXO1 was present in septic muscle, possibly allowing for increased nuclear translocation and activation of the transcription factor. To test whether this was the case, FOXO1 level and activity were determined in the nuclear fraction of EDL muscles 16 h after induction of sepsis. Results from that experiment showed that nuclear levels of FOXO1 as well as FOXO1 DNA binding activity were increased in skeletal muscle during sepsis (Fig 2A–C). Note that in these experiments, the presence of lamin A/C and the absence of SOD confirmed that the proteins studied here were representative of nuclear proteins.

We next determined muscle FOXO3a mRNA and protein levels after induction of sepsis in rats. Similar to FOXO1, FOXO3a mRNA levels were increased in EDL muscles of septic rats although the relative increase was less pronounced than for FOXO1 (Fig 3A and compare with Fig 1A). Both total and phosphorylated FOXO3a protein levels were increased in muscle of septic rats (Fig 3B–D). Because the relative increase in total and pFOXO3a (Thr 32) levels was similar, the ratio between pFOXO3a and total FOXO3a remained unchanged (Fig 3E). This observation suggests that sepsis may not result in increased nuclear translocation (activation) of FOXO3a. Indeed, Western blotting confirmed that nuclear levels of FOXO3a were not increased in muscle from septic rats (data not shown).

We next determined FOXO4 mRNA and protein levels in muscle of septic rats. In contrast to FOXO1 and 3a, FOXO4 mRNA and total and phosphorylated protein levels were not influenced by sepsis (Fig 4). As expected from these results, nuclear levels of FOXO4 were not increased in septic muscle (data not shown). Actually, nuclear FOXO4 levels were very low (hardly detectable) in muscles from both sham-operated and septic rats.

Taken together, the results described above suggest that sepsis results in increased expression and activity in skeletal muscle of FOXO1, increased expression (but not nuclear translocation) of FOXO3a, and no upregulation of FOXO4 expression. Because other studies suggest that an important function of FOXO factors in muscle atrophy is to regulate the transcription of the genes for the ubiquitin ligases atrogin-1 and MuRF1 (Sandri et al, 2004; Stitt et al, 2004; Zhao et al, 2007), we next determined mRNA levels for atrogin-1 and MuRF1 under the same experimental conditions as used here for the measurement of FOXO1, 3a and 4 expression. Muscle levels of atrogin-1 and MuRF1 mRNA were increased in septic rats (Fig 5), confirming previous results from this and other laboratories (Fareed et al, 2006; Frost et al, 2007; Glass, 2003; Poylin et al, 2008; Wray et al, 2003). Although there was a robust increase in mRNA levels for both ubiquitin ligases, the increase was most pronounced for MuRF1 (a 30-fold increase 16 h after CLP compared with an approximately 15-fold increase for atrogin-1 at the same time point).

Previous studies suggest that glucocorticoids are important mediators of sepsis-induced metabolic changes in skeletal muscle (Hall-Angeras et al, 1991; Hasselgren, 1999; Shakman et al, 2008; Sun et al, 2008; Tiao et al, 1996). Other studies suggest that glucocorticoids may regulate the expression and activity of FOXO transcription factors (Furuyama et al, 2003; Sandri et al, 2004; Stitt et al, 2004). The role of glucocorticoids in sepsis-induced upregulation of the FOXO1 transcription factor expression and activity, however, is not known. In order to test the role of glucocorticoids in increased FOXO expression in sepsis, we next treated rats with the glucocorticoid receptor antagonist RU38486 (Philibert, 1984). Treatment with this drug prevented sepsis-induced catabolic responses in skeletal muscle in previous studies (Hall-Angeras et al, 1991; Sun et al, 2008; Tiao et al, 1996). Because the results described above suggest that FOXO1 is particularly important in sepsis-induced muscle wasting (showing evidence of increased expression, nuclear translocation, and activity), the remainder of the experiments in the present study were focused on FOXO1. When rats were treated with RU38486, the sepsis-induced increase in FOXO1 expression was reduced (Fig 6A), suggesting that sepsis-induced upregulation of the FOXO1 transcription factor is, at least in part, regulated by glucocorticoids. Of note, FOXO1 mRNA levels were not reduced by RU38486 in sham-operated rats, suggesting that glucocorticoids are not needed to maintain basal levels of FOXO1. In additional experiments, treatment of rats with RU38486 prevented the sepsis-induced increase in muscle protein breakdown rates (Fig 6B). Because, in recent experiments, we found that the sepsis-induced expression of atrogin-1 and MuRF1 was reduced by RU38486 (Wray et al, 2003), the present observation of reduced FOXO1 expression and protein breakdown rates in RU38486-treated septic rats

suggests that there may be a link between FOXO1 and the regulation of ubiquitin-proteasome-dependent proteolysis in skeletal muscle during sepsis.

Although the results described above suggest that glucocorticoids participate in the regulation of FOXO1 expression during sepsis, we also wanted to test whether glucocorticoids alone can regulate FOXO1 expression in skeletal muscle. This was done by treating rats with dexamethasone, a treatment that we found in previous experiments to induce a catabolic response in skeletal muscle (Tiao et al, 1996; Yang et al, 2005a). Treatment of rats with dexamethasone resulted in increased muscle levels of FOXO1 mRNA (Fig 7A) as well as mRNA levels for atrogen-1 and MuRF1 (Fig 7B and C).

Although recent studies provided evidence for a link between FOXO transcription factors and activation of the atrogen-1 and MuRF1 genes (Sandri et al, 2004; Stitt et al, 2004; Waddell et al, 2008), apparently contradictory results have also been reported. For example, in a study by Kamei et al (2004), muscle-specific overexpression of FOXO1 resulted in muscle atrophy without concomitant changes in atrogen-1 and MuRF1 expression. Here, we tested the role of FOXO1 in dexamethasone-induced atrogen-1 and MuRF1 expression in cultured myotubes by using FOXO1 siRNA. Dexamethasone-treated myotubes are commonly used as an in vitro model of muscle wasting and show multiple metabolic changes typically seen in skeletal muscle during sepsis (Menconi et al, 2008). First, we wanted to validate the FOXO1 siRNA-transfected myotubes by determining the expression of FOXO1. When myotubes were transfected with FOXO1 siRNA, FOXO1 mRNA levels were reduced by approximately 50% (Fig 8A). FOXO3a mRNA levels were not influenced by FOXO1 siRNA (Fig 8B). Thus, transfecting the myotubes with FOXO1 siRNA resulted in a robust and specific downregulation of FOXO1 expression. Interestingly, protein degradation was reduced by approximately 15% in myotubes transfected with FOXO1 siRNA (Fig 8C), suggesting that basal protein degradation is at least in part regulated by FOXO1.

When myotubes were transfected with FOXO1 siRNA, dexamethasone-induced increase in FOXO1 protein levels was blunted (Fig 9A). Importantly, the dexamethasone-induced increase in atrogen-1 and MuRF1 mRNA levels was also blunted in myotubes transfected with FOXO1 siRNA, suggesting that the dexamethasone-induced upregulation of atrogen-1 and MuRF1 was at least in part regulated by FOXO1 (Fig 9B and C). Of note, basal atrogen-1 and MuRF1 mRNA levels were not influenced by FOXO1 siRNA, suggesting that the reduced basal protein degradation rates noted in FOXO1 siRNA transfected myotubes (see Fig 8C) did not reflect inhibited ubiquitin-proteasome-dependent proteolysis but may have reflected inhibition of other proteolytic pathways such as the autophagic/lysosomal pathway (Zhao et al, 2007).

Because CLP in rats did not result in increased nuclear levels of FOXO3a (see above) it is possible that FOXO3a is not involved in the regulation of atrogen-1 and MuRF1 expression, at least not during sepsis. To further test the potential role of FOXO3a in atrogen-1 and MuRF1 expression, we next transfected cultured myotubes with FOXO3a siRNA. This treatment resulted in an approximately 50% reduction of FOXO3a mRNA levels but did not influence FOXO1 mRNA levels (Fig 10A and B) consistent with a substantial and specific downregulation of FOXO3a in FOXO3a siRNA-treated myotubes. When these myotubes were exposed to dexamethasone, the increase in atrogen-1 and MuRF1 expression was not affected (Fig 10C and D), suggesting that, under the present experimental conditions, FOXO3a does not participate in the regulation of atrogen-1 and MuRF1.

DISCUSSION

In the present study, induction of sepsis in rats by CLP resulted in increased gene and protein expression as well as nuclear translocation and DNA binding activity of the transcription factor FOXO1 in skeletal muscle. The changes in FOXO1 expression and activity correlated with changes in the expression of atrogin-1 and MuRF1, important target genes for FOXO transcription factors in atrophying muscle (Sandri et al, 2004; Stitt et al, 2004; Waddell et al, 2008), suggesting (but not proving) that FOXO1 may be involved in the regulation of the ubiquitin ligases in skeletal muscle during sepsis. More direct evidence for a role of FOXO1 in the regulation of atrogin-1 and MuRF1 expression was observed in dexamethasone-treated myotubes in which FOXO1 expression was reduced by siRNA technique. It should be noted that although dexamethasone-treated myotubes are commonly used as an *in vitro* model of muscle wasting (Menconi et al, 2008), the physiological relevance of changes observed in cultured myotubes is not fully understood and results obtained in dexamethasone-treated myotubes need to be interpreted with caution. Nevertheless, taken together, the observations reported here are important from a clinical standpoint because they contribute to the understanding of the molecular mechanisms of muscle wasting in sepsis. The results also suggest that targeting FOXO1 may be of interest as part of a therapeutic strategy to prevent a clinically significant consequence of sepsis and perhaps other catabolic conditions as well.

Because activation of FOXO transcription factors is commonly associated with reduced phosphorylation of FOXOs, allowing for nuclear translocation of unphosphorylated FOXOs (Katoh, 2004; Van der Heide et al, 2004), the present finding of increased levels of pFOXO1 (Ser256) in septic muscle may seem paradoxical. However, since the muscle levels of total FOXO1 were increased to an even greater extent than the increase in pFOXO1 (Ser256), the net amount of unphosphorylated FOXO1 was increased (as indicated by reduced pFOXO1/FOXO ratio), resulting in nuclear translocation of the transcription factor. A similar seemingly paradoxical increase in pFOXO1 (Ser256) levels was reported in muscle wasting caused by starvation in mice (Furuyama et al, 2003). In that study as well, the increase in total FOXO1 protein levels was greater than the increase in pFOXO1 (Ser256) resulting in a net increase in unphosphorylated (activated) FOXO1. Because PI3K/Akt signaling is reduced in catabolic muscle (Sandri et al, 2004; Stitt et al, 2004), the increased levels of pFOXO1 (Ser 256) noticed here in septic muscle and during starvation (Furuyama et al, 2003) may reflect phosphorylation by kinases other than Akt.

The present finding of a selective activation of FOXO1 in skeletal muscle during sepsis is different from a recent study by Sandri et al (2004) in which results were consistent with activation of FOXO1, 3a, and 4 in dexamethasone- and starvation-induced atrophy of cultured muscle cells. In addition, in the same study (Sandri et al, 2004), overexpression of active FOXO3a in skeletal muscle *in vivo* resulted in muscle atrophy. In other, more recent, studies from the same group of investigators, overexpression of FOXO3a stimulated autophagy in skeletal muscle *in vivo* (Mammucari et al, 2007) and in cultured muscle cells (Zhao et al, 2007). One potential reason for these apparently contradictory results with regards to the role of individual FOXO transcription factors may be that whereas in our experiments, studies were performed in rats and in cultured L6 myotubes, a rat muscle cell line, in other studies (Mammucari et al, 2007; Sandri et al, 2004; Zhao et al, 2007), experiments were conducted in mice and in cultured C2C12 myotubes, a mouse cell line. Thus, it is possible that the role of individual FOXO transcription factors in muscle wasting is species-specific. It should also be noted that although the results reported here suggest that FOXO1 may be particularly important in sepsis-induced muscle wasting, our observations do not rule out the possibility that FOXO3a and 4 may be involved in the development of muscle atrophy in other catabolic conditions.

Although a study was published recently by Nystrom and Lang (2008) suggesting that sepsis upregulates the expression of FOXO transcription factors in skeletal muscle, the present experiments added important novel information. In the previous study (Nystrom and Lang, 2008), sepsis induced by CLP in mice resulted in a 2.5- to 3-fold increase in FOXO1 and 3a mRNA levels but the influence of sepsis on the expression of total and phosphorylated FOXO1 and 3a protein and on the nuclear translocation and activity of the transcription factors was not examined in those experiments. In addition, the correlation between changes in FOXO transcription factor mRNA levels and the expression of atrogin-1 and MuRF1 was not determined. Most importantly, the mechanism of sepsis-induced upregulation of FOXO transcription factors, including the role of glucocorticoids, was not defined (Nystrom and Lang, 2008).

In addition to the present study and the report by Nystrom and Lang (2008), Crossland et al (2008) reported that endotoxemia induced by intravenous infusion of lipopolysaccharide (LPS) in rats resulted in increased levels of FOXO1 mRNA and reduced levels of phosphorylated FOXO1 and 3a in skeletal muscle. Because metabolic changes induced by infusion of endotoxin and CLP may not be identical (Sachdeva et al, 2003), the implications of the findings reported by Crossland et al (2008) in the context of sepsis-induced muscle wasting are unclear. In fact, some of the changes induced by administration of endotoxin, e.g., the reduced levels of phosphorylated FOXO1 and 3a, were qualitatively different from the changes induced by sepsis in the current study. In addition, the influence of endotoxemia on the nuclear translocation and activity of the FOXO transcription factors was not examined in the study by Crossland et al (2008).

In addition to atrogin-1 and MuRF1 (Sandri et al, 2004; Stitt et al, 2004; Waddell et al, 2008), studies suggest that FOXO transcription factors may regulate the expression of other genes involved in muscle wasting, including autophagy-related genes (Mammucari et al, 2007; Zhao et al, 2007), myostatin (Allen and Unterman, 2007; Liu et al, 2007), and cathepsin L (Kamei et al, 2004). It is also possible that FOXO transcription factors contribute to the development of insulin resistance in skeletal muscle by upregulating the expression of pyruvate dehydrogenase kinase 4 (PDK4) (Crossland et al, 2008; Furuyama et al, 2003). PDK4 phosphorylates (inactivates) the pyruvate dehydrogenase complex, the rate-limiting step in carbohydrate oxidation (Alamdari et al, 2008). Thus, FOXO-regulated expression of PDK4 may be a mechanism of insulin resistance that is commonly seen in muscle wasting conditions and that contributes to the catabolic response in skeletal muscle.

Results in the present study suggest that sepsis-induced activation of FOXO1 is, at least in part, regulated by glucocorticoids. In addition, our results suggest that glucocorticoids by themselves can activate FOXO1 through a direct effect in skeletal muscle which is in line with previous observations in hydrocortisone-treated mice (Furuyama et al, 2003). A recent study provided evidence that dexamethasone-induced activation of FOXO1 in cultured C2C12 myotubes required binding to and activation of the glucocorticoid receptor (Zhao et al, 2009). Thus, the present and other observations support the concept that glucocorticoids are important mediators of the catabolic response in skeletal muscle during sepsis (Hasselgren, 1999; Shakman et al, 2008). Interestingly, there is evidence that in addition to FOXO1, other transcription factors involved in muscle wasting, including C/EBP β and δ , may also be regulated by glucocorticoids (Penner et al, 2002; Yang et al, 2005a, 2005b).

It should be noted that although glucocorticoids play an essential role in muscle wasting during sepsis and other catabolic conditions, additional mediators are probably involved as well. For example, multiple studies suggest that TNF α is an important regulator of protein balance in atrophying muscle (Frost et al, 2007; Li et al, 2003, 2005). Interestingly, in a recent study by Moylan et al (2008), treatment of cultured myotubes with TNF had no effect

on FOXO1 or 3a expression and nuclear localization but instead upregulated the expression of FOXO4. In additional experiments in the same report, silencing of the FOXO4 gene reduced the TNF-induced expression of atrogin-1. Thus, it is possible that different mediators of the catabolic response in skeletal muscle activate different mechanisms stimulating protein breakdown; glucocorticoids may upregulate atrogin-1 and MuRF1 (and possibly other muscle wasting-related genes) secondary to activation of FOXO1 whereas TNF may induce a catabolic response at least in part by activating FOXO4.

Transcription factors typically interact with nuclear cofactors when activating gene transcription. Of note, FOXO transcription factors can interact with PGC-1 α (Sandri et al, 2006) and p300/CBP (Perrot and Rechter, 2005) in the regulation of gene transcription. These observations are particularly important in the context of muscle wasting since both PGC-1 α (Lin et al, 2002) and p300/CBP (Yang et al, 2005b, 2007) participate in the regulation of muscle mass in various catabolic conditions. A recent study by Waddell et al (2008) suggests that the glucocorticoid receptor and FOXO1 synergistically activate the MuRF1 gene in muscle cells, further supporting the role of an interaction between FOXO transcription factors and other nuclear proteins in the regulation of muscle protein balance.

In summary, the present study provides evidence that FOXO1 expression and activity are increased in skeletal muscle during sepsis by a glucocorticoid-dependent mechanism. The results also support a role of FOXO1 in the regulation of atrogin-1 and MuRF1 expression in skeletal muscle. FOXO1 may be a target molecule in the treatment of sepsis-induced muscle wasting.

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Figure 1. Sepsis increases the expression of FOXO1 in rat skeletal muscle

(A) mRNA levels (arbitrary units, AU) in EDL muscles determined by real-time PCR 4, 8, and 16 h after cecal ligation and puncture (CLP) or sham-operation. (B) Representative Western blots of proteins from EDL muscles for phosphorylated (Ser 256) FOXO1 (p-FOXO1), total FOXO1 and α -tubulin in EDL muscles 16 h after CLP or sham-operation. (C) Densitometric quantifications of p-FOXO1 and (D) total FOXO1 protein levels normalized to α -tubulin levels. (E) Calculated p-FOXO1/total-FOXO1 ratios. Results are means $\pm$ SEM with $n = 6-8$ in each group. * $p < 0.05$ vs corresponding Sham group by ANOVA (panel A) or Student's t -test (panels C-E).

Figure 2. Sepsis increases nuclear FOXO1 protein levels and DNA binding activity in rat skeletal muscle

(A) Representative Western blots of nuclear proteins for FOXO1 and lamin A/C in EDL muscles 16 h after CLP or sham-operation. Nuclear and total tissue extracts were immunoblotted for the cytosolic protein superoxide dismutase (SOD) to validate the nuclear extractions. (B) Densitometric quantification of FOXO1 protein levels normalized to lamin A/C levels. (C) FOXO1 DNA binding activity in nuclear extracts from EDL muscles 16 h after CLP or sham-operation. Results are means $\pm$ SEM, with $n = 8$ in each group. * $p < 0.05$ vs Sham by Student's t -test.

Figure 3. Sepsis increases the expression of FOXO3a in rat skeletal muscle

(A) FOXO3a mRNA levels determined by real-time PCR in EDL muscles 4, 8, and 16 h after CLP or sham-operation. (B) Representative Western blots of proteins from EDL muscles for p(Thr 32)-FOXO3a, total FOXO3a, and α -tubulin 16 h after CLP or sham-operation. (C) Densitometric quantifications of p-FOXO3a and (D) total FOXO3a protein levels normalized to α -tubulin levels. (E) Calculated p-FOXO3a/total-FOXO3a ratios. Results are means $\pm$ SEM, with $n = 6-8$ in each group. * $p < 0.05$ vs corresponding Sham group by ANOVA (panel A) or Student's t -test (panels C-E).

Figure 4. FOXO4 expression is not influenced by sepsis in rat skeletal muscle

(A) FOXO4 mRNA levels determined by real-time PCR in EDL muscles 4, 8, and 16 h after CLP or sham-operation. (B) Representative Western blots of proteins from EDL muscles for p(Ser197)-FOXO4, total FOXO4, and α -tubulin 16 h after CLP or sham-operation. (C) Densitometric quantifications of p(Ser 197)-FOXO4 and (D) total FOXO4 protein levels normalized to α -tubulin levels. (E) Calculated p-FOXO4/total-FOXO4 ratios. Results are means $\pm$ SEM, with n = 6–8 in each group.

Figure 5. Sepsis increases the expression of atrogin-1 and MuRF1 in rat skeletal muscle
(A) Atrogin-1 and (B) MuRF1 mRNA levels determined by real-time PCR in EDL muscles 4, 8, and 16 h after CLP or sham-operation. Results are means $\pm$ SEM with $n = 6$ in each group. * $p < 0.05$ vs corresponding Sham group by ANOVA.

Figure 6. Sepsis-induced changes in FOXO1 expression and protein degradation in skeletal muscle are glucocorticoid-dependent

Sham-operated and septic (CLP) rats were treated with RU-38486 (RU, 10 mg/kg) or corresponding volume (0.3 ml) of vehicle administered intraperitoneally 2 h before sham-operation or CLP. (A) EDL muscles were harvested 16 h after CLP or sham-operation and FOXO1 mRNA levels were determined by real-time PCR. Results are means $\pm$ SEM with $n = 8$ in each group. (B) In a second set of experiments, protein degradation was determined in incubated EDL muscles 16 h after CLP or sham-operation. Results are means $\pm$ SEM with $n = 6-8$ in each group. * $p < 0.05$ vs all other groups by ANOVA. + $p < 0.05$ vs corresponding sham group by ANOVA.

Figure 7. The expression of FOXO1, atrogin-1, and MuRF1 is regulated by glucocorticoids in rat skeletal muscle

Rats were injected with 10 mg/kg of dexamethasone (DEX) intraperitoneally or a corresponding volume (1 ml) of solvent (control). Sixteen hours after injection, EDL muscles were excised and (A) FOXO1, (B) atrogin-1, and (C) MuRF1 mRNA levels were determined by real-time PCR. Results are means $\pm$ SEM with $n = 7$ in each group. * $p < 0.05$ vs corresponding control group by Student's t -test.

Figure 8. FOXO1 siRNA reduces FOXO1 mRNA levels and protein degradation in cultured L6 myotubes

Myotubes were transfected with non-targeting (non-specific, NS) or FOXO1 siRNA followed by determination of (A) FOXO1 mRNA, (B) FOXO3a mRNA, and (C) protein degradation. Results are means $\pm$ SEM with $n = 12$ in each group. * $p < 0.05$ vs NS siRNA by Student's t -test.

Figure 9. Silencing of FOXO1 in myotubes attenuates dexamethasone-induced increase in FOXO1 protein levels and expression of atrogen-1 and MuRF1

Myotubes were transfected with non-targeting (non-specific, NS) or FOXO1 siRNA, then treated with 1 μ M dexamethasone or a corresponding concentration of vehicle (control) for 24 h whereafter (A) FOXO1 protein levels were determined by Western blotting and mRNA levels for (B) atrogen-1 and (C) MuRF1 were determined by real-time PCR. In panel (A), each lane was generated by combining tissue from three 10-cm culture dishes. Almost identical results as observed here were seen in a duplicate experiments in which three 10-cm culture dishes were also used for each lane. In panel (B) and (C), results are means $\pm$ SEM with $n = 12$ in each group. * $p < 0.05$ vs corresponding control group by ANOVA. + $p < 0.05$ vs corresponding NS siRNA group by ANOVA.

Figure 10. Silencing of FOXO3a in myotubes does not influence dexamethasone-induced expression of atrogin-1 and MuRF1

Myotubes were transfected with non-targeting (non-specific, NS) or FOXO3a siRNA followed by determination of (A) FOXO3a mRNA and (B) FOXO1 mRNA. Myotubes were then treated with dexamethasone as described in Fig 9, where after mRNA levels for (C) atrogin-1, and (D) MuRF-1 were determined by real-time PCR. Results are means $\pm$ SEM, with $n = 8-10$ in each group. * $p < 0.05$ vs NS siRNA (panel A) by Student's t-test and vs corresponding control group (panels C and D) by ANOVA.