Uncoupled skeletal muscle mitochondria contribute to hypermetabolism in severely burned adults

Craig Porter,1,2 David N. Herndon,1,2 Elisabet Børseheim,3,4 Tony Chao,1,5 Paul T. Reidy,7 Michael S. Borack,5 Blake B. Rasmussen,6 Maria Chondronikola,1,5 Manish K. Saraf,1,2 and Labros S. Sidossis1,2,7

1Metabolism Unit, Shriners Hospitals for Children, Galveston, Texas; 2Department of Surgery, University of Texas Medical Branch, Galveston, Texas; <Arkansas Children’s Nutrition Center and Arkansas Children’s Hospital Research Institute, Little Rock, Arkansas; 4Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, Arkansas; 5Division of Rehabilitation Sciences, University of Texas Medical Branch, Galveston, Texas; 6Department of Nutrition and Metabolism, University of Texas Medical Branch, Galveston, Texas; and 7Department of Internal Medicine, University of Texas Medical Branch, Galveston, Texas

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Porter C, Herndon DN, Børseheim E, Chao T, Reidy PT, Borack MS, Rasmussen BB, Chondronikola M, Saraf MK, Sidossis LS. Uncoupled skeletal muscle mitochondria contribute to hypermetabolism in severely burned adults. Am J Physiol Endocrinol Metab 307: E462–E467, 2014. First published July 29, 2014; doi:10.1152/ajpendo.00206.2014.—Elevated metabolic rate is a hallmark of the stress response to severe burn injury. This response is mediated in part by adrenergic stress and is responsive to changes in ambient temperature. We hypothesize that uncoupling of oxidative phosphorylation in skeletal muscle mitochondria contributes to increased metabolic rate in burn survivors. Here, we determined skeletal muscle mitochondrial function in healthy and severely burned adults. Indirect calorimetry was used to estimate metabolic rate in burn patients. Quadriceps muscle biopsies were collected on two separate occasions (11 ± 5 and 21 ± 8 days postinjury) from six severely burned adults (68 ± 19% of total body surface area burned) and 12 healthy adults. Leak, coupled, and uncoupled mitochondrial respiration was determined in permeabilized myofiber bundles. Metabolic rate was significantly greater than predicted values for burn patients at both time points (P < 0.05). Skeletal muscle oxidative capacity, citrate synthase activity, a marker of mitochondrial abundance, and mitochondrial sensitivity to oligomycin were all lower in burn patients vs. controls at both time points (P < 0.05). A greater proportion of maximal mitochondrial respiration was linked to thermogenesis in burn patients compared with controls (P < 0.05). Increased metabolic rate in severely burned adults is accompanied by derangements in skeletal muscle mitochondrial function. Skeletal muscle mitochondria from burn victims are more uncoupled, indicating greater heat production within skeletal muscle. Our findings suggest that skeletal muscle mitochondrial dysfunction contributes to increased metabolic rate in burn victims.

SEVERE BURN TRAUMA results in a sustained pathophysiologic stress response (8–11). Hypermetabolism, an increase in resting metabolic rate, is a hallmark of this response (7, 9–11, 22) and persists for more than 1 yr post-injury (11). Furthermore, hypermetabolism is associated with greater cachexia and prolonged morbidity in burn survivors (8, 9), making its management clinically important. Although increased ATP turnover contributes to hypermetabolism following burn trauma, increased oxidative phosphorylation cannot fully explain this phenomenon. Indeed, increased ATP turnover accounts for ~50% of hypermetabolism in patients with severe burns (23). Subsequently, a complete biochemical understanding of hypermetabolism in burn patients is lacking.

Metabolic rate in humans is governed by the amount of oxygen required to support ATP production and thermoregulation. The vast majority (~90%) of whole body oxygen consumption occurs within mitochondria (16). This suggests that mitochondria play a causative role in post-burn hypermetabolism. Indeed, we have shown previously that coupled (ATP-producing) mitochondrial respiration is lower in skeletal muscle from burned children compared with healthy children (6). Furthermore, the ratio of coupled to uncoupled respiration was lower in burned children compared with healthy children (6), suggesting that mitochondrial thermogenesis is increased in response to burn trauma. In support of this supposition, it is well known that the hypermetabolic response to burn trauma is responsive to the use of occlusive wound dressings and elevations in ambient temperature (3–5, 21), indicating that altered skeletal muscle mitochondrial function may be an adaptive response that permits heat production at the expense of fuel oxidation.

Whereas mitochondria within other tissue depots, principally brown adipose tissue, are known to function primarily as heat-producing organelles, skeletal muscle mitochondria are generally considered to be well coupled, where >80% of oxygen consumption is coupled to ATP production (16). Although it is clear that well-coupled skeletal muscle are of paramount importance to normal physiological function following major thermal trauma, where patients are immobilized for prolonged periods and thus have little need for a high ATP-producing reserve, increased skeletal muscle mitochondrial thermogenesis may be advantageous with regard to thermoregulation. The aim of the current study was to investigate the impact of severe burn injury on skeletal muscle mitochondrial function and coupling control in adult humans. We hypothesize that severe burn injury alters skeletal muscle mitochondrial function, favoring mitochondrial thermogenesis. Our hypothesis offers a novel mechanistic explanation for the hypermetabolic response to severe thermal trauma.

MATERIALS AND METHODS

Patients and healthy volunteers. Severely burned adults admitted to the Blocker Burn Unit at the University of Texas Medical Branch for acute burn care were recruited for the purposes of the current study. Patients with burn wounds encompassing ≥40% of their total body
surface area (TBSA) were considered. All patients received standard burn care, including early fluid resuscitation and total wound debridement. This was followed by sequential grafting procedures to cover open wounds with autologous skin. Throughout this period, patients were fed 1.500 kcal/m² body area + 1.500 kcal/m² body area burned in the form of a low-fat enteral formula (82% carbohydrate, 3% fat, 15% protein) continuously through a nasogastric feeding tube (Vivonex T.E.N.; Nestle Health Science, Minneapolis, MN).

During the acute hospitalization period and following an overnight fast, muscle biopsy samples were collected from the m. vastus lateralis under local anesthesia (1% lidocaine) using a suction-adapted Bergström needle (1). Muscle biopsy samples were collected on two separate occasions during the acute hospitalization period approximately 1 (study 1) and 3 wk (study 2) postadmission. Indirect calorimetry was performed in burned patients to determine respiratory gas exchange. Because of clinical restrictions, we were not able to perform indirect calorimetry on one patient at the 1-wk postadmission time point and 1 patient at the 3-wk postadmission time point. When possible, indirect calorimetry measurements were performed on the same day as muscle biopsy sampling. This was not possible on two occasions where the nearest available measurement was used.

Twelve young healthy men were also recruited from the Galveston County, TX, area by means of advertisement to act as young healthy controls. Each participant reported to the Clinical Research Center at the University of Texas Medical Branch following an overnight fast, where a muscle biopsy was collected from the m. vastus lateralis under local anesthesia, as described above. All human research procedures were reviewed and approved by the Institution Review Board at the University of Texas Medical Branch. All patients and/or their legal guardians and healthy participants gave informed, written consent prior to participation.

Resting metabolic rate. Resting energy expenditure (REE) of burned patients was determined by indirect calorimetry (SensorMedics VMax 29, Yorba Linda, CA). REE was calculated from whole body oxygen consumption and carbon dioxide production rates using previously described equations (20). This was compared with predicted REE, which was estimated using the Harris-Benedict equations (17). This is a standard approach for estimating the degree of hypermetabolism in burn patients.

Muscle biopsy analysis. Approximately 10–20 mg of fresh skeletal muscle tissue was placed in an ice-cold (pH 7.1) preservation buffer (containing 10 mM Ca-EGTA, 0.1 mM free Ca²⁺, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl₂, 5.77 mM ATP, and 15 mM creatine phosphate) immediately upon collection. Maximal citrate synthase (CS) activity was determined in a Tris-HCl buffer (pH 8.3) containing acetyl-CoA, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), and oxaloacetate. The change in light absorbance relating to free CoA production and its reaction with DTNB was tracked at 412 nM in a spectrophotometer set in kinetic mode (BioTek Eon, Winooski, VT).

Total protein content. CS activity measurements were corrected for the protein content of the muscle lysate. Lysate protein concentration was determined using a modified version of the Bradford assay (2). Briefly, 10 μl of 5 mg/ml muscle lysates was incubated in 800 μl of protein quantification reagent (Bio-Rad, Hercules, CA) for 30 min at room temperature. Light absorbance was then read in a 200-μl aliquot of each sample at 595 nM (BioTek Eon), and protein concentrations were calculated from a 1 mg/ml bovine serum albumin standard curve.

Statistical analysis. All data are presented as group means ± SE unless stated otherwise. Differences in group means were detected using unpaired t-tests. Because three groups were compared for muscle biochemical measurements, a Bonferroni correction was applied. Therefore, for all muscle biochemical measurements, statistical significance was accepted when P ≤ 0.017. Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA).

RESULTS

Subject demographics. Demographics for patients and healthy volunteers are presented in Table 1. Patients and healthy volunteers were similar with regard to age, height, and weight. All patients and volunteers were male. Patients had massive burns encompassing on average 68 ± 19% of the TBSA, the majority of which were full thickness (3rd degree) burns (56 ± 25% TBSA). Body composition was not significantly different in burn victims compared with controls (Table 2), although bone mineral content was significantly lower in burn victims (P < 0.05).

Predicted and calculated REE. REE data for burn patients is presented in Fig. 1. Calculated REE was greater than predicted REE in the first study (43 ± 14%, P = 0.0075) and in the second study (45 ± 13%, P = 0.0154). This equated to an increased energy requirement of 845 ± 268 and 873 ± 236 kcal/day at the first and second study time points, respectively.

Skeletal muscle mitochondrial respiration. High-resolution respirometry data are presented in Fig. 2. State 2 leak respiration was significantly lower in burn victims compared with controls (Table 2), although bone mineral content was significantly lower in burn victims (P < 0.05).

Table 1. Patient demographics

<table>
<thead>
<tr>
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<th>Control (n = 12)</th>
<th>Burn (n = 6)</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>25 ± 3 (19–29)</td>
<td>30 ± 14 (21–57)</td>
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<tr>
<td>Height, cm</td>
<td>177 ± 7 (166–190)</td>
<td>180 ± 6 (173–191)</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>84 ± 0 (69–114)</td>
<td>88 ± 17 (71–113)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.9 ± 2.5 (21.5–30.6)</td>
<td>27.3 ± 4.8 (22.5–35.8)</td>
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<tr>
<td>Days post-burn biopsy</td>
<td>1</td>
<td>5 ± 2 (5–20)</td>
</tr>
<tr>
<td>Days post-burn biopsy</td>
<td>2</td>
<td>18 ± 8 (9–28)</td>
</tr>
<tr>
<td>Burn size (%TBSA)</td>
<td>68 ± 19 (45–99)</td>
<td>56 ± 25 (30–99)</td>
</tr>
<tr>
<td>Full-thickness burns</td>
<td>(%TBSA)</td>
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Data are means ± SD; ranges are reported in parentheses. TBSA, total body surface area.
tion tended to be lower in the burn group in study 1 vs. controls (16.5 ± 1.8 vs. 11.2 ± 2.6 pmol·s⁻¹·mg⁻¹, P = 0.11) and in the burn group in study 2 vs. controls (16.5 ± 1.8 vs. 10.5 ± 1.6 pmol·s⁻¹·mg⁻¹, P = 0.0498). However, neither burn group was statistically different from control. Coupled state 3 respiration was lower in burn patients in both study 1 (36.4 ± 3.5 vs. 19.7 ± 6.9 pmol·s⁻¹·mg⁻¹, P = 0.0279) and study 2 (36.4 ± 3.5 vs. 14.3 ± 4.3 pmol·s⁻¹·mg⁻¹, P = 0.0017) compared with control, although it was significantly lower only in the burn group in study 2 following the Bonferroni correction. Maximal coupled state 3 respiration (OXPHOS) was significantly lower in the burn group in study 1 (61.2 ± 5.9 vs. 27.1 ± 8.7 pmol·s⁻¹·mg⁻¹, P = 0.0047) and in study 2 (61.2 ± 5.9 vs. 19.1 ± 5.7 pmol·s⁻¹·mg⁻¹, P = 0.004). State 4o oligomycin-insensitive leak respiration was numerically but not significantly lower in burn patients compared with the control group in study 1 (14.3 ± 2.1 vs. 19.6 ± 2.7 pmol·s⁻¹·mg⁻¹, P = 0.223) and study 2 (9.9 ± 2.1 vs. 19.6 ± 2.7 pmol·s⁻¹·mg⁻¹, P = 0.033).

Skeletal muscle mitochondrial density. Skeletal muscle mitochondrial CS activity data are presented in Fig. 3. CS activity was used as a proxy of skeletal muscle mitochondrial density. CS activity was significantly lower in burn patients compared with control in muscle samples collected in study 1 (0.42 ± 0.09 vs. 3.91 ± 1.61 pmol·s⁻¹·mg⁻¹·CS activity⁻¹, P = 0.0062) and study 2 (0.42 ± 0.09 vs. 4.47 ± 1.79 pmol·s⁻¹·mg⁻¹·CS activity⁻¹, P = 0.0048). State 3 respiration normalized to CS activity was significantly higher in skeletal muscle from burn patients compared with controls in both study 1 (0.93 ± 0.18 vs. 5.86 ± 2.32 pmol·s⁻¹·mg⁻¹·CS activity⁻¹, P = 0.0126) and study 2 (0.93 ± 0.18 vs. 4.39 ± 1.76 pmol·s⁻¹·mg⁻¹·CS activity⁻¹, P = 0.0075). OXPHOS respiration normalized to CS activity was significantly greater in burn patients in study 1 compared with controls (1.66 ± 0.38 vs. 7.43 ± 2.47 pmol·s⁻¹·mg⁻¹·CS activity⁻¹, P = 0.005). OXPHOS respiration normalized to CS activity tended to be higher in burn patients in study 2 compared with controls (1.66 ± 0.38 vs. 5.39 ± 1.95 pmol·s⁻¹·mg⁻¹·CS activity⁻¹, P = 0.0195); however, this did not reach significance once the

**Table 2. DEXA analysis**

<table>
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<tr>
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<th>Control (n = 12)</th>
<th>Burn (n = 5)</th>
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<tbody>
<tr>
<td>Total body mass, kg</td>
<td>84 ± 12 (69–114)</td>
<td>83 ± 22 (65–121)</td>
</tr>
<tr>
<td>Bone mineral content, kg</td>
<td>3.3 ± 0.5 (2.7–4.1)</td>
<td>2.7 ± 0.2 (2.5–3.1)*</td>
</tr>
<tr>
<td>Fat-free mass, kg</td>
<td>64 ± 9 (51–78)</td>
<td>58 ± 11 (51–77)</td>
</tr>
<tr>
<td>Fat mass, kg</td>
<td>17 ± 7 (9–32)</td>
<td>22 ± 12 (13–41)</td>
</tr>
<tr>
<td>%Lean mass</td>
<td>20 ± 6 (10–28)</td>
<td>26 ± 8 (17–34)</td>
</tr>
<tr>
<td>Days post-burn</td>
<td></td>
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Data are means ± SD; ranges are reported in parentheses. *P < 0.01 vs. control.

Fig. 1. Predicted and measured resting energy expenditure (REE) in severely burned adults (n = 5). REE units are in kcal/day. **P < 0.05 vs. predicted; ***P < 0.01 vs. predicted.

Fig. 2. Mitochondrial respiration in permeabilized myofiber bundles from healthy control (n = 12; black bars), burned study 1 (n = 6; gray bars), and burned study 2 (n = 6; open bars) adults. Units are in pmol·s⁻¹·mg⁻¹·CS activity⁻¹. Leak (sample and respiration buffer), state 2 [+octanoyl-carnitine (1.5 mM), pyruvate (5 mM), malate (2 mM), and glutamate (10 mM)], state 3 [+ADP (5 mM)], oxidative phosphorylation (OXPHOS); + succinate (10 mM)), and state 4o [+oligomycin (5 µM)] respiration were determined sequentially in the sample tissue preparation. *P < 0.05 and **P < 0.01, respectively vs. healthy controls.

Fig. 3. Citrate synthase (CS) activity in muscle homogenates from healthy control (n = 12; black bar), burned study 1 (n = 6; light gray bar), and burned study 2 (n = 6; dark gray bar) adults. Acetyl-CoA production from CS is expressed in µmol·s⁻¹·g⁻¹ and corrected for the total protein concentration of the lysate. *P < 0.05 vs. control.
insensitive) (Fig. 5).

Mitochondrial respiration was linked to phosphorylation (oligomycin sensitive) and thermogenesis (oligomycin insensitive) (Fig. 5).

The development of chemical potential via electron transport chains results in a greater capacity for respiration per mitochondrion. Mitochondria are the cellular organelles responsible for almost all oxygen consumption in vivo (16). Therefore, we hypothesized that altered mitochondrial function mediates the increase in whole body oxygen consumption seen in burn victims. To test this hypothesis, we determined ex vivo mitochondrial respiration in permeabilized myofiber bundles from quadriceps muscle biopsies obtained from young healthy adults and adults with massive burns. Severely burned adults were hypermetabolic, where resting metabolic rate was ~40% above normal values. Furthermore, our data show that maximal coupled mitochondrial respiration was significantly lower in skeletal muscle of burned individuals, which was attributable largely to reduced mitochondrial density. However, both coupled and uncoupled respiration normalized to mitochondrial density were significantly greater in burn victims compared with healthy controls. This suggests that severe burn injury induces functional changes in skeletal muscle mitochondria, which result in a greater capacity for respiration per mitochondrion. Furthermore, the coupling of mitochondrial respiration to ATP production was significantly diminished in skeletal muscle mitochondria from burn victims, meaning that skeletal muscle ATP production is less efficient in burn victims, and more of the electrochemical potential generated by the mitochondria is lost as heat. Subsequently, our data show that uncoupling of oxidative phosphorylation in skeletal muscle mitochondria explains a portion of the hypermetabolic response to severe burns. These current findings offer a novel mechanistic explanation for hypermetabolism in burn victims, identifying mitochondria as a therapeutic target for interventions focusing on mitigating the stress response to burn injury.

Mitochondria play an integral role in ATP production, as explained by the chemiosmotic theory of oxidative phosphorylation (14). By transferring electrons along the electron transport chain to oxygen, protons are pumped from the mitochondrial matrix into the space enclosed by the inner and outer mitochondrial membrane. The development of chemical potential in the intramembrane space of the mitochondria can be transduced to ATP by ATP synthase. However, protons can re-enter the mitochondrial matrix independently of ATP synthase, a process that uncouples oxidative phosphorylation. In this instance, the mitochondrial membrane potential is dissipated as heat. In skeletal muscle, mitochondrial respiration is well coupled to ADP phosphorylation, owing to muscle tissues’ comparatively high need for ATP. However, like their counterparts in other cell types, skeletal muscle mitochondria are capable of inner membrane proton conductance. For example, rare inherited mitochondrial diseases such as Lufts’ syndrome and shock mitochondrial diseases (15).

DISCUSSION

Major burns result in a prolonged pathophysiological stress response (7, 9–11). Hypermetabolism is a hallmark of this response, which is associated with skeletal muscle wasting and prolonged morbidity. Mitochondria are the cellular organelles responsible for almost all oxygen consumption in vivo (16). Therefore, we hypothesized that altered mitochondrial function mediates the increase in whole body oxygen consumption seen in burn victims. To test this hypothesis, we determined ex vivo mitochondrial respiration in permeabilized myofiber bundles from quadriceps muscle biopsies obtained from young healthy adults and adults with massive burns. Severely burned adults were hypermetabolic, where resting metabolic rate was ~40% above normal values. Furthermore, our data show that maximal coupled mitochondrial respiration was significantly lower in skeletal muscle of burned individuals, which was attributable largely to reduced mitochondrial density. However, both coupled and uncoupled respiration normalized to mitochondrial density were significantly greater in burn victims compared with healthy controls. This suggests that severe burn injury induces functional changes in skeletal muscle mitochondria, which result in a greater capacity for respiration per mitochondrion. Furthermore, the coupling of mitochondrial respiration to ATP production was significantly diminished in skeletal muscle mitochondria from burn victims, meaning that skeletal muscle ATP production is less efficient in burn victims, and more of the electrochemical potential generated by the mitochondria is lost as heat. Subsequently, our data show that uncoupling of oxidative phosphorylation in skeletal muscle mitochondria explains a portion of the hypermetabolic response to severe burns. These current findings offer a novel mechanistic explanation for hypermetabolism in burn victims, identifying mitochondria as a therapeutic target for interventions focusing on mitigating the stress response to burn injury.

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drome suggest that uncoupled skeletal muscle mitochondria can have a significant impact on whole body energy expenditure in humans (12). Indeed, sufferers of Lufts’ syndrome sweat profusely and are unable to gain weight due to a massively elevated basal metabolic rate (12). Interestingly, skeletal muscle mitochondria from these patients are insensitive to ADP, oligomycin, and the proton-ionophore dinitrophenol (13), meaning that there is a high degree of inner membrane proton conductance in mitochondria from these patients and thus thermogenesis.

Intriguingly, data from the current study show that burn injury results in functional alterations in skeletal muscle mitochondria akin to those seen in Lufts’ syndrome. More specifically, mitochondria from burn patients appear less sensitive to ADP. Furthermore, inhibiting ATP synthase with oligomycin had a diminished (~40%) effect on mitochondrial respiration in muscle from burned individuals. This means that inner membrane proton conductance is maintained in mitochondria from burned individuals in the absence of ATP synthase activity. This alteration in skeletal muscle respiratory control is accompanied by a significant increase in whole body REE (~40%). Thus, for the first time, we demonstrate that skeletal muscle mitochondrial dysfunction contributes to hypermetabolism in burn victims. Our current findings extend the preliminary findings of Cree et al. (6), who reported a reduction in skeletal mitochondrial coupling control in burned children. With the use of a more detailed high-resolution respirometry protocol than that employed in the aforementioned study (6), we show conclusively that skeletal muscle mitochondria become uncoupled in response to major burns.

Mitochondrial volume/number and maximal coupled respiration per milligram of tissue were lower in the skeletal muscle of burn victims. However, these are maximal ex vivo measurements of oxidative capacity, reduced maximal oxidative capacity may not be limiting to ATP availability in vivo. Indeed, others have elegantly shown with the use of 31P-NMR spectroscopy that although skeletal muscle ATP production rates are around 40% lower in burned mice, ATP concentration is not different within skeletal muscle of burn- or sham-treated animals (15). Thus the respiratory control characteristics of skeletal muscle mitochondria following severe burn injury are of more physiological relevance than measurements of maximal oxidative capacity. An advantage of high-resolution respirometry is that the sensitivity of mitochondria to different substrates and inhibitors can be determined within the tissue sample. Calculating respiratory control ratios offers an index of mitochondrial function that is uninfluenced by mitochondrial density. Our current data show for the first time that human skeletal muscle mitochondria are insensitive to both ADP and oligomycin following severe burn injury; these two compounds should couple and uncouple oxidative phosphorylation, respectively. Therefore, our current data show intrinsic alterations in mitochondrial function in response to massive thermal trauma, where mitochondria exhibit a more thermogenic phenotype.

By determining maximal coupled oxidative phosphorylation respiration and uncoupled state 4o respiration, we were able to discern the proportion of mitochondrial oxygen consumption that was linked to thermogenesis or phosphorylation. In healthy individuals, approximately two-thirds of oxygen consumption was coupled to phosphorylation, with the remaining third attributable to thermogenesis. Strikingly, this was reversed in burn patients, where approximately one-third of mitochondrial respiration was coupled to phosphorylation, with two-thirds of total respiration being dissipated as heat.

Although our data demonstrate that mitochondrial physiology is altered in human skeletal muscle following burn injury, the molecular basis of this response requires further investigation. Previously, others have reported widespread reductions in the expression of transcripts involved in oxidative phosphorylation in skeletal muscle of burned rodents (15, 19). Furthermore, Tzika et al. (18) showed a significant increase in the expression uncoupling protein 2 expression in children with severe burns, suggesting that uncoupling protein expression in mitochondria may mediate uncoupling following severe burn injury.

We have shown for the first time that skeletal muscle mitochondrial coupling control is altered in burn victims, which is associated with elevated metabolic rate. Increased mitochondrial membrane proton conductance uncouples oxidative phosphorylation, resulting in greater heat production. We suggest that this may contribute to hypermetabolism in burn victims. These data may be applicable to other pathologies associated with adrenergic stress, inflammation, and rapid muscle cachexia such as cancer, sepsis, and HIV/AIDS. Further research should focus on elucidating the mechanisms responsible for proton leaks in skeletal mitochondria of burn victims, as the mitochondrialor may be a site of intervention for strategies aimed at mitigating the stress response to burn trauma.

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GRANTS

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DISCLOSURES

None of the authors have any relevant conflicts of interest to disclose, financial or otherwise.

AUTHOR CONTRIBUTIONS

C.P., D.N.H., E.B., B.B.R., and L.S.S. conception and design of research; C.P., T.C., P.R., M.B., M.C., and M.S. performed experiments; C.P., T.C., and M.C. analyzed data; C.P., D.N.H., E.B., T.C., and L.S.S. interpreted results of
experiments; C.P., P.R., and M.C. prepared figures; C.P. and L.S.S. drafted manuscript; C.P., D.N.H., E.B., T.C., P.R., M.B., B.B.R., M.C., M.S., and L.S.S. edited and revised manuscript; C.P., D.N.H., E.B., T.C., P.R., M.B., B.B.R., M.C., M.S., and L.S.S. approved final version of manuscript.

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