Hypermetabolism and Hypercatabolism of Skeletal Muscle Accompany Mitochondrial Stress Following Severe Burn Trauma

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Running Head: Muscle Metabolism after Burns

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Abstract

Burn trauma results in prolonged hypermetabolism and skeletal muscle wasting. How hypermetabolism contributes to muscle wasting in burn patients remains unknown. We hypothesized that oxidative stress, cytosolic protein degradation, and mitochondrial stress as a result of hypermetabolism contributes to muscle cachexia post-burn. Patients (n=14) with burns covering >30% of their total body surface area (TBSA) were studied. Controls (n=13) were young healthy adults. We found that burn patients were profoundly hypermetabolic at both the skeletal muscle and systemic levels, indicating increased oxygen consumption by mitochondria. In skeletal muscle of burn patients, concurrent activation of mTORC1 signaling and elevation in the fractional synthetic rate (FSR) paralleled increased levels of proteasomes and elevated fractional breakdown rate (FBR). Burn patients had greater levels of oxidative stress markers, as well as higher expression of mtUPR-related genes and proteins, suggesting that burns increased mitochondrial stress and protein damage. Indeed, upregulation of cyto-protective genes suggest hypermetabolism-induced oxidative stress post-burn. In parallel to mtUPR activation post burns, mitochondrial specific proteases (LONP1 and CLPP), and mitochondrial translocases (TIM23, TIM 17B, and TOM40) were upregulated, suggesting increased mitochondrial protein degradation and transport of pre-protein, respectively. Our data demonstrate that proteolysis occurs in both the cytosolic and mitochondrial compartments of skeletal muscle in severely burned patients. Increased mitochondrial protein turnover may be associated with increased protein damage due to hypermetabolism-induced oxidative stress and activation of mtUPR. Our results suggest a novel role for the mitochondria in burn-induced cachexia.

Keywords: Burn injury; Cachexia; hypermetabolism-induced oxidative stress; mitochondrial unfolded protein response; mitochondria proteases
INTRODUCTION

Burn trauma results in a profound hypermetabolic and hypercatabolic stress response that persists for several months after injury (12). Several studies have implicated catecholamines as the primary mediators of hypermetabolism (26, 67), in a state in which increased whole-body oxygen consumption supports greater ATP turnover and thermogenesis in critically injured burn patients. Skeletal muscle cachexia resulting from a chronic imbalance between protein synthesis and breakdown (12, 18), leads to significant long-term morbidity in burn survivors (67). While the degree of hypermetabolism and skeletal muscle catabolism are correlated in burn survivors (18), whether a common mechanism links burn-induced hypermetabolism and protein wasting remains unclear.

It has previously been shown that skeletal muscle becomes hypermetabolic in response to burns (68). Recent reports from our laboratory demonstrated that skeletal muscle mitochondria contribute to the hypermetabolic response in burn patients (49, 51, 60). Given their role in oxidative phosphorylation, mitochondria are a principal site of cellular reactive oxygen species (ROS) generation (42, 62). Respiratory complexes in the electron transport chain, with the exception of complex IV, may leak electrons to oxygen, forming superoxide anions \( (\text{O}_2^-) \) (12, 13, 62). The superoxide anion is a precursor to many other ROS and can activate other oxidative reactions (62, 64). A persistent elevation in mitochondrial respiration and superoxide production in skeletal muscle predisposes mitochondrial proteins to oxidation. Although mitochondria are normally capable of eliminating superoxide, sustained increases in the mitochondrial respiration and thus superoxide production in the skeletal muscle of burn victims may result in the chronic damage to mitochondrial proteins and activation of mitochondrial unfolded protein response (mtUPR) (11, 38, 41, 61).

Hypermetabolism-induced oxidative stress may contribute to mtUPR activation when the proteotoxic stress within the mitochondrion exceeds protein-folding capacity of chaperones (41,
44). When the protein-folding capacity of the chaperones is exceeded accumulation of misfolded or aggregated protein ensues. To protect the cellular compartments from the deleterious effects of protein misfolding and aggregation (21, 44), stressed cells use two mechanisms. They activate organelle-specific proteases such as LONP-1, CLPP, and YME1L1, to help degrade misfolded and damaged proteins (4, 44). In addition, mtUPR is activated to help increase chaperone capacity and re-establish homeostasis within the mitochondrial protein-folding environment (41, 44). This can be accomplished by transducing the stress response to the nucleus to help induce transcription factors responsible for proteostatic surveillance and synthesis of new peptide (32, 41).

Given their locality to the respiratory complexes, mitochondrial proteins are more susceptible to oxidative damage than cytoplasmic proteins (13, 62, 63). However, since most mitochondrial proteins are encoded in the nucleus, new proteins must be translocated from the cytoplasm via mitochondrial translocase proteins to replace damaged proteins (45, 61, 66). In this study, we hypothesized that persistent hypermetabolism-induced oxidative stress leads to cumulative oxidative damage of mitochondrial proteins and activation of the unfolded protein stress response. We also hypothesized that these result in an increase in mitochondrial protein turnover, which leads to an upregulation of mitochondrial translocase proteins and influx of replacement pre-proteins from cytoplasm (nucleus) of the skeletal muscle cell. Our results show that protein degradation takes place in both mitochondrial and cytosolic cellular compartments of skeletal muscle in burn patients. Therefore, our data provide a novel mechanistic association between burn-induced hypermetabolism and muscle wasting.
MATERIALS AND METHODS

**Subjects**: The study protocol was reviewed and approved by the Institutional Review Board (IRB) at the University of Texas Medical Branch (UTMB), Galveston, Texas. We recruited 14 adult burned patients who were admitted to the Blocker Burn Unit at UTMB and teenage children admitted to Shriners Hospitals for Children (Galveston, TX) with total burn surface area (TBSA) burn of >30% were recruited. Upon admission, all burn patients received standard burn care including fluid resuscitation and total wound excision. Sequential wound grafting was performed based on the individual need of the patients until burn wounds were closed. Patients were placed on a standard nutritional protocol and received a low-fat diet (82% carbohydrate, 3% fat, 15% protein) (Vivonex T.E.N.; Nestle Health Science, Minneapolis, MN) based on their burn size (1,500 kcal/m² body area + 1,500 kcal/m² body area burned). Nutrition was provided via nasogastric tube until patients were able to tolerate oral feeding. Resting energy expenditure (REE) was determined by indirect calorimetry (65) and compared to the Harris-Benedict derived REE (56) to determine the degree of systemic hypermetabolism.

We recruited 13 healthy control subjects from the Galveston County area via local advertisement to serve as unburned controls. These participants were admitted to the Clinical Research Center at the UTMB. Informed and written consent was obtained before participation in the study. After an overnight fast muscle biopsy was collected from *m. vastus lateralis* using a suction-adapted Bergstrom needle (6).

**High-resolution respirometry**: Skeletal muscle mitochondrial respiration (metabolism) was determined as initially described (48). Briefly, approximately 10mg of skeletal muscle was permeabilized by agitating myofiber bundles in 2 ml of mitochondrial preservation buffer containing 20 mM saponin at 4°C for approximately 30 min. Thereafter, myofiber bundles were washed in 2 ml of mitochondrial respiration buffer containing 0.5 mM EGTA, 3 mM MgCl₂, 60
mM K-lactobionate, 20 mM taurine, 10 mM KH$_2$PO$_4$, 20 mM HEPES, 110 mM sucrose, and 1
mg/ml essential fatty acid-free bovine serum albumin (pH 7.1) at 4°C for approximately 15 min.
Next, approximately 2–4 mg of myofiber bundles were blotted onto filter paper and weighed on
a precision micro-balance (Mettler-Toledo, Zaventem, Belgium) prior to being transferred to an
Oxygraph-O2K respirometer chamber (Oroboros Instruments, Innsbruck, Austria) where they
were suspended in 2 ml of mitochondrial respiration buffer. Respiration measurements were
made on the same day as sample collection, typically within 6 h of harvest. During experiments,
temperature was maintained at 37°C and O$_2$ concentration within 250–400 nmol/ml.
Mitochondrial O$_2$ flux was computed and recorded at 2–4 s intervals by DatLab software
(Oroboros Instruments, Innsbruck, Austria). Mitochondrial respiratory capacity and function were
assayed in permeabilized skeletal muscle samples by the addition of a saturating concentration
of substrates (1.5 mM octanoyl-L-carnitine, 5 mM pyruvate, 2 mM malate, 10 mM glutamate and
10 mM succinate), followed by the addition of adenosine diphosphate (5 mM), cytochrome C (10
mM), and the mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazin to a final
concentration of 5 mM. Resulting values were normalized using citrate synthase protein.

**Skeletal muscle protein kinetics study:** The fractional synthetic rate (FSR) and
breakdown rate (FBR) of mixed muscle proteins were determined according to the protocol
described previously (12). Briefly, baseline plasma samples were collected to determine
background isotopic enrichment prior to bolus injections of L-[ring-$^{13}$C$_6$] Phe (5.56 mg/kg) and L-
[$^{15}$N] Phe (5.40 mg/kg) administered intravenously at 0 and 30 min, respectively. Blood samples
were collected into heparinized sample tubes at various time points (5, 10, 15, 20, 29, 35, 40,
50, and 60 min) after the injection of the initial tracer bolus. Muscle samples were collected from
the *m. vastus lateralis* at 10 min and 60 min after the injection of the initial tracer bolus with the
aid of suction-adapted Bergstrom needle (6). Muscle samples were rinsed with saline to remove
all visible blood, and then rapidly frozen in liquid nitrogen.
Plasma and muscle phenylalanine enrichments were determined in tert-butyldimethylsilyl derivatives using an Agilent 6890 gas chromatograph-mass spectrometer. Ions were selectively monitored at mass-to-charge (m/z) ratios of 336, 337, 340, 342, and 346 for phenylalanine enrichment. Isotopic enrichments were expressed as tracer-to-tracer ratio. Muscle concentrations of free intracellular phenylalanine were calculated from the internal standard (46). The precursor-product method was utilized to calculate muscle FSR (Equation 1), while muscle FBR was calculated from the decay in intracellular and plasma enrichment (Equation 2) (70).

\[
\text{FSR} = \frac{EB(t) - EB(0)}{\int_{t_0}^{t_1} EF(t) \Delta t} \quad \text{Equation 1}
\]

\[
\frac{[EM(t_2) - EM(t_1)]_t}{\int_{t_1}^{t_2} EM(t) \Delta t} \frac{[EA(t) - EM(t_3)]_t}{\int_{t_1}^{t_2} EA(t) \Delta t} - \frac{[EM(t_2) - EM(t_1)]_t}{\int_{t_1}^{t_2} EM(t) \Delta t} \frac{[EA(t) - EM(t_3)]_t}{\int_{t_1}^{t_2} EA(t) \Delta t} \frac{\left(\frac{OM(t)}{t}\right)}{t} \quad \text{Equation 2}
\]

FSR and FBR data were obtained in healthy young men in a previous study by our group (16) and are presented here as healthy (normal) values to provide a comparison to data obtained in burn victims.

**Gene expression:** RNA was extracted from approximately 50 to 100 mg of muscle tissue from both control subjects and burn patients using the Pure Link RNA isolation kit according to manufacturer’s protocol (ThermoFisher Scientific, Waltham, MA). Isolated RNA was quantitated using a Biotek Take3 micro-volume plate reader (Biotek Instrument, Inc, VT). cDNA was synthesized using a High-Capacity RNA-to-cDNA Kit according to the manufacturer’s protocol (ThermoFisher Scientific, Waltham, MA). Gene expression was analyzed in both burn patients and healthy controls using quantitative PCR performed with the StepOne Plus real-time PCR system (Applied Biosystems Inc, Waltham, MA) and using Gene Expression master mix with the following TaqMan gene assays: Hs00998404_m1 (LONP1), Hs00204609_m1 (YME1L1), Hs00231457_m1 (NFE2L1), Hs00975961_g1 (NFE2L2), Hs00202227_m1 (KEAP1),
Hs00997642_m1(VCP/P97), Hs00427357_m1(PSMB1), Hs01002946_m1(PSMB2),
Hs00605652_m1(PSMB5), Hs01027360_g1(PSMA1), Hs01113429_m1(PSMD14),
Hs0160631_m1(PSMD1), Hs01029472_g1(PSMC5), Hs00426616_g1 (ACO2),
Hs00158095_m1(ACO1), Hs00533490_m1(SOD1), Hs00167309_m1(SOD2),
Hs00428953_g1(PRDX3), Hs00156308_m1(CAT), Hs00357891_S1(JUNB), Hs00232586_m1
(ATF6), Hs01922818_s1(CEBPγ), Hs00270923_s1(CEBPβ), Hs00358796_g1 (CHOP/DDIT3),
Hs00430663_g1(UBL5), Hs00269972_s1(CEBPα), Hs00177102_m1(JNK2/MAPK9),
Hs00195655_m1(CLPP), Hs01103582_s1(c-JUN), Hs01654720_g1 (HSP10),
Hs01036753_g1(HSP60), Hs03043881_g1(HsP90AB) and Hs02758991_g1(GAPDH)
(ThermoFisher Scientific, Waltham, MA). Expression of the target genes was normalized to that
of the house keeping gene, GAPDH. Relative gene expression data analyses were performed
using DataAssist™ Software Version 3.01(Life Technologies) to derive delta-delta CT values.
Final data are express in fold difference of burn patients to control.

**Western blotting analysis:**

**Antibodies:** Antibodies to LONP1 (no. 15440-1-AP), TIMM17B (no. 11062-1-AP), and TIM23
(no. 11123-1-AP) were from Proteintech (Rosemont, IL); PSMD14 (no. 7662S), SOD1 (no.
2770S), SOD2 (13194S), catalase (14097S), ACO2 (6922S), p-4EBP1(T37/46) (no. 2855S),
phospho-S6 ribosomal protein (Ser240/244) (no.5364S), p-mTOR (Ser 2448)(no. 5536S), P-p70
S6 kinase (T389) (no. 9234S), p-eEF2 (T56) (no.2331S), p-eIF2 alpha (S51) (no. 9721S), p-
eE2K (S366) (no.3691S), CHOP (no. 2895S), HSP60 (4869S), HSP90(4877S), and JunB (no.
3753S) were from Cell Signaling Technology (Denver, MA); NFE2L1/NrF1(sc-13031) were from
Santa Cruz (Dallas, TX); NFE2L2/NrF2 (ab62352), proteasome 20S α+β (ab22673),
proteasome 20S C2 (ab109530), YME1L1 (ab170123), 4-hydroxynonenal (ab48506),
nitrotyrosine (ab125106), TOM40 (ab51884), PRDX3 (ab73349), ATF-6(ab203119), rabbit
horseradish-peroxidase-labelled anti-mouse (ab6728), and donkey horseradish-peroxidase-
labelled anti-rabbit (ab6802) were from Abcam (Cambridge, MA); and GAPDH (no. G9545) were from Sigma-Aldrich (St. Louis, MO).

**Gel electrophoresis and protein detection:** Proteins were separated using SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Muscles samples were homogenized and protein was extracted using T-PER tissue extraction reagent (ThermoFisher Scientific, Rockford, IL) containing protease and phosphatase inhibitors. Twenty micrograms (20 µg) of the skeletal muscle homogenate protein from control and patient muscle samples was suspended in NuPAGE® LDS Sample Buffer, heated at 70°C for 10 min, rapidly cooled on ice, and then loaded onto 4-12 % Bis-tris gels or 3-8% Tris-acetate gel depending on the molecular weight of the protein of interest (ThermoFisher Scientific, Waltham, MA). Gels were run for 120 min at 100 V using the X-SureLock Mini-Cell electrophoresis system (Life Technologies, USA). NuPAGE 2-(N-morpholino) ethanesulfonic acid-sodium dodecyl sulfate (MES-SDS), MOPS (3-Morpholinopropane-1-sulfonic acid) or Tris-acetate SDS running buffer (Life Technologies, USA) was used depending on the molecular weight of protein of interest. Protein was then transferred to a nitrocellulose membrane using transfer-buffer (ThermoFisher Scientific, Waltham, MA) containing 20 % methanol at 20 V for 2 h. Blots were blocked using 5% (w/v) nonfat milk (BioRad, Hercules, CA) or bovine serum albumin (Cell Signaling) in TBS with 0.1% Tween 20 (TBST) for 1 h at room temperature. Membrane blots were then incubated with the antibody of interest in 5 % non-fat milk or BSA in TBST at 4°C overnight. Blots were washed three times with TBST for 15 min each before probing with either donkey anti-rabbit or rabbit anti-mouse secondary horseradish peroxidase conjugated antibodies (Abcam, Cambridge, MA) at a 1:10,000 dilution for 1 h at room temperature. Blots were washed with TBST, and visualized by autoradiography after incubation of blots in Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL) for 1 min at room temperature according to the manufacturer’s instructions. Autoradiographs were then scanned and band
areas were measured using ImageJ 1.48 software (NIH, Bethesda, MD). Resulting values were normalized to those obtained for GAPDH.

**Statistics:** Statistical analysis was performed and graphs created using GraphPad Prism version 6 (GraphPad Software, La Jolla). Student’s *t*-tests were used to compare controls and burn patient data, with *p* < 0.05 being considered significant. Both control and burn patient data are presented as mean ± standard error mean (SEM), with one exception. Data related to subject/patient characteristics are presented as mean ± standard deviation.
RESULTS

**Subject characteristics:** As shown in Table 1, healthy controls (n=13) and burn patients (n=14) did not differ with regards to age, weight, height, or body mass index (BMI) at the time of their admission to the intensive care unit. Burn patients had large injuries, encompassing 63 ± 22% of their TBSA, with full-thickness burn lesions encompassing 54 ± 23% of the TBSA. Both weight and BMI of burn victims declined significantly during hospitalization (P<0.05). On average, burn patients were hospitalized for 58±46 days.

*The hypermetabolic hypercatabolic response to burns:* The metabolic rate in burn patients was 43±7 % above normal (Fig. 1A). At the level of skeletal muscle, burn patients had greater coupled (P) and leak (L) mitochondrial respiration, indicating per mitochondria, O2 consumption was greater in burn patients compared to controls (Fig. 1B). Burn patients lost 18 ± 3 % (15 ± 3 kg) of their body mass during hospitalization (Fig. 1C; P<0.05 vs healthy controls). Lean body mass (Fig. 1D) and bone mineral content (Fig. 1E) were lower in burn victims at hospital discharge than in healthy controls.

Compared to a previously published (16) healthy control group, skeletal muscle protein turnover was significantly altered in burn patients. Skeletal muscle FSR was 1.6-fold higher in burn patients (0.086±0.013 %/h vs 0.06±0.006 %/h, P<0.05; Fig. 1F). Skeletal muscle FBR was 3.5-fold greater in burn patients than in healthy controls (0.354 ± 0.089 %/h vs. 0.100 ± 0.010 %/h P<0.001; Fig. 1F). Net protein balance (i.e., difference between FSR and FBR) was approximately 6-fold higher in burn patients (-0.268±0.091 %/h) than in healthy controls (-0.045 ± 0.010 %/h; Fig.1F).

*mTOR signaling:* Protein synthesis in burn patients and healthy controls was further explored through western blot analysis of the master growth regulator, mTOR and its targets p70S6K and 4E-BP1 (Fig. 2). Phosphorylation of mTOR at Ser2448 was significantly higher in
burn patients compared with healthy controls ($P<0.01$; Fig. 2A). In addition, phosphorylation of 4E-BP1 at Thr$^{37/46}$, S6rp at Ser$^{240/244}$, and p-eEF2 at Thr$^{56}$ was greater in burn patients ($P<0.05$; Fig. 2B-D). However, phosphorylation of p70S6K at Thr$^{389}$, Eef2k at Ser$^{366}$, and EeF2 at Ser$^{51}$ did not differ between the two groups ($P>0.05$; 2E, 2F, and 2G).

**Mitochondrial oxidative stress**: One of the primary objectives of this study was to determine whether hypermetabolism was associated with mitochondrial oxidative stress in skeletal muscle. We demonstrated that burn trauma induced up-regulation of SOD1, SOD2, and PRDX3 genes by 2.6-, 16.1, and 1.5-fold, respectively, compared to healthy controls ($P<0.001$, $P<0.05$, and $P<0.01$, respectively; Fig. 3A). Similarly, catalase gene expression was 5.2-fold greater in burn patients than in controls ($P<0.05$; Fig. 3A). SOD1, SOD2, and CAT proteins were all elevated in burn patients ($P<0.001$, $P<0.01$, and $P<0.05$ respectively; Fig. 3D-F). Conversely, PRDX3 protein, did not differ between groups ($P>0.05$; Fig. 3E). Nitrosylated tyrosine, a hallmark of amino acid oxidation and cell damage (54), was ~80% higher in burn patients compared with controls, ($P<0.01$, Fig. 3B). Levels of 4-hydroxynoneal (HNE), a by-product of lipid peroxidation, were not significantly different between burn patients and healthy controls (Fig. 3C).

**Aconitase**: Mitochondrial aconitase (ACO2) inactivation is a known source of hydroxyl radical formation. Cytosolic aconitase (ACO1) gene expression was 2.5-fold higher in burn patients ($P<0.01$; Fig. 3A). ACO2 mRNA levels were comparable between groups ($P>0.05$; Fig. 3A), but ACO2 protein levels were higher in burn patients than in controls ($P<0.001$; Fig. 3H).

**Mitochondrial unfolded protein response**: Stress response to oxidative stress or accumulation of misfolded proteins in the mitochondrial matrix and intermembrane space may result in the induction of mtUPR genes to help bolster chaperone capacity(44). Accumulation of unfolded proteins within mitochondrial matrix stimulates JNK2 and c-JUN to upregulate CHOP
CHOP subsequently activates chaperones to restore the mitochondrial protein-folding environment. Quantitative PCR analysis revealed that, compared to controls, burn patients had 1.5-fold higher JNK2 expression, 4.4-fold higher c-JUN expression, and 2.6-fold higher CHOP genes expression (P<0.05; Fig. 4A). Other factors needed for the activation of CHOP were elevated in burn patients. JUNB increased by 103.3-fold (P < 0.01 vs. control), CEBPβ by 4.0-fold (P < 0.01), CEBPα by 8.5-fold (P < 0.001), and CEBPγ by 3-fold (P>0.05; Fig. 4A). Furthermore, UBL5 was 2.5 fold higher in burn patients than in controls (P<0.001; Fig.4A), suggesting mitochondrial stress was transduced to the cytoplasm. The ultimate goal of the mtUPR is the upregulation of chaperones. Indeed, burn patients had 2.7-fold higher HsP90 mRNA levels, 3.7-fold higher HsP60 mRNA levels, and a 2.6-fold higher HsP10 mRNA (P<0.01for all; Fig. 4A). Burn patients had not only higher expression of mtUPR genes than controls, but also higher HSP90 and CHOP proteins (P<0.05; Fig.4B-C). HSP60 and JUNB were also expressed to a higher degree in burn patients, but this was not significant (P>0.05; Fig. 4D and F). ATF6 protein was slightly higher in burn patients (P=0.596; Fig. 4E).

**Cyto-protective transcription factors:** In response to oxidative stress, KEAP1/NFE2L2 signaling augments antioxidant capacity(23). NFE2L2 mRNA expression was 4.7-fold higher in burn patients than in controls, while KEAP1 was 1.8-fold higher (P<0.01; Fig. 5A). Surprisingly, NFE2L2 protein levels was lower in burn patients than in controls despite significant transcriptional up-regulation (P<0.05; Fig. 5C). Further, investigation of other genes revealed that NFE2L1 and VCP/P97 were 2.2- and 2.5-fold greater in burn patients than healthy controls, respectively (P<0.001; Fig. 5A). NFE2L1 protein levels were also lower in burn patients (P<0.01; Fig.5D), most likely since both NFE2L1 and NFE2L2 are from the same family of human genes encoding basic leucine zipper (bZIP) transcription factors.

**20S and 19S proteasomes:** Ubiquitin-mediated proteolysis is central to muscle atrophy (2). The 26S proteasome plays an important role in the degradation of ubiquitinated proteins,
and is composed of a 20S core and 19S regulatory subunits (35). 20S and 19S subunits mRNAs were upregulated in burn patients compared to healthy controls (Fig. 5B). Specifically, PSMB1 mRNA was increased by 2.6-fold (P < 0.001 vs. control), PSMB2 mRNA by 3.6-fold (P < 0.01), PSMB5 mRNA by 1.8-fold (P < 0.01), and PSMA1 by 3.0-fold (P<0.00; Fig. 5B). In addition, burn patients showed 2.3-fold higher expression of PSMD1 mRNA, 2.8-fold higher expression of PSMD14 mRNA, and 2.8-fold higher expression of PSMC5 mRNA (P<0.01; Fig. 5B). Interestingly, proteasomal protein expression showed a trend towards being greater in burn patients than in controls (Fig. 5E-G), though the increase was significant for only the α+β subunits of 20S (P<0.05; Fig.5G). Further, we found that mitochondrial respiration in skeletal muscle was significantly correlated with proteasome gene expression (r=0.71, P<0.01), suggesting a correlation between cellular respiration rates and the induction of proteolytic machinery in the cell cytosol.

Mitochondrial proteases (LONP1, CLPP and YME1L1) and translocase proteins:

LONP1 and CLPP are mitochondrial proteases responsible for degradation of oxidized or unfolded protein within the matrix, while YME1L1 is responsible for degrading damaged proteins in the intermembrane space (61). LONP1 and CLPP mRNA levels were 2.4 and 2.2-fold higher in burn patients compared with healthy controls, respectively (P<0.001; Fig.6A). Accordingly, burn patients had ~60% LONP1 protein abundance than healthy controls (P<0.01, Fig. 6B). YME1L1 mRNA was 2-fold higher in burn patients than in controls (P<0.01; Fig. 6A). This was accompanied by lower YME1L1 protein expression (P<0.01; Fig. 6C). Interestingly, we found that mitochondrial respiration in skeletal muscle was significantly correlated with LONP1 gene expression (r=0.86, P<0.001), suggesting a strong correlation between cellular respiration rates and in induction of mitochondrial proteases.

Mitochondrial membrane translocase proteins were also analyzed, as increased expression of proteins suggests increased mitochondrial protein demand in burn patients'
muscle. Expression of the inner membrane translocase protein 23 (TIM23) was ~50% higher in burn patients than in controls ($P<0.05$; Fig. 6D), while expression of the inner membrane translocase protein 17B (TIM17B) was ~55% higher in burn patients ($P<0.05$; Fig. 6E). Expression of the outer membrane translocase protein 40 (TOM40), the main constituent of the general import pore, was significantly greater in burn patients than in controls ($P<0.05$; Fig. 6F).
The present study demonstrates a novel association between hypermetabolism and skeletal muscle protein degradation in burn patients. Elimination of damaged proteins is mainly carried out by proteasomes (28-30), which are primarily found in the nucleus, endoplasmic reticulum (ER), and cytoplasm of the cell, but not in the mitochondria (43). In the context of severe burn injury and the profound hypermetabolism that accompanies this unique type of trauma, we hypothesized that mitochondrial oxidative stress and mtUPR also contribute to the marked muscle cachexia seen in burn patients. To address this hypothesis, we studied skeletal muscle protein turnover, markers of oxidative stress, mtUPR, and the proteolytic machinery residing in both the cytoplasmic and mitochondrial compartments of skeletal muscle in healthy individuals and severe burned patients exhibiting hypermetabolism and cachexia.

While both FSR and FBR of skeletal muscle proteins were elevated in burn patients compared to data previously published by our group from healthy young men (16), massively elevated skeletal muscle FBR was the primary contributor to the protein wasting phenotype post burn. This finding is in agreement with our previous findings using cross-limb arterial-venous balance methods (7, 19) and tracer bolus injections approaches (12). This previous study showed that skeletal muscle protein metabolism is profoundly altered in burn victims. Not surprisingly, in the ~8 weeks they were hospitalized after injury, burn patients lost around 15kg of body mass (~18% of their admit body mass). Indeed, burn patients had significantly less body mass and in particular lower lean mass and bone mineral content than healthy controls.

In agreement with our stable isotope data showing that FSR of skeletal muscle proteins is greater in burn patients than in healthy controls, the molecular machinery involved in protein anabolism is also upregulated in the skeletal muscle of burn patients. Phosphorylation of mTORC1 and its down-stream targets 4E-BP1 and S6rp was significantly greater in burn
patients. Burn patients exhibit a catabolic endocrine response and undergo prolonged denervation post-injury, making the observation of greater protein synthesis in skeletal muscle somewhat curious. However, we have previously shown that massive skeletal muscle proteolysis results in increase intracellular free amino acid concentrations within muscle cells (19). This chronic elevation in the concentration of intracellular amino acids, including leucine, may explain why anabolic signaling and protein FSR are greater in burn patients (12, 19, 47, 52). Nevertheless, increased skeletal muscle FSR in burn patients did not match the increase in skeletal muscle FBR, resulting in a greater net loss of amino acids than seen in healthy controls. It is important to note, the increased phosphorylation of eEF2 at Threonine 56 (p-eEF2-T56). eEF2 is a GTP-dependent translocase that is responsible for the movement of nascent peptidyl-tRNAs from the A-site to the P-site of the ribosome (22). p-eEF2-T56 is involved in the inhibitory regulation of this step during protein translation elongation. Therefore, phosphorylation of eEF2 at threonine 56 is inversely related to the rate of protein elongation, thereby contributing to the overall decrease of protein synthesis (24). This process might have been activated post-burn to meet the significant amount of energy needed for protein breakdown. Proteins degradation in the cytoplasm, the nucleus, and mitochondria requires pathways that are highly energy-dependent (15). Energy costs for protein turnover have been estimated to account for up to a third of the total energy production during cell replication (34). This heightened energy demand needed for protein turnover and resulting in reduced peptide elongation might have contributed to the disproportional ratio of FSR to FBR in burn patients.

Burn patients were profoundly hypermetabolic, with resting energy expenditure approximately 45% above normal values. Our data show that, in addition to exhibiting systemic hypermetabolism, burn patients experience hypermetabolism at the level of skeletal muscle. This indicates greater oxygen consumption by mitochondria of burn survivors. The increased
oxygen consumption may further exacerbate the rate of superoxide production in the mitochondrial matrix, leading to mitochondrial stress and protein damage (40).

Approximately, 0.2 to 0.5% of oxygen consumed by the mitochondrion leaked out as superoxide (3). This implies that post-burn hypermetabolism will increase exposure of mitochondria proteins to superoxide and possibly lead to oxidative protein damage in skeletal muscle mitochondria. Meanwhile, oxidative damage increases the likelihood of a protein being sequestered for a degradative fate. Oxidation disrupts protein structure and function, and it can result in the formation of protein aggregates that interfere with cell homeostasis (43, 53, 62). In the current study, we found that skeletal muscle of burn patients had an increased accumulation of nitrotyrosine, an indicator of greater protein oxidation. Within the mitochondrial matrix, SOD2 helps convert $\text{O}_2^-$ to $\text{H}_2\text{O}_2$ which can easily be detoxified by glutathione peroxidase but in the event of increased production of $\text{H}_2\text{O}_2$, hydroxyl radicals ($\cdot\text{OH}$) will be produced (3). The hydroxyl radical can indiscriminately react with nucleic acid, amino acids, and lipids. Tyrosine nitration occurs when tyrosine is exposed to a peroxynitrite anion ($\text{ONOO}^-\cdot$) and nitrogen dioxide ($\cdot\text{NO}_2$), both of which are by-products of nitric oxide (NO) metabolism in the presence of superoxide ($\text{O}_2^-$) and hydrogen peroxide ($\text{H}_2\text{O}_2$) (54). The marked hypermetabolism seen in burn patients likely augments mitochondrial $\text{O}_2^-$ production. Alternatively, burn-induced mitochondrial dysfunction may also increase ROS generation, which, in turn, may increase uncoupling in the mitochondria as a compensatory mechanism against mitochondrial oxidative stress (50). Increased expression of HNE, a marker of lipid peroxidation, further corroborates oxidative protein damage in burn patients. Accumulating evidence suggests that lipid peroxidation can form protein abducts and exacerbates mitochondria dysfunction (3, 55). Importantly, HNE-induced protein adducts are not reversible, and studies suggest that profound accumulation of HNE-induced adducts can decrease proteasomal activity(23). Our findings are consistent with this idea, that burn patients exhibit significant increase mRNA and protein levels
of proteasomes and antioxidants (catalase, SOD1, SOD2 and PRDX3). Up-regulation of these proteins may have occurred in response to increase oxidative stress and served as a preventive measure to limit oxidative damage to proteins post-burn (31,43, 53). Unabated ROS generation can result in accumulation of oxidative protein damage and resultant unfolded proteins response stress due to the proteotoxic stress within mitochondria. The pathological effects of accumulation of unfolded proteins are not limited to the subcellular compartment and are dangerous for the entire cell (41, 48). Any possible pathologic effects may be prevented through activation of the mtUPR and mitochondria proteases.

The mtUPR is induced when proteotoxic stress exceeds protein folding capacity by chaperones, and the proteotoxic stress is sensed and transduced to the nucleus to re-establish homeostasis within the mitochondrial protein-folding environment (32, 41, 44). Accumulation of unfolded proteins within mitochondria results in the upregulation of JNK2 and c-JUN transcription factors, further resulting in the stimulation of the transcription factors CHOP and CEBPβ (44). CHOP and C/EBPβ transcription factors bind to specific mtUPR promoter elements and activate target genes such as Hsp60 and CLPP (41, 44). Upregulation of JNK2, c-JUN, CHOP, C/EBPβ, C/EBPα, Hsp60, CLPP, JUNB, and ATF6 genes suggests that mtUPR is activated in skeletal muscle of burn patients. These data indicate profound proteotoxic stress within the mitochondria environment and most importantly, suggest that this stress is transduced to the nucleus to help combat the accumulation of unfolded proteins within skeletal muscle mitochondria in burn patients. Upregulation of both HSP60 and HSP10 chaperones further support our hypothesis that in response to proteotoxic stress new proteins have to be transported into the mitochondria to meet increased protein degradation. Specifically, prior study by Zhao and colleagues shows that accumulation of unfolded protein within the mitochondrial matrix results in the upregulation of CHOP, HSP60, HSP10, CLPP, and mtDnaJ (71). This further confirms the association of mitochondrial stress with burn trauma. Important to note is
that activation of UPR may result in profound mitochondrial dysfunction and possibly mitophagy (58). Interestingly, data showing mtUPR in burn patients are scarce. Most burn related studies have focused on ER stress (14, 25). Our data also suggest activation of ER stress in skeletal muscle of burn patients, as reflected by upregulation of ATF6 and CHOP mRNA and protein. Furthermore, CHOP has been shown to be activated during both mitochondrial proteotoxic stress and extreme ER stress (27, 71), this suggest the possibility of ER stress induction post-burn. Although, both CHOP and C/EBPβ contains AP-1 binding sites in their promoter, this a major requirement for the induction of mtUPR but not for ER stress (27). The upregulation of AP-1 (JUNB) mRNA by 100-fold strongly suggest activation of mtUPR after severe burn trauma. Therefore, our study is the first to show that burn patient skeletal muscle cells experience not only endoplasmic reticular stress but also activation of mtUPR stress. There seems to be a crosstalk between mitochondrial stress and ER stress besides the communication between the mitochondria and the nucleus. Furthermore, UBL-5 protein levels have been reported to be increased in response to acute or chronic stress related to unfolded mitochondrial protein stress to help activate mtUPR (10). In addition to performing this function, it can combine with an as yet to be characterized protein (DVE-1) when the stress is transduced to the cytoplasm to help upregulate the HSP60 gene (5, 10, 20, 27). Hence, UBL-5 mRNA upregulation suggests that, after burns, the stress response in skeletal muscle mitochondria is transduced to the cytoplasm to help activate the nuclear steps required for mounting a response to the threat of mitochondrial protein misfolding (5, 20).

For protein turnover within the cytoplasm to proceed during stress, stress-related transcription factors are activated (23). The transcription factors nuclear factor erythroid 2-related factor 2 (Nrf2/NFE2L2) and NFE2L1 regulate adaptive responses to oxidative stress (1). NFE2L2 and NFE2L1 are members of the CNC subfamily of bZIP transcription factors, which regulate the expression of a large number of antioxidants and cyto-protective genes (23, 36,
Specifically, NFE2L1 and NFE2L2 have been demonstrated to be important in the induction of proteasomes (23, 36, 37). Both prevent the accumulation of terminally misfolded or oxidatively damaged proteins by upregulating ubiquitin-proteasome system and antioxidant enzymes (23, 36). While NFE2L1 mediates the induction of genes encoding 26S proteasome subunits during proteasome inhibition, NFE2L2 induces 26S subunits only during oxidative stress after oxidative modification of KEAP-1 (53, 59). The 20S proteasome, which is responsible for the degradation of oxidized proteins, is induced specifically by NFE2L2 (17, 23).

A study by Lee et. al. showed that deletion of NFE2L1 impairs proteasome activation in the liver (36). The significant up-regulation of PSMB1, PSMB2, PSMB5, and PSMA1 proteasome genes (20S proteasome) found in burn patients suggests that upregulation of NFE2L1 and NFE2L2 was associated with cytoprotective functions. Concurrent up-regulation of 19S (PSMD1, PSMD14, and PSMC5) proteasomes, responsible for processing ubiquitinated proteins, strongly suggests that protein degradation was increased in burn patients due to protein damage. Our data are consistent with findings of Majetschak and colleagues, who observed elevated levels of circulating 20S proteasomes (39). Together, these findings support the notion that proteasomal activity plays a role in muscle wasting after burns. In addition, induction of valosin-containing protein (VCP/p97) is central in the degradation of damaged protein in the ER and helps in the induction of NrF1/NFE2L1 (31, 53, 69, 72). The increased expression of ATF6, a marker of ER stress, suggests that after burns the skeletal muscle ER experiences profound unfolded protein stress. This might have contributed to the greater expression of VCP/p97 genes post-burn.

The main responsibility of ubiquitin-proteasomal system is the maintenance of proteins within cytoplasm (23). Proteasomes are not present within the mitochondrial matrix, which is separated from the cytoplasm by a double membrane. Intriguingly, mitochondria are a major source ROS, making mitochondrial proteins susceptible to oxidative damage (42, 62). The untoward effects of persistent ROS generation within mitochondria are counteracted through
activation of a mitochondrial quality control system, including the ATPases Associated with diverse cellular Activities (AAA+) proteolytic family, to promptly remove oxidized proteins (43, 57). The AAA proteases YME1L1, CLPP, and LONP1 play an essential role in the removal of damaged mitochondrial proteins (38, 43, 63). Lon peptidase 1 (LONP1) degrades oxidized and misfolded proteins within the mitochondrial matrix, while CLPP is required for the activation of mtUPR, and degradation of misfolded proteins (20). YME1L1 is present within the mitochondrial intermembrane space, where it performs a similar function (4). Our data show that LONP1, CLPP, and YME1L1 mRNA levels were significantly elevated in burn patients. Elevated LONP1 and lower YME1L1 proteins further suggest that both proteins are activated in response to mitochondria protein stress. The higher level of YME1L1 mRNA might be a compensatory mechanism of the decreased YME1L1 protein expression to meet increased demand. These data also suggest that, after burns, increased accumulation of oxidized and misfolded proteins in skeletal muscle mitochondria, results in the induction of LONP1, CLPP and YME1L1. This response likely functions to prevent oxidized protein from creating toxic aggregates that would lead to gross cellular dysfunction (43). Overall, the activation of LONP-1, CLPP, YME1L1, and proteasomes support our hypothesis of increased protein turnover in skeletal muscle post-burn.

In addition to degrading misfolded or unfolded protein, LONP1 is one of the main proteases responsible for the degradation of oxidized protein within mitochondria (9, 63). Mitochondrial proteins with complex organization such as Fe/S cluster proteins are generally highly susceptible to oxidation-dependent degradation (43, 64). LONP1 preferentially degrades oxidatively inactivated mitochondrial aconitase (ACO2) because it contains Fe/S. This likely prevents extensive accumulation of oxidized proteins and aggregates (8). Mitochondrial aconitase (ACO2) inactivation is a major source of mitochondrial OH (11, 64). Mitochondrial aconitase protein levels were higher in burn patients, perhaps suggesting a compensatory response to the degradation of inactivated ACO2. Inactivation of mitochondrial aconitase ([4Fe-
4S•) by O− promotes the production of Fe^{2+} and H_2O_2, both of which can contribute to oxidative stress via the Fenton reaction and reduction mechanisms, respectively (13, 64).

Despite the predisposition of mitochondria to oxidative stress and protein damage, the mitochondrial genome encodes only 1% of the 1500 proteins needed for normal mitochondrial function (33, 66). The remaining mitochondrial proteins are synthesized in the cytoplasm as precursor proteins and transported into the mitochondrion via translocase protein complexes (66). Some of the translocase proteins that maintain the sorting and specificity of the polypeptide to be imported include TIM23, TIM17, and TOM40 complexes (33, 45). Elevated expression of TIM23, TIM17B, and TOM40 proteins in burn patients suggests that protein demand by skeletal muscle mitochondria is increased after injury, likely due greater mitochondrial proteolysis by LONP1, CLPP and YME1L1. In addition, mitochondrial stress-mediated export of peptides leads to nuclear translocation of ATFS-1 in complex with transcriptional regulators UBL-5 and DVE-1, as well as upregulation of chaperones, proteases, and mitochondrial import proteins (20). In response to decreased protein-folding load in mitochondria, cytosolic protein translation may be inhibited by activating a signaling cascade involving GCN-2-mediated phosphorylation of elf2α (20). In parallel with these signaling events, the rate of mitochondrial protein import may diminish in response to decreased cytosolic protein translation by upregulation of YME1L1-mediated degradation of TIM17A. Hence, this cascade may have contributed to the overall protein deficit in burn patients. The elevated translocase proteins further support a role for the mitochondrion in muscle protein wasting following burn trauma, where increased transport of cytosolic proteins replaces those damaged and degraded within the mitochondria, ultimately increasing protein turnover in both the cytoplasm and mitochondrion in skeletal muscle after burns.

The current study provides novel data indicating that skeletal muscle protein degradation in burn patients takes place in both the cytosol and mitochondria. Systemic and skeletal muscle
hypermetabolism (i.e., increased mitochondrial respiration) after burn was accompanied by increased oxidative stress and an mtUPR, as evidenced by higher levels of nitrotyrosine and SOD2, CHOP, JNK2 and Hsp60. Persistent mitochondrial superoxide leak creates a proteotoxic environment within the mitochondrion leading to increased oxidative stress damage and mtUPR, with subsequent degradation of mitochondrial proteins. Activation of mtUPR stress and UBL5 levels suggest that hypermetabolism induced-stress is not limited to mitochondria but may also be transduced to the cytoplasm where it contributes to protein degradation. Increased mitochondrial protease levels in the skeletal muscle of burn patients were accompanied by a concurrent increase in the abundance of mitochondrial translocase complexes, suggesting that new proteins were transferred into mitochondria to replace oxidatively damaged or misfolded proteins and subsequently degraded. The increase in translocase proteins, taken with the mtUPR activation, suggests a mechanism to help compensate for insufficient chaperones capacity and re-establish homeostatic protein folding within the mitochondrial compartment.

In summary, we provide evidence that burn-induced hypermetabolism is associated with skeletal muscle wasting. Oxidative stress and activation of mtUPR with subsequent degradation of proteins in both the mitochondria and cytosol of the skeletal muscle cell compartments contribute to muscle wasting post-burn. Thus, the mitochondrion is a cellular organelle of interest in terms of future strategies aimed at blunting muscle catabolism in burn patients.


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Author Contributions: JOO, CP, DNH, and LSS conception and design of research; JOO, DRA, and AP performed experiments; JOO, CP, TC, DRA, and AP analysed data; JOO, CP, DNH, RBB, ZT, and LSS interpreted results of experiments; JOO and CP prepared figures; JOO, CP, and LSS drafted manuscript; JOO, CP, DNH, TC, MC, DRA, AP, ZTA, RPT, RBB, and LSS edited and revised manuscript; JOO, CP, DNH, TC, MC, DRA, AP, ZTA, RPT, RBB, and LSS approved manuscript.
**Figure Legend:**

Figure 1. Effect of burn trauma on Resting energy expenditure (REE) (A), mitochondrial leak and coupled respiration (B), Body weight (C), Lean body mass, (D). Bone mineral content (BMC) (E), and the fractional Synthetic rate (FSR), fractional break down rate (FBR), and net balance (NB) of mixed muscle proteins (F). Healthy control FSR, FBR and NB data were taken from Gundermann D, Walker D, Reidy P, Borack M, Dickinson J, Volpi E, and Rasmussen B. Activation of mTORC1 signaling and protein synthesis in human muscle following blood flow restriction exercise is inhibited by rapamycin. Am J Physiol Endocrinol Metab 306: 1198-1204, 2014. Values are presented as mean ± SEM. P<0.05 vs. control; **P<0.01 vs. control; ***P<0.001 vs. control.

Figure 2. Effects of burn trauma on mTOR signaling in skeletal muscle. Phosphorylation of mTOR at Ser^{2448} (A), 4E-BP1 at Thr^{37/46} (B), S6rp at Ser^{240/244}(C), and eEF2 at Thr^{56} (D) was significantly greater in burn patients, while phosphorylation of p70S6K at Thr^{389} (E), Eef2k at Ser^{366} and (F), EeF2 at Ser^{51} (G) did not differ between groups. Data were normalized to a loading control (GAPDH) (n=4 healthy controls and n=5 burn patients).Values are presented mean ± SEM. P<0.05 vs. control.

Figure 3. Effect of burn trauma on skeletal muscle oxidative stress and related factors. Fold-difference in SOD1, SOD2, CAT, PRDX3, ACO1, and ACO2 mRNA levels between healthy controls and burn patients (n=8 per group) (A). Western blot analysis of nitrotyrosilated protein (B) 4-hydroxynonenal (HNE) (C) SOD1 (D) SOD2 (E) catalase (F), peroxidoxine-3 (G), and aconitase 2 (H). Data were normalized to a loading control
Figure 4. Effect of burn trauma on expression of factors related to the mitochondrial unfolded protein response. Fold-difference of mRNA levels of Jun-B, ATF-6, C/EBPγ, CHOP/DDIT3, HsP10, HsP60, HsP90, c-JUN, JNK2, UBL5 and C/EBPα between controls and burn patients (n=8 per group). Western blot analysis of HsP90 (B), CHOP (C) HsP60 (D), ATF6 (E), and Jun-B (F). Data were normalized to a loading control (GAPDH) (n=4 for control and n=5 for burn group). Values are presented as mean ± SEM. P<0.05 vs. control; **P<0.01 vs. control; ***P<0.001 vs. control.

Figure 5. Effect of burn trauma on expression of cytoprotective transcription factors and proteosomal subunits. Fold-difference in mRNA levels of Kelch-Like ECH-Associated Protein 1 (KEAP1), nuclear factor, erythroid 2 like 2 (NFE2L2), nuclear factor, erythroid 2 like 1 (NFE2L1), and valosin-containing protein (VCP/p97) between controls and burn patients (A) (n=8 per group). Fold-difference in the mRNA expression of 20S and 19S in controls and burn patients (B) (n=8 per group). Western blot analysis of NFE2L2 (NrF2) (C) NFE2L1 (NrF1) (D) PSMD14 (E), proteasome 20S C2 (F), and proteasome 20S α+β (G). Data were normalized to a loading control (GAPDH) (n=4 for control and n=5 for burn group). Values are presented as mean ± SEM. P<0.05 vs. control; **P<0.01 vs. control; ***P<0.001 vs. control.

Figure 6. Effect of burn trauma on expression of mitochondrial proteases and translocases. Fold difference in mRNA expression of lon peptidase 1 (LONP1), YME1 like 1 ATPase (YME1L1) and Caseinolytic Mitochondrial Matrix Peptidase Proteolytic
Subunit (CLpP) in controls and burn patients (A) (n=8 per group). Western blot analysis of LONP1 (B), YME1L1 (C), TIM23 (D), TIM17A (E), TIM17B (F), and TOM40 (G) Data were normalized to a loading control (GAPDH) (n=4 for control and n=5 for burn group). Values are presented as mean ± SEM. P<0.05 vs. control; **P<0.01 vs. control; ***P<0.001 vs. control.

Abbreviations: Phosphorylation of Mammalian Target of Rapamycin (mTOR) at Ser-2448 (p-mTOR (Ser\textsuperscript{2448})); Eukaryotic [translation] initiation factor 4E (eIF4E)-binding protein 1 at Thr37/46 (p-4E-BP1 (Thr\textsuperscript{37/46})); Phospho-S6 Ribosomal Protein at Ser240/244 (S6rp (Ser\textsuperscript{240/244})); Eukaryotic elongation-factor-2 kinase thr56 (eEF2 at Thr\textsuperscript{56}); Phospho-p70 S6 Kinase (Thr389) (p70S6K at Thr\textsuperscript{389}); Eukaryotic elongation factor-2 kinase (Ser366) (Eef2k at Ser\textsuperscript{366}); Eukaryotic initiation factor (eIF) 2-α (Ser51) (p-EeF2 at Ser\textsuperscript{51}); Glyceraldehyde 3-phosphate dehydrogenase (GAPDH); Kelch-Like ECH-Associated Protein 1 (KEAP1); Nuclear factor, erythroid 2 like 2 (NFE2L2); Nuclear factor, erythroid 2 like 1 (NFE2L1); Valosin-containing protein (VCP/p97); Proteasome non-ATPase regulatory subunit 14 (PSMD14); proteasome 20S α + β (P20S α+β); Proteasome subunit beta type-1(PSMB1); Proteasome subunit beta type-2 (PSMB2); Proteasome subunit beta type-5 (PSMB5); Proteasome subunit alpha type-1 (PSMA1); 26S proteasome non-ATPase regulatory subunit 1 (PSMD1); 26S protease regulatory subunit 8 (PSMC5); Superoxide dismutase 1(SOD1); Superoxide dismutase 2 (SOD2); Catalase (CAT); Peroxiredoxin 3 (PRDX3); Aconitase 1(ACO1); Aconitase 2 (ACO2); Lon peptidase 1 (LONP1); YME1 like 1 ATPase (YME1L1); Mitochondrial import inner membrane translocase subunit (TIM23); Mitochondrial import inner membrane translocase subunit Tim17-A (TIM17A); Mitochondrial import inner
membrane translocase subunit Tim17-B (TIM17B); Translocase of outer membrane 40 kDa subunit (TOM40); Activating transcription factor 6 (ATF6); CCAAT/enhancer-binding protein β (C/EBPβ); CCAAT/enhancer-binding protein α (C/EBPα); CCAAT/enhancer-binding protein γ (C/EBPγ); C/EBP homologous protein (CHOP/DDIT3); Caseinolytic Mitochondrial Matrix Peptidase Proteolytic Subunit (CLpP); 10 kDa heat shock protein, mitochondrial (HsP10); 60 kDa heat shock protein, mitochondrial (HsP60); Heat shock protein HSP 90 (HsP90); Ubiquitin-like protein 5 (UBL5); Transcription factor jun-B (JUN-B); c-Jun N-terminal protein kinase 2 (JNK2).
### Table 1. Subject Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control subjects (n = 13)</th>
<th>Burn patients (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>26.3 ± 4.7</td>
<td>33.9 ± 18.1</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.8 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Weight on Admission (Kg)</td>
<td>83.1 ± 14.5</td>
<td>83.5 ± 23.1</td>
</tr>
<tr>
<td>BMI on Admission (kg/m2)</td>
<td>25.4 ± 3.1</td>
<td>26.7 ± 5.4</td>
</tr>
<tr>
<td>Weight on Study date (Kg)</td>
<td>-</td>
<td>74.4 ± 17.5*</td>
</tr>
<tr>
<td>Length of stay (days)</td>
<td>-</td>
<td>58 ± 46</td>
</tr>
<tr>
<td>Days Post Burn (days)</td>
<td>-</td>
<td>18.8 ± 9</td>
</tr>
<tr>
<td>Weight at Discharge (Kg)</td>
<td>-</td>
<td>65.6 ± 15.7*</td>
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<tr>
<td>BMI at Discharge (kg/m2)</td>
<td>-</td>
<td>21.4 ± 3.7*</td>
</tr>
<tr>
<td>Burn size (%TBSA)</td>
<td>-</td>
<td>63 ± 22</td>
</tr>
<tr>
<td>Full-thickness burn (%TBSA)</td>
<td>-</td>
<td>54 ± 23</td>
</tr>
</tbody>
</table>

*P<0.05 vs. Admission measurement. TBSA, total body surface area.
Figure 1
Figure 2

A. p-mTOR (ser2448) GAPDH

B. p-4E BP1 (Thr37/46) GAPDH

C. p-S6rp (Ser240/244) GAPDH

D. p-4E2-T56 GAPDH

E. p-70 S6K (Thr389) GAPDH

F. p-4E (Ser51) GAPDH

G. p-4E2 (Ser51) GAPDH
Figure 3

A

Nitro-Tyrosine

B

C

D

E

F

G

H

SOD1
SOD2
CAT
PRDX3
ACON1
ACON2

GAPDH

Control Burn

SOD1
SOD2
CAT
PRDX3
ACON1
ACON2

GAPDH

Control Burn

SOD1
SOD2
CAT
PRDX3
ACON1
ACON2

GAPDH

Control Burn

SOD1
SOD2
CAT
PRDX3
ACON1
ACON2

GAPDH

Control Burn

SOD1
SOD2
CAT
PRDX3
ACON1
ACON2

GAPDH

Control Burn

SOD1
SOD2
CAT
PRDX3
ACON1
ACON2

GAPDH

Control Burn
Figure 4

A

Fold Difference from Control

JUNB ATF6 CEBP-CEBP/CHOP-Hsp10-Hsp60-Hsp90-JUN-JNK2-UBL5-CEBPA

B

Hsp90/GAPDH

Control Burn

90 kDa

37 kDa

C

CHOP/GAPDH

Control Burn

27 kDa

GAPDH

D

Hsp60/GAPDH

Control Burn

60 kDa

37 kDa

E

ATF6/GAPDH

Control Burn

P=0.0556

75 kDa

GAPDH

F

JunB/GAPDH

Control Burn

43 kDa

GAPDH
Figure 5

A

Fold Difference from Control

KEAP1 NFE2L2 NFE2L1 VCP/P97

B

Fold Difference from Control

PSMB1 PSMB2 PSMB5 PSMA1 PSMD1 PSMD14 PSMDC5

C

NFE2L2/GAPDH

0.0 0.5 1.0 1.5

D

NRF1/GAPDH

0.0 0.5 1.0 1.5

E

PIM1/GAPDH

Control Burn

PsMD14

GAPDH

140 kDa

Control Burn

120 kDa

37 kDa

Control Burn

24 kDa

F

Proteins for COL4A1

Control Burn

20 kDa

G

Proteins for COL4A1

Control Burn

30 kDa

PsMD14

GAPDH

37 kDa

PsMD14

GAPDH

37 kDa

PsMD14

GAPDH

37 kDa
**Figure 6**

(A) Bar graph showing fold difference from control for LONP1, YME1L1, and ClpP.

(B) Western blot analysis of LONP1 showing 106 kDa and 37 kDa bands.

(C) Western blot analysis of YME1L1 showing 86 kDa band.

(D) Western blot analysis of TIM23 showing 22 kDa band.

(E) Western blot analysis of TOM17B showing 18 kDa band.

(F) Western blot analysis of TOM40 showing 38 kDa band.