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Journal of Cellular Biochemistry 114:1294–1305 (2013)

Journal of Cellular Biochemistry

CaMKII Activity Is Reduced in Skeletal Muscle During Sepsis

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ABSTRACT

Exercise-induced muscle hypertrophy is associated with increased calcium/calmodulin-dependent protein kinase II (CaMKII) expression and activity. In contrast, the influence of muscle atrophy-related conditions on CaMKII is poorly understood. Here, we tested the hypothesis that sepsis-induced muscle wasting is associated with reduced CaMKII expression and activity. Sepsis, induced by cecal ligation and puncture in rats, and treatment of rats with TNFα, resulted in reduced total CaMKII activity in skeletal muscle whereas autonomous CaMKII activity was unaffected. The expression of CaMKIIδ, but not β and γ, was reduced in septic muscle. In additional experiments, treatment of cultured myotubes with TNFα resulted in reduced total CaMKII activity and decreased levels of phosphorylated glycogen synthase kinase (GSK)-3β, a downstream target of CaMKII. The present results suggest that sepsis-induced muscle wasting is associated with reduced CaMKII activity and that TNFα may be involved in the regulation of CaMKII activity in skeletal muscle. Decreased phosphorylation (consistent with activation) of GSK-3β may be a consequence of reduced CaMKII activity, indicating that inhibited CaMKII activity may be involved in the catabolic response to sepsis. J. Cell. Biochem. 114: 1294–1305, 2013. © 2012 Wiley Periodicals, Inc.

KEY WORDS: MUSCLE WASTING; CaMKII; SEPSIS; ATROGIN-1; MuRF1; GSK-3β

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a Ser/Thr kinase with a broad spectrum of substrates. The CaMKII family consists of four isoforms (α, β, γ, and δ). The structure and function of the CaMKII family members were reviewed in detail elsewhere [Soderling and Stull, 2001; Hudmon and Schulman, 2002; Sacchetto et al., 2005]. CaMKIIα is expressed almost exclusively in brain tissue [Means, 2000; Bayer and Schulman, 2001] whereas the other isoforms are expressed more widely [Soderling and Stull, 2001]. In skeletal muscle, a specific CaMKIIβ isoform ($CaMKII\beta_M$) is expressed together with CaMKIIγ and δ [Rose and Hargreaves, 2003; Sacchetto et al., 2005; Rose et al., 2006, 2007]. The CaMKII isoforms have a catalytic, an autoregulatory, and an association domain and form a complex of 6–12 subunits arranged in a spoke-wheel pattern with the association domains at the center and the catalytic domains radiating outwards [Hudmon and Schulman, 2002]. The α-CaMKII association protein, αKAP, which is an alternatively spliced isoform of the CaMKIIα gene lacking the catalytic domain, is highly expressed in skeletal muscle and plays an important role in targeting CaMKII to the sarcoplasmic reticulum (SR) or the nucleus [Bayer et al., 1996, 1998; O'Leary et al., 2006; Singh et al., 2009].

A unique feature of CaMKII is its autophosphorylation that occurs after the calcium/calmodulin-dependent activation. Thus, activation of CaMKII by the binding of calcium/calmodulin to the enzyme results not only in calcium-dependent phosphorylation of multiple target proteins but also in phosphorylation of the Thr 287 residue (Thr 286 in the α isoform) in the autoregulatory domain of CaMKII. This autophosphorylation generates calcium-independent autonomous CaMKII activity that persists even after calmodulin dissociates from the enzyme. Interestingly, the CaMKII (Thr287) autophosphorylation results in a substantial increase in the affinity for its activator, calcium/calmodulin (calmodulin trapping) [Miller and Kennedy, 1986; Meyer et al., 1992; Singla et al., 2001; Hudmon and Schulman, 2002; Sacchetto et al., 2005], suggesting that although calcium-dependent and autonomous CaMKII activities may be differentially regulated in various pathological and physiological

Grant sponsor: NIH; Grant number: R01 DK37908; Grant sponsor: Department of Clinical Medicine, Sapienza, University of Rome, Rome, Italy; Grant sponsor: Gobierno Vasco, Spain; Grant number: BFI2010-240.

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Manuscript Received: 25 May 2012; Manuscript Accepted: 27 November 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 13 December 2012

DOI 10.1002/jcb.24469 • © 2012 Wiley Periodicals, Inc.

conditions, changes in the two types of CaMKII activity are closely related.

Although CaMKII activity is particularly prominent in brain tissue [Erondu and Kennedy, 1985; Means, 2000; Bayer and Schulman, 2001], CaMKII regulates metabolism in other tissues as well, including skeletal muscle [Sacchetto et al., 2005]. Of note, muscle regeneration [Abraham and Shaw, 2006] and exercise-induced muscle hypertrophy [Fluck et al., 2000b; Rose and Hargreaves, 2003; Rose et al., 2007; Smith et al., 2008] are associated with increased CaMKII expression and activity. In addition, mitochondrial biogenesis [Chin, 2004] and insulin signaling [Brozinik et al., 1999; Wright et al., 2004; Illario et al., 2009] in skeletal muscle are regulated by CaMKII. In contrast, the influence of conditions characterized by muscle atrophy on CaMKII expression and activity is not well understood. Here, we tested the hypothesis that CaMKII expression and activity are reduced in skeletal muscle during sepsis, a condition that is characterized by significant wasting of skeletal muscle [Lecker et al., 2006; Aversa et al., 2011; Klaude et al., 2012], reduced biogenesis and function of mitochondria [Fredriksson and Rooyackers, 2007; Vanasco et al., 2008], and insulin resistance [Wang et al., 2006; Dhar and Castillo, 2011; Park et al., 2011]. In order to test this hypothesis, the expression of different CaMKII isoforms as well as total and autonomous CaMKII activity were determined in skeletal muscle from rats with muscle wasting caused by septic peritonitis. Our results suggest that sepsis-induced muscle wasting is associated with reduced total, but unchanged autonomous, CaMKII activity in skeletal muscle, in particular in white, fast-twitch skeletal muscle.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Three series of animal experiments were performed. In the first series of experiments, sepsis was induced in male Sprague–Dawley rats (50–60 g body weight) by cecal ligation and puncture (CLP) as described in detail previously [Tiao et al., 1994, 1997; Wray et al., 2003; Alamdari et al., 2010; Menconi et al., 2010]. The animals were obtained from Charles River Laboratories, Inc. (Wilmington, MA) and were specific pathogen-free. Rats were housed four per cage at 20°C both before and after the surgical procedures with a 12 h/12 h light/dark cycle. With rats under general anesthesia induced by pentobarbital (50 mg/kg administered i.p.) the abdomen was opened through a midline incision and the cecum was ligated below the ileocecal junction with a 3-0 silk ligature and punctured twice with an 18-gauge needle. Control rats underwent sham-operation consisting of 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. Analgesics were not given postoperatively in order to avoid the influence of analgesics on muscle metabolism. Animals had free access to water, but food was withheld after the surgical procedures to avoid the influence of differences in food intake on changes in muscle metabolism in sham-operated and septic rats. Sixteen hours after sham-operation or CLP and with rats under pentobarbital anesthesia, the white, fast-twitch extensor digitorum longus (EDL) and the red, slow-twitch soleus muscles were harvested, immediately frozen in liquid nitrogen, and stored at −80°C until analysis. After removal of the muscles and with the rats still under pentobarbital anesthesia, the rats were sacrificed by performing a thoracotomy and excising the heart. The septic model used here is clinically relevant because it resembles the situation in patients presenting with septic peritonitis caused by perforated viscus and intraabdominal abscesses. The model is associated with a mortality rate of 25–30% 16 h after CLP with some variability between experiments. Of note, small growing rats weighing 50–60 g were used in several previous reports to study the effects of sepsis on total and myofibrillar protein breakdown rates determined as release of tyrosine and 3-methylhistidine, respectively, from incubated EDL and soleus muscles [Hasselgren et al., 1989; Tiao et al., 1994]. Rats weighing 50–60 g have lower extremity muscles that are thin enough to allow for in vitro incubations with maintained tissue oxygenation and viability. Rats of the same size were used in the present experiments to make comparisons with previous studies possible.

In a second series of experiments, male Sprague–Dawley rats (50–60 g body weight) were treated with 10 mg/kg of dexamethasone (Sigma–Aldrich, St. Louis, MO) or corresponding volume of solvent (0.2 ml of 0.1% ethanol) administered intraperitoneally. Animals had free access to drinking water but food was withheld after injection of solvent or dexamethasone. Sixteen hours after treatment with dexamethasone or solvent, EDL muscles were harvested, immediately frozen in liquid nitrogen, and stored at −80°C until analysis. In previous studies, the same dose of dexamethasone as used here resulted in sepsis-like metabolic changes in skeletal muscle [Tiao et al., 1996; Alamdari et al., 2010; Menconi et al., 2010].

In a third series of experiments, male Sprague–Dawley rats (50–60 g body weight) were treated with three intraperitoneal injections of 100 μg/kg each of TNFα (Sigma–Aldrich) or corresponding volume of solvent (0.2 ml distilled sterile water). The injections were performed at 0, 7, and 14 h. EDL muscles were harvested 16 h after the first injection of solvent or TNFα and processed as described above. The protocol for TNFα treatment used here was based on previous studies in which treatment of rats with three doses of 100 μg/kg of TNFα resulted in stimulated muscle proteolysis [Zamir et al., 1992ab].

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

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; Gonnella et al., 2011]. When cells reached 80% confluence, they were removed by trypsinization (0.25% trypsin in phosphate buffered saline, PBS) and seeded in six-well culture plates in the presence of 10% fetal bovine serum (FBS) until they reached approximately 80% confluence, at which time the medium was replaced with Dulbecco's Modified Eagle's Medium (DMEM) containing 2% FBS for induction of differentiation into myotubes. After 5–7 days, when formation of myotubes was

observed, cytosine arabinoside (10 μM) was added to the culture medium for 48 h in order to remove any remaining dividing myoblasts. Myotubes were then treated for 24 h with 1 μM dexamethasone dissolved in ethanol (final concentration 0.1% in the culture medium) or corresponding concentration of vehicle whereafter they were harvested for determination of CaMKII activity. In another set of experiments, myotubes were exposed for 24 h to 100 ng/ml TNFα (Sigma–Aldrich) or corresponding volume of vehicle (10 μl distilled water per well) and then harvested for determination of CaMKII activity and cellular levels of phosphorylated CaMKII, glycogen synthase kinase (GSK) 3β, and serum response factor (SRF) by Western blotting.

REAL-TIME PCR

mRNA levels for atrogin-1, MuRF1, and CaMKIIβ, γ, and δ were determined by real-time PCR performed as described in detail recently [Menconi et al., 2008, 2010; Alamdari et al., 2010; Gonnella et al., 2011]. The sequences of the forward, reverse, and double-labeled oligonucleotides for atrogin-1 and MuRF1 used here were described recently [Menconi et al., 2008, 2010; Alamdari et al., 2010; Gonnella et al., 2011]. CaMKIIβ, γ, and δ mRNA levels were determined using the ABI TaqMan Gene Expression Assay (Applied Biosystems Assay ID Rn00572627_m1, Rn00592346_m1, and Rn00560913_m1, respectively). mRNA concentrations were normalized to the 18S mRNA levels and were expressed as arbitrary units (AU). 18S mRNA levels were not affected by sepsis (data not shown).

WESTERN BLOTTING

Total and nuclear muscle extracts were prepared as described by Rose et al. [2007]. Total and nuclear myotube fractions were prepared as described by Alamdari et al. [2012]. Nuclear fractions from muscles and myotubes were used for determination of phosphorylated serum response factor (p-SRF) whereas total muscle and myotube extracts were used for determination of other protein levels. Protein concentrations were determined by using the Bradford protein assay kit (Thermo Scientific, Rockford, IL) with bovine serum albumin as standard.

Western blotting was performed as described in detail recently [Menconi et al., 2008, 2010; Alamdari et al., 2010; Gonnella et al., 2011]. The following primary antibodies and the appropriate secondary antibodies were used for the immunoblotting: a rabbit polyclonal anti-human CaMKII antibody (#3362, Cell Signaling Technology, Danvers, MA), a rabbit polyclonal anti-human phospho-CaMKII (p-CaMKII) (Thr286/287) antibody (#3361, Cell Signaling Technology), a goat polyclonal anti-rat CaMKIIδ antibody (sc-5392, Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit polyclonal anti- human p-SRF(Ser103) antibody (#4261, Cell Signaling Technology), and a rabbit polyclonal anti human p-GSK-3β (Ser9) antibody (#9336, Cell Signaling Technology). Because a specific anti- α-CaMKII kinase anchoring protein (α-KAP) antibody is not commercially available, we used a CaMKII (M-176) rabbit polyclonal antibody raised against amino acids 303–478 at the *C*-terminus of CaMKIIα of mouse origin (sc-9035, Santa Cruz Biotechnology), that is an epitope located within the association domain of CaMKII. This antibody allows for the detection of a band at about 23 kDa in skeletal muscle, but not in rat brain [Rose et al., 2006], indicating that the band reflects the skeletal muscle non-kinase splice variant of the CaMKIIα gene called α-KAP which has a calculated molecular size of 25 kDa [Bayer et al., 1996, 1998]. A mouse monoclonal anti-rat α-tubulin antibody (Sigma–Aldrich) was used for loading control for whole muscle and myotube extracts. When nuclear extracts were analyzed, an anti-rat lamin A/C antibody (Cell Signaling Technology) was used for loading control. For determination of p-CaMKIIδ protein levels, co-immunoprecipitation was performed using a commercially available kit (Catch and Release, Millipore, Billerica, MA). A goat polyclonal anti-rat CaMKIIδ antibody (sc-5392, Santa Cruz Biotechnology) was used for pull-down and a rabbit polyclonal anti-human p-CaMKII (Thr286/287) antibody (#3361, Cell Signaling Technology) was used for immunoblotting. In the co-immunoprecipitation experiment, Western blotting for CaMKIIδ was performed of the initial extract that was used for subsequent pull-down to make certain that the amount of starting material (input) was the same in samples from sham-operated and septic rats. Immunoreactive protein bands were detected by using the Western Lighting kit for enhanced chemiluminescence detection (PerkinElmer, Inc., Waltham, MA) 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.

CaMKII ACTIVITY

To detect CaMKII activity in total muscle extract and in cells lysates a commercially available kit was used (SignaTECT Calcium/Calmodulin-Dependent Protein Kinase Assays System, Promega, Madison, WI) following the manufacturer's instructions. All assays were run in duplicate with calcium/calmodulin (activation buffer) or without calcium/calmodulin (control buffer) to measure maximal (total) and autonomous CaMKII activity, respectively. CaMKII activities were normalized to the amount of protein in each sample.

STATISTICS

Results are reported as means ± SEM. Statistical analysis was performed by using two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Sixteen hours after induction of septic peritonitis by CLP in rats, maximal (total) CaMKII activity was reduced by approximately 25% in EDL muscles (Fig. 1A) whereas autonomous CaMKII activity was unaffected (Fig. 1B). Both total and autonomous CaMKII activities were unchanged during sepsis in soleus muscles (Fig. 1C,D). In subsequent experiments, therefore, only EDL muscles were studied.

In order to ascertain that the EDL muscles in which CaMKII activity was reduced exhibited a catabolic response under the present experimental conditions, the expression of the muscle wasting-associated ubiquitin ligases atrogin-1 and MuRF1 was determined. Atrogin-1 and MuRF1 mRNA levels were increased 18- and 24-fold, respectively, in septic EDL muscles (Fig. 1E,F),

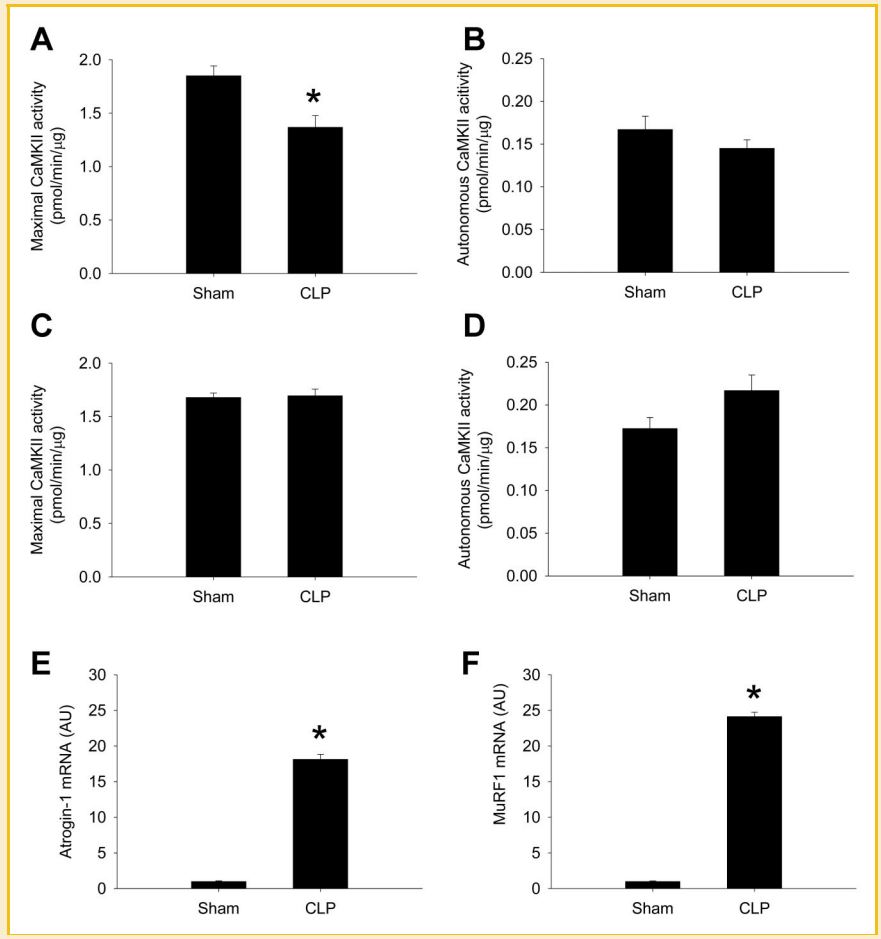

Fig. 1. Sepsis reduces maximal (total) CaMKII activity and upregulates the expression of the ubiquitin ligases atrogin-1 and MuRF1 in rat EDL muscles. Maximal (A) and autonomous (B) CaMKII activity in EDL muscles 16 h after sham-operation or CLP in rats. Maximal (C) and autonomous (D) CaMKII activity in soleus muscles 16 h after sham-operation or CLP in rats. Messenger RNA levels for atrogin-1 (E) and MuRF1 (F) in EDL muscles 16 h after sham-operation or CLP; mRNA levels were normalized to 18S mRNA levels and were expressed as arbitrary units (AU). Results are means ± SEM with n = 7 or 8 in each group. $^{*}P < 0.05$ versus sham by Student's *t*-test.

indicating that total CaMKII activity was reduced in muscles exhibiting evidence of an activated muscle wasting program.

In order to test whether the decreased CaMKII activity was associated with reduced expression of CaMKII, we determined mRNA levels for the CaMKIIβ, γ, and δ isoforms in EDL muscles from sham-operated and septic rats. The expression of the CaMKII isoforms was differentially regulated in EDL muscles during sepsis with unchanged expression of CaMKIIβ and γ and an approximately 25% reduction of CaMKIIδ mRNA levels (Fig. 2).

We next performed Western blotting of proteins extracted from EDL muscles. Western blotting using an anti-holoenzyme CaMKII antibody provided evidence for expression of CaMKIIβ_M, γ, and δ, but not α (Fig. 3A). The absence of detectable CaMKIIα is in line with previous studies suggesting that CaMKIIα protein is not expressed in skeletal muscle [Chin, 2004; Sacchetto et al., 2005]. Of note, total CaMKIIβ_M, γ, and δ protein levels were not different in EDL muscles from control and septic rats (Fig. 3A). Similarly, muscle levels of phosphorylated (Thr 286/287) CaMKII were not influenced by sepsis (Fig. 3B) which may in part explain why autonomous CaMKII activity was not affected by sepsis (see Fig. 1B).

Because unchanged CaMKIIδ protein levels in septic EDL muscles, as suggested in Figure 3A, may seem surprising in light of the reduced CaMKIIδ mRNA levels (see Fig. 2C), we performed Western blotting using a specific anti-CaMKIIδ antibody (as opposed to the anti-holoenzyme CaMKII antibody used in Fig. 3A). Also using this antibody, results suggested that sepsis did not influence total CaMKIIδ protein levels or levels of phosphorylated CaMKIIδ (Fig. 4A,B). Because a specific antibody against p-CaMKIIδ was not commercially available, co-immunoprecipitation was used to determine p-CaMKIIδ protein levels (Fig. 4B). In this experiment, an antibody against CaMKIIδ was used for pull-down (immunoprecipitation) and an antibody against phosphorylated total CaMKII was used for Western blotting. The CaMKIIδ antibody was used to perform Western blotting of the extracts used for the initial step of the co-immunoprecipitation procedure. The Western blot provided evidence that the amount of starting material (input) was similar in samples from septic and control (sham-operated) rats (upper panel in Fig. 4B). Western blotting of the immunoprecipitated proteins using an antibody against phosphorylated total CaMKII suggested that p-CaMKIIδ levels were not altered in septic muscle (lower panels in Fig. 4B).

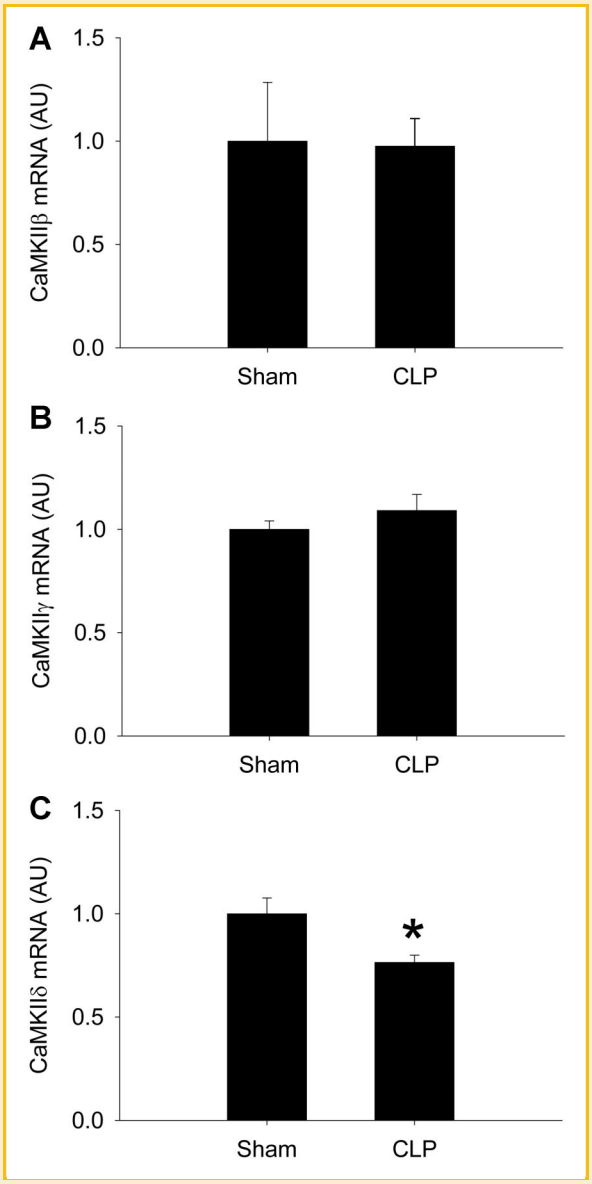

Fig. 2. Sepsis reduces the gene expression of CaMKIIδ in rat EDL muscles. Messenger RNA levels for CaMKIIβ (A), CaMKIIγ (B), and CaMKIIδ (C) determined by real-time PCR in EDL muscles 16 h after sham-operation or CLP in rats; mRNA levels were normalized to 18S mRNA levels and were expressed as arbitray units (AU). Results are means ± SEM with n = 7 or 8 in each group. *$P < 0.05$ versus sham by Student's *t*-test.

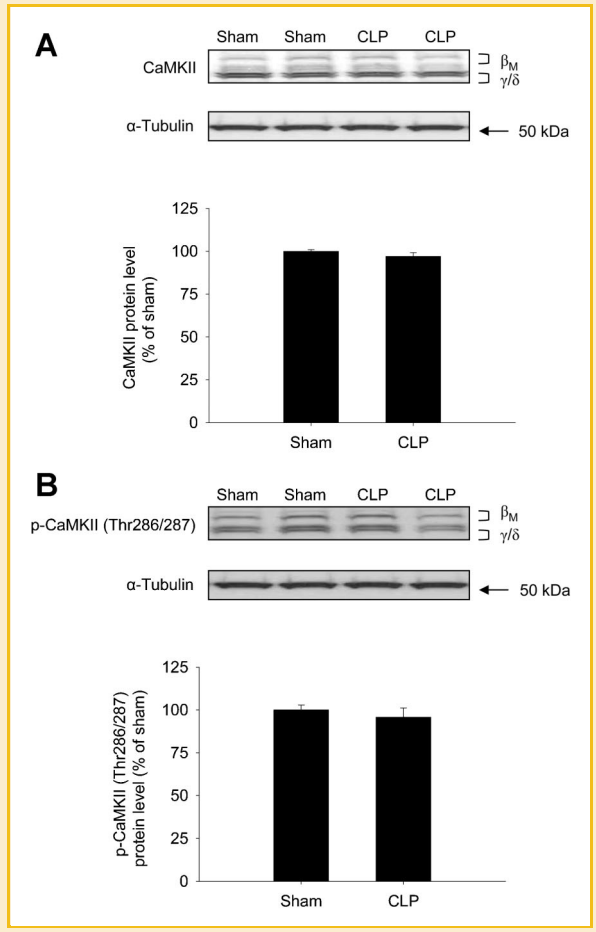

Fig. 3. CaMKII protein levels in rat EDL muscles are not influenced by sepsis. Western blotting of proteins from EDL muscles 16 h after sham-operation or CLP using an anti-holoenzyme CaMKII antibody (A). The identification of the different CaMKII isoforms was based on the molecular weights as indicated by the molecular weight ladder used for the Western blotting. Tissue levels of phosphorylated CaMKII (B) in EDL muscles 16 h after sham-operation or CLP determined by Western blotting using an anti phospho-CaMKII antibody as described in Materials and Methods Section. Levels of α-tubulin were determined as loading control. Representative Western blots and results from densitometric quantifications are shown. Results are means ± SEM with n = 8 in each group.

Taken together, the results described so far suggest that total CaMKII activity is reduced in the white, fast-twitch EDL muscle during sepsis and that this effect of sepsis is not caused by reduced amounts of CaMKII or decreased autophosphorylation of the enzyme. An alternative mechanism by which CaMKII activity may be regulated is altered intracellular trafficking of the enzyme. In order to test whether such a mechanism may be involved in sepsis-induced inhibition of CaMKII activity, we determined levels of αKAP in control and septic EDL muscles. We found that αKAP levels were reduced in skeletal muscle during sepsis (Fig. 5), raising the possibility that the intracellular trafficking of CaMKII was altered.

Members of the CaMKII family are multifunctional kinases, regulating the phosphorylation of multiple cellular proteins, including other kinases and transcription factors. The transcription factor SRF is an important substrate of CaMKII [Fluck et al., 2000a] and is involved in the regulation of muscle mass [Croissant et al., 1996; Soulez et al., 1996]. Previous studies suggest that decreased phosphorylation (reduced activity) of SRF may be involved in muscle atrophy [Croissant et al., 1996; Fluck et al., 2000b]. Based on those observations, we hypothesized that nuclear levels of phosphorylated SRF would be decreased in muscles from septic rats. Surprisingly, we found that the nuclear levels of phosphorylated SRF were increased, rather than decreased, in septic EDL muscles (Fig. 6) suggesting that any influence of reduced CaMKII activity on the phosphorylation of SRF was outweighed by increased activity of other kinases and/or reduced phosphatase activity.

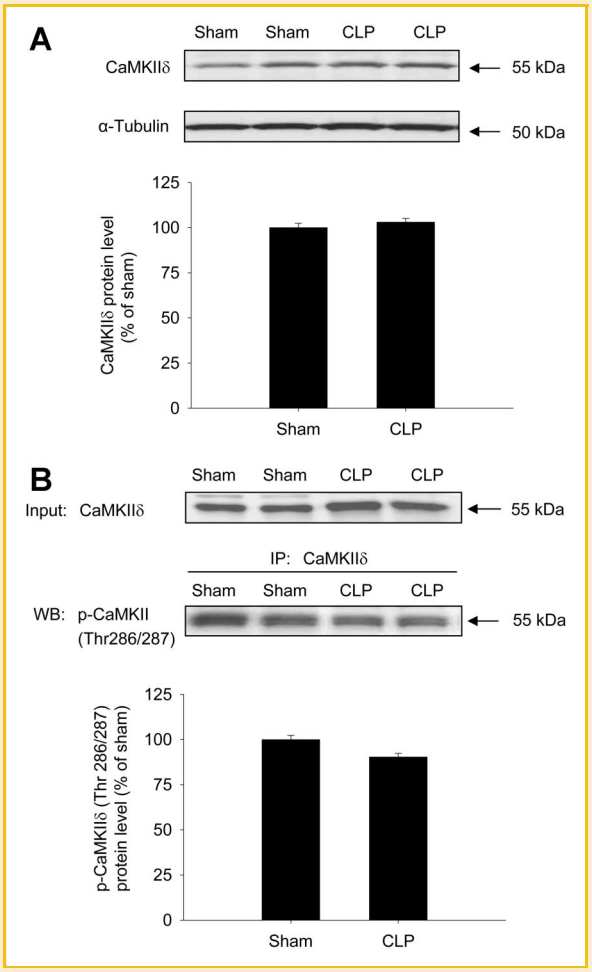

Fig. 4. Total and phosphorylated CaMKIIδ protein levels in rat EDL muscles are not altered during sepsis. A: Total CaMKIIδ protein levels in EDL muscles 16 h after sham-operation or CLP in rats. Representative Western blots are shown in the upper panels and densitometric quantifications are means ± SEM with n = 8 in each group. α-Tubulin levels were determined as loading control. B: p-CaMKIIδ levels were determined by co-immunoprecipitation as described in Materials and Methods Section. Muscle tissue extracts were immunoprecipitated (IP) using an anti-CaMKIIδ antibody and Western-blotted (WB) with an antibody directed against phosphorylated total CaMKII. Tissue extracts were probed with an antibody against CaMKIIδ to indicate equal input of CaMKIIδ before immunoprecipitation.

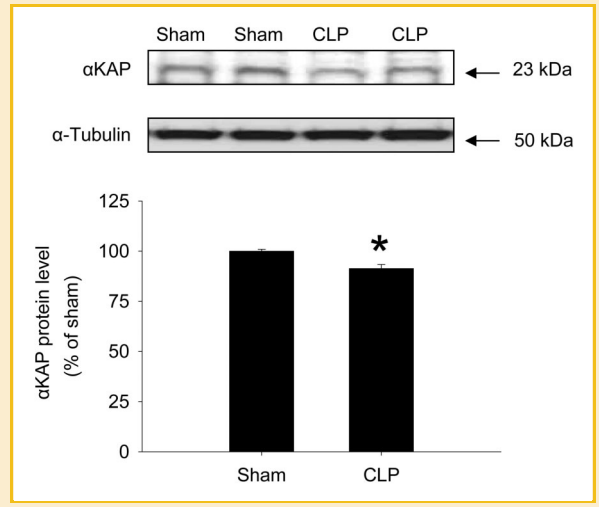

Fig. 5. Sepsis results in reduced αKAP levels in rat EDL muscles. αKAP protein levels were determined in EDL muscles 16 h after sham-operation or CLP in rats. Representative Western blots are shown in the upper panel and densitometric quantifications of immunoblots are shown in the lower panel. Results are means ± SEM with n = 8 per group. $^{*}P < 0.05$ versus sham by Student's *t*-test.

Metabolic changes in catabolic muscle are regulated by multiple mediators, including glucocorticoids [Schakman et al., 2008; Hasselgren et al., 2010] and TNFα [Ladner et al., 2003]. In order to test the potential involvement of these factors in the regulation of CaMKII activity, we treated cultured myotubes with dexamethasone or TNFα. Treatment of the myotubes with dexamethasone did not influence total or autonomous CaMKII activity (Fig. 7A,B) whereas treatment of the myotubes with TNFα reduced total, but not autonomous, CaMKII activity and decreased cellular levels of phosphorylated CaMKII (Fig. 7C–E). Of note, CaMKIIβ_M was the predominant CaMKII isoform in the cultured myotubes (Fig. 7E) whereas CaMKIIγ and δ seem to be the predominant isoforms in rat EDL muscles (see Fig. 3).

Although the experiments in cultured myotubes suggest that glucocorticoids alone do not regulate CaMKII activity (see Fig. 7A,B) and that TNFα downregulates total, but not autonomous CaMKII activity in skeletal muscle (see Fig. 7C,D), additional experiments were performed in order to test the relevance of these observations in vivo. When rats were treated with dexamethasone in vivo, total and autonomous CaMKII activities were unchanged in EDL muscles (Fig. 8A,B). In contrast, treatment of rats with TNFα resulted in an approximately 50% reduction of maximal CaMKII activity in EDL muscles but did not give rise to statistically significant changes in autonomous CaMKII activity (Fig. 8C,D). From a qualitative standpoint, these results were similar to those observed in vitro, suggesting that results in dexamethasone- and TNFα-treated myotubes are relevant for the in vivo situation, at least with regards to the regulation of CaMKII activity.

We reported previously that sepsis results in activation (reduced phosphorylation) of GSK-3β in skeletal muscle [Evenson et al., 2005], but the influence of TNFα on the phosphorylation of GSK-3β in muscle cells is not known. Here, we found that treatment of cultured myotubes with TNFα decreased cellular levels of p-GSK-3β (Ser 9; Fig. 9A). This observation raises the possibility that TNFα activates GSK-3β in skeletal muscle and that this effect of TNFα is associated with inhibited CaMKII activity. In contrast, TNFα did not influence the phosphorylation of SRF (Fig. 9B), suggesting that TNFα-induced decrease in CaMKII activity does not result in a general inhibition of the phosphorylation of potential target proteins.

DISCUSSION

The present study is important because it provides novel information about the influence of sepsis on the expression and activity of

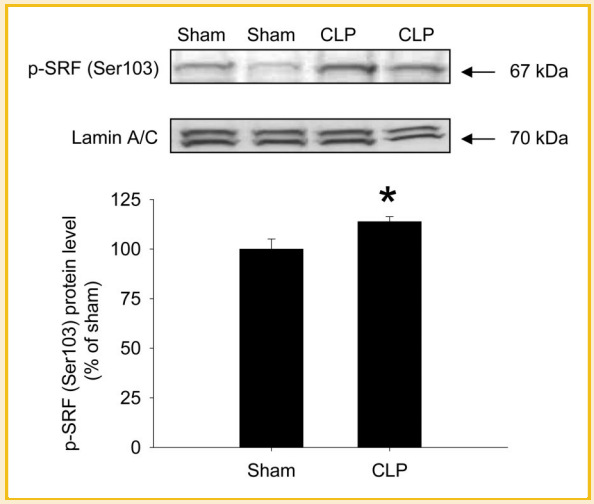

Fig. 6. Sepsis results in increased levels of phosphorylated serum response factor (SRF) in rat EDL muscles. Nuclear levels of p-SRF(Ser 103) determined by Western blotting of proteins from EDL muscles 16 h after sham-operation or CLP in rats. Representative Western blots are shown in the upper panel. Lamin A/C protein levels were determined for loading control. Densitometric quantifications are shown in the lower panel with results being means ± SEM and n = 8 in each group. $^{*}P < 0.05$ versus sham by Student's *t*-test.

CaMKII. Because CaMKII activity is increased in conditions characterized by muscle hypertrophy and stimulated mitochondrial biogenesis and function [Fluck et al., 2000b; Rose and Hargreaves, 2003; Rose et al., 2007; Smith et al., 2008], we hypothesized that sepsis, which is characterized by muscle wasting and reduced mitochondrial biogenesis and function [Lecker et al., 2006; Fredriksson and Rooyackers, 2007; Vanasco et al., 2008; Aversa et al., 2011; Klaude et al., 2012], may be associated with reduced CaMKII activity. Our results supported that hypothesis by demonstrating that sepsis inhibits CaMKII activity in skeletal muscle, in particular in white, fast-twitch skeletal muscle. In addition, our results suggest that TNFα may be more important than glucocorticoids for the regulation of CaMKII activity in skeletal muscle.

The CaMKII activity rates reported here in the intact muscles were similar to several previous reports [Fluck et al., 2000b; Rose and Hargreaves, 2003; Rose et al., 2007] but higher CaMKII activities have also been reported [Abraham and Shaw, 2006]. It should be noted that the basal CaMKII activity in myotubes was higher in the TNFα experiment than in the dexamethasone experiment in the present study. Although we can not offer a definitive explanation for this observation, it is possible that the difference reflected different solvents being added to the myotubes (0.1% ethanol in the dexamethasone experiment versus distilled water in the TNFα experiment).

Although the present report provides the first evidence that sepsis-induced muscle wasting is associated with reduced CaMKII activity, other studies suggest that CaMKII activity is inhibited in muscle from mice with muscular dystrophy [Abraham and Shaw, 2006]. When CaMKII was measured in different subcellular fractions, results suggested that CaMKII activity was reduced in the myofibrillar fraction but not in the cytosolic or membrane fraction of dystrophic muscles [Abraham and Shaw, 2006]. Those observations support the concept that CaMKII activity is regulated not only by tissue specific expression but also by discrete localization within the cell [Fluck et al., 2000a; Hudmon and Schulman, 2002; O'Leary et al., 2006; Singh et al., 2009]. Because, in our study, CaMKII activity was measured in whole tissue extracts, it is not known whether sepsis regulated CaMKII activity differentially at specific cellular sites. Our finding of reduced expression of αKAP in septic muscle, however, suggests that targeting of CaMKII to sarcoplasmic membranes and nuclei may have been reduced, possibly resulting in decreased CaMKII activity in those fractions [Bayer et al., 1996, 1998; O'Leary et al., 2006; Singh et al., 2009].

Of note, in the study by Abraham and Shaw [2006], CaMKIIδ protein levels were reduced in dystrophic muscle fibers, a finding that differs from our observation of unchanged CaMKIIδ protein levels in septic muscle. These observations suggest that reduced CaMKII protein levels may be involved in inhibited CaMKII activity in some, but not all, muscle wasting conditions. The result in the present study of reduced CaMKIIδ mRNA but unchanged CaMKIIδ protein levels suggest that the degradation of CaMKIIδ was reduced in skeletal muscle during sepsis. Regulation of CaMKII expression and activity by proteolytic processing of the enzyme was reported previously [Kwiatowski and King, 1989].

The lack of response of CaMKII activity to dexamethasone in cultured myotubes and in dexamethasone-treated rats observed in the present study was surprising considering previous reports of dexamethasone-induced muscle atrophy in cultured myotubes [Menconi et al., 2008] and in dexamethasone-treated rats [Tiao et al., 1996; Alamdari et al., 2010; Menconi et al., 2010]. We can not offer a definitive explanation for these apparently contradictory observations at present but the findings suggest that downregulation of CaMKII activity may not be necessary for glucocorticoid-induced activation of an atrophy program.

In previous studies from our laboratory, treatment of cultured myotubes with KN62 or KN93 inhibited the increase in proteasome activity caused by the calcium ionophore A23187 [Menconi et al., 2004] and the increase in protein degradation caused by dexamethasone [Evenson et al., 2004]. Because KN62 and KN93 are commonly used as CaMKII inhibitors [Brozinik et al., 1999], our previous results were interpreted as indicating that calcium- and glucocorticoid-induced muscle proteolysis is at least in part regulated by increased CaMKII activity. Although our previous reports were apparently contradictory to the present results, a number of factors need to be considered when our previous studies are compared with the current report. First, CaMKII activity was not measured in our previous reports and it is therefore not known if the kinase activity was increased. Second, even though K62 and K93 are commonly used as CaMKII inhibitors, studies suggest that the compounds are not completely CaMKII specific but may influence metabolic activities by CaMKII-independent mechanism(s) as well [Witczak et al., 2010]. Third, recent studies suggest that the role of CaMKII may be different in calcium ionophore-mediated gene expression and metabolic changes than in changes induced by different pathophysiological conditions [Macpherson et al., 2002]. Thus, it is not inconceivable that CaMKII is differentially regulated

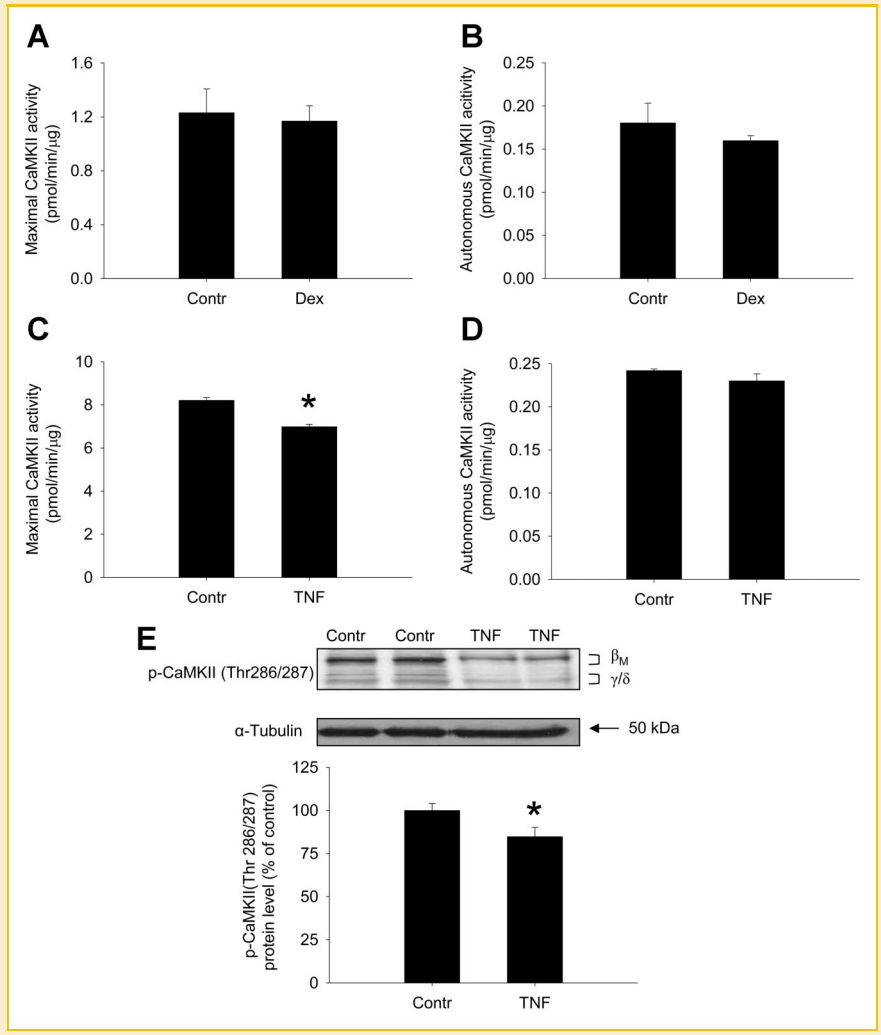

Fig. 7. Treatment of cultured myotubes with TNFα, but not dexamethasone, reduces total CaMKII activity. Total (maximal) (A) and autonomous (B) CaMKII activity in cultured L6 myotubes treated with 1 μM dexamethasone for 24 h. Total (C) and autonomous (D) CaMKII activity in cultured L6 myotubes treated with 100 ng/ml of TNFα for 24 h. p-CaMKII levels (E) in cultured L6 myotubes treated with 10 ng/ml of TNFα for 24 h. Results are means ± SEM with n = 6–8 in each group. $^{*}P < 0.05$ versus control (Contr).

in myotubes treated with A23187 [Menconi et al., 2004] and in muscle during sepsis and myotubes treated with TNFα (present study).

Even though CaMKII phosphorylates a large number of protein substrates [Hudmon and Schulman, 2002; Sacchetto et al., 2005], in the present study, we focused on the phosphorylation of two proteins that are involved in the regulation of muscle mass, i.e., GSK-3β and SRF. Multiple studies suggest that activation of GSK-3β is involved in muscle atrophy [Rommel et al., 2001; Vyas et al., 2002; Evenson et al., 2005]. GSK-3β is activated by reduced phosphorylation [Cross et al., 1995; Doble and Woodgett, 2003] suggesting that inhibited activity of kinases regulating GSK-3β phosphorylation may be a potential mechanism of muscle wasting. A recent study suggests that CaMKII is particularly important for GSK-3β phosphorylation [Song et al., 2010]. We reported recently that p-GSK-3β (Ser 9) levels were reduced in skeletal muscle from septic rats [Evenson et al., 2005]. The present study expanded our previous observation by finding evidence that TNFα may be involved in the inhibition of GSK-3β phosphorylation in skeletal muscle. Because previous studies suggest that TNFα plays an important role in sepsis-induced muscle wasting [Zamir et al., 1992ab] and that p-GSK-3β (Ser9) levels are reduced in skeletal muscle during sepsis [Evenson et al., 2005], the present findings of reduced CaMKII activity in skeletal muscle during sepsis and after treatment with TNFα suggest (but do not prove) that TNFα-mediated inhibition of CaMKII activity may be involved in sepsis-induced activation (reduced phosphorylation) of GSK-3β and muscle wasting.

Given previous observations suggesting that decreased activation (reduced phosphorylation) of SRF may be involved in muscle atrophy [Croissant et al., 1996; Fluck et al., 2000a,b], increased, rather than decreased, levels of p-SRF (Ser 103) in septic EDL muscles and unchanged levels of p-SRF (Ser 103) in TNFα-treated myotubes were surprising findings in the present study. Of note,

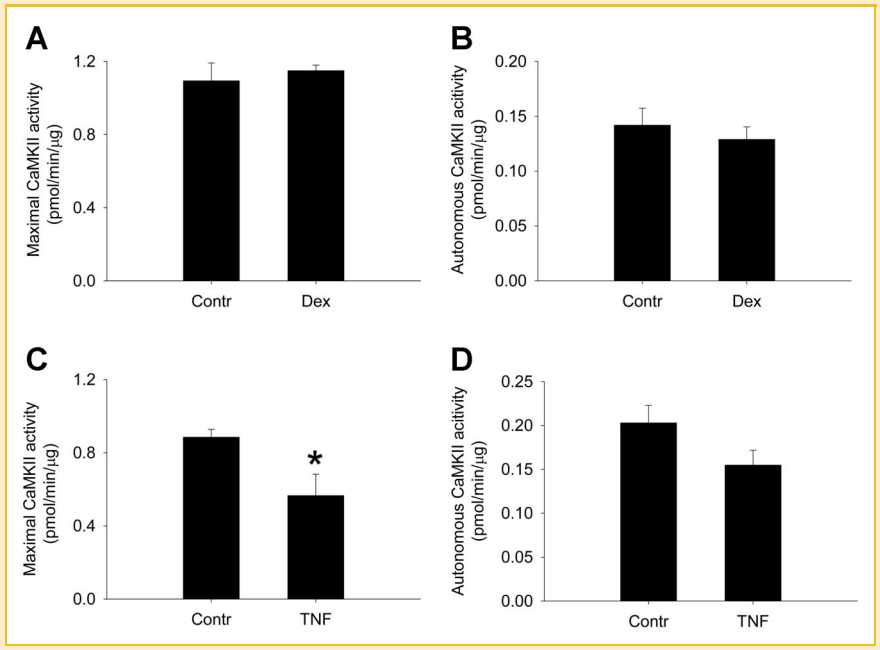

Fig. 8. Treatment of rats with TNFα, but not dexamethasone, results in reduced total CaMKII activity in EDL muscles. Maximal (A) and autonomous (B) CaMKII activity in EDL muscles 16 h after treatment of rats with 10 mg/kg of dexamethasone or corresponding volume of solvent (Contr) administered intraperitoneally. Maximal (C) and autonomous (D) CaMKII activity in EDL muscles from rats treated with three intraperitoneal doses of 100 μg/kg of TNFα or corresponding volumes of solvent (Contr). Results are means ± SEM with n = 6–8 in each group. $^{*}P < 0.05$ versus control by Student's *t*-test.

recent studies suggest that in addition to Ser 103, Thr 160 is also phosphorylated by CaMKII [Fluck et al., 2000a]. In addition, kinases other than CaMKII, for example CaMKIV, MAPKAP kinase 2, and a growth factor-induced kinase, phosphorylate SRF [Rivera et al., 1993; Heidenreich et al., 1999; Fluck et al., 2000a]. These observations raise the possibility that reduced phosphorylation of SRF residues other than Ser 103 as well as kinases other than CaMKII may be involved in the regulation of SRF phosphorylation in septic and TNFα-treated muscle.

In a recent report by Stephens et al. [2010], total and phosphorylated CaMKIIβ protein levels were slightly higher in muscle from cancer patients with high weight loss than in patients with low weight loss. This finding led the authors to suggest that CaMKIIβ may be a molecular biomarker of cancer cachexia and to speculate that CaMKIIβ may "subdue normal maintenance" of skeletal muscle mass, possibly secondary to inhibition of protein synthesis (or, alternatively, the increased CaMKIIβ levels were suggested to be a compensatory response to inhibited protein synthesis). Although the report by Stephens et al. [2010] may seem to contradict the results in the present study, several aspects need to be taken into account when the results reported by Stephens et al. [2010] are interpreted. First, although the difference in total CaMKII protein levels between patients with high and low weight loss was statistically significant ($P = 0.04$), the difference in p-CaMKII levels between the groups was not statistically significant ($P = 0.07$). Second, the difference in total CaMKIIβ levels, although statistically significant, was small why its biological role is difficult to assess. Most importantly, CaMKII activity was not measured in the study by Stephens et al. [2010] and, as illustrated by the results in the present study, changes in CaMKII activity are not necessarily accompanied by corresponding changes in gene or protein expression of the different CaMKII isoforms. The influence of cancer cachexia on CaMKII activity in human muscle remains to be determined. In recent experiments in our laboratory, an experimental rat cancer model was associated with reduced CaMKII activity [Aversa et al., in preparation].

The present study has several limitations that need to be taken into account when the results are interpreted. First, experiments were performed in a rat model of sepsis and in cultured myotubes and the influence of sepsis in human patients on muscle CaMKII expression and activity remains to be determined. Second, although TNFα reduced CaMKII activity both in cultured myotubes and in muscle in vivo, the exact role of TNFα in sepsis-induced inhibition of CaMKII activity remains to be determined. Finally, the mechanistic role of reduced CaMKII activity in muscle wasting during sepsis needs to be defined in future studies.

ACKNOWLEDGMENTS

This work was supported in part by NIH R01 DK37908 (P.O.H.). Z.A. was supported in part by the Department of Clinical Medicine, Sapienza, University of Rome, Rome, Italy. E.C. was supported in part by Gobierno Vasco, Spain (BFI2010-240).

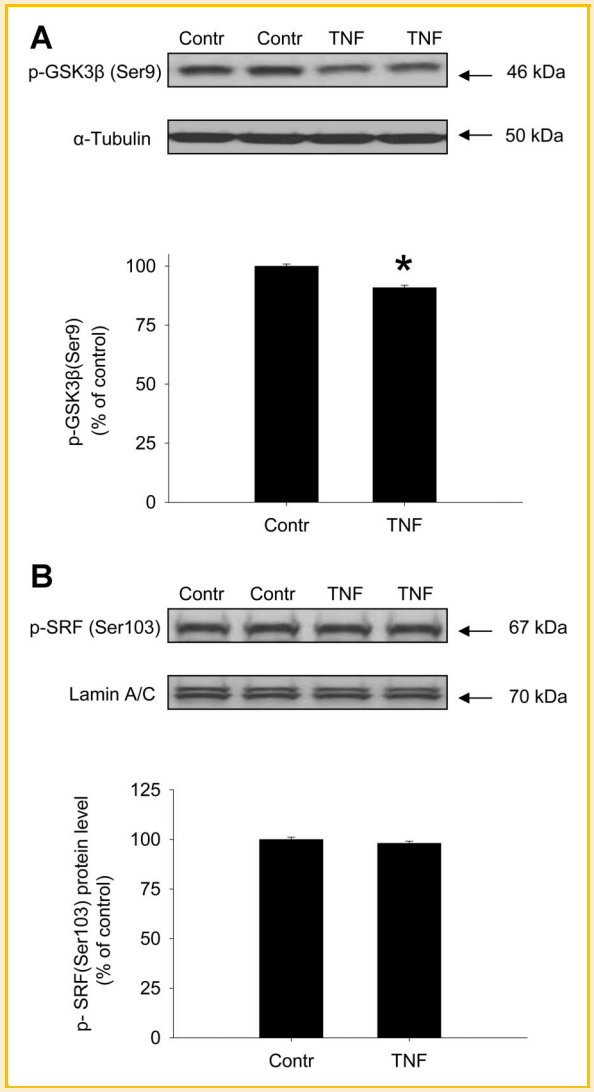

Fig. 9. Treatment of cultured myotubes with TNFα reduces cellular levels of phosphorylated GSK3β but does not influence cellular levels of phosphorylated SRF. Cellular levels of p-GSK3β(Ser 9) (A) and p-SRF(Ser 103) (B) in cultured L6 myotubes treated with 10 ng/ml of TNFα for 24 h. p-GSK3β levels were determined in whole cell extracts and p-SRF levels were determined in nuclear extracts. α-Tubulin and lamin A/C levels were determined as loading controls for total and nuclear extracts, respectively. Representative Western blots are shown in the upper panels and densitometric quantifications are shown in the lower panels. Results are means ± SEM with n = 8 in each group. $^{*}P < 0.05$ versus control (Contr).

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