Protein metabolism and gene expression in skeletal muscle of critically ill patients with sepsis

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ABSTRACT

Muscle wasting negatively affects morbidity and mortality in critically ill patients. This progressive wasting is accompanied by, in general, a normal muscle PS (protein synthesis) rate. In the present study, we investigated whether muscle protein degradation is increased in critically ill patients with sepsis and which proteolytic enzyme systems are involved in this degradation. Eight patients and seven healthy volunteers were studied. In vivo muscle protein kinetics was measured using arteriovenous balance techniques with stable isotope tracers. The activities of the major proteolytic enzyme systems were analysed in combination with mRNA expression of genes related to these proteolytic systems. Results show that critically ill patients with sepsis have a variable but normal muscle PS rate, whereas protein degradation rates are dramatically increased (up to 160%). Of the major proteolytic enzyme systems both the proteasome and the lysosomal systems had higher activities in the patients, whereas calpain and caspase activities were not changed. Gene expression of several genes related to the proteasome system was increased in the patients. mRNA levels of the two main lysosomal enzymes (cathepsin B and L) were not changed but, conversely, genes related to calpain and caspase had a higher expression in the muscles of the patients. In conclusion, the dramatic muscle wasting seen in critically ill patients with sepsis is due to increased protein degradation. This is facilitated by increased activities of both the proteasome and lysosomal proteolytic systems.

INTRODUCTION

Skeletal muscle wasting is a common feature in different diseased states, e.g. cancer, sepsis, burns and diabetes, but is seen in its worst form in patients treated in the ICU (intensive care unit). Independent of their initial diagnosis, most patients treated in the ICU for more than 24 h develop a syndrome called multiple organ failure in which vital organ systems start failing and need to be supported in the ICU for the patients to survive. Wasting of muscle protein in these patients can be as much as 10% per week in the initial weeks of ICU treatment [1] and severely compromises muscle functionality both in the ICU and long after discharge from the unit. Previous
experiments from our group [2,3] indicate that protein fractional synthesis rates in leg skeletal muscle on average is not different in ICU patients compared with healthy individuals but is far more variable. These observations indicate that muscle wasting is mainly related to protein degradation in this patient group.

In muscle cells, there are four main systems of proteolytic enzyme pathways involved in protein degradation: the ubiquitin–proteasome pathway, the autophagy–lysosome system, calpains and caspase 3. Myofibrillar proteins are degraded by the ubiquitin–proteasome pathway, and most cellular proteins degraded by this pathway must first be polyubiquitinated in order to be recognized by the 26S proteasome [4]. There are three classes of enzymes involved in the initial steps of the proteasome pathway: E1 proteins that activate ubiquitin, E2 proteins that are ubiquitin-conjugating enzymes and E3 protein ligases that transfer ubiquitin from the conjugating system to the protein substrate [5]. Proteins are then degraded in the 20S core of the 26S proteasome by three different proteases into small peptides [6]. The autophagy–lysosome system is implicated in the degradation of extracellular constituents, and the turnover of cytoplasmic constituents and of cellular organelles like mitochondria. Autophagosomes are formed as double-membrane vesicles around cellular constituents, and the vesicles fuse with lysosomes where their contents are degraded. Cathepsins B, L, D and H are the major proteolytic enzymes in lysosomes and primarily determine the proteolytic capacity of this organelle [7]. Calpains and caspase 3 are believed to be involved in the initial step of myofibrillar breakdown by releasing actin and myosin filaments from the myofibrils. Skeletal muscle contractile proteins exist as actomyosin complexes which cannot be degraded by the proteasome but have to be released from the sarcomere as monomers first [8].

The aim of the present study was to elucidate the mechanisms leading to the dramatic muscle protein loss in septic patients. To achieve this, we have used various methods to measure PB (protein breakdown) in ICU patients and healthy controls. We also measured PS (protein synthesis) to confirm previous results using a different method. Arteriovenous differences, of phenylalanine and 3-methylhistidine tracers, over leg muscles were used to quantify both PS and PB of phenylalanine and 3-methylhistidine tracers, over using a different method. Arteriovenous differences, ICU patients and healthy controls. We also measured various methods to measure PB (protein breakdown) in loss in septic patients. To achieve this, we have used mechanisms leading to the dramatic muscle protein loss first [8].

MATERIAL AND METHODS

Patients and subjects

ICU patients ($n = 8$) with sepsis and/or septic shock at admittance were included in the study. Patients with severe liver failure, undergoing dialysis or with impaired coagulation not allowing muscle biopsies were excluded from the study. The characteristics of the patients on the study day are given in Table 1. According to the SOFA (Sepsis Organ Failure Assessment) scores, all of the patients suffered from multiple organ failure. All of the patients were sedated, one with midazolam and the others with propofol, together with intermittent doses of analgesics, and all patients required mechanical ventilation. At the time of the study, all patients were circulatorily stabilized, although all but one patient required vasopressor support. Intravenous short-term corticosteroid treatment was given to seven patients and one patient was given corticosteroid substitution. Antibiotics were given to six patients and one patient was treated with more than one. Antifungal therapy was given to three patients. At the time of study one of the patients had an indwelling thoracic epidural catheter with continuous infusion of local anaesthetics and short-acting opioids. Total parental nutrition, enteral nutrition or a combination were given continuously to all patients according to the individual caloric goals (range 0.8–1.1 kcal/kg of body weight per h). All patients received intravenous insulin to keep blood glucose between 4 and 8 mmol/l. One patient died in the ICU (day 40 of ICU stay). The rest survived ICU and the hospital stay and were still alive 6 months after completion of the study.

The control group consisted of eight healthy volunteers; no data were available from one due to technical problems with the tracer infusion. The patient data were therefore compared with data of seven healthy volunteers with a mean age of 25 years (range 21–29) consisting of five male and two female subjects with normal BMI (body mass index).

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethical Committee of Karolinska Institutet, Stockholm, Sweden. All subjects, or for the ICU patients, close relatives, gave informed consent to participate in the studies after receiving both oral and standardized written information approved by the Ethical Committee.

Study protocol

The controls reported to the research laboratory in the morning after an overnight fast (16 h). Patients were in the ICU unit already for at least one day before inclusion into the study.

Catheters were inserted in the radial artery and in the cubital veins for the controls, whereas in the patients existing arterial and central vein catheters were
used. Baseline blood samples were taken from the artery. Subsequently, a primed continuous infusion of \([^{2}H_{5}]\)phenylalanine (prime, 0.5 mg/kg of body weight; infusion, 0.5 mg/kg of body weight per h) and 3-\([^{2}H_{3}]\)methylhistidine (prime, 0.01 mg/kg of body weight; infusion, 0.01 mg/kg of body weight per h) was started. At the same time parenteral nutrition (Kabiven, Fresenius) was started in the volunteers at 1 kcal/kg of body weight per h. After 2 h, an additional catheter was placed in the femoral vein. Blood samples from the femoral vein and the radial artery were collected simultaneously at 220, 230 and 240 min after the start of the infusion. Blood samples were centrifuged at 2000 \(g\) for 15 min to obtain plasma which was stored at \(-80^\circ C\) until analysis. Immediately before and after the sampling period, blood flow to the leg was measured by venous occlusion plethysmography with a minimum of ten readings, as described in detail previously [9]. Muscle biopsies were taken at 220 and 240 min, immediately frozen in liquid nitrogen and thereafter stored at \(-80^\circ C\). Muscle biopsies were taken from the vastus lateralis using a Bergstrom needle. The biopsies were taken using local anaesthesia (lidocaine) and were confined to the skin and fascia only.

**Amino acid analyses**

Plasma samples were analysed for concentration and tracer enrichment of phenylalanine- and 3-methylhistidine by HPLC (Waters Alliance 2690 and Waters fluorescence detector 474) and GC–MS (Agilent 5973n) respectively.

For HPLC analysis, plasma was deproteinized with 3 % SSA (5-sulfosalisylic acid-2-dihydrate) containing 200 \(\mu\)M norvaline as an internal standard. The HPLC analysis has been described in detail previously [10]. Sample preparation and GC–MS analysis to measure plasma \([^{2}H_{5}]\)phenylalanine and 3-\([^{2}H_{3}]\)methylhistidine have been described in detail previously [11].

Analyses of muscle tissue-free concentrations of phenylalanine and 3-methylhistidine were performed as described before [10]. For analyses of tissue-free \([^{2}H_{5}]\)phenylalanine in muscle a modification of the method used to analyse plasma \([^{2}H_{5}]\)phenylalanine was utilized. The muscle sample was freeze-dried and pulverized. Fat, connective tissue and blood were carefully removed from the muscle fibres, which were subsequently weighed again. Muscle samples were homogenized in 6 % SSA using a mini-bead beater (Biospec Products, Bartlesville, OK, U.S.A.). After centrifugation, the amino acids in the supernatant were purified using ion-exchange chromatography. Furthermore, the samples were derivatized and analysed on the GC–MS as described for plasma samples [11]. The samples were analysed five times and the median value used for calculations to minimize the variation.

**Enzyme measurements**

**Preparation of muscle biopsies**

Approx. 90 mg of muscle tissue was homogenized in a glass homogenizer in 1 ml of buffer A [50 mM Tris/HCl, pH 7.5, 1 mM DTT (dithiothreitol), 1 mM EDTA, 5 mM MgCl\(_{2}\), 250 mM sucrose and 10 % glycercol]. The homogenate was centrifuged at 700 \(g\) to remove cell debris. The resulting supernatant was thereafter centrifuged at 16,300 \(g\). The supernatant was frozen in aliquots at \(-80^\circ C\) and used for determination of protease and caspase 3 activity. Before determination of protease activities in the two fractions protein content was measured using the BioRad protein assay. The pellet suspension was first freeze–thawed three times.

**Proteasome activity**

Chymotrypsin-like activity of the proteasome fraction was measured using the fluorogenic peptide substrate SUC-LIVY-AMC [succinyl-Leu-Leu-Val-Tyr-AMC (7-amido-4-methylcoumarin)] (Sigma) [12]. Then 10 \(\mu\)l of the supernatant (approximately 10 \(\mu\)g of protein) was
incubated in 100 μl of buffer (50 mM Tris/HCl, pH 7.5, 1 mM ATP, 5 mM MgCl2, 1 mM and 150 μM LLVY) in microplates. Standard curves were prepared using AMC (Sigma). Fluorescence was measured continuously over 1 h at 37 °C in a FLOUstar OPTIMA (BMG Labtechnologies) spectrophotometer at λex = 380 nm and λem = 460 nm. Proteolytic activity was calculated from the increment of the curves from samples and standards and are expressed as pmol of AMC released/μg of protein per min.

Cathepsin B and L activities

Activity of the lysosome fraction was measured using Z-Arg-Arg-AMC (Sigma) for cathepsin B and Z-Phe-Arg-AMC (Sigma) for cathepsin L [13]. For cathepsin B activity, 10 μl of pellet suspension (approximately 2 μg of protein) was incubated in 100 μl of buffer (0.1 M sodium phosphate, pH 6, 1 mM EDTA, 2 mM cysteine and 250 μM Arg-Arg-AMC). For cathepsin L activity, 10 μl of pellet suspension (approximately 2 μg of protein) was incubated in 100 μl of buffer (0.1 M sodium acetate, pH 5.5, 1 mM EDTA, 1 mM DTT and 250 μM Phe-Arg-AMC). Standard curves were prepared using AMC. Fluorescence and proteolytic activity was measured and calculated as described for the proteasome activity.

Caspase 3 activity

Activity was measured using Ac-DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-AMC; Sigma) as substrate [14]. Aliquots of the supernatant (30 μg of protein) were incubated in 100 μl of reaction buffer (100 mM Hepes, pH 7.5, 10 % sucrose and 1 mM DTT) containing 90 μM DEVD-AMC at 30 °C for 1 h. Parallel incubations were done in the presence of 30 μM Ac-DEVD-CHO (N-acetyl-Asp-Glu-Val-Aldehyde; Sigma) a caspase 3 inhibitor. Fluorescence and proteolytic activity were measured and calculated as described for the proteasome activity. Activity in the presence of Ac-DEVD-CHO was subtracted from the activity in the presence of Ac-DEVD-AMC alone and expressed as fluorescence units/μg of protein per min.

Calpain activity

Approx. 50 mg of muscle tissue was homogenized in a buffer (20 mM Tris/HCl, pH 8, 5 mM EDTA, 10 mM 2-mercaptoethanol, 2.5 μM E-64 [trans-epoxysuccinyl-l-leucylamido-4-guanidino]butane), 2 mM PMSF and 0.1 mg/ml trypsin inhibitor). The homogenate was centrifuged 19900g for 30 min. Protein concentration in the supernatant was determined as described above. Calpain activity in the supernatant was determined using BODIPY FL-casein (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid-labelled casein) (Molecular Probes) as substrate [14]. Then, 30 μg of supernatant protein was incubated in a buffer containing 20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 100 mM KCl, 1 mM DTT, 10 mM CaCl2 and 4.6 μg/ml of BODIPY FL-casein in a total volume of 220 μl. Parallel incubations were done in the absence of calcium and in the presence of 10 mM EDTA. Fluorescence was measured continuously over 1 h at 26 °C at λex = 485 nm and λem = 520 nm. Proteolytic activity was calculated from the increment of the curves obtained and expressed as fluorescence units/μg of protein per min. By subtracting the activity curve in the absence of calcium from the activity in the presence of calcium, the calcium-dependent activity, i.e. calpain activity, was obtained.

Gene expression

Selected genes involved in the different proteolytic enzyme systems were analysed using real-time PCR. Total RNA was isolated from muscle samples using TRIzol® (Invitrogen) and quantified using a nano-drop spectrophotometer. cDNA was prepared from 1 μg of RNA using random hexamer primers and reverse transcription reagents (Applied Biosystems) in a final volume of 40 μl. Oligonucleotide primers were designed using a primer design centre (http://www.probelibrary.com/) and synthesized by Invitrogen. To avoid amplification of nuclear DNA the primers were designed to amplify across exon–exon boundaries. The cycle threshold values (dCT) were calculated based on correction to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and statistical analysis was applied to these raw data. The linear value of each sample (2-dCT) was calculated and this value from each ICU patient was then compared with the mean of all the control values to obtain an estimate of the mean increase/decrease and the variation between samples [15].

Calculations

Skeletal muscle protein kinetics over the leg was performed using both a two- and a three-pool model. In the two-pool model, the kinetics are estimated as the rate of appearance (estimate for PB) and rate of disappearance (estimate for PS), whereas in the three-pool model the additional muscle measurements allows for calculations of actual PB and synthesis rates.

The NB (net balance) of phenylalanine and 3-methylhistidine (nmol/min per 100 ml of leg volume) across the leg was calculated as:

\[
NB = (C_A - C_V) \times F
\]

where \(C_A\) and \(C_V\) are the arterial and venous concentrations respectively (nmol/ml) and \(F\) is the plasma flow (ml/min per 100 ml of leg volume).

The \(R_a\) (rate of appearance) and \(R_d\) (rate of disappearance) of phenylalanine (nmol/min per 100 ml of leg volume) across the leg were calculated utilizing the two-pool model [11]:

\[
R_a = C_V \times [1 - (E_V/E_A)] \times F
\]
\[ R_d = NB + R_a \]

where \( E_V \) and \( E_A \) are enrichments of the plasma phenylalanine tracer in the vein and artery respectively (atom percent excess).

Leg skeletal muscle PS and PB (nmol/min per 100 ml of leg volume) rates were calculated utilizing a three-pool model for phenylalanine [16]:

\[
PS = (C_A \times E_A - C_V \times E_V) \times F/E_M
\]

\[
PB = PS - NB
\]

where \( E_M \) is enrichment of the free phenylalanine tracer in muscle (atom percentage excess). The mean enrichment from the two biopsies was used for calculation.

The \( R_a \) of 3-methylhistidine over the leg was calculated utilizing a two-pool model [11]:

\[
R_a = C_A \times [(E_A/E_V) - 1] \times F
\]

**Statistical methods**

Student’s \( t \) tests were used to compare values between ICU patients and controls. Results are means ± S.D.

**RESULTS**

**Muscle protein kinetics**

The ICU patients had approx. 160% higher muscle PB compared with the controls as calculated from both two-pool and three-pool models (Figure 1). The NB of phenylalanine concentration across the leg was negative in the patients, whereas the controls had a NB not significantly different from zero (Figure 1). PS in leg muscle did not differ between ICU patients and controls as calculated from both two-pool \( R_d \) and three-pool PS (Figure 1). In addition, the \( R_a \) of 3-methylhistidine was significantly higher in the patients compared with controls.

**Proteolytic activities**

Proteasome activity was 44% higher in the ICU patients as compared with the controls (Figure 2). In addition lysosomal proteolytic activities were higher in the patients compared with the controls: cathepsin B by 200% and cathepsin L by 150% (Figure 3). In the ICU patients, the proteasome activity correlated significantly with both cathepsin B activity (\( R = 0.929, P < 0.001 \)) and cathepsin L activity (\( R = 0.860, P < 0.001 \)), whereas this correlation was not seen in the controls. The calpain activity did not differ between patients and controls (Figure 4). In addition, the caspase 3 activity did not differ significantly between the patients and the controls (Figure 4).
Correlations between *in vitro* and *in vivo* measurements

In the patients all of the *in vitro* proteolytic activities correlated significantly with the *in vivo* measurements of \( R_a \) and PB using the phenylalanine tracer. Proteasome activity correlated with \( R_a \) (\( R = 0.708, P < 0.05 \)) and PB (\( R = 0.783, P < 0.05 \)). Cathepsin B correlated with \( R_a \) (\( R = 0.818, P < 0.02 \)) and PB (\( R = 0.771, P < 0.05 \)). Cathepsin L correlated with \( R_a \) (\( R = 0.851, P < 0.01 \)) and PB (\( R = 0.815, P < 0.02 \)). In the controls proteasome activity correlated significantly with \( R_a \) (\( R = 0.973, P < 0.001 \)) and PB (\( R = 0.962, P < 0.001 \)), whereas no correlation was found between cathepsin activities and the *in vivo* measurements. \( R_a \) of 3-methylhistidine, however, did not show any correlation with the *in vitro* measurements of the proteolytic activities in either the patients or the controls.

Gene expression

Real-time QPCR (quantitative PCR) was used to determine whether genes related to different pathways for skeletal muscle protein turnover were activated. We chose genes related to the proteasome pathway, the autophagy–lysosome system, calpain and caspase (Figure 5). In the ICU patients, there was a general tendency for up-regulation of expression of the genes investigated when compared with the controls. Expressions were significantly higher in patients compared with controls for atrogin-1 (by 200 %, \( P = 0.02 \)), ubiquitin (by 200 %, \( P = 0.004 \)), Bnip3 (Bcl2/adenovirus E1B 19 kDa interacting protein 3) (by 800 %, \( P = 0.02 \)), caspase 3 (by 300 %, \( P = 0.005 \)) and m-calpain (by 200 %, \( P = 0.02 \)) (Figure 5). Although cathepsin B and L activities were significantly elevated in the patients mRNA levels were not significantly different between patients and controls. Conversely, calpain and caspase 3 mRNA levels were enhanced in the patients but the enzyme activities remained at the same levels as in the controls.

DISCUSSION

The present study is the first to assess muscle wasting in septic patients from *in vivo* kinetics measurements to gene expression. In addition, these experiments were done during continuous parenteral feeding, which is clinical practice, in both the patients and the controls. The results clearly show that in these critically ill patients the loss of muscle mass is the result of increased protein degradation rather than decreases in synthesis. It also shows that both the proteasome and the lysosomal systems are activated but that gene expression does not always support the kinetic measurements.
**Protein kinetics**

Earlier studies from our group have shown that in vivo PS in skeletal muscle from patients with sepsis does not differ from healthy controls when measured as fractional synthesis rates using incorporation of tracer phenylalanine [2,3]. In another study with similar patients, we demonstrated an enhanced proteasome activity in leg and respiratory muscles [12]. The present study confirms that PS in skeletal muscle is unaltered in septic patients showing that muscle wasting in these patients is mainly due to increased protein degradation. Here, we used an in vivo model measuring plasma flow of tracer phenylalanine over the leg with three different approaches (Figure 1). $R_a$ and $R_d$ were utilized to assess protein kinetics in leg muscle using a two-pool model [11]. The results showed that $R_a$ was higher in the patients compared with the controls, indicating an increase in PB, whereas $R_d$ indicated that PS was the same in both groups. In the three-pool model, it is possible to calculate the PS and PB rates of the leg muscle protein [16]. This model also showed that compared with the controls the patients had an increased PB and equal PS. The third model, a measure of the $R_a$ of 3-methylhistidine that is a marker of contractile protein PB rates, showed an enhanced efflux rate in the patients compared with the controls. These differences are not the result of the age difference between our control subjects and the patients, as human aging is characterized by a decreased or maintained muscle PS and, if anything, a decreased muscle PB [17,18].

In the present study, we also investigated the proteolytic activities in all four main degradation pathways in leg muscle biopsies: proteasome activity, activities of lysosomal cathepsins B and L, and of calpain and caspase 3. In addition, we measured mRNA levels of proteins in respective pathways. We think it is important to measure both protein activity and gene expression in the same samples, as protein levels or activities are not always regulated on the transcriptional level but also on the post-transcriptional level.

**Ubiquitin–proteasome pathway**

There are a number of studies, using animal models for sepsis, which have shown that in skeletal muscle the ubiquitin–proteasome pathway is up-regulated during sepsis, both at the mRNA level of different components in the pathway and proteolytic activities [19–24]. However, studies of molecular mechanisms of muscle atrophy in human sepsis are few and the data from these studies are not as comprehensive as the data from animal experiments. In accordance with the animal models for sepsis, elevated expression of ubiquitin in skeletal muscle from septic patients was found [25–28]. Furthermore, an induction of atrogin-1 [25], increased mRNA levels of cathepsin B [26] and the proteasome subunit HC3 [28] and enhanced proteolytic activity of the proteasome [12,29] have been demonstrated in septic patients. Muscle biopsies from septic patients in the present study showed increased proteolytic activity of the proteasome as well as enhanced mRNA levels of factors linked to the induction of the ubiquitin–proteasome pathway (ubiquitin and atrogin-1, but not MuRF1), thus indicating an enhanced capacity for PB (Figures 2 and 5). Atrogin-1 and MuRF1 (muscle RING-finger protein 1) are ubiquitin-ligases and increased mRNA levels have been observed in a number of animal models of muscle atrophy, including burn injury, diabetes mellitus, denervation, unweighting and sepsis [30]. However, studies of these two so-called atrogins in human inflammatory states are few and there have been inconsistent findings of mRNA levels of the two genes. In COPD (chronic obstructive pulmonary disease) both an elevation and no significant change of atrogin-1 and MuRF1 gene expressions have been reported [31,32].

**Autophagy–lysosome pathway**

Recent data from animal experiments in vitro and in vivo have indicated that the autophagy–lysosome system is also involved in muscle atrophy and that this system is co-ordinated with the ubiquitin–proteasome system. Furthermore, the induction of the two degradation systems is mediated by transcription factor FoxO3 (forkhead box O3) [33,34]. In addition, results from a human study on ventilator-induced diaphragm disuse in patients after cerebrovascular accidents point in this direction [35]. Earlier studies of models for sepsis in rats have measured enhanced levels in muscle of cathepsin B activity [36,37] and an elevation of cathepsin L mRNA and protein levels [38]. There are also a few human studies indicating an up-regulation of the lysosomal system in skeletal muscle in catabolic states. In trauma patients, increased levels of cathepsin D mRNA were found [39] and increased levels of cathepsin B mRNA and enzymatic activity were observed [40]. Cathepsin B mRNA levels were increased in skeletal muscle of patients with early lung cancer [41]. In patients with sepsis immunolabelling of cathepsin B was enhanced in atrophic muscle fibres [26]. In our present study, we found a dramatic increase in activity of lysosomal cathepsin B and L in leg muscle of the septic patients as compared with the controls (Figure 3). However, there was no difference in the mRNA levels of the cathepsins between patients and controls (Figure 5). Studies using animal models of muscle wasting also found limited effects on mRNA levels of cathepsins but large increases in the enzymatic activities, suggesting that post-transcriptional processes may be mainly responsible for the activation of cathepsins [42–44]. In accordance with the animal and human studies mentioned above [33,35] we found increased levels of Bnip3 mRNA in the patients (Figure 5). Bnip3 is a factor that recruits the autophagy machinery on mitochondria [45]. Although both cathepsin B and L are capable of degrading several myofibrillar proteins [7],
skeletal muscle contains few lysosomes in comparison with other organs such as the liver or spleen. Even if not ruled out, it is unlikely that these organelles are involved in the turnover of myofibrillar proteins. Mitochondria are turned over via the autophagy–lysosome system and are a better candidate for degradation after the activation of this system [46]. Previously, we found a mitochondrial content that was 30–40% lower in skeletal muscle of ICU patients with sepsis and that this was not the result of decreased biogenesis [15,47]. The co-ordinated induction of the two main proteolytic pathways presumably leaves the mitochondrial to myofibrillar composition ratio relatively normal but with reduced strength and endurance capacity due to the loss of myofibrillar components and mitochondria [46]. In addition, our results also indicate that the proteasome and autophagy–lysosome systems are activated simultaneously since there was a significant positive correlation between proteasome and lysosomal cathepsin activities in the patients but not in the controls, suggesting a common control system such as FoxO3.

Calpain and caspase systems

There is increasing evidence that calpain and/or caspase 3 are involved in the initial degradation of myofibrillar proteins [8,48]. Since myofibrils are too large to be engulfed by proteasomes, myofilaments must first be released from the myofibrils prior to degradation by the proteasome system. In contrast to the results of cathepsins, the ICU patients in the present study had elevated levels of mRNA of both m-calpain and caspase 3 but the activities of the two proteases did not differ between the study groups (Figures 4 and 5). The question is whether this discrepancy of activity compared with expression levels depends on methodological difficulties, due to low enzyme activities in the muscle extracts, or reflects a high turnover of the enzymes. Animal models for sepsis have come to different conclusions. In septic rats increased mRNA levels of m- and µ-calpain were found but no change in calpain activity [49]. Wei et al. [14] found increased calpain activity in septic rats but unchanged levels of caspase 3 mRNA and enzyme activity. In two other studies of rodent models for sepsis, increased levels of both enzyme activity and protein levels of caspase 3 and calpain were observed [48,50]. So far there have been no studies carried out on calpain and caspase 3 in human sepsis but a study of patients with acute quadriplegic myopathy found enhanced immunoreactivity for calpain in atrophic muscle fibres [51].

General conclusions

In the present study, we show that the dramatic loss of skeletal muscle in critically ill patients with sepsis-induced multiple organ failure is due to an increased protein degradation rather than a decreased PS. We also see that the patients continuously loose muscle protein despite being fed, in contrast with the controls that did not lose muscle protein but were in net protein balance. This increased protein degradation is facilitated by increased activities of the proteasome and lysosomal systems but not of calpain and caspase. The results also show that measuring mRNA levels will give indications of pre- or post-translational adaptations but are not alternative measurements for enzyme activity or kinetic analyses.

AUTHOR CONTRIBUTION

Maria Klaude performed the experiments and data analyses of the proteolytic enzymes, and participated in the study design. Maiko Mori participated in the clinical studies, performed the experiments and data analyses of the protein kinetics, and participated in the study design. Inga Tjäder performed the clinical studies and compiled the patient data. Thomas Gustafsson performed the experiments and data analyses of gene expression. Jan Wernerman participated in study design and clinical studies. Olav Rooyackers participated in the study design and supervised the clinical studies. Maria Klaude compiled the data and wrote the paper. Maiko Mori, Inga Tjäder, Thomas Gustafsson, Jan Wernerman and Olav Rooyackers all reviewed and revised the paper.

ACKNOWLEDGEMENT

We thank Ms Viveka Gustafsson for excellent nursing assistance.

FUNDING

This work was supported by the Swedish Medical Research Council [grant numbers 04210 and 14244].

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