C/EBP DNA-binding activity is upregulated by a glucocorticoid-dependent mechanism in septic muscle

GAIL PENNER,1 GYU GANG,1 XIAOYAN SUN,1,2 CURTIS WRAY,1 AND PER-OLOF HASSELGREN1
1Department of Surgery, University of Cincinnati, Cincinnati 45267-0558; and 2Shriners Hospitals for Children, Cincinnati, Ohio 45229-3095

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Penner, Gail, Gyu Gang, Xiaoan Sun, Curtis Wray, and Per-Olof Hasselgren. C/EBP DNA-binding activity is upregulated by a glucocorticoid-dependent mechanism in septic muscle. Am J Physiol Regulatory Integrative Comp Physiol 282: R439–R444, 2002; 10.1152/ajpregu.00512.2001.—Sepsis-induced muscle cachexia is associated with increased expression of several genes in the ubiquitin-proteasome proteolytic pathway, but little is known about the activation of transcription factors in skeletal muscle during sepsis. We tested the hypothesis that sepsis upregulates the expression and activity of the transcription factors CCAAT/enhancer binding protein (C/EBP)-β and -δ in skeletal muscle. Sepsis was induced in rats by cecal ligation and puncture, and control rats were sham operated. C/EBP-β and -δ DNA-binding activity was determined by electrophoretic mobility shift assay and supershift analysis. In addition, C/EBP-β and -δ nuclear protein levels were determined by Western blot analysis. Sepsis resulted in increased DNA-binding activity of C/EBP, and supershift analysis suggested that this reflected activation of the β- and δ-isotypes of C/EBP. Concomitantly, C/EBP-β and -δ protein levels were increased in the nuclear fraction of skeletal muscle. In additional experiments, we tested the role of glucocorticoids in sepsis-induced activation of C/EBP-β and -δ by treating rats with the glucocorticoid receptor antagonist RU-38486. This treatment inhibited the sepsis-induced activation of C/EBP-β and -δ, suggesting that glucocorticoids participate in the upregulation of C/EBP in skeletal muscle during sepsis. The present results suggest that C/EBP-β and -δ are activated in skeletal muscle during sepsis and that this response is, at least in part, regulated by glucocorticoids.

Muscle wasting in these conditions is associated with increased expression of several genes in the ubiquitin-proteasome proteolytic pathway (25, 26). In addition, there is evidence that mRNA levels for calpains are increased in skeletal muscle during sepsis, possibly reflecting the role of calcium-calpain-dependent release of myofilaments from the sarcomere before ubiquitination and degradation by the 26S proteasome (30).

Despite the fact that the expression of several genes that are involved in the regulation of protein breakdown is increased in cachectic muscle, little is known about the activation of transcription factors in skeletal muscle. Sequence analysis of genes that are upregulated in septic muscle, such as the genes for calpains and members of the ubiquitin-proteasome pathway (12, 13, 15, 22, 23, 31), demonstrated that the promoter regions of several of these genes contain binding sites for transcription factors commonly associated with inflammation, including nuclear factor-κB (NF-κB), activating protein-1 (AP-1), and CCAAT/enhancer binding protein (C/EBP) (Fig. 1). In a recent study we found that sepsis in rats influenced the activity of AP-1 and NF-κB in skeletal muscle (17). In contrast, the regulation of C/EBP in skeletal muscle during sepsis is poorly understood.

The C/EBP family of transcription factors consists of at least six isoforms: C/EBP-α, β, γ, δ, ε and C/EBP-homologous protein-10 (CHOP-10) (1, 14, 19). The different isoforms form homo- or heterodimers at a leucine zipper region before binding to DNA. The composition of the dimers and abundance of the isoforms vary between different tissues and conditions. Among the C/EBP family members, there is evidence that C/EBP-β and -δ are particularly important for the inflammatory response (19).

The purpose of the present study was to test the hypothesis that sepsis upregulates the expression and activity of C/EBP-β and -δ in skeletal muscle. Because in other studies we found evidence that glucocorticoids regulate sepsis-induced muscle cachexia (8, 9, 24), we also examined the potential role of glucocorticoids in C/EBP activation in septic muscle. This was done by treating rats with the glucocorticoid receptor antagonist RU-38486 (18). Results reported here provide evidence that sepsis upregulates the expression and activity of C/EBP-β and -δ in skeletal muscle and that...
Phenylmethylsulfonyl fluoride, protease inhibitor cocktail (Sigma Chemical, St. Louis, MO; P8340), and phosphatase inhibitor cocktail I (Sigma, P2850) to all buffers. To increase the amount of nuclear protein, muscles from five rats were pooled for each electrophoretic mobility shift assay (EMSA). The nuclear preparations were examined under a light microscope, and only preparations containing even, round nuclei were used for further analysis. The nuclear proteins were extracted from the nuclei into buffer containing 75 mM HEPES (pH 7.5), 60 mM KCl, 0.42 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 40% glycerol, 0.5 mM dithiothreitol (DTT), 0.5 mM spermidine, and 0.5 mM spermine on ice for 30 min. The samples were centrifuged at 16,000 g for 20 min, and the supernatants were saved as the nuclear extracts. A Bio-Rad Bradford protein assay kit was used to determine protein concentrations in the nuclear extracts (Bio-Rad Laboratories, Hercules, CA).

EMSA. Oligonucleotide encoding the sequence for C/EBP binding (5'-TGC AGA TTG CGC AAT CTG CA) (Santa Cruz Biotechnology, Santa Cruz, CA) was end labeled with [γ-32P]ATP using T4 polynucleotide kinase (Amersham Pharmacia Biotech, Piscataway, NJ). End-labeled probe was purified from unincorporated [γ-32P]ATP using a purification column (Bio-Rad Laboratories) and recovered in Tris-EDTA buffer, pH 7.4. Nuclear protein (20 μg) was incubated in buffer containing 12% glycerol (vol/vol), 12 mM HEPES, pH 7.9, 4 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM DTT, 25 mM KCl, 5 mM MgCl2, and 0.04 μg/μl poly(dI.dC) (Boehringer Mannheim, Indianapolis, IN). Labeled probe was added, and the samples were incubated for 30 min on ice. Where indicated in results, an excess (15×) of unlabeled consensus or mutant oligonucleotide (5'-TCG AGA GCC TAG TCT CTG CA; nucleotide substitutions underlined; Santa Cruz Biotechnology) was added to test the specificity of the EMSA. For supershift reactions, 1 μl of rabbit polyclonal or mouse monoclonal antibody to C/EBP-β or rabbit polyclonal antibody to C/EBP-β (Santa Cruz Biotechnology) was added 30 min after addition of the radiolabeled probe with an additional 15 min of binding time at room temperature. Samples were subjected to electrophoretic separation on a nondenaturing 5% polyacrylamide gel at 100 V in 0.5x TBE buffer (1 x TBE = 89 mM Tris-borate, 2 mM EDTA, pH 8.3). Gels were dried and analyzed by exposure to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis. Nuclear proteins (50 μg) were separated electrophoretically on an 8–16% Tris-glycine gel (Novex, San Diego, CA). The proteins were transferred to blots...
nitrocellulose membrane, and Western blot analysis was performed using a polyclonal antibody to C/EBP-β or C/EBP-δ (Santa Cruz Biotechnology).

RESULTS

C/EBP DNA-binding activity in EDL muscle was increased 4 h after induction of sepsis by CLP and remained increased throughout the duration of the experiment (Fig. 2, left). At earlier time points (1 and 2 h), there was no difference in C/EBP DNA-binding activity between septic and sham-operated rats (data not shown). The C/EBP binding activity in muscles from control rats was higher at 16 h than at earlier time points, most likely reflecting the fact that these rats were subjected to trauma (laparotomy) and fasting. The relative difference in C/EBP DNA-binding activity between sham-operated and septic rats was most pronounced at 4–8 h. When an excess of unlabeled wild-type C/EBP oligonucleotide was added to the reaction, the C/EBP band on the EMSA was obliterated, whereas a mutant C/EBP oligonucleotide did not affect the C/EBP band. This result confirms the specificity of the EMSA.

In previous studies we found that the catabolic response to sepsis in rats was more pronounced in the white fast-twitch EDL than in the red slow-twitch soleus muscle (11, 27). To examine whether C/EBP binding activity was differentially regulated in different types of skeletal muscle, we next determined the influence of sepsis on C/EBP activity in soleus muscle. Results from this experiment showed that C/EBP DNA-binding activity was upregulated in soleus muscle at the same time points as in the EDL muscle (Fig. 2, right).

To test whether the C/EBP-β and -δ isoforms were involved in the sepsis-induced activation of C/EBP, supershift analysis was performed. When an antibody against C/EBP-β was used, supershift analysis indicated that this isoform was at least in part responsible for the basal C/EBP binding activity in EDL muscles (Fig. 3A). Sepsis resulted in upregulation of C/EBP-β binding activity noted at 4 h and persisting throughout the septic course. Sham operation as well resulted in increased C/EBP-β activity. Supershift analysis showed that C/EBP-δ DNA-binding activity was low under basal conditions and was upregulated in EDL muscles during sepsis (Fig. 3A). Because of the low basal C/EBP-δ activity, the relative difference between muscles from sham-operated and septic rats was more pronounced for C/EBP-δ than for C/EBP-β.

Supershift analysis of the EMSA from soleus muscle showed a similar pattern as seen in the EDL muscle. Thus there was evidence of C/EBP-β DNA-binding activity under basal conditions and upregulation of both C/EBP-β and -δ during sepsis (Fig. 3B).

An additional way to examine the involvement of the different isoforms in the activation of C/EBP is to determine the expression of C/EBP-β and -δ proteins. Results from experiments in which Western blot analysis was performed on nuclear proteins were consistent with the supershift analysis, i.e., C/EBP-β and -δ pro-

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**Fig. 2.** C/EBP DNA-binding activity in extensor digitorum longus (EDL; left) and soleus muscles (right) at different time points after sham operation or cecal ligation and puncture (CLP) in rats. Numbers above the lanes indicate hours after sham operation or CLP. Wild-type and mutant indicate competition reactions in which an excess of nonlabeled wild-type or mutant C/EBP oligonucleotide was added to the electrophoretic mobility shift assay (EMSA) reaction. When the EMSAs were subjected to densitometry and the arbitrary unit for muscles from sham-operated rats was set to 1, the corresponding units for EDL muscles at 4, 8, and 16 h after CLP were 1.9, 1.7, and 1.2 and for soleus muscles were 3.2, 3.0, and 2.2, respectively.

**Fig. 3.** Supershift analysis of EMSAs performed in EDL (A) and soleus muscles (B) at different time points after sham operation or CLP in rats. Supershift was induced by the addition of a polyclonal antibody to C/EBP-β or C/EBP-δ to the EMSA reaction.
DISCUSSION

In the present study, sepsis induced by CLP in rats resulted in increased C/EBP DNA-binding activity in skeletal muscle determined by EMSA. Supershift analysis as well as determination of protein levels by Western blotting suggested that the β- and δ-isomers of C/EBP were involved in this response to sepsis. The sepsis-induced activation of C/EBP was reduced by the glucocorticoid receptor antagonist RU-3846, suggesting that glucocorticoids were at least in part responsible for the C/EBP activation in septic muscle. To our knowledge, this is the first report of upregulated C/EBP activity in skeletal muscle during sepsis (or in any condition associated with muscle catabolism). The results are important because they contribute to the understanding of the molecular regulation of sepsis-induced muscle cachexia and suggest that transcription factor(s) involved in the inflammatory response in other tissues may be activated in skeletal muscle as well.

Increased levels of the C/EBP-β and δ proteins as noted here were reported in liver and intestinal mucosa of endotoxemic mice in previous studies by Papaconstantinou and co-workers (2, 3). It should be noted that the abundance of the proteins is not the only factor accounting for their activity. Mitogen-activated protein kinase (MAPK)-dependent phosphorylation of C/EBP-β (28) and probably of C/EBP-δ as well (21) is an additional mechanism accounting for upregulation of C/EBP DNA-binding activity. This may explain why in the present study the inhibition by RU-3846 of C/EBP-β and δ DNA-binding activity was more pronounced than the reduction of C/EBP-β and δ protein levels. It is possible that the glucocorticoid receptor antagonist inhibited C/EBP DNA-binding activity both by reducing the amounts of the C/EBP-β and δ isoforms and by inhibiting signaling pathway(s). In several previous reports, three different isoforms of C/EBP-β were detected by Western blot analysis (2, 3, 6, 7). The sizes of the different isoforms varied somewhat between different tissues and cell types, but the largest C/EBP-β isoform was reported to be ~36 kDa, similar to the size found in the present study. A recent report provided evidence that the smaller C/EBP-β isoforms may be products of C/EBP-β degradation generated during preparation of tissues (4). Because of this, and because only minimal amounts of smaller C/EBP-β isoforms (30 and 20 kDa) were noticed in the present experiments, only changes in the larger C/EBP-β isoform were reported here.

**Fig. 4.** C/EBP-β and δ protein levels in the nuclear fraction of EDL and soleus muscles at different time points after sham operation or CLP determined by Western blot analysis.

**Fig. 5.** The effect of RU-3846 on C/EBP DNA-binding activity (top) and C/EBP-β and δ protein levels in the nuclear fraction (bottom) in EDL muscle 16 h after sham operation or CLP. Groups of rats were treated with 10 mg/kg of RU-3846 or corresponding volume of vehicle by gavage 2 h before sham operation or CLP. Supershift was induced by adding a monoclonal antibody to C/EBP-β or a polyclonal antibody to C/EBP-δ to the EMSA reaction as indicated above the gels.
We tested the potential role of glucocorticoids in sepsis-induced C/EBP activation by treating rats with the glucocorticoid receptor antagonist RU-38486. In previous reports from this laboratory, RU-38486 prevented the increase in ubiquitin-proteasome-dependent proteolysis as well as the upregulation of the gene expression of ubiquitin in skeletal muscle during sepsis (8, 24). Thus the effect of RU-38486 on C/EBP activation paralleled the effects on proteolysis and ubiquitin gene expression, suggesting that C/EBP may be involved in the regulation of sepsis-induced muscle cachexia. It should be noted, however, that the present finding of a similar upregulation of C/EBP in the white, fast-twitch EDL and the red, slow-twitch soleus muscle differed from the more pronounced effect of sepsis on ubiquitin-proteasome-dependent proteolysis in white, fast-twitch muscle (11, 27). This observation is consistent with the concept that C/EBP regulates genes that are involved in sepsis-induced metabolic changes other than, or in addition to, ubiquitin-dependent proteolysis and that may occur in different types of skeletal muscle.

Although the present experiments focused on the influence of sepsis on C/EBP activation, it is likely that other transcription factors are also activated in skeletal muscle in this condition. In fact, we recently reported that AP-1 DNA-binding activity was increased early in sepsis but was subsequently downregulated. Taken together with the present results, the observations suggest that sepsis regulates the expression and activity in skeletal muscle of multiple transcription factors commonly involved in the inflammatory response. An important question, of course, is which genes in skeletal muscle are regulated by C/EBP and the other “inflammatory” transcription factors and which of these genes (if any) are involved in the development of muscle cachexia during sepsis. There is evidence that genes that are activated during inflammation are often regulated by multiple transcription factors and that the transcription factors interact with each other during inflammation. For example, NF-κB and C/EBP binding sites are frequently found in close proximity to each other, and the two transcription factors can interact physically and functionally (16). Such an interaction has been found to be important for the regulation of the genes for several cytokines and acute-phase proteins (29). When we analyzed the promoter regions of several genes in the ubiquitin-proteasome proteolytic pathway using the MatInspector V2.2 program (20) (see Fig. 1), the results revealed that the promoter of the rat ubiquitin gene has binding sites for C/EBP and NF-κB within 64 base pairs of each other, the proteasome C3 subunit gene promoter has NF-κB and C/EBP binding sites within 17 base pairs of each other, and the ubiquitin-conjugating enzyme E24 gene promoter has NF-κB and C/EBP binding sites within 21 base pairs of each other. These observations provide further (albeit circumstantial) evidence that C/EBP (and NF-κB) may be involved in the regulation of genes that are activated in cachectic muscle. Further studies are needed to test the role of C/EBP (and other transcription factors) in the regulation of specific genes in cachectic muscle. Although the present study and a recent study from our laboratory (17) only provide indirect evidence that C/EBP, NF-κB, and AP-1 may be involved in sepsis-induced muscle cachexia, the observations are important because they provide the first evidence that these transcription factors are activated in skeletal muscle in vivo during sepsis.

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