Role of IGF-I and the TNFα/NF-κB pathway in the induction of muscle atrogenes by acute inflammation

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Schakman O, Dehoux M, Bouchuari S, Delaere S, Lause P, Decroly N, Shoelson SE, Thissen JP. Role of IGF-I and the TNFα/NF-κB pathway in the induction of muscle atrogenes by acute inflammation. Am J Physiol Endocrinol Metab 303: E729–E739, 2012. First published June 26, 2012; doi:10.1152/ajpendo.00060.2012.—Several catabolic states (sepsis, trauma, cancer, etc.) associated with acute inflammation are characterized by a loss of skeletal muscle due to accelerated proteolysis. The main proteolytic systems involved are the autophagy and the ubiquitin-proteasome (UPS) pathways. Among the signaling pathways that could mediate proteolysis induced by acute inflammation, the transcription factor NF-κB, induced by TNFα, and the transcription factor forkhead box O (FOXO), induced by glucocorticoids (GC) and inhibited by IGF-I, are likely to play a key role. The aim of this study was to identify the nature of the molecular mediators responsible for the induction of these proteolytic systems in response to acute inflammation caused by LPS injection. LPS injection robustly stimulated the expression of several components of the autophagy and the UPS pathways in the skeletal muscle. This induction was associated with a rapid increase of circulating levels of TNFα together with a muscular activation of NF-κB followed by a decrease in circulating and muscle levels of IGF-I. Neither restoration of circulating IGF-I nor restoration of muscle IGF-I levels prevented the activation of autophagy and UPS genes by LPS. The inhibition of TNFα production and muscle NF-κB activation, respectively, by using pentoxifylline and a repressor of NF-κB, did not prevent the activation of autophagy and UPS genes by LPS. Finally, inhibition of GC action with RU-486 blunted completely the activation of these atrogenes by LPS. In conclusion, we show that increased GC production plays a more crucial role than decreased IGF-I and increased TNFα in the induction of the proteolytic systems caused by acute inflammation.

insulin-like growth factor I; tumor necrosis factor-α; nuclear factor-κB; proteolysis; autophagy system; ubiquitin-proteasome system; glucocorticoids; sepsis; skeletal muscle

SEVERAL CATABOLIC STATES (sepsis, trauma, cancer, etc.) are associated with acute inflammation due to the release of proinflammatory cytokines. Acute inflammation leads to accelerated skeletal muscle proteolysis and atrophy associated with an increased risk of morbidity and mortality. The two main proteolytic systems involved are the autophagy (62) and the ubiquitin-proteasome (UPS) systems (9, 62), two proteolytic pathways regulated by the transcription factors nuclear factor-κB (NF-κB) (3) and forkhead box O (FOXO)1 and -3a (38, 48, 64). However, the mechanisms responsible for their induction in response to acute inflammation remain poorly understood.

Considerable evidence indicates that insulin-like growth factor (IGF)-I could contribute to muscle atrophy induced by acute inflammation. Indeed, IGF-I is a growth factor that stimulates the development of muscle mass by increasing protein synthesis and myogenesis while decreasing proteolysis and apoptosis (14, 36). Moreover, both circulating and muscle expression of IGF-I are decreased in sepsis (5, 11, 29). Interestingly, IGF-I inhibits muscle proteolysis caused by the autophagy and the ubiquitin-proteasome pathways through the PI3K/Akt/FOXO pathway (33). Finally, IGF-I administration inhibits muscle atrophy induced by thermal injury or glucocorticoids (10, 47, 49, 50).

Tumor necrosis factor (TNF)α, a major proinflammatory cytokine, could also play a critical role in muscle atrophy encountered in acute inflammation. Indeed, in vitro (35) and in vivo (4) TNFα is able to induce muscle atrophy by stimulating the expression of different component of UPS (34). Furthermore, acute inflammation mimicked by lipopolysaccharide (LPS) injection induces a rapid increase of circulating TNFα (5), and elevated circulating levels of TNFα are associated with muscle atrophy in humans (42). Finally, inhibition of TNFα production by torbaftylline decreases proteolysis mediated by UPS (6), and blockade of TNFα by antibodies (8) reduces the loss of lean body mass caused by cancer.

NF-κB, the main transcription factor induced by TNFα, could be a crucial mediator implicated in muscle atrophy induced by acute inflammation. Indeed, NF-κB muscle activation by IKKβ overexpression is sufficient to increase proteolysis mediated by muscle ring finger (MuRF)1 and to induce muscle atrophy (3). In addition, sepsis is associated with activation of NF-κB in skeletal muscle (43). Finally, inhibition of NF-κB prevents proteolysis in TNFα/interferon-γ (IFNγ)-treated myotubes (28) and muscle atrophy caused by denervation, disuse (i.e., hindlimb unloading) (3, 59) and pulmonary inflammation (20).

Finally, many observations point out the role of glucocorticoids (GC) in muscle atrophy induced by acute inflammation. Indeed, GC are sufficient to induce muscle atrophy (49) resulting from a stimulation of protein breakdown and from an inhibition of protein synthesis (18, 37, 58). GC stimulate, via FOXO, the autophagy and UPS pathways (22). Moreover, circulating levels of GC are increased in sepsis (32). Finally, inhibition of the production or action of GC respectively by adrenalectomy or by treatment with an inhibitor of GC receptor (RU-486) attenuates muscle protein breakdown associated with sepsis (21).
In light of all of these observations, the aim of our work was to determine the respective roles of IGF-I, TNFα, NF-κB, and GC in the induction of the genes involved in skeletal muscle atrophy (“atrogenes”) caused by acute inflammation. Induction of atrogens was evaluated by measuring the expression of several genes regulating (FOXO1 and -3a) or involved in the autophagy (LC3B, Gabarapl1, and Bnip3) and in the UPS (atrogin-1, MuRF1) pathways in an acute inflammation model induced by intraperitoneal injection of LPS.

MATERIALS AND METHODS

Expression Plasmids and DNA Preparations

As previously described (49), plasmid pM1-hIGF-I was constructed by inserting a 770-bp human IGF-I cDNA in the pM1 expression vector (Roche Molecular Biochemicals, Indianapolis, IN). Plasmids were amplified in Escherichia coli top 10 F’ (Invitrogen, Carlsbad, CA) and purified with an EndoFree plasmid giga kit (QIAGEN, Hilden, Germany). Plasmids were stocked at -20°C before starting the experiment. Access to animal chow was available only between 6:00 PM and 9:00 AM, whereas access to water was unrestricted.

Animals

Six-week-old male Wistar rats (Janvier Breeding, Le Genest St-Isle, France) and 10-week-old male C57Bl6 mice (wild-type mice, Janvier Breeding and muscle-specific expression of IkB SuperRepressor (MISR) mice, previously described by Cai et al. (3)) were all housed for 1 wk under standardized conditions of light (12:12-h light-dark cycle) and temperature (22 ± 2°C) before starting the experiment. Six-week-old male Wistar rats (Janvier Breeding, Le Genest St-Isle, France) and 10-week-old male C57Bl6 mice (wild-type mice, Janvier Breeding and muscle-specific expression of IkB SuperRepressor (MISR) mice, previously described by Cai et al. (3)) were all housed for 1 wk under standardized conditions of light (12:12-h light-dark cycle) and temperature (22 ± 2°C) before starting the experiment. Access to animal chow was available only between 6:00 PM and 9:00 AM, whereas access to water was unrestricted. The experiments were conducted and the animals were cared for in accordance with the directives of the Institutional Animal Care and Use Committee of the University of Louvain.

In Vivo Experimental Design

Experimental design 1: role of decreased circulating IGF-I in LPS-induced atrogenes. Rats were randomly divided into four different groups: control (n = 10), LPS (n = 8), IGF-I (n = 10), and LPS + IGF-I (n = 7). The LPS and LPS + IGF-I groups were injected with LPS (750 μg/100 g body wt ip; serotype 012:7:B8, Sigma-Aldrich, St. Louis, MO), whereas the control and the IGF-I groups were injected with an equivalent volume of saline buffer. In parallel, animals from IGF-I and LPS + IGF-I groups received three subcutaneous injections of IGF-I (375 μg/100 g body wt sc; Genentech, San Francisco, CA) respectively 0, 4, and 8 h after LPS or saline injection, whereas animals from the two other groups received three subcutaneous injections of saline buffer. Twelve hours after LPS injection, animals were killed, blood was collected and serum was stored at −20°C until analysis. Tibialis anterior (TA) muscles were dissected, snap-frozen in liquid nitrogen, and stored at −80°C until processing.

Experimental design 2: role of decreased muscle IGF-I in LPS-induced atrogenes. Rats were randomly divided into two different groups: control (n = 5) and LPS (n = 10). The LPS group was injected ip with LPS as described in protocol 1, and the control group was injected with an equivalent volume of saline buffer. Seven days before LPS injections, each rat was anesthetized with a mixture of 75 mg/kg ketamine (Ketalar; Pfizer, Oslo, Norway) and 15 mg/kg xylazine hydrochloride (Rompun; Bayer, Fernwald, Germany) administered by ip injection. Plasmids solutions (1 μg/μl) were injected in 10 different sites (total volume per muscle = 100 μl) in each TA muscle, and then muscles were electroporated using the electroporation conditions previously described (49). One TA muscle was injected with the pM1-hIGF-I plasmid and the contralateral TA muscle with the empty pM1 plasmid. Twelve hours after LPS injection, animals were killed, and TA muscles were dissected, snap-frozen in liquid nitrogen, and stored at −80°C until processing.

Experimental design 3: role of increased circulating TNFα in LPS-induced atrogenes. To confirm increased circulating levels of TNFα in response to LPS and its inhibition by pentoxifylline (Pentox), rats were randomly divided into four different groups: control (n = 5), LPS (n = 4), Pentox (n = 5) and LPS + Pentox (n = 5). The LPS and LPS + Pentox groups were injected ip with LPS as described in protocol 1, whereas the two other groups were injected with an equivalent volume of saline buffer. One hour before LPS or saline injections, animals from group Pentox and LPS + Pentox received a single intraperitoneal injection of pentoxifylline (10 mg/100 g body wt ip; Sigma-Aldrich, St. Louis, MO), whereas the two other groups received an equal amount of saline buffer. Animals were killed 90 min after LPS injections to collect blood, and serum was stored at −20°C.

To assess the role of TNFα in LPS-induced atrogenes, rats were randomly divided into four different groups: control (n = 5), LPS (n = 3), Pentox (n = 5), and LPS + Pentox (n = 4). The LPS and LPS + Pentox groups were injected ip with LPS as described in protocol 1, whereas the two other groups were injected with an equivalent volume of saline buffer. One hour before LPS or saline injections, animals from group Pentox and LPS + Pentox received a single ip injection of pentoxifylline as described before, whereas the two other groups received an equivalent amount of saline buffer. Animals were killed 12 h after LPS injections, and TA muscles were removed, snap-frozen in liquid nitrogen, and stored at −80°C until processing.

Experimental design 4: role of NF-κB activation in LPS-induced atrogenes. To determine the activation of NF-κB in response to LPS injection, MISR and C57Bl6 wild-type mice were divided into two different groups: control (MISR n = 3; C57Bl6 n = 5) and LPS (MISR n = 9; C57Bl6 n = 3). The LPS group was injected ip with LPS as described in protocol 1, and the control group was injected with an equivalent volume of saline buffer. Two hours after LPS or saline injections, animals were killed, and TA muscles were removed, snap-frozen in liquid nitrogen, and stored at −80°C until processing.

To assess the role of NF-κB in LPS-induced atrogenes, MISR and C57Bl6 wild-type mice were divided into two different groups: Control (MISR n = 5; C57Bl6 n = 6) and LPS (MISR n = 5; C57Bl6 n = 5). The LPS group was injected ip with LPS as described in protocol 1, and the control group was injected with an equivalent volume of saline buffer. Twelve hours after LPS or saline injections, animals were killed, and TA and extensor digitorum longus (EDL) muscles were removed, snap-frozen in liquid nitrogen, and stored at −80°C until processing.

Experimental design 5: role of GC increase in LPS-induced atrogenes. C57Bl6 wild-type mice were randomly divided into four different groups: control (n = 4), LPS (n = 6), RU-486 (n = 4), and LPS + RU-486 (n = 6). The LPS and LPS + RU-486 groups were injected ip with LPS as described in protocol 1, whereas the control and the RU-486 groups were injected with an equivalent volume of saline buffer. Fifteen minutes before LPS or saline injections, animals from RU-486 and LPS + RU-486 groups received a single ip injection of RU-486 (5 mg/mice; Sigma-Aldrich), whereas the two other groups received an equal amount of DMSO solution. Twelve hours after LPS injection, animals were killed, and TA and EDL muscles were dissected, snap-frozen in liquid nitrogen, and stored at −80°C until processing.

mRNA Analysis by Real-time Quantitative PCR

Total RNA was isolated from the EDL muscle for mice and TA muscle for rats, using TRIzol reagent as described by the manufacturer. Recovery was 1 μg/mg of muscle. Reverse transcription and real-time quantitative (RTQ)-PCR were done as previously described (9). Briefly, SYBR Green RTQ-PCR analyses were carried out using
the following cycle parameters: 10 min at 95°C followed by 40 cycles of 1 min at 60°C and 15 s at 95°C. For each gene, RTQ-PCR was conducted in duplicate with a 25-µl reaction volume containing 5 ng of cDNA and the SYBR Green mix. To ensure the quality of the measurements, each plate included for each gene a negative control (sample replaced by RNase-free water) and a positive control consisting of a pool of cDNA from positive samples. The threshold cycle (Ct) from a positive sample was used to calculate the interassay coefficient of variation (CV). For each gene, the CV was calculated as standard deviation/mean of the Ct determined on five different plates and with different mixes. Accession numbers and the sequences of primers used were as follows: ubiquitin: NM017314 (fw: GATCCAGGCAGGGATGATGTTC) used as reporter gene. Primers were tested in order to avoid primer dimers, self-priming formation, or unspecific amplification.

**Circulating IGF-I and TNFα Concentrations**

After serum extraction, circulating IGF-I was measured by RIA as described previously (7, 55). Quantification of circulating TNFα was assessed on serum with an ELISA kit (R&D) using specific antibodies and according to the manufacturer instructions.

**Statistical Analysis**

Results are presented as means ± SE. Statistical analyses were performed using one-way ANOVA followed by a Newman-Keuls multiple comparison test. Statistical significance was set at P < 0.05.

**RESULTS**

**Prevention of Circulating and Muscle IGF-I Decline Fails to Prevent LPS-Induced Atrogenes**

Confirming previous results obtained by our group (5, 9), LPS injection in rats led to a reduction of systemic IGF-I concentrations by ~36% (CTRL 77.7 ± 3.1 nM vs. LPS 49.8 ± 1.9 nM, P < 0.001; Fig. 1A). This decline was associated with a marked induction of Foxo1 (7.5-fold, P < 0.001) and Foxo3a mRNA (3.5-fold, P < 0.001) (Fig. 1B), two transcription factors regulating UPS and autophagy proteolytic pathways (38, 48, 53). Moreover, LPS treatment upregulated significantly the expression of several components of these two proteolytic pathways such as atrogin-1 and MuRF1, two major ubiquitin ligases involved in muscle proteolysis (atrogin-1 12.5-fold, P < 0.001 and MuRF1 18-fold, P < 0.001; Fig. 1C) and several components of autophagy (LC3B 6-fold, P < 0.001; Gabarapl1 4-fold, P < 0.001; and Bnip3 2.5-fold, P < 0.001; Fig. 1D). The selection of the 12-h time point was based on the time course previously performed. Indeed, we showed that the induction of atrogen expression peaked about 12 h after LPS and declined thereafter (9). Decrease in muscle mass and protein content were not observed 12 h after LPS injection (data not shown). The absence of skeletal muscle atrophy after LPS injection was probably due to the relatively early time point considered to assess muscle mass. To determine whether the systemic IGF-I decrease observed in LPS-treated rats was involved in the induction of atrogenes, we administrated IGF-I systematically to normalize circulating levels. Interestingly, IGF-I replacement after LPS injection produced a complete normalization of circulating IGF-I concentrations (LPS + IGF-I 77.9 ± 4.9 nM vs. CTRL 77.7 ± 3.1 nM; Fig. 1A) without any alteration of glycemia (data not shown). However, normalization of circulating IGF-I by exogenous IGF-I did not or very marginally prevented the induction of the UPS, autophagy, and FOXO genes by LPS (Fig. 1, B–D). This observation indicates that the reduction of circulating IGF-I induced by LPS is not mandatory for the induction of atrogenes.

**Local Production and thus Autocrine Actions of IGF-I seem to Play an Important Role in the Regulation of Muscle Mass in Physiological (52) and in Some Pathological Conditions (49).** As shown in Fig. 2A, LPS treatment strongly decreased muscle IGF-I expression by ~80% (P < 0.001). To evaluate whether this muscle IGF-I decrease (5) is involved in the induction of muscle protein contents were determined using Bradford’s protein assay (Bio-Rad, Munich, Germany). Equal amounts of proteins (20 μg for NF-kB, 25 μg for LC3B, and 60 μg for MuRF1) were resolved by SDS-polyacrylamide gel 10% electrophoresis and transferred to hydrophobic PVDF membranes (Amersham Hybond-P; GE Healthcare, Pittsburgh, PA). Membranes were probed with anti-p65 (1:1,000; Santa Cruz Biotechnology, Heidelberg, Germany), anti-MuRF1 (1:200; R&D Systems, Minneapolis, MN), anti-LC3B (1:1,000; Cell Signaling Technology, Danvers, MA), and anti-GAPDH (1:3,000; Cell Signaling Technology), followed by a horseradish peroxidase-coupled secondary antibody, anti-rabbit (1:40,000; Amersham, GE Healthcare) or anti-goat (1:80,000; Dako, Glostrup, Denmark) and developed by a chemiluminescence-based detection system (Amersham ECL Plus, GE Healthcare). Developed film was scanned and analyzed as previously described (50).
atrogenes, we locally overexpressed IGF-I by electroporation gene transfer. Interestingly, IGF-I overexpression was able to completely prevent the decline of muscle IGF-I expression induced by LPS (Fig. 2A). As observed for systemic IGF-I administration, normalization of muscle IGF-I by overexpression did not prevent the induction of the UPS, autophagy, and FOXO genes by LPS (Fig. 2, B–D). All together, these observations indicate that neither systemic IGF-I nor muscle IGF-I decline is responsible for the induction of atrogenes in LPS-treated rats.

**Prevention of Circulating TNFα Increase Fails to Prevent LPS-Induced Atrogenes**

Proinflammatory cytokines such as TNFα are thought to play an important role in muscle atrophy observed in several conditions (6, 8). As shown in Fig. 3A, LPS administration in rats strongly increased the circulating levels of TNFα. To investigate the role of circulating TNFα increase in the induction of atrogenes induced by LPS, we inhibited the production of TNFα by pentoxyfilline. Although pentoxyfilline almost completely inhibited TNFα induction (~92%, P < 0.001; Fig. 3A), activation of muscle UPS and autophagy genes as well as FOXO genes in response to LPS was not prevented (Fig. 3, B–D). The time point selected to assess circulating TNFα was based on the time course previously performed. Indeed, we showed that the circulating levels of TNFα peaked ~2 h after LPS injection, to decline soon after and return to normal after ~12h (5). These results suggest that the increase of circulating TNFα does not play a major role in the induction of atrogenes in LPS-treated rats.

**Prevention of NF-κB Activation Fails to Prevent LPS-Induced Atrogenes**

Much evidence suggests that NF-κB activation is a crucial step in the muscle atrophy encountered in several pathological conditions and in particular in denervation, disuse (i.e., hindlimb unloading), and cancer (3, 59). In the muscle of LPS-treated mice, we showed the activation of NF-κB characterized by the translocation of the p65 subunit from the cytoplasm to the nucleus (Fig. 4A). Indeed, the nucleus/cytoplasm ratio of p65 was increased about threefold (P < 0.01) in muscle of LPS-treated mice (Fig. 4A). The translocation of p65 from the cytoplasm to the nucleus results from the release of NF-κB from its inhibitory complex formed with IκBα. The degradation of IκBα promotes translocation of NF-κB from the cytoplasm to the nucleus. To determine whether this activation...
is involved in the induction of atrogenes, we used MISR mice overexpressing an undegradable form of IκBα specifically in the muscle. As assessed by Western blot, IκBα overexpression totally inhibited NF-κB translocation from cytoplasm to the nucleus and thus NF-κB activation. Although NF-κB activation was completely inhibited (Fig. 4A), induction of muscle UPS, autophagy, and FOXO genes in response to LPS was not prevented (Fig. 4, B–F). The time point selected to assess NF-κB activation was based on the time course previously performed. Indeed, we showed a maximal induction of NF-κB between 2 and 4 h after LPS injection (data not shown). These results suggest that NF-κB muscle activation is not required for induction of atrogenes in LPS-treated mice.

**Inhibition of Glucocorticoid Receptor Totally Blunts LPS-Induced Atrogenes**

Increased circulating levels of GC are known to play a central role in several models of muscle atrophy (21, 23, 56). To determine whether the circulating GC increase observed in LPS-treated mice is involved in the induction of atrogenes, we administered RU-486, an antagonist of GC receptor. To evaluate the efficacy of RU-486 treatment in repressing muscle GC action, we measured muscle expression levels of REDD1. Indeed, a recent study has described that REDD1 muscle expression is closely correlated with muscle GC receptor activity (27). As shown in Fig. 5A, LPS administration strongly increased REDD1 muscle expression (17-fold, \( P < 0.001 \)), indicating that GC receptor is strongly activated in acute inflammation and that this activation was totally blocked by RU-486 treatment. Interestingly, inhibition of GC action totally blunted induction of muscle UPS, autophagy, and FOXO genes in response to LPS (Fig. 5, B–F). These results indicate that GC are mandatory for the induction of atrogenes in LPS-treated mice. The fact that TNFα expression was still induced in muscle of LPS-treated mice injected with RU-486 suggests the conclusion that this cytokine is not sufficient by itself to initiate the atrogene upregulation (Fig. 5G).

**DISCUSSION**

The present study shows that acute inflammation induced by intraperitoneal LPS injection activates several genes involved in muscle atrophy, or atrogenes. Most of these atrogenes belong to the autophagy and UPS pathways, the two major pathways of protein degradation. Inhibition of GC action totally blunts induction of atrogenes in LPS-treated mice, suggesting that GC are mandatory for the induction of atrogenes. The fact that TNFα expression was still induced in muscle of LPS-treated mice suggests that this cytokine is not sufficient by itself to initiate the atrogene upregulation. These results support the idea that GC are key regulators of muscle atrophy in response to acute inflammation.
proteolytic systems responsible for the muscle atrophy observed in many catabolic conditions. Moreover, we show that the induction of these atrogenes is associated with a decrease of IGF-I, a crucial muscle anabolic growth factor, together with an activation of the TNFα/NF-κB catabolic pathway. These hormonal changes could therefore contribute to the activation of these atrogenes. However, neither IGF-I restoration nor TNFα/NF-κB inhibition prevents the induction of the autophagy and UPS pathways in response to LPS. More interestingly, only GC blockade is able to fully blunt the induction of these two proteolytic systems. Taken together, our investigations demonstrate for the first time in vivo that neither activation of the TNFα/NF-κB pathway nor IGF-I decrease is responsible for induction of the atrogenes by acute inflammation. In contrast, the increased GC production seems to play a major role in the induction of these atrophy genes.

The activation of a common transcriptional program responsible for muscle atrophy has been demonstrated in many systemic diseases, such as cancer cachexia, severe insulinopenia, and denervation (31, 46). In our study, we show that acute inflammation is also associated with the activation of this family of genes. Indeed, LPS injection induced expression of several genes involved in the autophagy (LC3B, Gabarapl1, Bnip3) and UPS (atrogin-1, MuRF1, ubiquitin, E214kDa) proteolytic systems. In acute inflammation, as in other systemic diseases (31), atrogin-1 and MuRF1 are the most strongly induced atrogenes. There is considerable evidence that the protein breakdown observed in acute inflammation is mediated mainly by these two proteolytic systems. Indeed, in vitro and in vivo, inhibition of these two pathways by specific inhibitors blocks proteolysis induced by acute inflammation (24, 57, 62). Moreover, it has been recently demonstrated that expression of a dominant negative form of FOXO, a transcription factor regulating autophagy and UPS genes, inhibits the induction of several atrogenes together with muscle fiber atrophy caused by sepsis (45). These data indicate, therefore, that induction of the atrogenes through Foxo activation is a crucial step in the process leading to muscle atrophy observed in sepsis. Furthermore, these observations suggest that these transcriptional changes may serve as useful biomarkers to assess the muscle atrophy process observed in acute inflammation.

IGF-I is known as an important muscle growth factor which stimulates the development of muscle mass (14). Circulating or muscle IGF-I concentrations are often decreased in many catabolic conditions characterized by muscle atrophy, suggesting that it could contribute to the activation of these atrogenes.

Fig. 3. Prevention of circulating TNFα increase failed to inhibit LPS-induced atrogenes in rat TA muscle. A: circulating TNFα concentrations increased markedly 90 min after LPS injection (750 μg/100 g body wt ip). Pentoxyfilline administration (10 mg/100 g body wt, 1 h before LPS injection) almost completely blunted the release of TNFα induced by LPS. B: 12 h after LPS injection, inhibition of TNFα production by pentoxyfilline (10 mg/100 g body wt) failed to prevent induction of Foxo genes in response to LPS. C: inhibition of TNFα production by pentoxyfilline failed to prevent, or very marginally prevented, induction of UPS genes in response to LPS. Results are means ± SE (3–5 rats per group). Statistical analyses were performed using 1-way ANOVA followed by a Newman-Keuls multiple comparison test. Statistical significance was set at P < 0.05 (**P < 0.001, *P < 0.01, +P < 0.05 vs. CTRL ***P < 0.001, °P < 0.05 vs. LPS).
Indeed, we have previously shown that muscle atrophy caused by dexamethasone, which is associated with decreased muscle IGF-I concentrations, may be prevented by restoration of normal local IGF-I concentrations (49). However, restoration of circulating and muscle IGF-I to control levels is not sufficient to prevent LPS-induced atrogenes. These findings are consistent with previous reports indicating that IGF-I fails to prevent protein breakdown in sepsis (25, 61). The failure of IGF-I to prevent the induction of atrogenes in acute inflammation suggests the role of proinflammatory cytokines in this resistance to the IGF-I actions. Indeed, there is some evidence indicating that TNFα inhibits the action of IGF-I and insulin on muscle protein metabolism (16, 54, 60, 63). Moreover, pretreatment with pentoxyfilline, an inhibitor of TNFα production, restores the muscle responsiveness of protein degradation to inhibition by insulin in the early phase of sepsis (60). Furthermore, in the later phase of sepsis, when the production of proinflammatory cytokines vanishes, protein degradation in muscles from septic rats became more susceptible to the inhibitory action of insulin (60). Because of this IGF-I resistance, the induction of atrogenes is not prevented by the restoration of normal IGF-I concentrations in muscles from septic rats.
tance, supraphysiological IGF-I levels might be needed to block LPS-induced atrogenes. However, recent experiments performed by our group in muscle IGF-I transgenic (mTr-IGF-I) mice showed that even high muscle IGF-I levels (39) are not able to prevent atrogene induction after LPS injection (data not shown). All together, these observations indicate that administration of IGF-I alone fails to blunt atrogene induction caused by acute inflammation. Therefore, inhibition of proinflammatory cytokines (i.e., TNFα) could be required to restore IGF-I muscle responsiveness and thus its ability to inhibit
muscle atrogene induction. Nevertheless, some authors observed that local IGF-I administration partially attenuated muscle atrophy caused by sepsis (40). This observation may be explained by the fact that muscle protein synthesis, in contrast to protein degradation, remains sensitive, at least in vivo, to the stimulation by IGF-I even in septic animals (25, 40).

Many studies point to TNFα as an important mediator involved in muscle atrophy caused by sepsis. Indeed, circulating TNFα levels are increased during chronic and acute inflammation and TNFα, by stimulating proteolysis and inhibiting protein synthesis, is able to induce muscle atrophy both in vitro (35, 51) and in vivo (4, 13). In this study, we show that increased circulating TNFα is not involved in the induction of the muscle atrophy gene program observed in the acute phase of inflammation. Our data are in line with a previous study showing that inhibition of TNFα using a TNF-binding protein did not prevent the increase of atrogin-1 and MurF1 expression in septic rats (17). Moreover, it has been shown in arthritic rats, a model of chronic inflammation, that inhibition of TNFα using a soluble TNF receptor linked to polyethylene glycol prevented neither induction of atrogin-1 and MurF1 expression nor muscle atrophy observed in this model (19). Interestingly, the fact that TNFα upregulates atrogin-1 expression in a Foxo4-dependent, but Foxo1/3-independent, manner reinforces the notion that TNFα did not play a direct role in sepsis-induced muscle atrophy gene program (34). Indeed, only Foxo1 and -3 are upregulated in the skeletal muscle response to sepsis (41, 53). The persistence of the activation of the atrogenes despite the complete suppression of circulating TNFα increase is not sufficient to exclude the role of its main transduction pathway, namely NF-κB, in muscle atrophy caused by acute inflammation.

The NF-κB transcription factor is induced in skeletal muscle in many pathological conditions such as cancer cachexia, disuse atrophy, and sepsis (1). There is clear evidence that inhibition of NF-κB induction prevents muscle atrophy observed after denervation and in cancer cachexia (3). However, the role of muscle NF-κB in the activation of the atrogenes in sepsis has not yet been clearly demonstrated. It has been shown that the inhibition of sepsis-induced muscle proteolysis by curcumin is associated with inhibition of muscle NF-κB (44). However, this inhibitor suffers from a lack of specificity. Moreover, the effect of curcumin on NF-κB is still controversial. Indeed, it has been shown that curcumin blocks LPS-induced loss of muscle mass without altering the effect of LPS on muscle NF-κB (26). Using a specific inhibitor of muscle NF-κB, we show that the activation of the atrogenes by LPS is independent of NF-κB. Interestingly, the fact that NF-κB activation is totally blocked by using a specific muscle inhibitor demonstrates that NF-κB activation by LPS occurs mainly in muscle cells and not in immune cells localized in the muscle. In contrast to our results, recent reports have shown that NF-κB activation is required to induce MuRF1 expression and diaphragm muscle atrophy in acute pulmonary inflammation (12, 20, 30). The discrepancy between the present work and these studies could be easily explained by a crucial difference in the experimental design. Indeed, in these publications demonstrating the role of muscle NF-κB in the regulation of atrogenes during sepsis, LPS was administrated through the trachea into the lungs, a mode of administration that differs from our protocol (ip injection). This difference in the administration route can make a difference, as circulating levels of LPS are increased only with ip and not with tracheal administration, as recently reported (12). Our results do not exclude, however, the role of NF-κB activation outside of the muscle, in particular in immune cells or neurons, in the induction of the atrogenes.

The role of GC in the stimulation of proteolysis during sepsis has been recognized for many years. Indeed, Hasselgren et al. (21) have shown that inhibition of GC by RU-486, an antagonist of the GC receptor, attenuates both the induction of several atrogenes and the accelerated muscle proteolysis in sepsis. Our results confirm and extend these results to autophagy in acute inflammation. Interestingly, as RU-486 treatment did not impair the induction of the TNFα/NF-κB pathway in sepsis (43), our results confirm the hypothesis that TNFα/NF-κB pathway induction is not sufficient in vivo to induce the atrogenes in the absence of GC. Because RU-486 blunted not only the atrogenes but also the FOXO gene expression, one might speculate that inhibition of FOXO is directly responsible for the inhibition of these atrogenes. This hypothesis is confirmed by a recent study showing that expression of a dominant negative form of FOXO inhibits the induction of several atrogenes together with muscle fiber atrophy caused by sepsis (45). Thus, we can conclude that GC are necessary and sufficient to induce FOXO genes and to activate the transcription of autophagy and UPS genes in acute inflammation. Our observation is in full agreement with recent work showing that GC contribute largely to the muscle atrophy induced by systemic inflammation, either peripheral or central (2). However, it does not exclude the possibility for GC to synergize with cytokines acting directly on muscle to promote atrophy.

Collectively, our results showed that circulating TNFα and muscle NF-κB are unlikely to be the major direct mediators of the activation of the atrogenes caused by acute inflammation. Moreover, we could not find any evidence for the role of circulating or local IGF-I in the activation of this program. In contrast with studies suggesting that the direct action of pro-inflammatory cytokines on muscle cells is sufficient to induce proteolysis, we have demonstrated that GC are required for...
acutet inflammation to cause the activation of the atrogens. However, these observations do not exclude the role of cytokines in this process. Indeed, cytokines released by LPS-stimulated macrophages or neurons may act indirectly, in particular by stimulating the corticotrope axis, leading to the release of GC into the circulation (2).

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**


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