Expression of uncoupling protein 3 is upregulated in skeletal muscle during sepsis

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Sun, Xiaoyan, Curtis Wray, Xintian Tian, Per-Olof Hasselgren, and James Lu. Expression of uncoupling protein 3 is upregulated in skeletal muscle during sepsis. Am J Physiol Endocrinol Metab 285: E512–E520, 2003.—Uncoupling protein 3 (UCP3) is a member of the mitochondrial protein family that function by uncoupling oxidative phosphorylation from ATP synthesis (5). This process is important for thermogenesis and regulation of energy balance. Among the uncoupling protein (UCP) family members, UCP3 is expressed primarily in skeletal muscle (6, 50).

The influence of various pathophysiological conditions on the expression of UCP3 was recently reported. Interestingly, upregulated UCP3 expression in skeletal muscle was observed during various conditions characterized by muscle atrophy, including hyperthyroidism (13, 17), treatment with glucocorticoids (GCs) (17), cancer cachexia (46), and LPS administration (59). Although mechanisms regulating the expression of UCP3 are not completely understood, there is evidence that a concomitant increase of free fatty acids (FFA) and lipid oxidation (26, 55), as well as GCs (17), participates in mediation of skeletal muscle UCP3 during these pathological conditions.

Sepsis is another condition characterized by skeletal muscle cachexia, mainly reflecting increased degradation of proteins by the ubiquitin-proteasome proteolytic pathway (19, 34). Multiple lines of evidence suggest that sepsis-induced muscle wasting is mediated by GCs (52). Thus the metabolic consequences of sepsis in skeletal muscle resemble those of several other conditions characterized by upregulated expression of UCP3. Despite these facts, the influence of sepsis on the expression of UCP3 in skeletal muscle is not known.

In the present study, we tested the hypothesis that sepsis increases the expression of UCP3 in skeletal muscle and that GCs and FFA at least in part regulate this effect. We found that sepsis induced by cecal ligation and puncture (CLP) in rats increased both gene and protein expression of UCP3 in different types of skeletal muscle. In addition, the sepsis-induced increase of UCP3 was prevented by treatment of the rats with the glucocorticoid receptor (GR) antagonist RU-38486. Our experiments also demonstrate upregulated UCP3 gene expression in skeletal muscle of normal rats after treatment with GCs or FFA. The fact of GC- and FFA-induced UCP3 gene expression was confirmed by ex vivo treatment of incubated skeletal muscles, which supports our hypothesis that sepsis-induced UCP3 gene expression is at least partly mediated by GCs and FFA.

MATERIALS AND METHODS

Experimental animals. In the first series of experiments, sepsis was induced in male Sprague-Dawley rats (40–60 g body wt) by CLP, as described previously (52, 54). Control rats underwent sham operation, i.e., laparotomy and manipulation, but no ligation or puncture of the cecum. All rats were resuscitated with 10 ml/100 g body wt of normal saline administered subcutaneously on the back at the time of experimental operation.

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surgery to prevent hypovolemia and septic shock. Rats had free access to drinking water after the surgical procedures, but food was withheld to avoid the influence of any differences in food intake between the groups of rats on UCP3 expression. The extensor digitorum longus (EDL) and soleus muscles were harvested 16 h after CLP or sham operation.

The muscles were immediately frozen in liquid nitrogen and stored at −70°C until further analysis. The septic model for this study has been used in several previous reports both from our laboratory and others to study sepsis-related metabolic changes (8, 52, 54). The model is clinically relevant, because it results in hyperdynamic, hypermetabolic sepsis 16–18 h after CLP and resembles the situation in many surgical patients with sepsis caused by intra-abdominal abscess and devitalized tissue. Small rats were used here to make possible comparisons with previous reports in which we examined the influence of sepsis on protein metabolism in incubated muscles from rats with a similar body size (52, 54). Muscles from rats of this size (40- to 60-g body wt) are small enough to allow for measurement of protein turnover rates during incubation ex vivo (20).

In the second series of experiments, the role of GCs in sepsis-induced changes in UCP3 expression was tested. The rats were treated with 10 mg/kg of the RU-38486 administered by gavage 2 h before sham operation or CLP, as described previously (18, 52). Other rats received a corresponding volume (0.5 ml) of vehicle by gavage. In previous experiments, treatment of rats with RU-38486 prevented several sepsis-induced metabolic changes in skeletal muscle, including increased protein breakdown (18) and upregulated expression and activity of the ubiquitin-proteasome proteolytic pathway (53).

In the third series of experiments, the direct role of GCs on UCP3 expression was tested. Dexamethasone (DEX; Sigma Chemical, St. Louis, MO) was suspended in an aqueous solution (10 mg/ml) of 0.4% Tween 80–0.5% carboxymethyl cellulose-0.9% benzyl alcohol. Rats received a subcutaneous injection of DEX (100 mg/kg body wt). Muscle UCP3 levels were measured 2 h after injection of DEX. The vehicle and dose of DEX were chosen on the basis of previous experiments in which an identical protocol resulted in plasma corticosterone (CORT) levels that were similar to those observed in septic rats (16, 32, 53, 54).

In the fourth series of experiments, the direct role of FFA on UCP3 expression was tested. The normal rats received an intraperitoneal injection of 0.1% M KCl, 0.05 M Tris-HCl (pH 7.4), 5 mM MgCl2, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The homogenates were centrifuged at 650 g for 10 min, and the supernatants were centrifuged at 14,000 g for 10 min. The pellets were resuspended in 0.1 M KCl. Aliquots (50 μg protein) were separated electrophoretically on an 8–16% Tris-Glycine gel (Novex, San Diego, CA). The proteins were transferred to an immunoblot polyvinylidene fluoride membrane (Bio-Rad, Richmond, VA), and Western blot analysis was performed by using a rabbit polyclonal antibody to a 14-amino acid human UCP3 antibody used here recognizes both the long (312 amino acids) and short (275 amino acids) isoforms of UCP3 (52). Blots were quantified by using PhosphoImager and Molecular Dynamics ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Results were expressed as arbitrary units after normalization to GAPDH mRNA levels.

In the first series of experiments, the influence on protein expression and activity of the ubiquitin-proteasome proteolytic pathway (53).

RESULTS

Northern blot analysis. Muscle RNA was extracted using the method described by Chomczynski and Sacchi (10). Northern blot analysis was performed as described previously in detail from our laboratory (52–54). A 924-bp cDNA probe was synthesized by RT-PCR by use of primers based on the published sequence of the rat UCP3 gene (38). The sense and antisense primers used for the synthesis of the UCP3 cDNA probe were 5′-ATG GTC GGA CCT CCA TCA GAA GTG-3′ and 5′-AAA TGG AGA TTC CCG CAG TAC CTG-3′, respectively. The cDNA probe was labeled by random priming with [α-32P]dCTP (Amersham) to a specific radioactivity of ~1 × 109 dpm/μg DNA. After hybridization with the UCP3 probe, gels were stripped and rehybridized with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe to control for equal loading of the lanes. The GAPDH cDNA probe (452 bp) was synthesized by RT-PCR with the following sense and antisense primers: sense, 5′ - TCG TCT CAT AGA CAA GAT G - 3′; antisense, 5′ - GAA GCC CAT GCC AGT GAG GTT - 3′, as described previously (52–54). Blots were quantified by using PhosphoImager and Molecular Dynamics ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Results were expressed as arbitrary units after normalization to GAPDH mRNA levels.

Western blot analysis. Muscles were homogenized in buffer containing 0.1 M KCl, 0.05 M Tris-HCl (pH 7.4), 5 mM MgCl2, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The homogenates were centrifuged at 650 g for 10 min, and the supernatants were centrifuged at 14,000 g for 10 min. The pellets were resuspended in 0.1 M KCl. Aliquots (50 μg protein) were separated electrophoretically on an 8–16% Tris-Glycine gel (Novex, San Diego, CA). The proteins were transferred to an immunoblot polyvinylidene fluoride membrane (Bio-Rad, Richmond, VA), and Western blot analysis was performed by using a rabbit polyclonal antibody to a 14-amino acid human UCP3 COOH-terminal peptide (AB3046; Chemicon International, Temecula, CA) as primary antibody (1:1,000 dilution) and a horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) as secondary antibody (1:20,000 dilution). The UCP3 antibody used here recognizes both the long (312 amino acids) and short (275 amino acids) isoforms of UCP3 described previously (6, 50). Blots were developed by an enhanced chemiluminescence detection system (Amersham) and quantified by densitometry (Molecular Dynamics). To test for equal loading of the gels, immunoblotting was also performed for β-actin by use of a rabbit monoclonal anti-β-actin antibody (Sigma). The molecular mass of the proteins was assessed by using protein molecular mass standards (Bio-Rad). After quantification, the results were expressed as arbitrary units based on UCP3-to-β-actin ratios.

Plasma CORT and FFA levels. At 16 h after CLP or sham operation, or at 2 h after RU-38486 administration, the rat blood was collected by heart puncture for determination of CORT and FFA levels. Plasma CORT and FFA concentrations were measured using radioimmunoassay kits (ICN Biochemicals, Costa Mesa, CA) and the NEFA C kit (Wako Chemicals, Dallas, TX), as described previously (36, 52).

Statistical analysis. Results are presented as means ± SE. Student’s t-test or ANOVA was used for statistical analysis as appropriate.

RESULTS

The plasma CORT and FFA levels were increased by 142% (Fig. 1A) and 71% (Fig. 1B), respectively, at 16 h after CLP treatment; these results are concomitant to the previous reports in septic rats (36, 52), Escherichia coli O111:B4, and other bacterial pathogens. The differences in food intake between the groups of rats on UCP3 expression were statistically significant (p < 0.05). The differences in food intake between the groups of rats on UCP3 expression were statistically significant (p < 0.05).
coli-injected rats (34), and LPS-treated rats (2). RU-38486 partially blocked the sepsis-induced increase of circulating FFA (Fig. 1C).

Sepsis resulted in a two- to threefold increase in UCP3 mRNA levels in skeletal muscle, with a more pronounced increase in EDL (a white, fast-twitch muscle) than in soleus muscle (a red, slow-twitch muscle) (P < 0.05, Fig. 2). As in previous reports (6), UCP3 mRNA appeared as two transcripts, with the sizes of 2.5 and 2.8 kb. Both transcripts were upregulated in parallel and were analyzed together using densitometry for quantification. To examine whether the increased mRNA levels were associated with elevated UCP3 protein levels, Western blot analysis was performed. Similar to the elevation of mRNA levels, the UCP3 protein levels were upregulated two- to threefold in muscles from septic rats, with a somewhat more prominent increase noticed in the EDL compared with the soleus (P < 0.05, Fig. 3). The distribution of UCP3 in EDL and soleus muscle was examined. The UCP3 levels were found to differ considerably from one muscle type to another. UCP3 protein level in EDL was 2.3-fold higher than in soleus, and mRNA level in EDL was 1.7-fold higher than in soleus. Therefore, the relative amounts of UCP3 protein and mRNA in the two muscles tested were comparable. Those results correlate with previous reports about distribution of UCP3.
protein and mRNA in mouse and various human muscles (22, 30).

To test the potential role of GCs in the sepsis-induced increase in UCP3 expression, rats were treated with RU-38486 before sham operation or CLP. This treatment prevented the sepsis-induced upregulation of UCP3 gene and protein expression in both EDL and soleus muscle (Fig. 4). Although this observation suggests that the increased expression of muscle UCP3 in septic rats was at least in part regulated by GCs, it is not known whether the result reflected a direct effect of GCs. To further examine the regulation of UCP3 by GCs, incubated muscles from normal rats were administered DEX in vitro. This treatment resulted in increased mRNA and protein (Fig. 5) levels of UCP3 in both EDL and soleus muscles, suggesting that UCP3 is directly regulated by GCs in skeletal muscle. DEX treatment increased the mRNA level of UCP3 in our

Fig. 4. UCP3 mRNA levels in EDL (A) and soleus (B) muscles of rats 16 h after sham operation or CLP shown in representative Northern blots (top) and quantifications by densitometry (bottom). UCP3 protein levels in EDL (C) and soleus (D) muscles of rats 16 h after sham operation or CLP shown in representative Western blots (top) and quantifications by densitometry (bottom). Groups of rats were treated by gavage with 10 mg/kg of RU-38486 or a corresponding volume of vehicle 2 h before sham operation or CLP. Results are means ± SE; n = 7 in each group. S, sham; V, vehicle; C, CLP; R, RU-38486. *P < 0.05 among all treatment groups by ANOVA in each panel.

Fig. 5. UCP3 mRNA levels in EDL (A) and soleus (B) muscles and UCP3 protein levels in EDL (C) and soleus (D) muscles in rats 2 h after vehicle or dexamethasone (DEX) treatment. Representative Northern (A and B) and Western (C and D) blots and quantifications by densitometry are shown (top and bottom, respectively). Results are means ± SE; n = 7 in each group. *P < 0.05, vehicle vs. DEX by Student’s t-test.

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experiment, which is supported by evidence from a previous report (17).

Because sepsis is characterized by an increase of circulating GCs as well as FFA, the following experiments were performed to measure the mRNA and protein levels of UCP3 in the skeletal muscles of FFA-treated rats as well. The results showed that both mRNA and protein levels of UCP3 in rat EDL and soleus muscles were increased by FFA treatment (Fig. 6). The mRNA levels of UCP3 were also increased by FFA treatment, a result that was supported by the previous reports (51, 55).

To confirm the direct effect of GCs, as well as FFA, on UCP3 gene expression, we next introduced ex vivo incubated normal rat muscles to treatments of FFA, DEX, or FFA + DEX. The results showed that the mRNA and protein levels of UCP3 were increased in both EDL and soleus muscles (Fig. 7), with the strength of these effects in the order of FFA + DEX > FFA > DEX, suggesting that sepsis-induced UCP3 expression may be at least in part directly regulated by both GCs and FFA.

In additional experiments, we measured muscle ATP and PCr levels after 2.5 and 5 h of incubation (data not shown) to determine whether or not anoxia during incubation altered our experimental results. There was no observable difference in the muscle ATP and PCr levels, suggesting that oxygen supply during the incubation period was sufficient. The experiments with time-extended incubation of isolated skeletal muscles have also been reported previously (10, 24, 60).

**DISCUSSION**

In the present study, sepsis resulted in increased gene and protein expression of UCP3 in rat skeletal muscle. This effect of sepsis was prevented by treatment with the GR antagonist RU-38486, suggesting that sepsis-induced upregulation of UCP3 was at least in part mediated by GCs. Further support for the role of GCs in the regulation of muscle UCP3 was established in experiments wherein treatment in vivo of normal rats and ex vivo of incubated muscles with DEX increased UCP3 mRNA and protein levels. Evidence for a role of GCs in the regulation of muscle UCP3 was provided by other studies as well (17).

Although GCs may be an important mediator of UCP3 expression in skeletal muscle, it is possible that other mechanisms are involved as well. For example, increased levels of FFA and stimulated lipid oxidation result in increased gene expression of UCP3 in skeletal muscle in vivo (55) and in cultured myotubes in vitro (26, 51). Interestingly, sepsis is commonly associated with increased circulating levels of GCs as well as FFA (current report and Refs. 35, 36, and 52). Indeed, the availability of fatty acids likely influenced the expression of skeletal muscle UCP3 in the current study, as evidenced by a strong association between plasma FFA concentration and UCP3 expression that was observed in septic rats (Figs. 1B, 2, and 3). Compared with controls, UCP3 expression in FFA-treated rats in vivo and incubating muscles ex vivo was approximately two- and threefold greater. The elevation of sepsis on UCP3 protein and mRNA levels was not completely blocked by RU-38486. Thus it is possible that, during sepsis, muscle UCP3 expression is regulated by multiple factors, including, but not necessarily limited to, GCs and lipid oxidation. The mechanisms underlying the sepsis-induced rise of circulating CORT and FFA

![Fig. 6. UCP3 mRNA levels in EDL (A) and soleus (B) muscles and UCP3 protein levels in EDL (C) and soleus (D) muscles after vehicle or FFA treatment in rats. Representative Northern (A and B) and Western (C and D) blots and quantification by densitometry are shown (top and bottom, respectively). Results are means ± SE; n = 7 in each group. *P < 0.05, vehicle vs. FFA by Student’s t-test.](image-url)
levels and their association with UCP3 gene expression remain unknown.

UCP3 belongs to a family of mitochondrial membrane proton transporter proteins that uncouple oxidation from ATP production, resulting in increased heat production (5, 6, 50). These proteins, therefore, are important for muscle and whole body thermogenesis. Although skeletal muscle is an important site of regulated energy homeostasis and thermogenesis (49), the role of the UCPs in the regulation of energy metabolism in skeletal muscle is unclear. For example, a substantial increase in UCP3 mRNA and protein levels in skeletal muscle during starvation was not accompanied by changes in mitochondrial energy coupling (7, 31). Studies have suggested that UCP3 does not serve in a thermogenic capacity in LPS-treated mice (59). However, UCP3 may function as a regulator of lipids as a fuel substrate, rather than as mediator of regulatory thermogenesis in food-deprived rats (45). In addition, the results of our current UCP3 study do not correlate with previous results from this laboratory in which the septic rat demonstrated minimal or no difference in skeletal muscle ATP concentrations (1). Further study is warranted to assess the possibility that UCP3 regulates energy metabolism in septic skeletal muscle.

Increased gene expression of UCP3 in skeletal muscle was reported previously in several conditions characterized by muscle atrophy, such as muscle unloading and denervation (12, 13), starvation (17), hyperthyroidism (13, 17, 41), fasting (17), cancer (46), LPS treatment (59), and treatment with GCs (17). It is characterized by rapid and progressive loss of body weight and tissue wasting, particularly in skeletal muscle, under those cachexia conditions that are associated with marked alterations in endocrine and metabolic homeostasis. Acceleration of tissue protein breakdown accounts for most of the cachectic response (19). Muscle wasting is also associated with enhanced protein turnover rates (19). In particular, skeletal muscle hypercatabolism involves hyperactivation of the ATP-ubiquitin-dependent proteolytic system (37). In addition, muscle cachexia tends to develop during the late stages of sepsis. Thus preventing muscle wasting in septic patients is of potential clinical interest. The present study is the first report of upregulated skeletal muscle UCP3 gene and protein expression during sepsis. Indeed, we have recently demonstrated that sepsis-induced skeletal muscle proteolysis is mediated through a GR pathway (52). This may offer a clue that UCP3 would involve regulation of muscle cachexia during sepsis via GCs. This concept is supported by the fact that the GR has been known to interact with the proinflammatory transcription factors AP-1 and C/EBP. The DNA-binding sites of the transcription factors can be found in the promoter of mouse UCP3 gene (58). Consequently, some relationship probably exists between GR, AP-1, C/EBP, and UCP3 in sepsis-induced skeletal muscle proteolysis. In considering previous reports of both UCP3 gene activity (26, 51, 55) and skeletal muscle proteolysis (56) regulation by FFA, we suggest that UCP3 may regulate the muscle cachexia during sepsis. Additional experiments are needed, however, to test the relationship connecting UCP3 and muscle cachexia.
Our results suggest that GCs mediate both UCP3 (17) gene and protein expression and myofibrillar protein catabolism during sepsis-induced skeletal muscle proteolysis (52–54). This observation was more pronounced in white, fast-twitch EDL than in red, slow-twitch soleus muscles (21). These findings are consistent with the concept that increased expression of UCP3 may be involved in sepsis-induced muscle cachexia. Although the reason for the difference in sensitivity between different types of skeletal muscle is not known, a similar preferential increase in protein degradation and UCP3 gene expression in fast-twitch muscle was noted in other catabolic conditions as well, including cancer (3, 37, 46), fasting (17, 30, 57), denervation (12, 13, 40), GC treatment (17, 43), and burn injury (15). These observations suggest that some metabolic disparity acts to preferentially preserve protein in oxidative muscles under catabolic conditions and that UCP3 is more involved in amino acid metabolism under sepsis, because sepsis alters muscle glutamine concentration and transport to a greater extent in EDL muscle (29).

Increased circulating FFA levels associated with prominent UCP3 gene expression in EDL muscle may suggest that a positive feedback mechanism could exist mainly in glycolysis, possibly as an adaptive mechanism for glycolysis after sepsis (29) or other pathophysiological states where fatty acid oxidation by muscle is partially substituted by glycolysis. It has been demonstrated that UCP3 stimulates glucose transport and GLUT4 translocation to the skeletal muscle cell surface by activating a phosphoinositide 3-kinase-dependent pathway (25). In addition, mice that overexpress skeletal muscle UCP3 have an increased glucose clearance rate and strikingly reduced fat deposition (11). It is conceivable that the increased expression of UCP3 and GLUT4 (47) in the fast-twitch glycolytic muscles during sepsis could cause impaired glucose tolerance followed by lipid accumulation.

The high susceptibility of fast-twitch glycolytic muscles to sepsis might be a defensive response to maintain ATP levels for tissue survival. In addition, others (23) have shown an increase in UCP3 gene expression in skeletal muscle (gastrocnemius, but not soleus) together with similar changes in mRNA content of different enzymes related to lipid catabolism. These results suggest a functional difference in muscle cachexia between fast- and slow-twitch muscles under the septic condition. The present data also indicate that the GC- and FFA-dependent increase in UCP3 during sepsis is more in fast-twitch glycolytic muscles than in slow-twitch oxidative muscles. In particular, the notable induction in the level of the UCP3 protein in the atrophied fast-twitch muscle with sepsis strongly suggests increased metabolic capacity and thermogenic ability, which may lead to the consumption of excess energy as fat and an induction of glycolysis and proteolysis in skeletal muscle.

Fast-twitch fibers compared with slow-twitch fibers have an extensive sarcoplasmic reticulum (SR), with the Ca$^{2+}$-ATPase estimated to be three- to fourfold higher (14). Fast-twitch muscle fibers experience a much more rapid decline of ATP and total adenine nucleotide pool than muscles of predominant slow-twitch fibers during ischemia (27). One explanation for this difference may be that Na$^+$-K$^+$-ATPase is more dependent on glycolysis for ATP production in EDL than in soleus. The soleus has a greater number of mitochondria and, presumably, a greater capacity to produce ATP oxidation than the EDL (28). UCP3 expression was increased in both EDL and soleus muscle, although a slight change in soleus suggests that upregulated UCP3 in septic muscle is important not only for regulating glycolysis or proteolysis but for other metabolic consequences of sepsis, such as lipolysis. Our results also suggest that GCs might induce lipolysis to increase circulating FFA during sepsis. To this end, these results, together with those garnered from the literature, support the notion that sepsis upregulates UCP3 and that this UCP contributes at least in part to the development of skeletal muscle cachexia by hypercortisolism and the enhancement of FFA oxidation.

Although our results provide strong evidence for upregulated expression of UCP3 in skeletal muscle during sepsis, several limitations need to be considered when the data are interpreted. First, it is not known from the present results whether the increased mRNA levels for UCP3 represent increased transcription of the UCP3 gene or increased stability of the transcript or a combination of these changes. Second, it is not known whether the elevated UCP3 protein levels are accompanied by increased UCP3 activity. Indeed, the exact function of muscle UCP3 is not known at present. Although questions remain to be answered with regard to the regulation and function of the UCP3 gene in septic muscle, the present study is important, because it provides the first evidence that sepsis results in a strong upregulation of muscle UCP3 gene and protein expression. This conclusion, together with previous similar observations in other conditions characterized by muscle atrophy, suggests that further studies are warranted to examine the potential role of UCP3 in muscle cachexia.

DISCLOSURES

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