Impaired autophagy, chaperone expression, and protein synthesis in response to critical illness interventions in porcine skeletal muscle

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Submitted 23 October 2012; accepted in final form 4 April 2013

Banduseela VC, Chen YW, Kultima HG, Norman HS, Aare S, Radell P, Eriksson LI, Hoffman EP, Larsson L. Impaired autophagy, chaperone expression, and protein synthesis in response to critical illness interventions in porcine skeletal muscle. Physiol Genomics 45: 477–486, 2013. First published April 9, 2013; doi:10.1152/physiolgenomics.00141.2012.—Critical illness myopathy (CIM) is characterized by a preferential loss of the motor protein myosin, muscle wasting, and impaired muscle function in critically ill intensive care unit (ICU) patients. CIM is associated with severe morbidity and mortality and has a significant negative socioeconomic effect. Neuromuscular blocking agents, corticosteroids, sepsis, mechanical ventilation, and immobilization have been implicated as important risk factors, but the causal relationship between CIM and the risk factors has not been established. A porcine ICU model has been used to determine the immediate molecular and cellular cascades that may contribute to the pathogenesis prior to myosin loss and extensive muscle wasting. Expression profiles have been compared between pigs exposed to the ICU interventions, i.e., mechanically ventilated, sedated, and immobilized for 5 days, with pigs exposed to critical illness interventions, i.e., neuromuscular blocking agents, corticosteroids, and induced sepsis in addition to the ICU interventions for 5 days. Impaired autophagy as well as impaired chaperone expression and protein synthesis were observed in the skeletal muscle in response to critical illness interventions. A novel finding in this study is impaired core autophagy machinery in response to critical illness interventions, which when in concert with downregulated chaperone expression and protein synthesis may collectively affect the proteostasis in skeletal muscle and may exacerbate the disease progression in CIM.

Critical illness myopathy (CIM) is an acquired myopathy frequently observed among critically ill intensive care unit (ICU) patients (24, 32, 43, 45). CIM is characterized by progressive muscle weakness, muscle atrophy, preferential loss of myosin, and disorganization of myofibrils, which are common in the acute phase of the disease (44, 55, 58, 60, 79). In addition to severe wasting and impaired muscle function, which may delay respirator weaning, the impaired neuromuscular function associated with critical illness has significant negative consequences on quality of life for a long time after hospital discharge (20, 29, 32). There is accordingly a significant need for better understanding of the underlying mechanism behind CIM (32). Currently, the etiology of CIM is thought to be multifactorial with several associated independent risk factors (25, 84). Immobilization, mechanical ventilation, sepsis, and exposure to corticosteroids and neuromuscular blocking agents are all implicated as major risk factors in CIM (11, 23, 45). Exact onset of the disease is unknown, and diagnosis is further complicated by the use of analgesia and sedatives; however, despite an acute phase of the disease well-characterized by weakness, muscle atrophy, and myosin loss, the early cellular mechanisms and molecular cascades of an ICU stay prior to a manifestation of myopathy remain to be elucidated.

Skeletal muscle is continuously remodeled as an adaptive mechanism to stress. Muscle proteins including the sarcomeric components are typically in a dynamic state of synthesis, degradation, assembly, and maintenance (39). Therefore, the protein homeostasis (proteostasis) or protein quality control is a critical and essential requirement for proper function of skeletal muscle (12, 39). Chaperones play a key role in proteostasis by either refolding nonnative proteins or targeting them for degradation (4, 18). The ubiquitin proteasome system (UPS), as well as various autophagic pathways, is directly involved in the degradation arm of proteostasis (37, 89). Skeletal muscle specific disruption of autophagy-lysosome system results in profound atrophy, myofibrillar disorganization, and age-dependent drop in force (54).

Using a porcine ICU model we have previously shown that 5 days of immobilization and mechanical ventilation result in upregulation of the UPS and oxidative stress-responsive genes, as well as upregulation of heat shock proteins that may play a protective role in response to immobilization and mechanical ventilation in the ICU (6). In the present study the expression profile of porcine skeletal muscles exposed to neuromuscular blocking agents, corticosteroids, and induced sepsis for 5 days [hereafter critical illness interventions (CII)] was compared with that of pigs that were immobilized and mechanically ventilated [hereafter ICU interventions (ICU-I)] for the same period. The aim of this study was to decipher the immediate cellular and molecular cascades by isolating the collective effect of neuromuscular blocking agents, corticosteroids, and induced sepsis on gene expression and protein expression, which may otherwise be masked by the background effect of...
immobilization and mechanical ventilation. The results indicate that the core autophagy machinery, as well as the chaperone expression, is severely impaired in the porcine skeletal muscle in response to CII.

MATERIALS AND METHODS

Animals and tissue collection. Eight female domestic pigs (Sus scrofa, average body wt 26.5 kg) were included in this study after approval from the ethical committee on animal research at the Karolinska Institute, Stockholm, Sweden (Dnr N71/98, N54/02, and N75/04). All pigs originated from the same farm (Vallrumbs Lantbruk, Ransta, Sweden) and were kept in 12-square meter pens with hay, straw, and wood shavings as bedding material. They were housed at 18–19°C and relative humidity of 45–55% under normal day-night rhythm with liberal access to feed (Smågrisfoder Solo 331; Lantmännen, Stockholm, Sweden), water, and environmental enrichment. Food, but not water, was withheld for 12 h before induction of anesthesia. The pigs were sedated with medetomidine (1 mg/ml Domitor vet; Orion Pharma, Stockholm, Sweden) and Zolazepam (Zoletil 250; Reading, Carros, France) before an intravenous access was prepared, and 100 mg of ketamine (vet 50 mg/ml Ketaminol; Intervet, Boxmeer, Netherlands) was administered iv to induce anesthesia. Following tracheostomy, all animals were mechanically nor- moventilated by volume controlled ventilation (Servo 900A ventilator; Siemens-Elema, Solna, Sweden) by adjusting the FiO₂ to 0.21–0.30 and an inspired tidal volume of 10 ml/kg, respiratory rate of ~20 breaths/min at an I:E ratio of 1:2, and inspiratory rise time of 5–10%. During the 5-day study period, the settings were adjusted to avoid high airway pressures and risk of barotraumas while maintaining arterial normoxia and normocarbma as guided by repetitive arterial blood gas analysis (ABL; Radiometer, Copenhagen, Denmark). Sedation was titrated to promote immobilization and allow ventilator synchrony by inhibiting spontaneous breathing activity. Anesthesia was maintained and adjusted through a novel anesthetic conserving device (AnaConda; Sedena Medical, Sundbyberg, Sweden), which permits administration of an inhaled anesthetic via a syringe pump to the inspired gas mixture, as previously described (70). During the 5-day study period isoflurane (Abbott Laboratories, Chicago, IL) was delivered at 0.8–1.3% end-tidal concentration and supplemented by intravenous bolus doses of morphine and ketamine as needed to provide adequate depth of anesthesia.

Core body temperature (blood) was maintained in the range of 38.5–40°C by a servo-controlled heating pad. Animals received 2,000–4,000 ml/day as a continuous intravenous infusion of a crys- talloid solution (Ringer acetate; Fresenius Kabi, Stockholm, Sweden) at a rate of 2–4,000 ml/24 h, the infusion rate being adjusted to maintain stable blood pressure and urinary output throughout the experimental period. In addition an intravenous glucose infusion (25 mg glucose/ml, Rehdyrex, Fresenius Kabi) was administered at 1–1.5 ml/kg/24 h to maintain stable blood pressure and urinary output throughout the experimental period. In addition an intravenous glucose infusion (25 mg glucose/ml, Rehdyrex, Fresenius Kabi) was administered iv at a rate of 1–1.5 ml/kg/h and titrated to maintain blood glucose between 4 and 8 mmol/l to decrease the effects of catabolism. Each animal received streptomyacin 750 mg/day prophylactic and 600 mg/day benzylpenicillin (Streptocillin Vet; Boeringer-Ingelheim, Hellerup, Denmark). Arterial blood gas analysis and electrolytes and blood glucose levels were monitored regularly and kept in the normal range throughout the study period.

In addition to the above treatments (ICU-I group), four pigs (CII group) received a neuromuscular blocking agent as a continuous infusion of 25 mg/h/racuronium (Esmeron; Organon, Bxell, The Netherlands) throughout the study period. Corticosteroids were administered to the same four pigs, as bolus doses of hydrocortisone (Solu-Cortef, Pfizer), 50 mg three times per day, during the study period. Endotoxemia was induced on the first day in the same four pigs, by administration of a continuous infusion of Escherichia coli endotoxin (serotype O26:B6; Sigma Chemicals, St. Louis, MO) at a rate titrated to physiological effects (mean blood pressure decrease of >30% and pulmonary artery occlusion pressure increase 50%) with a mean total dose of 8 μg/kg.

Muscle biopsies from m. biceps femoris were obtained from the ICU-I and CII groups on day 5. The biopsies were frozen in liquid propane cooled by liquid nitrogen and stored at −80°C for extraction of RNA and protein.

Expression profiling. Total RNA (3 μg) from the muscle samples were extracted and processed to generate biotin-labeled cRNA as previously described (19). Each sample was then hybridized to Af- fymetrix Porcine Genome Array (Affymetrix, Santa Clara, CA), which contains 23,937 probes representing 20,201 genes. Standard operating procedure and quality control were done as previously described (19). Muscle samples from two groups (four animals in the ICU-I group and four animals in the CII group) were profiled. All profiles have been made publicly accessible via National Center for Biotechnology Information Gene Expression Omnibus (no GSE16348 and GSE24239; http://www.ncbi.nlm.nih.gov/geo) and the Children’s National Medical Center Public Expression Profiling Resource (http://pepr.cnmcresearch.org).

Microarray data normalization and analysis. Analysis of the gene expression data was carried out in the freely available statistical computing language R (http://www.r-project.org) using packages available from the Bioconductor project (http://www.bioconductor.org). The raw data were normalized by the robust multiarray average (RMA) (33) method first suggested by Li and Wong (48). To search for the differentially expressed genes between the samples from the different days an empirical Bayes moderated t-test was then applied (81), using the “limma” package (80). A linear model was fitted to the data, and the effect of ICU-I group vs. CII group was estimated. To address the problem with multiple testing, the P values were adjusted according to Benjamini and Hochberg (8). Significant probe sets with an adjusted P value < 0.05 and those with greater than a twofold change were further investigated. Published putative human homologs were further analyzed with database for annotation, visualization and integrated discovery (DAVID) web-based functional annotation tool (http://david.abcc.ncifcrf.gov/) for functionally categorizing up- and down-regulated transcripts (31, 85). Functional categories were refined to improve the interpretative value of data. Genesis cluster analysis software was used to generate expression plots (83). Selected functional categories with up- and downregulated transcripts were graphically presented and tabulated in Fig. 1.

Total protein quantification. Quantification of total protein was performed using NanoOrange protein Quantification Kit (Molecular Probes, Eugene, OR) according to manufacturer’s instructions. Briefly, biopsy samples homogenized in 8 M urea buffer were cen- trifuged, sonicated, and allowed to react with diluted NanoOrange protein Quantification Kit (Molecular Probes, Eugene, OR) according to manufacturer’s instructions. Total protein quantification was performed using NanoOrange protein Quantification Kit (Molecular Probes, Eugene, OR) according to manufacturer’s instructions. Total protein quantification was performed using NanoOrange protein Quantification Kit (Molecular Probes, Eugene, OR) according to manufacturer’s instructions. Total protein quantification was performed using NanoOrange protein Quantification Kit (Molecular Probes, Eugene, OR) according to manufacturer’s instructions. Total protein quantification was performed using NanoOrange protein Quantification Kit (Molecular Probes, Eugene, OR) according to manufacturer’s instructions. Total protein quantification was performed using NanoOrange protein Quantification Kit (Molecular Probes, Eugene, OR) according to manufacturer’s instructions.
and using ECL Advance Western blotting detection kit (RPN 2135, GE Healthcare) according to the manufacturer’s instructions. Autoradiograms (28-9068-36, GE Healthcare) were exposed to the protein blots until a measurable signal intensity was achieved. The autoradiograms were scanned (16 bit, grayscale) using a color image scanner (1,600 dpi, EPSON Expression 1600 Pro), and the intensity volumes were measured (ImageQuant TL, version 7, 2007, GE Healthcare). We used rolling ball (radius = 200 pixels) background subtraction method before measuring the intensity volume on each autoradiogram. All signal intensity volumes were normalized to respective actin signal intensity volumes.

Enzyme-histochemistry and fiber cross-sectional area measurements. Cryosections (serial, transverse, 10 μm thick) were stained for myofibrillar ATPase after acid (pH 4.35, 4.60) and alkaline (pH 10.30) preincubations. Fibers types (type I, IIA, and IIB) were classified according to pH sensitivity of myofibrillar ATPase. Fiber cross-sectional area (CSA) was measured on myofibrillar ATPase stained sections (pH 4.6) using a microscope (Zeiss Axiovert; Carl Zeiss, Oberkochen, Germany) combined with a computer assisted image analysis system (Videoplan; Kontron Bildanalyse, Munich, Germany).

Statistics. Student t-test (unpaired) was used to determine statistical significance using Sigma Plot 9 (Systat Software). Values are means ± SE. Statistical outliers were removed by the extreme studentized deviate method. Statistical significance level was set at P < 0.05. The Mann-Whitney rank sum test was carried out when equal variances failed.

RESULTS

Downregulated transcripts and related proteins. A total of 54 probe sets were downregulated in the CII group compared with the ICU-I group. A summary of selected downregulated transcripts, functional groups, and fold change are presented in Fig. 1A. Downregulated transcripts and related proteins associated with each functional group are discussed below.

**Autophagy and cytoskeleton.** Dyneins and kinesin superfam-
ily (KIF) motor proteins are associated with microtubule de-
pendent plus-end and minus-end directed intracellular trans-
port, respectively (30, 86). Cytoplasmic dynein light chain 1
(DCL1), a subunit of retrograde motor protein, is required for
autophagy and clearance of aggregate prone proteins (7, 67).

Microtubule-associated protein 1A (MAP-1A) is a microtu-
bule-stabilizing protein that interacts with microtubule-associ-
ated protein light chain 3 (LC3) to modulate the shape of
microtubules (56). LC3 plays a critical role in autophagosome
membrane biogenesis where cytosolic LC3-I is converted to
membrane-bound LC3-II lipidated form. The LC3-I to LC3-II
conversion by phosphatidylethanolamine (PE) conjugation is a
universal cellular marker for autophagosome formation (36)
(See Fig. 2).

**Oxidative stress responsive.** As the expression profile showed downregulation of several cytoskeletal molecules that are also associated with autophagy, we hypothesized that autophagy may be affected in the skeletal muscle in response to CII (Fig. 1A). Multiple autophagy genes including LC3B transcription are induced by the FOXO3 in
skeletal muscle (92). Moreover, transcription-dependent induc-
tion of autophagy in skeletal muscle is mediated by FOXO3
directly binding to the LC3 promoter (53). Thus we investi-

![](https://physiolgenomics.physiology.org/doi/figure/10.1152/physiolgenomics.00141.2012)

Fig. 1. Graphical representation of gene expression in each functional category. Downregulated (A) and upregulated (B) major functional categories are represented by expression values and fold change. Expression values reflect the variation from median gene expression. Reference key indicates positive expression in red and negative expression in blue. Maximum expression is set to ± 3.0.

![Graphical representation of gene expression in each functional category.](https://physiolgenomics.physiology.org/doi/figure/10.1152/physiolgenomics.00141.2012)
Impaired skeletal muscle proteostasis in a porcine ICU model.

Fig. 2. Schematic presentation of select genes in autophagosome formation. LC3 is cleaved by ATG4 (autophagy related 4), which results in LC3-I. ATG7 conjugates ATG5 to ATG12 and is also involved in conjugating membrane lipid phosphatidylethanolamine (PE) to LC3-I in conversion of LC3-II. ATG5-ATG12-ATG16 complex is necessary for autophagosome formation. LC3-II mediates membrane elongation by associating with autophagosomal membrane. Adaptor protein p62 directly binds to ubiquitinated (Ub) proteins and acts as a receptor for Ub proteins. By binding to LC3-II, p62 facilitates autophagy by localizing in autophagic compartments, transporting Ub proteins and organelles for degradation. (Illustration was designed with pathway builder from http://www.proteinlounge.com and modified with Adobe Illustrator.)

Gated the protein levels of LC3B-I and LC3B-II. The conversion of LC3B-I to LC3B-II by PE conjugation was impaired in the CII group (Fig. 1A), and the LC3B-II/LC3B-I conversion ratio was decreased in the CII group, indicating an impaired autophagosome formation in the skeletal muscle in response to CII (Fig. 3A). Importantly, only LC3B-II was decreased at the protein level, clearly demonstrating that LC3B-I protein expression was not affected in the CI group (Figs. 3A and 4A). ATG7 (autophagy related 7) conjugates ATG5 to ATG12 and also is responsible for the key step of conjugating PE to LC3-I in converting to LC3-II. ATG5–ATG12 is a key protein complex involved in catalyzing LC3-I to LC3-II conversion. Therefore, ATG7 and ATG5 (ATG5–ATG12 complex) protein expression was investigated. ATG7 and ATG5 protein expression was reduced in the CII group, suggesting an impaired autophagy activation cascade (Figs. 3A and 4A). Adaptor protein p62/SQSTM1 facilitates autophagic turnover by binding directly to LC3 and polyubiquitinated proteins. This interaction is necessary for the autophagic degradation of p62/SQSTM1 positive inclusion bodies (63). Thus, we tested for the p62/SQSTM1 protein expression, and p62/SQSTM1 was decreased at the protein level in the skeletal muscle of the CI group (Fig. 3A and 4A). Impaired core autophagy machinery (decreased LC3-I to LC3-II conversion, ATG7, ATG5, and p62/SQSTM1; see Fig. 2) in response to critical illnesses interventions is a novel finding that has not been documented before in either CIM patients or in ICU experimental animal models.

Endoplasmic reticulum chaperones, heat shock proteins, and protein metabolism. Several chaperones including endoplasmic reticulum (ER) chaperones and ER membrane proteins were downregulated at the RNA level as indicated by the expression profile (Fig. 1A). Among them, calnexin is a key ER chaperone that regulates the glycoprotein folding and quality control through calnexin cycle (52). Heat shock protein (HSP)47 plays a similar role as an ER chaperone that is indispensable for correct folding of collagen and in preventing aggregate formation of misfolded collagen in ER (34). CRYAB (α-B crystallin) is a small heat shock protein that not only plays a protective mechanism in preventing denaturation and aggregation of unfolded proteins but is also specifically localized in the sarcomere and interacts with titin and actin (71). CRYAB is a particularly important chaperone in skeletal muscle as it has been associated with several myopathies including desmin-related myopathy (71). HSPA4 is a member of HSP family 110, which is related to the HSP70 family and is thought to function in concert with other chaperones.

Chaperones are generally involved in preventing protein misfolding and aggregation of secretory proteins. Since the expression profile data suggest a severely affected and stressed ER, we first examined if BiP/GRP78 protein expression were affected and would follow the same trend of chaperones in the CII group. BiP/GRP78 is a member of HSP70 chaperone family, which regulates ER stress sensors and unfolded protein response (UPR) (10). Surprisingly BiP/GRP78 was not affected in the CII group at the protein level (Figs. 3B and 4B). We then examined the total EIF2A (T-EIF2A) and its phosphorylation status to determine if ER stress and UPR were present in the CII group. Phosphorylated EIF2A (P-EIF2A) is an adaptive response to ER stress and is one of the three major pathways of UPR (77). Increased phosphorylation of EIF2A was observed in the intervention group, determined by P-EIF2A:T-EIF2A (Figs. 3B and 4B). CRYAB (small HSP) and HSPA4 (HSP110 family) were also examined at the protein level and were decreased in the CII group, following the trend indicated by the expression profile (Figs. 1A, 3C, and 4C).
Furthermore, we examined the protein expression of HSP70, one of the key chaperone families involved in proteostasis. HSP70 was also decreased at protein level (Figs. 3C and 4C). Since a number of chaperone localizations and autophagy were impaired, the effect on chaperone-mediated autophagy (CMA) was investigated by measuring the protein expression of LAMP-2A. LAMP-2A acts as a receptor as well as a component of the autophagic-cargo translocation complex in CMA (22). LAMP-2A was decreased in the CII group, indicating that CMA was also impaired in response to CII (Figs. 3C and 4C). Decreased LAMP-2A protein expression and decreased chaperone expression in skeletal muscle are novel findings in the ICU set-up in response to CII.

Oxidative stress responsive. An array of oxidative stress-responsive genes was downregulated at RNA level as indicated by expression profile in the intervention group (Fig. 1A). We examined the protein expression of SOD2 by immunoblotting, and the SOD2 was decreased in response to CII (Figs. 3D and 4D).

Sarcomeric myosins. MYH7 and MYL2 code for the B/slow (type 1) myosin heavy chain isoform and the regulatory myosin light chain, respectively (75). Loss of myosin in CIM is well documented, and the present results of a downregulation of myosin at the transcriptional level are in agreement with previous studies in ICU patients as well as in ICU experimental animal models (44, 59, 60, 62). The myosin-actin ratios at the protein level remained unchanged despite the downregulation at RNA level during the study period (61).

Upregulated transcripts and related proteins. A total of 101 probe sets were upregulated in CII group compared with the ICU-I group. A summary of selected upregulated transcripts, functional groups, and fold change is presented in Fig. 1B. Upregulated transcripts and related proteins associated with each functional group are discussed below.

Protein metabolism. Anykrin repeat SOCS box 11 (ASB11) belongs to the family of suppressor of cytokine signaling (SOCS) and mediate protein degradation through Elongin C-cullin-SOCS box (ESC) type complexes, which act as E3 ligases (65). Eukaryotic initiation factor 4E binding protein 1 (EIF4EBP1) acts as a translational repressor by binding to EIF4E and inhibit protein synthesis. PDZRN3 (PDZ ring finger 3) function as an E3 ligase, regulating surface levels of muscle-specific receptor tyrosine kinase (51). TTP is an mRNA binding protein that binds to AU-rich elements in proinflammatory mediators and limits their expression via mRNA decay (74).

Of the genes related to protein metabolism, we examined protein level of the translational repressor EIF4EBP1, as it was upregulated in the expression profile (Fig. 1B). Hypophosphorylated EIF4EBP1 represses the translation of protein synthesis in skeletal muscle that is reversed by the hyperphosphorylation of EIF4EBP1 by activation of the IGF1/AKT/mTOR pathway (14). Total EIF4EBP1 (T-4EBP1) protein expression was increased in response to CII (Fig. 3E). The phosphorylation status of EIF4EBP1 was examined, since phosphorylated EIF4EBP1 (T-4EBP1) indicates an activated protein synthesis cascade. Phosphorylated-to-total protein ratio of the EIF4EBP1 (P-4EBP1/T-4EBP1) was decreased in the CII group, indicating a decreased kinase activity and thereby indicating an impaired protein synthesis in response to CII in skeletal muscle (Fig. 4E). Atrogin-1 (FBXO32) is a muscle-specific E3-ubiquitin ligase that is involved in muscle atrophy (13), and the protein expression of Atrogin-1 did not change in response to CII (Figs. 3E and 4E).

Early growth response. Early growth response 1 (EGR1) initiates a defense mechanism against oxidative stress in skeletal muscle by activating its downstream targets SIRT1 and SOD2 (64). FOS family of transcription factors heterodimerize with JUN proteins and constitute AP-1 (activating protein 1) transcription factor complex. The AP-1 complex is induced in response to oxidative stress (94). JUNB inhibits autophagy via an mTOR-independent pathway that requires AP-1 transcription and leads to cell death in response to starvation (90).

Protein kinase activity. Activin receptor 2B (ACVR2B) acts as a signaling receptor for myostatin, a potent negative regulator of muscle mass (46). The myosin light chain kinase family of proteins is emerging as a diverse group of regulatory proteins involved in protein turnover and autophagy (26). Tribbles 1 is involved in regulating MAPK signaling cascade (40). Increased expression of ACVR2B as demonstrated by the expression profile was confirmed at the protein level by immunoblotting (Figs. 3E and 4E).

Skeletal muscle related. p27kip1 (CDKN1B) is a cyclin-dependent kinase inhibitor that controls cell cycle progression. FOXO1 and FOXO3a induce p27kip1 transcription and decrease proliferation of myoblast precursor cells (47). Mitochondrial creatine kinase (CKMT2) is closely associated with energy metabolism, and
CKMT upregulation has been reported in response to oxidative stress, as a compensatory mechanism against low energy state (76). Four and a half LIM domains 3 is a protein with LIM motif, which forms a complex with MYOD in vitro, suppressing MYOD-mediated transcription and myotube formation (21). Myeloid leukemia factor 1 (MLF1) is highly expressed in skeletal muscle. Overexpression of MLF1 in skeletal muscle results in nonpathogenic aggregates (49). Myomesin 2 is a key protein involved in maintenance of sarcomeric integrity and stability.

Fiber CSA. Fiber CSA of fiber types, type I, IIA, and IIB did not change in porcine skeletal muscle in response to CII during the study period (Fig. 5). The mean CSAs for the ICU-I group were 810 ± 60 (type I), 920 ± 20 (IIA), and 1,320 ± 140 (IIB) μm², and the mean CSAs for the CII group were 780 ± 60 (type I), 810 ± 60 (IIA), and 1,370 ± 110 (IIB) μm². Despite the significant changes at RNA and protein levels, fiber CSA was maintained in the early phase in response to CII.

**DISCUSSION**

Dysregulated autophagy and chaperone expression have been reported in many pathobiological conditions including different myopathies and neurodegenerative diseases (18, 72, 73). However, the relevance and impact of these processes on immediate molecular and cellular events in critical illness-associated muscle weakness have not been elucidated. In our previous study we used a porcine model to analyze the temporal effect of 5 days of immobilization and mechanical ventilation on limb...
muscle (6). Using this model we demonstrated that chaperones, UPS, cathepsins, and oxidative stress response genes were up-regulated, while transcripts of sarcomeric proteins, extracellular matrix proteins, and mitochondrial/oxidative phosphorylation genes were down-regulated (6). Analysis of the porcine masticatory muscle, which is reported to be spared in CIM patients, followed upregulated chaperone expression similar to that previously described in the limb muscle, which confirms our hypothesis that chaperones play a protective mechanism in skeletal muscle in ICU setup (1). In the present study we demonstrate that core autophagy machinery and chaperone expression are impaired simultaneously in response to CII in skeletal muscle.

The impaired conversion of LC3B-I to LC3B-II and the decreased protein expression of ATG7, ATG5, and p62 indicate that the core lysosomal autophagy system is profoundly impaired in skeletal muscle in response to CII. It has been previously shown that skeletal muscle-specific ATG7-null mice show an age-dependent impairment of force production in addition to atrophying muscle and impaired autophagy. Disorganized myofibrils with misaligned Z-line, swollen mitochondria, and dilated sarcoplasmic reticulum have been reported in these mice (54). The skeletal muscle-specific ATG5-deficient mice show impaired autophagy, muscle loss, protein aggregates, and accumulation of abnormal membranous structures (66). p62/SQSTM1 is an LC3 interacting protein that is essential for autophagic degradation (63). p62/SQSTM1 knockdown results in impaired conversion of LC3-I to LC3-II in a model of cardiac proteinopathy (93). Recently it was shown that LC3-II preferentially binds to p62 and efficiently localize to autophagic compartments (50). However, skeletal muscle-specific ATG7 and ATG5 knockout mouse models show ubiquitin and p62-positive inclusions (54, 66), an observation that does not fit with the decreased p62 protein expression observed in this study. Recently, decreased autophagic vacuoles and increased membranous structures were reported in postmortem skeletal muscle biopsies of critically ill patients, suggesting insufficient activation of autophagy (87). Autophagy actively maintains cellular and protein homeostasis by removing non-native proteins as well as damaged cellular components including mitochondria (mitophagy) and ER (reticulophagy) (38, 42). Therefore autophagy inhibition in skeletal muscle could lead to dysfunctional mitochondria and ER, which in turn could lead to a new cycle of exacerbated ER stress and oxidative stress, eventually triggering apoptosis.

Expression profile analyzed by microarray indicates that several chaperones including ER chaperones were downregulated at RNA level in the CII group. We hypothesized that the ER stress sensor BiP/GRP78 would be increased in the CII group, indicating ER stress in these muscles, but there was no change in BiP/GRP78 protein expression. However, the phosphorylated EIF2A/total EIF2A ratio was increased, suggesting that UPR is initiated in the CII group. In stressed conditions, BiP/GRP78 dissociates from transmembrane protein PKR-like endoplasmic reticulum kinase (PERK), which results in autophagy and subsequent EIF2A hyperphosphorylation. Phosphorylated EIF2A results in attenuation of protein synthesis. During this translational attenuation, only select mRNA such as ATF4 are translated. Prolonged ER stress causes ATF4 to induce apoptosis via C/EBP homologous protein. Moreover, skeletal muscle-specific attenuation of ATG7 expression results in a profound increase in BiP/GRP78, as well as phosphorylated EIF2A at the protein level (54).

We demonstrate that CRYAB (small HSP family), as well as HSP70 and HSPA4 (HSP110 family), is indeed decreased at the protein level. CRYAB is associated with preventing aggregate formation by binding to unfolded proteins. A missense mutation in CRYAB (CRYABR120G) causes desmin-related myopathy with intracellular aggregates and muscle weakness (88). HSP70 is a key chaperone family involved in recognition of nonnative proteins in collaboration with HSP40 cochaperones and subsequent refolding or even disaggregation by cooperating with HSP100 (16). Previously, we and others have shown that upregulation of chaperones is associated with a protective role in skeletal muscle in response to immobilization (6, 78).

Moreover, the decrease in LAMP-2A protein expression in the CII group strongly suggests that CMA is impaired in the CII group. LAMP-2-deficient mice develop a progressive myopathy of skeletal and cardiac muscles (57). Force generation and Z disk integrity is compromised due to Z disk disintegration and the rupture of sarcomeric structures in these mice (3).

Oxidative damage by reactive oxygen species (ROS) modifies protein structures or fragment proteins, which leads to an accumulation of misfolded protein (35). ROS and oxidative damage have been implicated in a range of disorders. Oxidative stress-responsive genes act in defense against oxidative stress and maintain redox balance. Conversely, deficiency in oxidative stress response mechanisms exacerbates the oxidative damage (15). In our previous study, using immobilized and mechanically ventilated pigs, we have shown that oxidative stress-responsive genes are upregulated in the expression profile (6). In this study, in response to CII, oxidative stress-responsive genes were downregulated, suggesting that protection from ROS is compromised in these pigs. In fact, skeletal muscle-specific ATG7-null mice show increased oxidative stress and upregulation of oxidative stress-responsive genes (54). Moreover, p62 is shown to stabilize nuclear factor erythroid 2-related factor 2 (NRF2) and activate NRF2 target genes including oxidative stress-responsive genes (41). We have observed decreased protein expression of p62 with par-
allel downregulation of oxidative stress-responsive genes that associate impaired oxidative response in response to CII with p62/NRF2 pathway in skeletal muscle.

The increased expression of the translational repressor EIF4EBP1 at the RNA and protein levels and the decrease in phosphorylated EIF4EBP1 indicate that protein synthesis is attenuated, inhibiting the IGF/AKT/mTOR signaling axis (14, 68). Together with EIF2A-mediated attenuation of protein synthesis via BiP/PERK axis, the CII exerts a drastic negative impact on skeletal muscle protein synthesis. IGF/ AKT/mTOR pathway is inhibited by glucocorticoid (dexamethasone), leading to attenuation of protein synthesis and activation of FOXO transcription factors and muscle-specific ubiquitin ligases (82). Increased protein expression of ACVR2B suggests increased myostatin signaling, which could further downregulate protein synthesis in the CII group. However, the protein expression of muscle-specific E3 ubiquitin ligase atrogin-1 did not change in response to CII. Unchanged atrogin-1 expression, together with impaired autophagy, indicates that protein degradation pathways do not positively contribute to proteolysis in the early phase in ICU. On the other hand, proteasome inhibition results in muscle atrophy, loss of sarcomeric architecture, vacuolization, and aggregate formation, suggesting that basal level of UPS activity is required for maintaining skeletal muscle cellular architecture (27).

Despite the profound molecular and cellular changes in response to CII, the fiber CSA was maintained in the CII group, indicating that muscle fiber atrophy does not occur in the early phase of ICU stay, which is in accordance with our previous observations in the porcine ICU model (1, 61). This observation is not surprising and could be partly explained by the impaired autophagy and unchanged E3 ligase, atrogin-1 protein expression. Similar observations have been reported in patients with CIM, indicating that onset of the disease and atrophy of the muscle occur at a later phase of ICU stay, ranging from at least 4 days to 1 wk after the admission to ICU (2, 84). Myosin-actin ratios were maintained in these muscles during the 5-day study period despite the downregulation of myosin type I at RNA level, suggesting that major contractile proteins are not affected at this early phase of exposure to CII, and atrophy is an unlikely event at this early time point.

ER plays a key role in autophagosome formation by providing a platform for expansion of autophagosomal membranes and emergence of fully formed autophagosomes (5). ER stress and UPR trigger autophagy, which suggest a close interplay between the two systems and may play a regulatory role in protein quality control and proteostasis (9, 91).

The proteostasis network is a complex system that integrates the diverse chaperone systems with UPS and autophagy lysosome system (28). Since the expression profile and the protein expression results suggest a general and ER-specific breakdown of chaperone RNA expression and protein expression, taken together with profound impairment in autophagy lysosome system, we propose that proteostasis may be the key process affected in skeletal muscles during critically illness.

Conclusion

Skeletal muscle undergoes continuous remodeling as an adaptive response to physiological, mechanical and metabolic stresses. Proteostasis, or protein quality control, plays a crucial physiological role in the maintenance of skeletal muscle and sarcomere, in response to various stresses. Chaperones are not only involved in folding and refolding of misfolded or damaged proteins, but also in targeting the terminally misfolded or irreversibly damaged proteins for degradation. On the other hand, autophagy plays a major role in cellular homeostasis as well proteostasis in skeletal muscle. Thus, taking that knowledge together with the novel findings in this study, we conclude that the CII impairs autophagy, chaperone expression, and protein synthesis and may adversely affect overall proteostasis in skeletal muscle, leading to long-term catastrophic cellular and molecular events.

ACKNOWLEDGMENTS

We are grateful to Klas Kullander and Kalicharan Patra for providing excellent laboratory facilities. We thank Ted Ebendal and Charlotte Israelsson for critically reviewing the manuscript and offering valuable suggestions. We are grateful to Yvette Hedström, Ann-Marie Gustafson, and Annika Kylberg for excellent technical assistance. We thank Rongye Shi for generating array data.

GRANTS

This study was supported by grants from the Swedish Research Council (6851), the European Commission (Myoage, EC Ft CT-223756, and COCT CM 1001), Association Française Contre les Myopathies, the National Centre for Medical Rehabilitation Research, Uppsala University Hospital to L. Larsson and Karolinska Foundations and Stockholm Council to P. Radell and L. I. Eriksson.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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