Proteomic analysis of altered protein expression in skeletal muscle of rats in a hypermetabolic state induced by burn sepsis

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mRNA profiling has been extensively used to study muscle wasting. mRNA level changes may not reflect that of proteins, especially in catabolic muscle where there is decreased synthesis and increased degradation. As sepsis is often associated with burn injury, and burn superimposed by sepsis has been shown to result in significant loss of lean tissues, we characterized changes in the skeletal-muscle proteome of rats subjected to a cutaneous burn covering 20 % of the total body surface area, followed 2 days later by sepsis induced by CLP (caecal ligation and puncture). EDL (extensor digitorum longus) muscles were dissected from Burn-CLP animals ($n = 4$) and controls (sham-burned and sham-CLP-treated, $n = 4$). Burn-CLP injury resulted in a rapid loss of EDL weight, increased ubiquitin-conjugated proteins and increased protein carbonyl groups. EDL protein profiles were obtained by two-dimensional gel electrophoresis using two immobilized pH gradient strips with overlapping pH range covering a pH 3–8 range. Seventeen spots were significantly altered in the Burn-CLP compared with the control group, representing 15 different

proteins identified by peptide mass fingerprinting. The identities of three proteins including transferrin were further confirmed by liquid chromatography–tandem MS. The significant changes in transferrin and HSP27 (heat-shock protein 27) were verified by Western-blot analysis. HSP60, HSP27 and HSP β 6 were down-regulated, along with HSP70, as detected by Western blotting. Six metabolic enzymes related to energy production were also down-regulated. A simultaneous decrease in chaperone proteins and metabolic enzymes could decrease protein synthesis. Furthermore, decreased HSPs could increase oxidative damage, thus accelerating protein degradation. Using cultured C2C12 myotubes, we showed that H₂O₂-induced protein degradation *in vitro* could be partially attenuated by prior heat-shock treatment, consistent with a protective role of HSP70 and/or other HSPs against proteolysis.

Key words: burn sepsis, heat-shock protein, metabolic enzyme, muscle atrophy, proteomic analysis, skeletal muscle.

INTRODUCTION

Loss of skeletal-muscle mass is a hallmark of the characteristic catabolic response to cancer, sepsis, and severe burns and trauma [1]. Increased net proteolysis may be beneficial in the acutely ill patient since the released amino acids can in principle serve as the substrate for energy production as well as the synthesis of proteins involved in immune defence and the acute-phase response [2]. However, sustained loss of lean body mass is debilitating and considered a major factor leading to pulmonary complications and poor prognosis in general. The mechanisms of accelerated proteolysis involve the activation of multiple cellular protease pathways, most notably the ubiquitin–proteasome system [1]. For example, inhibition of the proteasome [3,4], or a genetic deletion of either of two ubiquitin ligases, MuRF1 (muscle RING finger 1) and MAFbx (muscle atrophy F-box) [5], attenuates muscle atrophy induced by sepsis, disuse or denervation. The activation of protease pathways is believed to be the result of a combination of effects involving inflammatory cytokines [especially TNF α (tumour necrosis factor α)], glucocorticoids, myostatin and oxidative stress, although these factors can be disease-specific [1,6]. Inflammatory mediators act, at least partially, via activation of the transcription factor NF- κ B (nuclear factor κ B), which has recently been shown to up-regulate pathways involved in muscle protein degradation and down-regulate those involved in protein synthesis. For example, NF- κ B effects on target genes include

the up-regulation of ubiquitin-conjugating enzyme UbcH2, which is involved in proteasome-dependent proteolysis, and the down-regulation of MyoD, a transcription factor essential for myofibrillar synthesis [7–10].

Since protein degradation is also critical to many normal physiological functions such as cell growth, metabolism, disposal of misfolded proteins and the immune response, blocking proteolytic pathways, such as the proteasome–ubiquitin system, may have harmful side effects [1]. In the search for potential new targets for clinical intervention, the global transcriptional profile of catabolic skeletal muscle has been widely used to identify differentially expressed genes in response to cytokine treatment [11], aging (sarcopenia) [12,13], disuse [5,14–16], fasting [17] and several disease conditions (e.g. diabetes, uraemia and cancer) [18]. These investigations have led to the discovery of potential candidates (e.g. MuRF1 and MAFbx) for therapeutic intervention and provide a more in-depth view of muscle atrophy at the mRNA level. However, mRNA level changes may not necessarily reflect that of the corresponding proteins, especially in catabolic muscle where there is suppressed protein synthesis and increased protein degradation. In contrast with the extensive global mRNA analysis literature data, only two proteomic analyses of muscle atrophy have been reported [19,20]. In these two studies, hindlimb suspension and denervation were used to induce muscle atrophy and the proteins were separated by two-dimensional gel electrophoresis, followed by Coomassie Blue staining. A limited

Abbreviations used: CLP, caecal ligation and puncture; DMEM, Dulbecco's modified Eagle's medium; DNPH, dinitrophenylhydrazine; DTT, dithiothreitol; EDL muscle, extensor digitorum longus muscle; HS, horse serum; HSP, heat-shock protein; IPG, immobilized pH gradient; MAFbx, muscle atrophy F-box; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; MS/MS, tandem MS; mt-GrpE#1, mitochondrial GrpE protein homologue 1; MuRF1, muscle RING finger 1; MyBP-H, myosin-binding protein H; nano-LC–ESI, nano-liquid chromatography–electrospray ionization; NF- κ B, nuclear factor κ B; SAGE, serial analysis of gene expression; SOD-1, superoxide dismutase-1; TNF α , tumour necrosis factor α .

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number of proteins were found to be altered, including metabolic proteins and chaperone proteins.

Clinical data have shown that approx. 50% of the patients with severe burns were complicated by infection and ventilatory support was needed partially due to the weakness of the respiratory muscles [21]. Significant body weight loss and skeletal-muscle wasting were observed in previous studies using a rat model of 30% full-thickness burn followed by bacterial infection [22,23]. In the present study, we used two IPG (immobilized pH gradient) strips with overlapping pH range covering a pH 3–8 range and Sypro Ruby staining to obtain protein profiles in skeletal muscle in a rat model of cutaneous burn injury with superimposed infection. The main objective of the present study was to identify the differentially expressed proteins that are functionally involved in muscle atrophy in a clinically relevant model of burns with septic complications. We found that many chaperone proteins and metabolic enzymes were down-regulated co-ordinately, suggesting that impaired cellular functions are temporally associated with the increased protein turnover. We hypothesized that the decrease in chaperone proteins may sensitize to oxidative stress, an important inducer of muscle atrophy, as shown by *in vivo* and *in vitro* studies [24–29]. Consistent with this notion, we show that heat-shock response (induction of chaperone expression) *in vitro* protects against H_2O_2 -induced protein degradation in C2C12 cultured myotubes.

EXPERIMENTAL

Materials

Unless otherwise specified, all the reagents for two-dimensional gel electrophoresis including IPG strips, mineral oil, Biolyte ampholytes pH 3–10, acrylamine, bisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, ammonium persulphate, glycine, Tris base, urea, CHAPS and glycerol were purchased from Bio-Rad (Hercules, CA, U.S.A.). Unless specified, all other reagents were obtained from Sigma (St. Louis, MO, U.S.A.).

Animal model of thermal injury and sepsis

All procedures involving animals were conducted in accordance with National Research Council (Washington, DC, U.S.A.) guidelines, and approved by the Subcommittee on Research Animal Care at the Massachusetts General Hospital. Prior to experiments, male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, U.S.A.) weighing 100–125 g were acclimatized for 1 week under a 12 h light/dark cycle with free access to food and water. The animals were randomly divided into four groups of four each: Sham-Sham, Burn-Sham, Sham-CLP and Burn-CLP. Burn-CLP animals were first subjected to a full-thickness cutaneous burn injury covering 20% of the total body surface area. For this purpose, the animals were anaesthetized by an intraperitoneal injection of 62.5 mg/kg of ketamine and 12.5 mg/kg of xylazine. The back of the animal was shaved and immersed in boiling water for 10 s. The animal was immediately resuscitated with 10 ml of saline solution intraperitoneally. Two days later, animals were anaesthetized again, and underwent CLP (caecal ligation and puncture). The operation was carried out by performing a midline laparotomy to expose the caecum, ligating the caecum below the ileocaecal valve, puncturing three times with an 18-gauge needle, squeezing out a small amount of faeces from the puncture site, returning the caecum to the peritoneal cavity, and closing the incision in layers. Immediately after the CLP procedure, each animal was resuscitated with 10 ml of saline solution intraperitoneally. After each procedure, animals were monitored for pain and given buprenorphine (0.05–0.1 mg/kg subcutaneously

Figure 1 Effect of systemic injury on rat EDL muscle wet weight

Sham-burn or burn injury was administered, followed by a sham-CLP or CLP operation 2 days later. Muscles were harvested on post-burn day 4. * $P < 0.05$; ** $P < 0.001$ versus Sham-Sham by Student's *t* test.

every 8–12 h) as long as required. Controls consisted of: Sham-Sham animals, which were sham-burned on day 0 and then sham-CLP-operated on day 2; Burn-Sham animals, which were burned on day 0 and then sham-CLP-operated on day 2; Sham-CLP animals, which were sham-burned on day 0 and then CLP-operated on day 2.

Muscle protein sample preparation

The fast-twitch EDL (extensor digitorum longus) muscle, which was reported to be preferentially lost in response to burn injury or sepsis [30,31], was dissected from both legs on post-burn day 4 and stored at -80°C for later analysis. Burn-CLP caused the most significant EDL muscle weight loss (Figure 1); thus all subsequent two-dimensional gel electrophoresis and Western-blot analyses were performed on the Burn-CLP and the control Sham-Sham groups only. EDL muscles were ground in a mortar containing liquid nitrogen, and a small portion was solubilized in a solution containing 8 M urea, 4% (w/v) CHAPS, 40 mM Tris and 65 mM DTT (dithiothreitol) through sonication. The debris was removed through centrifugation, and the protein concentrations in the supernatant were measured in triplicate by a Coomassie dye-based protein assay (Bio-Rad) in a 96-well plate format using BSA as the standard.

Two-dimensional gel electrophoresis and image analysis

Two-dimensional gel electrophoresis was performed as described previously with minor modifications [32]. Briefly, 50 μg of protein from each sample was mixed with 125 μl of rehydration buffer containing 8 M urea, 2% CHAPS, 10 mM DTT and 0.2% (pH 3–10) carrier ampholytes, and then loaded overnight on to linear pH 3–6 or pH 5–8 IPG strips through passive in-gel rehydration. After isoelectric focusing with a maximum of 5000 V for a total of 12000 V $\cdot$ h at 20°C and a two-step equilibration {15 min incubation in DTT buffer [6 M urea, 2% (w/v) SDS, 0.5 M Tris/HCl, pH 6.8, and 1% DTT], and then 15 min in iodoacetamide buffer (6 M urea, 2% SDS, 0.5 M Tris/HCl, pH 6.8, and 2.5% iodoacetamide)}, the IPG strips were positioned on 10–15% (w/v) polyacrylamide gels for the second-dimension separation at 200 V. The gel was stained with Sypro Ruby fluorescent dye according to the manufacturer's instructions (Molecular Probes, Eugene, OR, U.S.A.). The stained gel was scanned with a Fluor-S MultiImager system (Bio-Rad) and analysed with PDQuest software (version 7.0; Bio-Rad). During image analysis, the spots in each image were automatically detected first, and then manually edited to correct for artefacts. One matched set was created for analysing gel images with pI 3–6, and one for gel images with pI 5–8 with each matched set containing eight images

(four Sham-Sham and four Burn-CLP). Individual spot volumes (the integration of density over area) were normalized to the volume of all the spots on the gel image to correct for image-to-image variation. The normalized data of each matched set were exported to Microsoft Excel, where differences between corresponding protein spots in the Burn-CLP and Sham-Sham groups were identified using a Student's *t* test with $P < 0.05$ as a criterion for statistical significance.

MALDI-TOF (matrix-assisted laser-desorption ionization-time-of-flight) MS

The significantly altered spots were then excised from the gels with a robotic ProteomeWorks™ spot cutter (Bio-Rad) and digested with 10 μ l of 12.5 ng/ μ l sequencing-grade trypsin (Promega, Madison, WI, U.S.A.) overnight at 37°C. Tryptic peptides were extracted twice with 15 μ l of a solution consisting of 5% (v/v) formic acid and 50% (v/v) acetonitrile for 20 min, and the pooled extracts were processed with ZipTips according to the manufacturer's instructions. A 1.5 μ l portion of a 1:1 mixture of the desalted peptides and matrix solution [1% (w/v) α -cyano-4-hydroxycinnamic acid solution in 50% acetonitrile and 50% of a 0.1% (w/v) trifluoroacetic acid solution] were applied to the sample target plate. Peptide mass fingerprints were then obtained from a MALDI-TOF mass spectrometer (Reflectron) with a sum of at least ten spectra, each consisting of ten laser shots. Identification was performed by matching 40–60 (depending on the signals) peak values generated by ProteinLynx against the NCBI non-redundant database using the MS-Fit program (<http://prospector.ucsf.edu/ucshtml4.0/msfit.htm>). In general, a mass tolerance of ± 0.1 Da, one missed trypsin cleavage, and fixed modification of carboxyamidomethylcysteine, and the species (*Rattus norvegicus*) were selected as matching parameters. If necessary, the estimated experimental molecular mass was applied to increase the confidence of identification.

Nano-LC-ESI (nano-liquid chromatography-electrospray ionization) MS/MS (tandem MS)

Tryptic peptides were loaded on to a 2 μ g capacity peptide trap (CapTrap; Michrom Bioresources, Auburn, CA, U.S.A.) in 0.1% trifluoroacetic acid and separated by capillary LC using a capillary column (75 μ m $\times$ 5 cm $\times$ 3 μ m; LC Packings, Amsterdam, The Netherlands) at 150 nl/min delivered by an Agilent 1100LC pump (400 μ l/min) and a flow splitter (Accurate; LC Packings). A mobile-phase gradient was run using mobile phase A (2% acetonitrile/0.1% formic acid) and B (80% acetonitrile/0.1% formic acid) from 0 to 10 min with 0–20% B followed by 10–150 min with 20–70% B and 150–180 min with 70–100% B. The resolved peptides were analysed on a ThermoFinnigan Advantage ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, U.S.A.) after ESI using end-coated spray SilicaTip tip (inner diameter 75 μ m, tip inner diameter 15 μ m; New Objective) held at a spray voltage of 1.8 kV. After acquisition of the peptide parent ion mass, zoom scans and MS/MS spectra of parent peptide ions above a signal threshold of 2×10^4 were recorded with dynamic exclusion for a duration of 2 min, using Xcalibur 1.3 data acquisition software (ThermoFinnigan).

Peptide sequences were identified using Sequest algorithm embedded in BioWorks software (version 3.1) against indexed SWISS-PROT protein sequence database. Modifications that were taken into consideration included methionine oxidation and alkylation of cysteine with iodoacetamide. The search results were filtered by X_{corr} (scoring value for peptide assignment to a particular spectra; the value indicates the degree of certainty associated

with each peptide identification) versus charge with 1.5 for singly charged ions, 2.0 for doubly charged ions and 2.5 for triply charged ions. A protein was considered identified when a minimum of two peptides were matched.

Western-blot analysis

The expression levels of ubiquitin-conjugated proteins, SOD-1 (superoxide dismutase-1), HSP70 (heat-shock protein 70), HSP27 and transferrin in EDL muscle tissue were evaluated by Western blotting. All the primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Protein extract (40 μ g) from each animal was separated by SDS/PAGE. The separated proteins were transferred on to a nitrocellulose membrane with a Bio-Rad transfer cell unit according to the manufacturer's instructions. The blot was blocked with 5% (w/v) dried milk in Tris-buffered saline solution (pH 7.6) containing 0.05% Tween-20 (TBS/T), and probed with a 1:250 dilution of rabbit polyclonal anti-(human polyubiquitin), rabbit polyclonal anti-(human SOD-1), mouse monoclonal anti-(mouse HSP70), goat polyclonal anti-(human HSP27) or rabbit polyclonal anti-human antibody for 1 h at room temperature (20°C) with gentle shaking. After three 5 min washes, the blot was incubated for 1 h with the corresponding goat anti-rabbit, goat anti-mouse or bovine anti-goat HRP (horseradish peroxidase)-conjugated secondary antibody (1:5000 dilution), washed, developed with the Pico chemiluminescence kit (Pierce, Rockford, IL, U.S.A.) and imaged using a GS525 phosphorimager (Bio-Rad). The images for HSP27 and transferrin were recorded on an X-ray film and scanned using a GS-800 densitometer (Bio-Rad). The band volume was analysed with Quantity One software (Bio-Rad).

Measurement of protein carbonyls

The muscle content of protein carbonyl groups, a sensitive index of oxidative damage to proteins, was determined by derivatizing the protein sample with DNP (dinitrophenylhydrazine) and measuring the bound DNP through a 96-well ELISA kit (Zenith Technology, Dunedin, New Zealand) according to the manufacturer's instructions.

Cell culture and protein degradation measurement

C2C12 myoblasts were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). Cells were seeded in 12-well dishes and cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin and streptomycin under a 5% CO₂ atmosphere at 37°C. Prior to reaching confluence, the medium was replaced with DMEM containing 2% (v/v) HS (horse serum) to induce myotube formation. After 4 days of differentiation, the formed myotubes were labelled with 1.0 μ Ci of [³H]tyrosine/ml for 2 days in DMEM containing 2% HS. Cells were then washed twice with 2% HS/DMEM containing 2 mM non-radioactive tyrosine (experimental medium) and cultured in the same medium. Heat-shock treatment was conducted for 2 h at 42°C while control cells were maintained at 37°C. Following 4 h recovery, the cells were washed once and treated with H₂O₂ in experimental medium for 1 h at 37°C. The H₂O₂-treated cells were then washed once and incubated in fresh experimental medium at 37°C for protein degradation measurement. The rate of protein degradation was expressed as percentage protein degradation over a 24 h incubation period. The percentage protein degradation was calculated by the medium radioactivity (released from myotubes) divided by the medium radioactivity plus myotube radioactivity. The cell lysate (lysis buffer: 0.5 M NaOH and 0.1% Triton X-100) and

Figure 2 Two-dimensional profiles of EDL muscles from control (Sham-Sham) and Burn-CLP rats

Protein extracts (50 μ g) were separated with two overlapping two-dimensional gels and visualized with Sypro Ruby dye. Arrows point to the 17 protein spots that were found to be significantly altered. The altered spots that overlap on the pl 3–6 and pl 5–8 images are marked with the same identifying number and count as one regulated spot. Results shown are for one representative sample in each group.

medium radioactivities were measured using a Beckman LS6500 scintillation counter.

RESULTS

Muscle wet weight and protein content

On post-burn day 4, burn injury followed by sham-CLP decreased the average EDL muscle weight, although not significantly (Figure 1). Sham-burn injury followed by CLP-induced sepsis caused a more pronounced EDL muscle weight decrease ($P < 0.05$). Burn injury followed by CLP caused the most EDL muscle weight loss, with a 30 % decrease ($P < 0.001$). Despite profound EDL loss, the overall protein concentration was similar between the Burn-CLP (0.142 ± 0.006 g/g wet weight) and Sham-Sham (0.141 ± 0.008 g/g wet weight) groups, suggesting that the muscle weight decrease was not due to a change in water content. Since the 'double-hit' injury (Burn followed by CLP) caused the most muscle atrophy, the subsequent analysis of the altered expression of proteins in EDL muscle was done with the Burn-CLP group only, and compared with the Sham-Sham control group.

Differentially expressed proteins revealed by two-dimensional gel-based proteomic analysis

Two-dimensional analysis was performed on EDL muscle from Burn-CLP and Sham-Sham rats to identify protein changes associated with muscle wasting. The protein sample from each animal was separated using two IPG strips with overlapping pH range covering a pH range of 3–8, and visualized with Sypro Ruby fluorescent dye. Representative two-dimensional gel images from each group are shown in Figure 2. Quantitative image analysis identified 17 protein spots (highlighted with black arrows) that were statistically significantly changed. These 17 spots were found to represent 15 different proteins (Table 1). All the spots were reliably identified through MALDI-TOF MS, and the identities of the spots for transferrin, ATP synthase β -chain and

parvalbumin α were further confirmed using nano-LC-ESI MS/MS analysis (Table 1). Figure 3(B) represents a typical MS/MS spectrum for a peptide with a matched mass peak 1539.68 in the peptide mass 'fingerprinting' spectrum (Figure 3A) of transferrin obtained from MALDI-TOF MS. Among all the differentially regulated proteins unveiled by two-dimensional analysis, only two proteins were up-regulated, actin and MyBP-H (myosin-binding protein H).

The proteins with altered expression were classified into four functional categories, as shown in Table 1. Transferrin was down-regulated by approx. 40 %. This decrease was expected, as transferrin is a well-known negative acute-phase protein [33,34]. All the proteins within the categories of chaperone proteins and metabolic enzymes were down-regulated, suggesting that these proteins may be co-ordinately regulated. The chaperone proteins consist of three HSPs, HSP60, HSP27 and HSP β 6 (previously known as p20), and an HSP70 co-chaperone mt-GrpE#1 (mitochondrial GrpE protein homologue 1). HSPs play a major role in cellular homeostasis, including protecting against oxidative stress [35], assisting in protein synthesis [36] and repairing misfolded proteins [37]. Thus the decreased expression of these proteins may contribute to increasing net EDL muscle protein degradation in the Burn-CLP group. Some structural proteins were down-regulated, such as myosin regulatory light chain 2, a subunit of myosin. Other subunits of myosin, such as myosin light chain 1, showed a decreasing trend that was not statistically significant. In contrast, actin, an important component of myofibrillar proteins, was up-regulated. A striking 3-fold increase was also detected for MyBP-H, a myofibrillar constituent with unclear functional annotation.

Although identifying the expected change in transferrin provides some validation of our differential proteomic approach, to further confirm the fidelity of our two-dimensional data, Western blotting was performed for two proteins: transferrin and HSP27. The qualitative and quantitative results are shown in Figure 4, and are consistent with the two-dimensional data in Table 1.

Table 1 Significantly regulated proteins in EDL skeletal muscle following burn-CLP treatment based on two-dimensional gel images

Spot no.	Identity	SWISS-PROT accession no.	No. of peptides matched	Coverage (%)	Theoretical pI/molecular mass (kDa)*	Normalized spot volume (p.p.m.)†	
						Sham-Sham	Burn-CLP‡
1–3	Acute-phase protein Transferrin (serotransferrin)§	P12346	16/6	24.6/13.1	7.4/76.7	11 340 ± 1110	7012 ± 1170
4	Chaperone proteins HSP60	P19226	20	37.0	5.9/61.0	6780 ± 720	4560 ± 480
8	HSP27	P42930	8	34.0	6.5/22.9	8060 ± 490	4092 ± 840
12	GrpE protein homologue 1	P97576	5	21.0	8.6/24.3	2770 ± 460	1200 ± 450
13	HSP β 6	P97541	5	50.0	6.4/17.5	7230 ± 1180	3090 ± 1080
6	Metabolic enzymes ATP synthase β -chain	P10719	28/12	54.8/33.4	5.3/56.3	40 570 ± 5260	26 540 ± 5280
9	Triosephosphate isomerase	P48500	4	19.4	6.9/26.7	5980 ± 490	4611 ± 600
10	ATP synthase D chain P31399		4	29.0	6.2/18.8	10 260 ± 1950	6790 ± 950
15	Cytochrome c oxidase polypeptide Va	P11240	5	22.0	6.1/16.1	17 950 ± 2440	9400 ± 1400
16	Cytochrome c oxidase polypeptide Vb	P12075	8	37.0	7.7/13.9	6570 ± 1340	3260 ± 880
17	Fatty acid-binding protein	P07483	4	39.0	5.9/14.8	11 440 ± 2420	6480 ± 2090
5	Structural proteins MyBP-H	O88599	14	49.0	6.3/52.6	1290 ± 560	3920 ± 410
7	Actin, α skeletal muscle	P68136	10	24.7	5.2/41.8	190 690 ± 35 260	253 280 ± 31 150
11	Myosin regulatory light chain 2	P04466	15	77.4	4.8/18.8	120 610 ± 8590	90 460 ± 2230
14	Parvalbumin α	P02625	5/8	50.5/65.9	5.0/12.0	86 590 ± 10 460	118 090 ± 12 910

* The theoretical values of pI and molecular mass are based on the protein precursor.

† The values for spots 6, 7, 11, 14 and 15 were obtained from the pI 3–6 two-dimensional images and the values for the other spots from the pI 5–8 two-dimensional images, and each value is the mean $\pm$ S.D. for data from four animals in each group.

‡ $P < 0.05$ versus Sham-Sham.

§ The normalized volume of this protein is the sum of the three individual spots.

|| The values were from nano-LC-MS/MS analysis.

Down-regulated HSP70 detected by Western blotting

One important finding from the above two-dimensional analysis is that HSP60 and several small HSPs were down-regulated in the EDL muscle of Burn-CLP rats, and a direct protective effect of the heat-shock response against increased protein turnover has recently been demonstrated both *in vivo* and *in vitro* [38,39]. However, several high-molecular-mass HSPs were not on the list of the altered proteins revealed by two-dimensional analysis, probably due to relatively low expression levels and/or semi-quantitative limitation associated with two-dimensional analysis. Thus Western-blot analysis was carried out to measure the expression of other important members of the HSP family, namely HSP70, HSP90 and HSP105. Using an antibody against both the inducible (HSP72) and constitutive (HSP73) forms of HSP70, an approx. 40% decrease was observed in the expression level of HSP70 in the Burn-CLP group compared with the Sham-Sham control (Figure 5). No significant changes in the expressions of HSP90 or HSP105 were observed (results not shown).

Increased ubiquitin-conjugated proteins and oxidative stress

Increased protein degradation is often mediated by the ubiquitin-proteasome pathway, and increased levels of ubiquitinated proteins have been observed in many different cases of muscle atrophy, including sepsis [40], fasting and denervation [41]. We quantified ubiquitin-conjugated proteins on Western blots by integrating the density of all bands of >43 kDa, in accordance with previous reports [40,41]. The data show an approx. 2-fold increase in ubiquitin-conjugated proteins in EDL muscle in the Burn-CLP group as compared with the Sham-Sham control group (Figure 6).

An increased level of oxidized proteins, as measured by protein carbonyl-group content in skeletal muscle, has been reported after burn injury alone [25] or sepsis alone [42,43]. In this animal model of burn injury superimposed with sepsis induced by CLP, an increase in protein carbonyl groups of EDL muscle was also observed on post-burn day 4 (Figure 7A). Since oxidized proteins are known to be preferentially and rapidly degraded [27], their increased levels are consistent with the EDL muscle loss and increased levels of ubiquitinated proteins in the Burn-CLP group.

In prior studies, oxidative injury in sepsis and disuse atrophy models has been attributed to increased SOD-1 activity and a decrease in glutathione peroxidase and catalase, which together can contribute to elevate H_2O_2 levels [28,43]. Using Western-blot analysis, we also observed an increase in the level of SOD-1 in EDL muscle isolated from Burn-CLP rats (Figure 7B).

Heat shock attenuates H_2O_2 -induced protein degradation in cultured myotubes

On the basis of the findings above that muscle atrophy induced by burn injury and sepsis was correlated with evidence for increased oxidative stress (likely due to increased H_2O_2 generation) and decreased expression of HSPs (impaired defence mechanisms), a well-defined muscle cell culture system was used to examine whether the heat-shock response is protective against H_2O_2 -induced protein degradation. The cumulative degraded proteins over 24 h were measured starting at approx. 8 h (2 h heat-shock treatment + 4 h recovery + 1 h H_2O_2 treatment + ~ 1 h washing and handling) after the removal of labelling isotope. The period used for heat-shock treatment and recovery was designed to induce HSP expression before adding H_2O_2 . The short-lived proteins are completely degraded within this 8 h period, so that the

Figure 3 Identification of transferrin by peptide mass ‘fingerprinting’ and peptide sequencing (A) A total of 16 peptide mass peaks (grey line) matched the theoretical mass of the tryptic peptides of transferrin with a mass tolerance < 0.1 Da. (B) The sequence of one representative peptide DQYELLCLDNTR (corresponding to the peak mass 1539.68 in (A)) was generated by the MS/MS spectrum obtained using a ThermoFinnigan ion-trap mass spectrometer. All the matched Y and B ions were singly charged, with the exception of the *m/z* 761.4 value, which matched doubly charged B-12 ion.

Figure 4 Western blotting detection of transferrin (A) and HSP27 (B) to confirm their changes observed by two-dimensional analysis

A 40 µg portion of EDL muscle protein from each animal was loaded for immunoblotting. The quantified band volume was used to represent the relative amount of the protein, and is shown in the lower panel. Results are the means ± S.D. for three animals in each group. The upper panel shows one representative blot (lane 1, Sham-Sham; lane 2, Burn-CLP). *P < 0.05 versus Sham-Sham by Student's *t* test.

data primarily reflect degradation of the long-lived proteins. Heat shock alone (2 h at 42 °C) slightly increased protein degradation from 37.6 ± 0.7 % to 38.9 ± 0.4 % (*P* < 0.01 by Student's *t* test), consistent with a previous report that hyperthermia activates the

Figure 5 Effect of burn-CLP on HSP70 expression

Western-blot analysis of HSP70 was carried out on 40 µg of EDL muscle protein. The quantified band volume is used to represent the relative amount of the protein. Results shown represent the means ± S.D. for three animals in each group. The upper-right panel shows a representative blot (lane 1, Sham-Sham; lane 2, Burn-CLP). *P < 0.05 versus Sham-Sham by Student's *t* test. kD, kDa.

proteasome pathway [44,45]. In order to isolate the effect of heat shock on H₂O₂-induced protein degradation, the measured protein degradation following H₂O₂ treatment in either control or heat-shock group was normalized to the corresponding average value in the absence of H₂O₂. The results are depicted in Figure 8.

Figure 6 Effect of Burn-CLP on protein ubiquitination

Western-blot analysis of ubiquitin-conjugated proteins was carried out on 40 μ g of EDL muscles, and the quantified volume for all the bands of > 43 kDa was used to represent the relative content of ubiquitin-conjugated proteins. Results shown represent the means $\pm$ S.D. for three animals per group. The upright corner shows one representative blot (lane 1, Sham-Sham; lane 2, Burn-CLP). * P < 0.05 versus Sham-Sham by Student's t test.

Figure 7 Effect of Burn-CLP on oxidative damage

(A) Protein carbonyl-group levels expressed as the means $\pm$ S.D. for data from EDL muscles from four animals in each group. (B) SOD-1 levels measured by Western blotting in 40 μ g of EDL muscle protein extracts. The quantified band volume was used to represent the relative amount of the protein. Results shown are the means $\pm$ S.D. for three animals in each group. The upper-right panel shows one representative blot (lane 1, Sham-Sham; lane 2, Burn-CLP). * P < 0.05 versus Sham-Sham by Student's t test.

Figure 8 Effect of H₂O₂ and heat shock on protein degradation in C2C12 myotubes

Cells were labelled with [³H]tyrosine for 48 h, subjected to heat shock (42 °C for 2 h, followed by 4 h recovery at 37 °C) or incubated at 37 °C (control), and then treatment with H₂O₂ for 1 h. The protein degradation was then measured as described in the Experimental section. Results shown are means $\pm$ S.D. normalized to the values for the 0 mM H₂O₂ in the heat-shock and control groups. The baseline values for protein degradation (in the absence of H₂O₂) were 37.6 $\pm$ 0.7 % in the control group and 38.9 $\pm$ 0.4 % in the heat-shock group. n = 4 in each group.

Treatment with H₂O₂ increased protein degradation in C2C12 myotubes in a concentration-dependent manner (P < 0.001 by two-factor ANOVA F test). The increase was smaller than that described in the previous reports [23,24], most likely

Scheme 1 Summary of the potential roles of the differentially regulated proteins identified in the present study within the proteolytic and protein synthetic pathways

Mediators (e.g. TNF) released following severe injury induce oxidative stress, which leads to increased oxidized proteins (more susceptible to degradation) and up-regulation of the ubiquitin-proteasome pathway. These mediators also down-regulate HSPs, which are normally protective against oxidative stress and participate in repair mechanisms for oxidized proteins, further exacerbating oxidative stress-induced proteolysis. A decrease in HSPs and metabolic enzymes, both of which are essential for protein synthesis, may explain, at least partially, decreased protein synthesis after severe injury. —, negative effect; +, positive effect; ↓, protein or cellular event down-regulated; ↑, protein or cellular event up-regulated.

due to the fact that in the other studies the measurement technique incorporated the effect of H₂O₂ on the short-lived proteins. Nevertheless, there was a significant reduction in H₂O₂-induced protein degradation in heat-shocked cells (P < 0.001 by two-factor ANOVA F test). The increased resistance of heat shock-treated myotubes to H₂O₂-induced protein degradation is consistent with a protective role of HSPs against oxidative stress.

DISCUSSION

In the present study, a proteomic approach was utilized to study the global differential protein expression profile in the rat EDL muscle undergoing a 'double-hit' injury consisting of a cutaneous burn injury followed by sepsis induced by CLP. As mentioned earlier, only two proteomic analyses of skeletal muscle in the context of muscle wasting have been reported previously [19,20], and they had found a relatively smaller number of proteins to be regulated following either denervation or hindlimb suspension. In the present study, we found more protein changes, which may be due to the greater severity of the insult, the greater number of animals used per group, and/or the sensitivity of the staining method. It is worth noting that the lesser amount of protein sample used for small size gels (8 cm $\times$ 10 cm) in the present study could be compensated by using two IPG strips with narrow and overlapping pH range. Interestingly, three down-regulated proteins found in the disuse/denervation studies were similarly altered in our burn sepsis model, namely the chaperone protein HSP66, the metabolic enzyme ATP synthase β -chain and fatty-acid-binding protein. These similar observations suggest some other common molecular events in addition to the activation of proteolysis pathway occurring in muscle atrophy, regardless of the pathology responsible for muscle wasting [18]. Taken together, our results suggest potential mechanisms for explaining increased protein degradation and decreased protein synthesis in muscle-wasting conditions (Scheme 1).

Cumulative evidence from previous *in vivo* and *in vitro* experiments along with our *in vitro* data (Figure 6) show that oxidative stress is an important trigger of muscle wasting [24–29]. Therapeutic effects of antioxidants on muscle atrophy have been reported in animal models and in AIDS patients [24,46–48]. Hindlimb unloading and CLP injury have been shown to elevate SOD-1 and decrease catalase and glutathione peroxidase activities in atrophying muscle, probably causing increased H_2O_2 levels [28,43]. Consistent with these findings, we found increased SOD-1 levels in EDL muscle of Burn-CLP rats. Moderate or severe muscle atrophy has been observed in transgenic mice overexpressing SOD-1, highlighting the central role of SOD-1 in oxidative stress-related muscle atrophy [49].

One consequence of treatment with oxidative stress is the oxidative modification of proteins, as shown in our results and other studies [25,42,43]. This modification can lead to increased protein degradation, as the oxidized proteins are more susceptible to proteolysis [27]. In addition, the treatment with oxidative stress such as H_2O_2 has recently been shown to activate the ubiquitin–proteasome pathway [8,26,29]. Prior studies have demonstrated that small HSPs are able to counteract the deleterious effects of oxidative stress through a glutathione-dependent pathway [50–53]. We detected the down-regulation of several small HSPs in EDL muscle of Burn-CLP rats. The down-regulation of HSP70 and its co-chaperone GrpE could also contribute to an increased susceptibility to oxidative stress, since these chaperones are thought to attenuate the proteolysis of oxidized proteins by binding to them and assisting in their repair [39]. Furthermore, a recent study showed that the heat-shock response suppresses inflammation-related oxidative stress [54]; our *in vitro* data also demonstrated that the heat-shock response reduces protein degradation stimulated by H_2O_2 .

While increased proteolysis has often been implicated in muscle wasting, there has been less attention paid to mechanisms that could lead to decreased protein synthesis. In the present study, down-regulation of proteins classified as chaperones, as well as the decrease in metabolic enzymes that are needed for intracellular energy production, may both contribute to decreased protein synthesis. One putative function of HSPs is to act as molecular chaperones to facilitate the assembly and translocation of nascent proteins [37]. A decrease in HSP70 has been implicated in the slower elongation of nascent peptides and initiation of translation in a model of unloading-induced muscle atrophy [55,56]. Another evidence for the role of HSPs in assisting protein synthesis is that increased HSP levels are associated with skeletal-muscle hypertrophy induced by exercise, as well as smooth-muscle cell overgrowth in atherosclerosis [57–59]. Therefore it is very likely that the decrease in several HSPs found in the Burn-CLP rat could slow the process of protein synthesis. The down-regulation of several metabolic enzymes may lead to less energy production in the cell, which in turn could hamper protein translation, an ATP-requiring process. Indeed, a marked decrease in muscle ATP levels has been previously detected in severely injured patients and animals [23,60,61]. It is worth noting that the ubiquitin–proteasome proteolytic system is also energy-dependent. Whether the limited amount of ATP produced in wasting muscle is selectively used for proteolysis rather than protein synthesis warrants further investigation.

Many altered HSPs reported in the present study exhibited changes similar to published mRNA level data in many muscle atrophy models. For example, a down-regulation of HSP70 has been previously reported by mRNA profiling study of disuse-induced muscle atrophy [14,16]. A microarray study of sarcopenia by Lee et al. [13] also showed a 2-fold decrease of HSP70 in muscle of aged mice. DnaJ-like protein, functioning as a co-

chaperone of HSP70 similarly to mt-GrpE#1, was found to be decreased at the mRNA level in rat soleus muscle after hindlimb suspension [16]. A decrease in mRNA HSP β 6 as well as α B-crystallin, a small HSP highly homogeneous to HSP β 6, was reported in denervation-induced atrophying muscle [62,63]. These results and our results suggest that a co-ordinated down-regulation of chaperone proteins may represent a common mechanism for muscle atrophy. On the other hand, the alterations in HSP60 and HSP27 levels detected by two-dimensional analysis in the present study were not previously reported. Either these two chaperones do not respond at the transcriptional level, or only change in more severe cases of inflammatory disease, which the ‘double-hit’ burn-CLP injury model tries to emulate.

Decreased mRNA expression of metabolic enzymes involved in energy production has been reported in various conditions associated with muscle atrophy. Lecker et al. [18] have reported a set of metabolic genes down-regulated at the mRNA level in four different atrophy-inducing states: fasting, tumour bearing, uraemia and diabetes mellitus. Transcriptional profiling of human muscle from older individuals, as well as a study using SAGE (serial analysis of gene expression) technique to analyse immobilization-induced atrophy, showed that many genes involved in energy metabolism were down-regulated, including ATP synthase and cytochrome *c* oxidase [12,15], both of which were found to decrease in the present study. One response to infection is the decreased fatty acid oxidation in multiple tissues including muscle [64], which could be the consequence of the decreased expression of fatty acid binding proteins in these tissues [65]. We also detected a decrease in fatty acid binding protein in EDL muscle tissue from Burn-CLP rats. In general, a down-regulation of several metabolic enzymes has been observed at both the mRNA and protein levels in different muscle wasting models, suggesting that mitochondrial dysfunction may occur as part of the pathophysiology.

Many changes in the myofibrillar proteins reported in the present study are consistent with data obtained in other muscle atrophy models. The significant decrease in myosin regulatory light chain 2 observed in Burn-CLP is consistent with mRNA level changes observed in four different muscle atrophy models [18]. The average spot volumes of other types of myosin light chain were also less in Burn-CLP animals even though the difference was not statistically significant. Both types of myosin heavy chain are large molecules of > 220 kDa, which is not within the resolving range of SDS/PAGE. The parvalbumin is specifically expressed in fast-twitch muscle and its known function is to aid rapid relaxation of muscle through binding cytosolic Ca^{2+} . The increased expression of this protein in our study is consistent with similar mRNA changes observed in other muscle atrophy models [15,18]. Possessing strong affinity ability to Ca^{2+} , increased parvalbumin may account for the abnormally high intracellular Ca^{2+} content associated with Duchenne muscular dystrophy disease [66].

Actin, one major component of myofibrillar proteins, was expected to decrease along with myosin, the other major component of myofibrillar proteins. Surprisingly, an increase in actin was found in the present study, which may be the result of the normalization method (individual spot normalized to total spot volume) used during image analysis. A decrease in myosin accounting for one large proportion of the total protein content may result in an increase in normalized values of actin, the other large proportion of the total proteins. Published mRNA profiles in several other muscle atrophy models did not show any changes in actin, except for one study on hindlimb suspension, which reported a 2.5-fold increase [16]. The only substantially increased protein detected in this study is MyBP-H. Its strong affinity for

myosin suggests possible roles in myofibril assembly and in the regulation of contraction [67]. A substantial increase in MyBP-H accompanied with decreased myosin may suggest its role in the degrading process of myosin. The previous mRNA data are inconsistent, as MyBP-H decreased in one microarray analysis of fasting condition [17] and MyBP-C increased in two different SAGE studies of disuse condition [15,16]. To interpret these differences, further studies are needed to examine whether the alteration of myosin binding proteins is dependent on atrophy phenotype and/or different between mRNA and protein levels.

Finally, the limitations of two-dimensional gel-based proteomic analysis should be mentioned. Even though the protein staining method has been improved, the detection limit of the relatively new Sypro Ruby dye is still in the nanogram range. Another limitation is that membrane proteins and proteins with $pI > 10$ or $pI < 3$ are difficult to work with through two-dimensional gel-electrophoretic separation. These are obstacles that may explain that no signalling molecules and ubiquitin ligases were found to be changed in the present study. Nevertheless, the co-ordinated down-regulation of chaperone proteins and metabolic enzymes reported here suggests that impaired cellular functions may contribute to the mechanisms of muscle wasting in response to severe injury.

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REFERENCES

- Lecker, S. H., Solomon, V., Mitch, W. E. and Goldberg, A. L. (1999) Muscle protein breakdown and the critical role of the ubiquitin–proteasome pathway in normal and disease states. *J. Nutr.* **129**, 227S–237S
- Hasselgren, P. O. (2000) Catabolic response to stress and injury: implications for regulation. *World J. Surg.* **24**, 1452–1459
- Tawa, Jr, N. E., Odessey, R. and Goldberg, A. L. (1997) Inhibitors of the proteasome reduce the accelerated proteolysis in atrophying rat skeletal muscles. *J. Clin. Invest.* **100**, 197–203
- Hobler, S. C., Tiao, G., Fischer, J. E., Monaco, J. and Hasselgren, P. O. (1998) Sepsis-induced increase in muscle proteolysis is blocked by specific proteasome inhibitors. *Am. J. Physiol.* **274**, R30–R37
- Bodine, S. C., Latres, E., Baumhueter, S., Lai, V. K., Nunez, L., Clarke, B. A., Poueymirou, W. T., Panaro, F. J., Na, E., Dharmarajan, K. et al. (2001) Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* **294**, 1704–1708
- Jackman, R. W. and Kandarian, S. C. (2004) The molecular basis of skeletal muscle atrophy. *Am. J. Physiol. Cell Physiol.* **287**, C834–C843
- Guttridge, D. C., Mayo, M. W., Madrid, L. V., Wang, C. Y. and Baldwin, Jr, A. S. (2000) NF- κ B-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia. *Science* **289**, 2363–2366
- Li, Y. P., Lecker, S. H., Chen, Y., Waddell, I. D., Goldberg, A. L. and Reid, M. B. (2003) TNF- α increases ubiquitin-conjugating activity in skeletal muscle by up-regulating UbcH2/E220k. *FASEB J.* **17**, 1048–1057
- Langen, R. C., Schols, A. M., Kelders, M. C., Wouters, E. F. and Janssen-Heininger, Y. M. (2001) Inflammatory cytokines inhibit myogenic differentiation through activation of nuclear factor- κ B. *FASEB J.* **15**, 1169–1180
- Cai, D., Frantz, J. D., Tawa, Jr, N. E., Melendez, P. A., Oh, B. C., Lidov, H. G., Hasselgren, P. O., Frontera, W. R., Lee, J., Glass, D. J. and Shoelson, S. E. (2004) IKK β /NF- κ B activation causes severe muscle wasting in mice. *Cell* **119**, 285–298
- Alon, T., Friedman, J. M. and Socci, N. D. (2003) Cytokine-induced patterns of gene expression in skeletal muscle tissue. *J. Biol. Chem.* **278**, 32324–32334
- Welle, S., Brooks, A. I., Delehanty, J. M., Needler, N. and Thornton, C. A. (2003) Gene expression profile of aging in human muscle. *Physiol. Genomics* **14**, 149–159
- Lee, C. K., Klopp, R. G., Weindrich, R. and Prolla, T. A. (1999) Gene expression profile of aging and its retardation by caloric restriction. *Science* **285**, 1390–1393
- Bey, L., Akunuri, N., Zhao, P., Hoffman, E. P., Hamilton, D. G. and Hamilton, M. T. (2003) Patterns of global gene expression in rat skeletal muscle during unloading and low-intensity ambulatory activity. *Physiol. Genomics* **13**, 157–167
- St-Amand, J., Okamura, K., Matsumoto, K., Shimizu, S. and Sogawa, Y. (2001) Characterization of control and immobilized skeletal muscle: an overview from genetic engineering. *FASEB J.* **15**, 684–692
- Cros, N., Tkatchenko, A. V., Pisani, D. F., Leclerc, L., Leger, J. J., Marini, J. F. and Dechesne, C. A. (2001) Analysis of altered gene expression in rat soleus muscle atrophied by disuse. *J. Cell. Biochem.* **83**, 508–519
- Jagoe, R. T., Lecker, S. H., Gomes, M. and Goldberg, A. L. (2002) Patterns of gene expression in atrophying skeletal muscles: response to food deprivation. *FASEB J.* **16**, 1697–1712
- Lecker, S. H., Jagoe, R. T., Gilbert, A., Gomes, M., Baracos, V., Bailey, J., Price, S. R., Mitch, W. E. and Goldberg, A. L. (2004) Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. *FASEB J.* **18**, 39–51
- Isfort, R. J., Hinkle, R. T., Jones, M. B., Wang, F., Greis, K. D., Sun, Y., Keough, T. W., Anderson, N. L. and Sheldon, R. J. (2000) Proteomic analysis of the atrophying rat soleus muscle following denervation. *Electrophoresis* **21**, 2228–2234
- Isfort, R. J., Wang, F., Greis, K. D., Sun, Y., Keough, T. W., Farrar, R. P., Bodine, S. C. and Anderson, N. L. (2002) Proteomic analysis of rat soleus muscle undergoing hindlimb suspension-induced atrophy and reweighting hypertrophy. *Proteomics* **2**, 543–550
- Rue, III, L. W., Cioffi, W. G., Mason, Jr, A. D., McManus, W. F. and Pruitt, Jr, B. A. (1995) The risk of pneumonia in thermally injured patients requiring ventilatory support. *J. Burn Care Rehabil.* **16**, 262–268
- Marano, M. A., Moldawer, L. L., Fong, Y., Wei, H., Minei, J., Yurt, R., Cerami, A. and Lowry, S. F. (1988) Cachectin/TNF production in experimental burns and *Pseudomonas* infection. *Arch. Surg.* **123**, 1383–1388
- Fong, Y. M., Minei, J. P., Marano, M. A., Moldawer, L. L., Wei, H., Shires, III, G. T., Shires, G. T. and Lowry, S. F. (1991) Skeletal muscle amino acid and myofibrillar protein mRNA response to thermal injury and infection. *Am. J. Physiol.* **261**, R536–R542
- Buck, M. and Chojkier, M. (1996) Muscle wasting and dedifferentiation induced by oxidative stress in a murine model of cachexia is prevented by inhibitors of nitric oxide synthesis and antioxidants. *EMBO J.* **15**, 1753–1765
- Fagan, J. M., Ganguly, M., Stockman, H., Ferland, L. H. and Toner, M. (1999) Posttranslational modifications of cardiac and skeletal muscle proteins by reactive oxygen species after burn injury in the rat. *Ann. Surg.* **229**, 106–114
- Gomes-Marcondes, M. C. and Tisdale, M. J. (2002) Induction of protein catabolism and the ubiquitin–proteasome pathway by mild oxidative stress. *Cancer Lett.* **180**, 69–74
- Grune, T., Reinheckel, T., Joshi, M. and Davies, K. J. (1995) Proteolysis in cultured liver epithelial cells during oxidative stress. Role of the multicatalytic proteinase complex, proteasome. *J. Biol. Chem.* **270**, 2344–2351
- Lawler, J. M., Song, W. and Demaree, S. R. (2003) Hindlimb unloading increases oxidative stress and disrupts antioxidant capacity in skeletal muscle. *Free Radical Biol. Med.* **35**, 9–16
- Li, Y. P., Chen, Y., Li, A. S. and Reid, M. B. (2003) Hydrogen peroxide stimulates ubiquitin-conjugating activity and expression of genes for specific E2 and E3 proteins in skeletal muscle myotubes. *Am. J. Physiol. Cell Physiol.* **285**, C806–C812
- Tiao, G., Lieberman, M., Fischer, J. E. and Hasselgren, P. O. (1997) Intracellular regulation of protein degradation during sepsis is different in fast- and slow-twitch muscle. *Am. J. Physiol.* **272**, R849–R856
- Fang, C. H., Li, B. G., Tiao, G., Wang, J. J., Fischer, J. E. and Hasselgren, P. O. (1998) The molecular regulation of protein breakdown following burn injury is different in fast- and slow-twitch skeletal muscle. *Int. J. Mol. Med.* **1**, 163–169
- Duan, X., Yarmush, D. M., Jayaraman, A. and Yarmush, M. L. (2004) Dispensable role for interferon gamma in the burn-induced acute phase response: a proteomic analysis. *Proteomics* **4**, 1830–1839
- Schreiber, G., Tsykin, A., Aldred, A. R., Thomas, T., Fung, W. P., Dickson, P. W., Cole, T., Birch, H., De Jong, F. A. and Millard, J. (1989) The acute phase response in the rodent. *Ann. NY Acad. Sci.* **557**, 61–85, discussion 85–86
- Gabay, C. and Kushner, I. (1999) Acute-phase proteins and other systemic responses to inflammation. *N. Engl. J. Med.* **340**, 448–454
- Arrigo, A. P. (1998) Small stress proteins: chaperones that act as regulators of intracellular redox state and programmed cell death. *Biol. Chem.* **379**, 19–26
- Nelson, R. J., Ziegelhoffer, T., Nicolet, C., Werner-Washburne, M. and Craig, E. A. (1992) The translation machinery and 70 kd heat shock protein cooperate in protein synthesis. *Cell* **71**, 97–105
- Gething, M. J. and Sambrook, J. (1992) Protein folding in the cell. *Nature (London)* **355**, 33–45
- Luo, G., Sun, X., Hungness, E. and Hasselgren, P. O. (2001) Heat shock protects L6 myotubes from catabolic effects of dexamethasone and prevents downregulation of NF- κ B. *Am. J. Physiol. Regulatory Integrative Comp. Physiol.* **281**, R1193–R1200

- 39 Naito, H., Powers, S. K., Demirel, H. A., Sugiura, T., Dodd, S. L. and Aoki, J. (2000) Heat stress attenuates skeletal muscle atrophy in hindlimb-unweighted rats. *J. Appl. Physiol.* **88**, 359–363
- 40 Tiao, G., Fagan, J., Roegner, V., Lieberman, M., Wang, J. J., Fischer, J. E. and Hasselgren, P. O. (1996) Energy-ubiquitin-dependent muscle proteolysis during sepsis in rats is regulated by glucocorticoids. *J. Clin. Invest.* **97**, 339–348
- 41 Wing, S. S., Haas, A. L. and Goldberg, A. L. (1995) Increase in ubiquitin–protein conjugates concomitant with the increase in proteolysis in rat skeletal muscle during starvation and atrophy denervation. *Biochem. J.* **307**, 639–645
- 42 Fagan, J. M., Ganguly, M., Tiao, G., Fischer, J. E. and Hasselgren, P. O. (1996) Sepsis increases oxidatively damaged proteins in skeletal muscle. *Arch. Surg.* **131**, 1326–1331, discussion 1331–1332
- 43 Ritter, C., Andrades, M., Frota Junior, M. L., Bonatto, F., Pinho, R. A., Polydoro, M., Klamt, F., Pinheiro, C. T., Menna-Barreto, S. S., Moreira, J. C. and Dal-Pizzol, F. (2003) Oxidative parameters and mortality in sepsis induced by cecal ligation and perforation. *Intensive Care Med.* **29**, 1782–1789
- 44 Morita, S., Tsujinaka, T., Yano, M., Ebisui, C., Morimoto, T., Fujita, J., Ogawa, A., Taniguchi, M., Shiozaki, H. and Monden, M. (1996) Temperature-dependent enhancement of proteolysis in C2C12 myotubes in association with the activation of 26S proteasome. *Biochem. Biophys. Res. Commun.* **228**, 813–818
- 45 Luo, G. J., Sun, X. and Hasselgren, P. O. (2000) Hyperthermia stimulates energy-proteasome-dependent protein degradation in cultured myotubes. *Am. J. Physiol. Regulatory Integrative Comp. Physiol.* **278**, R749–R756
- 46 Shabert, J. K., Winslow, C., Lacey, J. M. and Wilmore, D. W. (1999) Glutamine-antioxidant supplementation increases body cell mass in AIDS patients with weight loss: a randomized, double-blind controlled trial. *Nutrition* **15**, 860–864
- 47 Kondo, H., Kodama, J., Kishibe, T. and Itokawa, Y. (1993) Oxidative stress during recovery from muscle atrophy. *FEBS Lett.* **326**, 189–191
- 48 Appell, H. J., Duarte, J. A. and Soares, J. M. (1997) Supplementation of vitamin E may attenuate skeletal muscle immobilization atrophy. *Int. J. Sports Med.* **18**, 157–160
- 49 Rando, T. A., Crowley, R. S., Carlson, E. J., Epstein, C. J. and Mohapatra, P. K. (1998) Overexpression of copper/zinc superoxide dismutase: a novel cause of murine muscular dystrophy. *Ann. Neurol.* **44**, 381–386
- 50 Baek, S. H., Min, J. N., Park, E. M., Han, M. Y., Lee, Y. S., Lee, Y. J. and Park, Y. M. (2000) Role of small heat shock protein HSP25 in radioresistance and glutathione-redox cycle. *J. Cell Physiol.* **183**, 100–107
- 51 Mehlen, P., Kretz-Remy, C., Preville, X. and Arrigo, A. P. (1996) Human hsp27, *Drosophila* hsp27 and human alphaB-crystallin expression-mediated increase in glutathione is essential for the protective activity of these proteins against TNFalpha-induced cell death. *EMBO J.* **15**, 2695–2706
- 52 Mehlen, P., Hickey, E., Weber, L. A. and Arrigo, A. P. (1997) Large unphosphorylated aggregates as the active form of hsp27 which controls intracellular reactive oxygen species and glutathione levels and generates a protection against TNFalpha in NIH-3T3-ras cells. *Biochem. Biophys. Res. Commun.* **241**, 187–192
- 53 Preville, X., Salvemini, F., Giraud, S., Chaufour, S., Paul, C., Stepien, G., Ursini, M. V. and Arrigo, A. P. (1999) Mammalian small stress proteins protect against oxidative stress through their ability to increase glucose-6-phosphate dehydrogenase activity and by maintaining optimal cellular detoxifying machinery. *Exp. Cell Res.* **247**, 61–78
- 54 Okada, M., Hasebe, N., Aizawa, Y., Izawa, K., Kawabe, J. and Kikuchi, K. (2004) Thermal treatment attenuates neointimal thickening with enhanced expression of heat-shock protein 72 and suppression of oxidative stress. *Circulation* **109**, 1763–1768
- 55 Ku, Z., Yang, J., Menon, V. and Thomason, D. B. (1995) Decreased polysomal HSP-70 may slow polypeptide elongation during skeletal muscle atrophy. *Am. J. Physiol.* **268**, C1369–C1374
- 56 Thomason, D. B. (1998) Translational control of gene expression in muscle. *Exercise Sport Sci. Rev.* **26**, 165–190
- 57 Kilgore, J. L., Timson, B. F., Saunders, D. K., Kraemer, R. R., Klemm, R. D. and Ross, C. R. (1994) Stress protein induction in skeletal muscle: comparison of laboratory models to naturally occurring hypertrophy. *J. Appl. Physiol.* **76**, 598–601
- 58 Patton, W. F., Erdjument-Bromage, H., Marks, A. R., Tempst, P. and Taubman, M. B. (1995) Components of the protein synthesis and folding machinery are induced in vascular smooth muscle cells by hypertrophic and hyperplastic agents. Identification by comparative protein phenotyping and microsequencing. *J. Biol. Chem.* **270**, 21404–21410
- 59 Thompson, H. S., Maynard, E. B., Morales, E. R. and Scordilis, S. P. (2003) Exercise-induced HSP27, HSP70 and MAPK responses in human skeletal muscle. *Acta Physiol. Scand.* **178**, 61–72
- 60 Ardawi, M. and Salleh, M. (1988) Skeletal muscle glutamine production in thermally injured rats. *Clin. Sci.* **74**, 165–172
- 61 Askanazi, J., Carpentier, Y. A., Michelsen, C. B., Elwyn, D. H., Furst, P., Kantrowitz, L. R., Gump, F. E. and Kinney, J. M. (1980) Muscle and plasma amino acids following injury. Influence of intercurrent infection. *Ann. Surg.* **192**, 78–85
- 62 Inaguma, Y., Hasegawa, K., Kato, K. and Nishida, Y. (1996) cDNA cloning of a 20-kDa protein (p20) highly homologous to small heat shock proteins: developmental and physiological changes in rat hindlimb muscles. *Gene* **178**, 145–150
- 63 Atomi, Y., Yamada, S. and Nishida, T. (1991) Early changes of alpha B-crystallin mRNA in rat skeletal muscle to mechanical tension and denervation. *Biochem. Biophys. Res. Commun.* **181**, 1323–1330
- 64 Feingold, K. R., Staprans, I., Memon, R. A., Moser, A. H., Shigenaga, J. K., Doerrler, W., Dinarello, C. A. and Grunfeld, C. (1992) Endotoxin rapidly induces changes in lipid metabolism that produce hypertriglyceridemia: low doses stimulate hepatic triglyceride production while high doses inhibit clearance. *J. Lipid Res.* **33**, 1765–1776
- 65 Memon, R. A., Bass, N. M., Moser, A. H., Fuller, J., Appel, R., Grunfeld, C. and Feingold, K. R. (1999) Down-regulation of liver and heart specific fatty acid binding proteins by endotoxin and cytokines *in vivo*. *Biochim. Biophys. Acta* **1440**, 118–126
- 66 Gailly, P. (2002) New aspects of calcium signaling in skeletal muscle cells: implications in Duchenne muscular dystrophy. *Biochim. Biophys. Acta* **1600**, 38–44
- 67 Gilbert, R., Cohen, J. A., Pardo, S., Basu, A. and Fischman, D. A. (1999) Identification of the A-band localization domain of myosin binding proteins C and H (MyBP-C, MyBP-H) in skeletal muscle. *J. Cell Sci.* **112**, 69–79

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