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Skeletal muscle dysfunction is associated with derangements in mitochondrial bioenergetics (but not UCP3) in a rodent model of sepsis

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Zolfaghari PS, Carré JE, Parker N, Curtin NA, Duchen MR, Singer M. Skeletal muscle dysfunction is associated with derangements in mitochondrial bioenergetics (but not UCP3) in a rodent model of sepsis. Am J Physiol Endocrinol Metab 308: E713–E725, 2015. First published February 24, 2015; doi:10.1152/ajpendo.00562.2014.—Muscle dysfunction is a common feature of severe sepsis and multiorgan failure. Recent evidence implicates bioenergetic dysfunction and oxidative damage as important underlying pathophysiological mechanisms. Increased abundance of uncoupling protein-3 (UCP3) in sepsis suggests increased mitochondrial proton leak, which may reduce mitochondrial coupling efficiency but limit reactive oxygen species (ROS) production. Using a murine model, we examined metabolic, cardiovascular, and skeletal muscle contractile changes following induction of peritoneal sepsis in wild-type and Ucp3−/− mice. Mitochondrial membrane potential (Δψm) was measured using two-photon microscopy in living diaphragm, and contractile function was measured in diaphragm muscle strips. The kinetic relationship between membrane potential and oxygen consumption was determined using a modular kinetic approach in isolated mitochondria. Sepsis was associated with significant whole body metabolic suppression, hypothermia, and cardiovascular dysfunction. Maximal force generation was reduced and fatigue accelerated in ex vivo diaphragm muscle strips from septic mice. Δψm was lower in the isolated diaphragm from septic mice despite normal substrate oxidation kinetics and proton leak in skeletal muscle mitochondria. Even though wild-type mice exhibited an absolute 26 ± 6% higher UCP3 protein abundance at 24 h, no differences were seen in whole animal or diaphragm physiology, nor in survival rates, between wild-type and Ucp3−/− mice. In conclusion, this murine sepsis model shows a hypometabolic phenotype with evidence of significant cardiovascular and muscle dysfunction. This was associated with lower Δψm and alterations in mitochondrial ATP turnover and the phosphorylation pathway. However, UCP3 does not play an important functional role, despite its upregulation.

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MUSCLE DYSFUNCTION IS A COMMON feature of severe sepsis and other critical illnesses, and a major cause of both prolonged intensive care stay (21) and long-term disability (7, 34). Neuropathic and myopathic features are well recognized (7, 38, 40, 50), yet the underlying pathophysiology remains incompletely understood. A net catabolic state, impaired cellular and calci-
The aim of our study was to determine changes in metabolism, muscle function, and the influence of UCP3 in an established rodent model of sepsis. We hypothesized that sepsis leads to metabolic suppression and muscle dysfunction as a result of decreased capacity of mitochondrial ATP synthesis, and that UCP3, an uncoupling protein restricted to muscle and adipose tissue, offers protection to ROS-induced muscle contractile dysfunction at the expense of mitochondrial efficiency.

GLOSSARY

ΔΨm Mitochondrial membrane potential
TMRE Tetramethylrhodamine methyl ester
TPMP Methyltriphenylphosphonium
UCP Uncoupling protein
$V_O^2$ Whole body oxygen consumption
$V_{CO^2}$ Whole body carbon dioxide production
WT Wild-type

METHODS

Mouse Septic Model

All experiments were performed under the UK Animals (Scientific Procedures) Act 1986 with approval from the University College London Ethics Committee. Ten-week-old male C57 black mice were purchased from Charles River (Margate, UK) and maintained in the university animal facility until they reached 25–35 wk of age. They were housed in cages (maximum 6/cage) with standard bedding, environment-enhancing objects, and free access to water and chow diet. Cages were maintained at room temperature with 12:12-h light-dark cycles. Comparison was made against age-matched litter mice from three Ucp3−/− homozygote breeding pairs [originally produced by Gong et al. (29) and back-crossed 10 generations with wild-type C57 mice]. Confirmatory genotyping was carried out using tail snips. All Ucp3−/− mice were kept in individually ventilated cages with littermates of the same sex kept in the same cage after weaning. Mice were allowed to acclimate to the laboratory for 1 wk before the start of experiments.

The model of sepsis has been described in detail elsewhere (67). In summary, sepsis was induced by intraperitoneal injection of diluted cecal slurry (20 ml/kg) under a brief period of isoflurane anesthesia. Sham mice received 20 ml/kg of 0.9% saline intraperitoneally. As septic mice stop eating, a further sham-starved group was added to account for this potential confounding factor. Starvation commenced after intraperitoneal injection of saline. Mice were allowed to acclimate to the laboratory for 1 wk before the start of experiments.

All mice received 0.9% saline 10 ml/kg subcutaneously at time 0 h, and 50 ml/kg of 5% dextrose/0.1% saline solution at 6, 18, 30, and 42 h. Initial experiments observed 72-h survival, serum biochemical markers of organ failure, blood gases, and echocardiography (Vivid 7; GE Healthcare, Chalfont St. Giles, Bucks, UK) at 24 h. Clinical variables comprising behavior, appearance, temperature, and acidemia were used to score sepsis severity (67). Severity scoring was carried out by two observers, one of whom was blinded to the randomization process. The scores given by the two observers were consistently equal. Subsequent studies are outlined below.

Whole Animal Metabolism

Whole body metabolic rate of sham and septic mice was measured for 24 h in individual metabolic chambers (Oxymax; Columbus Instruments, Columbus, OH). Gas samples from each box were sampled for 90 s at 8-min intervals. Oxygen consumption ($V_O^2$) and CO$_2$ production ($V_{CO^2}$) were calculated using standard formulas. The respiratory exchange ratio was calculated as the ratio of $V_{CO^2}$ to $V_O^2$. Values near 1 indicate a predominance of carbohydrate metabolism while values approaching 0.7 indicate fatty acid oxidation (36). Rectal temperature was measured at 0, 6, 18, and 24 h, and animals were weighed at 0- and 24-h time points.

Assessment of Cardiac Function

Echocardiography was performed at 0, 6, and 24 h in sham and septic wild-type and Ucp3−/− mice using a 14-MHz probe connected to a Vivid 7 Dimension device as previously described (67). Aortic blood flow velocity was measured in the proximal ascending aorta immediately before the bifurcation of the right carotid artery using pulse-wave Doppler. Stroke volume was calculated by multiplying the velocity time integral from six consecutive cycles (equivalent to one respiratory cycle) by aortic cross-sectional area (using an aortic diameter of 0.26 cm). Average peak-to-peak distance and maximum velocity over six consecutive systolic cycles were used to measure heart rate and peak velocity, the latter being a marker of left ventricular contractility (12). Cardiac output was calculated as the product of stroke volume and heart rate.

Western Blotting for UCP3 Protein

UCP3 protein abundance was measured in snap-frozen heart and skeletal muscle tissue at 10 and 24 h following induction of sepsis in the wild-type mice. After SDS protein precipitation, 10 μg protein were separated on a 12% SDS polyacrylamide gel and transferred to nitrocelullose. Membranes were probed overnight at 4°C with the primary antibody (Rabbit anti-UCP3: PA1-055 Pierce antibodies; ThermoFisher Scientific, Loughborough, Leics, UK) at 1:1,000 dilutions in blocking buffer. Each sample was loaded two times in the same gel, and each gel was repeated one time. Following incubation with secondary antibody (goat-anti-rabbit antibody: DAKO, Ely, Cambs, UK), cross-reacted proteins were visualized by enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, Bucks, UK). Densitometry measurements were made using Image J image processing and National Institutes of Health analysis software. A single control sample was used in each gel, and all results were normalized to this control allowing comparison between samples in different gels. α-Tubulin (1:5,000 dilution; Abcam 7291 Cambridge, Cambs, UK) was used to ensure equal loading of gels and transfer of proteins from gel to membrane. Each membrane was also Coomassie stained to ensure equal loading and transfer.

Diaphragm Preparation

At 24 h, under terminal isoflurane anesthesia, a midline laparotomy was performed, and the diaphragm was excised en bloc (still attached to ribs laterally) and pinned (taut without excess stretch) at its edges in a petri dish filled with physiological saline [containing (in mmol/l): 145 Na$^+$, 5 K$^+$, 5 Ca$^{2+}$, 1 Mg$^{2+}$, 25 HCO$_3^-$, 118 Cl$^-$, 1 SO$_4^{2-}$, 1 PO$_4^{3-}$, 10 glucose, 10 pyruvate, and equilibrated with 95% O$_2$ and 5% CO$_2$ (pH 7.4)]. Tubocurarine (2.5 μm) was added to stop spontaneous neuromuscular junction activity. Diaphragm strips (~1 mm wide) were dissected using stereomicroscopy; a strip consisted of muscle fibers with the ribs at one end and central tendon at the other. Strips were allowed to rest in physiological saline for at least 30 min before experimentation. All experiments were performed within 5 h of tissue harvest.

Force and Power Measurements

Aluminium foil T-clips were attached to the rib and central tendon at the ends of the diaphragm strips using cyanoacrylate gel glue. The muscle strips were transferred to a temperature-controlled (23°C) Perspex bath through which physiological saline was continuously circulated. At one end the strip was attached to a combined motor and force transducer (model 300B; Cambridge Technology, Watertown, MA) and the other end to a hook mounted on a micrometer allowing alteration of resting muscle length. Electrical stimulation (MultiStim System-D330; Digitimer, Welwyn Garden City, Herts, UK) was delivered by two platinum electrodes placed in the bath just above and...
below the belly of the muscle fibers. A program written in TestPoint (Keithley Instruments, Bracknell, Berks, UK) controlled stimulation and motor arm position and recorded force, length, and stimulation. A DAS-1800AO Series A/D board (Keithley Instruments) was used. The computer program could be altered to achieve complex protocols such as force recording during repeated phased cyclical tetanic stimulations while making controlled changes to muscle length.

Following system calibration and zeroing, optimal stimulation voltage (at 96 Hz) was found for the muscle strip; these stimulus parameters were used throughout the experiment. The length/tension relationship was explored by increasing the muscle length in 0.2- to 0.4-mm increments to identify L₀, the muscle length at which filament overlap was optimal for isometric tetanic force development. A single 350-ms tetanic stimulation was used to measure the maximal achievable tetanic isometric force. The maximum force generated was expressed relative to muscle cross-sectional area (CSA) to take account of muscle size.

\[
\text{CSA} = 4.9 \times d \times M/L_0
\]

where 4.9 is the wet-to-dry ratio (65), \(d\) is density (assumed to be 1 mg/mm³), \(M\) is dry mass of the muscle, and \(L_0\) is muscle length as defined above. \(M\) and \(L_0\) were measured at the end of the experiment.

The ability of the muscle to produce work and power was measured using a pattern of sinusoidal movement and stimulation that mimics diaphragm muscle action in vivo. This pattern was described and optimized by Stevens and Faulkner for diaphragm muscle strips from C57 black mice (59). The amplitude of the length change was ±0.4 mm. Stimulation was applied for ~30% of each movement cycle and was approximately centered on the time at which the muscle length was longest. The maximum power was measured in one cycle of movement at a frequency of 4 Hz. The initial muscle strip length was incrementally increased to find the optimal range. To measure the effects of fatigue on power, cycles of length change with stimulation were repeated over a 1-min period. The frequency of movement was 2 Hz, which is equivalent to 120 breaths/min, close to the average respiratory rate measured in septic mice.

The work performed by the muscle strip is the “positive work” performed during muscle shortening and contraction, minus the “negative work” to bring the muscle back to its original length (59). We evaluated net work as follows: passive force was recorded during muscle shortening and contraction, minus the “negative work”.

\[
\text{Net work} = \int (F - P) \, \text{d}L
\]

where \(F\) is the active force and \(P\) is the passive force. The net work performed during the movement cycle was evaluated as the stimulated record subtracted from the stimulated record to give the active force value.

Isolation of Mitochondria from Total Skeletal Muscle

At the 24-h time point, lower limb, back, and respiratory skeletal muscle were taken from wild-type fed sham, starved sham, and septic mice, with clinical severity scoring performed beforehand. Mitochondria were then isolated as described by Bhattacharya et al. (8) at 4°C. In brief, muscles were rapidly removed, weighed, and placed in ice-cold isolation solution (100 mM KCl, 50 mM Tris-HCl, and 2 mM EGTA, pH 7.4 at 4°C). The muscle was minced, and the muscle slurry was washed and protease-digested in digestion buffer [(in mM) 100 KCl, 50 Tris·HCl, 2 EGTA, 1 ATP, 5 MgCl₂·6H₂O, 0.5% defatted BSA, and protease type VIII (245.7 U/100 ml; Sigma P 5380), pH 7.4]. The mixture was Dounce homogenized and spun for 10 min at 490 g. Supernatant was filtered and spun in two to three stages for 10 min each at 10,400 g. The final pellet (mitochondria) was resuspended in a small volume of isolation medium (0.6–0.8 ml), and protein concentration was measured using a BCA protein assay kit (ThermoFisher Scientific).

To determine percentage recovery of mitochondria from skeletal muscle homogenate from all three groups of mice, citrate synthase activity was assayed at each step of the isolation process by spectrophotometric determination of oxaloacetate-dependent coenzyme A coupled reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) at 412 nm (57), in an assay adapted for 96-well plates.

Respiratory Activity of Isolated Mitochondria and Modular Kinetic Analysis

Respiratory activity of isolated mitochondria was determined using a Clark electrode chamber (Rank Brothers, Bottisham, Cambs, UK) surrounded by a thermostatically controlled water jacket at 30°C and calibrated with air-saturated assay medium. Mitochondria (0.35 mg/ml) were incubated in assay medium [(in mM) 120 KCl, 5 KH₂PO₄, 3 HEPES, 1 EGTA, and 0.3% (wt/vol) defatted BSA, pH 7.4].

Respiratory control ratios were calculated as state 3 respiratory rate after addition of ADP (200 μM) divided by the state 4 rate after ADP phosphorylation with 5 mM pyruvate and 2 mM malate as substrates. Ratios of 3–10 have been quoted as acceptable respiratory control ratios (51, 58). FCCP (1–3 μM) was added at end-study to measure maximal oxygen consumption rate; a threefold rise in respiratory rate has been used to indicate good mitochondrial quality (58).
Modular Kinetic Analysis

This technique allows interrogation of mitochondrial pathways that generate \( \Delta \psi_m \) (substrate oxidation), and pathways that utilize/dissipate it (proton leak and ATP turnover) (10, 15, 47, 48). The conceptual division of mitochondrial respiration into these three kinetic modules is further detailed in Fig. 1. The modules are divided into a substrate oxidation module (\( \Delta \psi_m \) producing); an ATP turnover module, comprising phosphorylation and associated transport activity (\( \Delta \psi_m \) dissipating); and a proton leak module (\( \Delta \psi_m \) dissipating). Substrate oxidation and proton leak kinetics are determined directly, whereas ATP turnover is derived by subtracting proton leak from total \( \Delta \psi_m \) dissipating pathway activity. Studies were performed in a specially adapted 7-ml Clark electrode chamber that simultaneously measures oxygen consumption and \( \Delta \psi_m \) using a methyltriphenylphosphonium (TPMP\(^+\)) electrode in the presence of nigericin (0.1 \( \mu \)M) to abolish the pH gradient. Following addition of mitochondria (0.35 mg/ml) to the assay medium [(in mM) 120 KCl, 5 KH\(_2\)PO\(_4\), 3 HEPES, 1 EGTA, 0.3% (wt/vol) defatted BSA, and 0.1 \( \mu \)M nigericin; pH 7.4], the TPMP\(^+\) electrode was calibrated with five sequential 0.5-\( \mu \)M additions of TPMP. Mitochondria were energized with 8 mM succinate (+5 \( \mu \)M rotenone). The starting oxygen level and the zero point were used for two-point calibration of the Clark electrode (100 and 0% points). FCCP was added at the end of all experimental runs to correct for any drift of the TPMP\(^+\) electrode. Because of the large number of mitochondria needed for each of the protocols, mitochondria isolated from two mice in the same group were pooled. Each protocol was run two times, and the results were averaged.

Because the kinetic response of each module to an effector reflects a change in the common intermediate, \( \Delta \psi_m \), the kinetic curves of modules that consume protonmotive force are independent of the respiratory substrate used to generate that \( \Delta \psi_m \) (2). Succinate (8 mM) was used as a substrate in all experiments, since mitochondrial energization is more readily achieved than with NADH-linked substrates (41). The kinetic dependency of oxygen consumption towards \( \Delta \psi_m \) was described for each kinetic pathway module by modulating \( \Delta \psi_m \) using effectors targeted towards another module within the system. Substrate oxidation kinetics were measured in the presence of oligomycin (1.3 \( \mu \)M) by sequential depolarization with FCCP (0.2–1 \( \mu \)M). Proton leak kinetics were determined in the presence of 1.3 \( \mu \)M oligomycin by sequential additions of malonate (0.1–2.3 mM). The kinetics of total \( \Delta \psi_m \)-dissipating activity (proton leak + ATP turnover reactions) were measured under state 3 conditions (2 mM ADP) by sequential additions of malonate (0.1–2.3 mM). ATP turnover kinetics (phosphorylation and transport reactions) were subsequently calculated by subtraction of proton leak rate from the state 3 respiratory rate at the corresponding value of \( \Delta \psi_m \). FCCP (1 \( \mu \)M) was added at the end of all experimental runs to correct for drift of the TPMP\(^+\) electrode.

To test for statistical significance, respiration rates between flanking values of \( \Delta \psi_m \) were interpolated so that, for each curve, oxygen consumption at \( \Delta \psi_m \) of \(-170 \text{ mV} \) was calculated. This value was the highest \( \Delta \psi_m \) common to all conditions. The higher the value of \( \Delta \psi_m \), the higher the flux of protons through the various \( \Delta \psi_m \)-consuming modules, and therefore the more likely to see a result emerging through the inherent noise of the experimental setup. Additionally, uncoupling proteins (UCP3 in particular) may require a high \( \Delta \psi_m \) to become active (48). One-way ANOVA was used to test for difference in \( \text{VO}_2 \) between the groups at \( \Delta \psi_m \) of \(-170 \text{ mV} \).

**Statistical Analysis**

Statistical analysis was performed using SPSS 18.0 (IBM) software. Data were normally distributed, unless stated. Student’s t-test, Mann-Whitney U-tests, and ANOVA (with and without repeated-measures calculations) were used to test for statistical significance with the alpha error set to <5% (\( P < 0.05 \)). Tukey and Dunnet post hoc tests were used to define significance.

**RESULTS**

**Whole Body Physiology and Skeletal Muscle Function**

**Mortality and whole animal metabolism.** Mortality rates of the septic wild-type (\( n = 11 \)) and Ucp3\(^{-/-}\) (\( n = 12 \)) mice at 24-, 48-, and 72-h time points were similar, at \( \sim 20, 70, \) and 73%, respectively (Fig. 2). No deaths occurred in either wild-
type \((n = 10)\) or \(Ucp3^{-/-}\) \((n = 9)\) sham groups. Septic mice developed a mixed respiratory and metabolic acidosis with biochemical evidence of acute kidney and liver injury (Table 1). The degree of organ dysfunction and acidosis correlated with severity of illness and mortality.

Sepsis produced a large and rapid drop in core temperature and metabolic rate with good correlation between temperature and \(\dot{V}O_2\) (\(R^2 = 0.95\)) (Fig. 3, A–C). Responses were similar in both wild-type and \(Ucp3^{-/-}\) groups. In both genotypes, the sham-fed mice lost a small amount of weight over the first 24 h (\(2 \pm 1\%\) SE body weight change; \(P = 0.08\)). This was more pronounced in the starved sham mice (\(8 \pm 1\%\) SE body weight change; \(P = 0.0001\)). However, the septic mice gained weight (\(5 \pm 1\%\) SE body weight; \(P = 0.001\)) because of a combination of renal dysfunction and fluid sequestration in body cavities (pleural effusions and ascites) and subcutaneous tissues evident at postmortem examination (Fig. 3 D).

### Cardiac function.

Echocardiography was performed in 16 sham and 13 severe septic wild-type mice, and 9 sham and 7 severe septic \(Ucp3^{-/-}\) mice (Fig. 4). Compared with baseline values, there was a marked reduction in cardiac output at 6 and 24 h in the severe septic mice in both genotypes (\(P < 0.02\)).

### Table 1. Blood gas and serum biochemistry at 24 h in wild-type mice following induction of sepsis with comparison against sham controls

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>pCO₂, kPa</th>
<th>pO₂, kPa</th>
<th>Lactate, mmol/l</th>
<th>glucose, mmol/l</th>
<th>urea, mmol/l</th>
<th>creatinine, µmol/l</th>
<th>AST, IU/l</th>
<th>ALT, IU/l</th>
</tr>
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<tbody>
<tr>
<td>sham</td>
<td>7.33 ± 0.03</td>
<td>4.3 ± 0.3</td>
<td>13.4 ± 4.2</td>
<td>2.4 ± 0.9</td>
<td>11.7 ± 4.0</td>
<td>6.5 ± 2.0</td>
<td>10 ± 6</td>
<td>61 ± 30</td>
<td>24 ± 14</td>
</tr>
<tr>
<td>septic</td>
<td>6.96 ± 0.12*</td>
<td>8.5 ± 4.1*</td>
<td>12.2 ± 4.3</td>
<td>5.0 ± 1.2*</td>
<td>14.9 ± 2.4*</td>
<td>34 ± 28*</td>
<td>30 ± 15*</td>
<td>266 ± 95*</td>
<td>77 ± 28*</td>
</tr>
</tbody>
</table>

Values are means ± SD. Hb, hemoglobin; AST, aspartate transaminase; ALT, alanine aminotransferase; IU international unit. *\(P < 0.05\).
within-group ANOVA, but no statistical significance between the two genotypes). Decreases were noted in left ventricular contractility (measured as peak flow velocity), stroke volume, and heart rate. No difference was seen between wild-type and Ucp3/H11002/H11002 groups.

Diaphragm muscle force and power. Measurements were made on diaphragm strips from both wild-type (9 fed sham, 8 starved sham, 9 septic wild type) and Ucp3/H11002/H11002 (6 fed sham, 8 starved sham, 7 septic) mice. The average ages (±SE) were 35 ± 2 wk for wild-type mice and 35 ± 3.5 wk for Ucp3/H11002/H11002 mice (P = 0.92), with average weights of 32 ± 4 and 34 ± 3 g, respectively (P = 0.048). At 24 h, maximal tetanic isometric force generated by diaphragm strips from septic wild-type mice was 28% lower than that produced by either fed or starved sham mice (P < 0.05) (Fig. 5A). Similarly, maximal power generated during a single-length cycle at 4 Hz (mimicking 240 breaths/min, typical for normal mice) was 36 and 28% lower in muscle strips from septic mice than from fed and starved sham mice, respectively (P < 0.05 1-way ANOVA) (Fig. 5B). Similar results were seen in the muscle strips from Ucp3/H11002/H11002 animals (Fig. 5, A and B). The work loop profiles generated by all sets of muscle strips showed a similar contraction-relaxation profile (Fig. 5C).

The effect of fatigue on power output was assessed during cycles of movement and stimulation repeated for 1 min. A movement frequency of 2 Hz was used to mimic the respiratory rate of 120 breaths/min in a septic mouse. A characteristic pattern of power fatigue was seen: an initial rapid decline was followed by a plateau phase, and then a second rapid decline to a lower plateau (Fig. 5D). The initial power in the fatigue test (Fig. 5D) was about one-half of the maximum power (shown in Fig. 5B) because of the difference in movement frequency, 2 and 4 Hz, respectively. For both genotypes, the power output throughout the fatigue test was lower in the septic group than in either of the sham groups. For both genotypes, the duration of the first plateau ended sooner in the septic group (35 ± 3 cycles) compared with both fed sham (41 ± 6 cycles) and starved sham (41 ± 4 cycles) strips (P = 0.02).

In summary, sepsis resulted in severe hypothermia and metabolic suppression associated with cardiovascular dysfunction, lower muscle force and power generation, and earlier fatigue. Because no differences were seen between wild-type and Ucp3/H11002/H11002 mice, this implies that UCP3 does not affect the response to sepsis either at the level of the whole animal or at the level of muscle (dys)function. Further experiments were thus performed using only wild-type animals.

Mitochondrial Physiology

A detailed analysis of mitochondrial function was performed to determine whether mitochondrial dysfunction, potentially including increased proton leak, could underlie the muscle dysfunction described above. Studies were performed using wild-type mice only.
**UCP protein abundance.** UCP3 protein expression in diaphragms taken from wild-type sham \((n = 6)\) and severe septic mice \((n = 9)\) at 24 h was assessed by Western blot. UCP3 protein abundance increased by 26 ± 6% (SE) compared with sham \((P < 0.05)\). The α-tubulin densitometry signal decreased in septic wild-type mouse diaphragms by 22 ± 7% at 10 h \((P = 0.016)\) and by 12 ± 6% at 24 h (not significant). The ratio of UCP3 to α-tubulin change was 47 ± 13% \((P < 0.05; \text{Fig. 6})\). Coomassie staining confirmed equal protein loading and transfer. UCP2 was not detectable in these samples using three different commercially available antibodies.

**Mitochondrial membrane potential in diaphragm muscle strips.** Muscle strips from nine fed sham and nine septic wild-type mice were compared simultaneously. TMRM loading took 45 min to reach steady state before imaging. A typical double-row and subsarcolemmal high-intensity fluorescence pattern was observed in all muscle cells, representing interbrillar and subsarcolemmal groups of mitochondria (Fig. 7, A–C). Baseline TMRM fluorescence in diaphragms from wild-type septic mice was significantly lower than diaphragms from sham mice, suggesting a decrease in \(\Delta\psi_m\) (Fig. 7D). The ATP synthase inhibitor oligomycin was then added to observe changes in \(\Delta\psi_m\) following inhibition of mitochondrial ATP production. This differentiates between states where mitochondria are depolarized as a result of high ATP production and flux through the respiratory chain, from other states where the ATP synthase switches into its native ATPase activity (17, 28). In the diaphragm strips, oligomycin increased TMRM fluorescence, with a greater relative increase above baseline in the septic group (increase of 30 ± 5% in septic diaphragm vs. 16 ± 8% in sham; \(P < 0.001\)). Absolute values of fluorescence postoligomycin were not statistically different between the two groups \((P = 0.1)\), suggesting the differences are attributable to a difference in proton flux through the \(F_0F_1\)-ATP synthase. The addition of FCCP resulted in complete dissipation of \(\Delta\psi_m\) in all specimens examined, confirming unquenched mitochondrial TMRM signal (24).

**Citrate synthase activity and respiratory control of isolated mitochondria from skeletal muscle.** Mitochondria were isolated from muscles of 8 fed sham, 8 starved sham, and 13 severe septic wild-type mice (clinical severity 5–6). Fractional recovery (mean ± SD) of citrate synthase activity at each step of the mitochondrial preparation process compared with the initial muscle homogenate was similar in all three groups, with a final recovery in the mitochondrial pellet of 43 ± 11% (fed sham), 38 ± 23% (starved sham), and 41 ± 9% (septic) \((P = 0.48)\).
Oxygen consumption rate was significantly lower in mito-
chondrial sham, and 120/H11006 septic mice: for a given mem-
bane potential (170 mV), the/H11002 (P with both fed sham (230
slurry (n
Fig. 6. E720 SKELETAL MUSCLE MITOCHONDRIAL BIOENERGETICS IN SEPSIS

DISCUSSION

The respiratory control of the final mitochondrial pellet mea-
sured using the NADH-linked substrates pyruvate and malate
was also similar (3.0 ± 0.6 fed sham, 3.5 ± 0.9 starved sham,
2.9 ± 0.5 septic; P = 0.47).

Modular kinetic analysis of isolated skeletal muscle mitochondria. The effect of sepsis on the kinetic behavior of processes that govern ΔΨm was examined using modular kinetic analysis in 8 fed sham, 8 starved sham, and 13 severe septic mice. Substrate oxidation (ΔΨm-producing) and proton leak (ΔΨm-consuming) kinetics were determined directly, whereas ATP turnover (ΔΨm consuming) was derived by subtracting proton leak from total ΔΨm-dissipating pathway activity. Proton leak kinetics were similar in all three groups (Fig. 8, A and B). However, the kinetic behavior of total ΔΨm-dissipating processes (Fig. 8, C and D) and ATP turnover processes (Fig. 8, E and F) was significantly affected in the septic mice: for a given membrane potential (~170 mV), the oxygen consumption rate was significantly lower in mito-
bhordia from septic animals (207 ± 26 fed sham, 157 ± 53 starved sham, and 120 ± 30 septic nmol oxygen·min⁻¹·mg⁻¹; P < 0.05). Whereas the substrate oxidation kinetic curves of the septic and fed sham mice were similar (Fig. 8, G and H), the curve for the starved sham group (316 ± 19 nmol oxygen·min⁻¹·mg⁻¹) was significantly different compared with both fed sham (230 ± 17) and septic (312 ± 22) groups (P < 0.05), with oxygen consumption rates lower at all values of ΔΨm.

In this rodent model of sepsis and multiorgan failure, we have shown early muscle dysfunction and fatigue associated with metabolic suppression, lower ΔΨm and phosphorylation capacity, but no change in mitochondrial proton leak despite an increase in UCP3 protein abundance. These changes were unaffected by the presence or absence of UCP3 protein.

The pathophysiology of multiorgan failure and muscle dys-
function in established sepsis is complex and multifactorial. Recent interest has focused on bioenergetic dysfunction: increased production of reactive oxygen species results in disrup-
tion of metabolic processes and cell damage (12, 13, 61), whereas decreased transcription of mitochondrial proteins af-
fects respiratory capacity (18, 32). A decrease in cell metab-
olism may also occur for other reasons, e.g., secondary to a decrease in thyroid activity associated with critical illness (9).

Recent publications have highlighted limitations of mouse models of sepsis and the lack of translation of successful treatments from animal models to humans (31, 56). Indeed, significant disparity was reported between mice and humans in their gene expression profile response to inflammatory condi-
tions, including sepsis (56). We reported marked differences in the metabolic response to sepsis between rats and mice (67). As seen in the present study, mice showed an early and severity-dependent hypometabolic, hypothermic phenotype with early myocardial depression. This presentation is seen in ~10% of cases of human sepsis and is associated with a two- to threefold increased risk of mortality (4, 19). However, the ability to define the roles of specific genes by genetic manip-
ation and to study fully intact biological systems in complex diseases justify an ongoing role for rodent models in defining pathophysiological mechanisms (46). We did make consider-
able efforts to refine and optimize our murine septic model. These included using older mice equivalent to 30- to 40-yr-old humans (63), injecting a standardized septic inoculum, provid-
ing regular fluid administration to limit hypovolemia-induced tissue hypoperfusion, and performing a block randomization for group allocation in experiments (67).

Metabolic suppression and hypothermia are well-described phenomena in septic mice, and are influenced by age, septic insult, and ambient temperature (22, 54, 62, 67). These included using older mice equivalent to 30- to 40-yr-old humans (63), injecting a standardized septic inoculum, provid-
ing regular fluid administration to limit hypovolemia-induced tissue hypoperfusion, and performing a block randomization for group allocation in experiments (67).

Metabolic suppression and hypothermia are well-described phenomena in septic mice, and are influenced by age, septic insult, and ambient temperature (22, 54, 62, 67). We previously demonstrated that rewarming of septic mice to normothermia failed to increase metabolic rate (67), implying a primary metabolic suppression rather than a reactive response to hypother-

UCP3 is upregulated in sepsis (60, 66), prompting specula-
tion about the role of this protein in decreasing mitochondrial coupling efficiency. This may, however, act as a negative feedback loop to reduce ROS production (11). We set out to establish the importance of functional consequences of this increase in UCP3 expression, but instead report no detectable differences in the responses of Ucp3⁻/⁻ mice to sepsis. Rather than detecting effects on mitochondrial proton leak, our find-
ings suggest that processes related to mitochondrial phosphor-
lization may be affected.

Previous studies in healthy Ucp3⁻/⁻ mice also found little difference in thermoregulation and oxygen consumption rate compared with their wild-type controls (6, 64). Despite a fivefold increase in skeletal muscle UCP3 in mice given intraperitoneal endotoxin, Yu et al. in fact described a drop in core temperature to 30°C (66). While we too observed an increase in skeletal muscle UCP3, we found no impact of the lack of UCP3 protein upon metabolic changes, temperature, cardiovascular and muscle function, or mortality in sepsis.
Similarly, although skeletal muscle mitochondria isolated from healthy *Ucp3<sup>-/-</sup>* mice showed more coupled respiration and increased ROS production (64), we could find no differences in force, power, and response to repeated stimulation between diaphragm muscle strips isolated from septic wild-type and *Ucp3<sup>-/-</sup>* mice. The original description of the *Ucp3<sup>-/-</sup>* mouse showed no compensatory upregulation of other UCPs (64), although it is conceivable that such upregulation may be seen with sepsis. In line with previous work, we were unable to detect UCP2 in muscle from either sham or septic mice using a number of commercially available UCP2 antibodies (data not shown).

We imposed repeated work-loop cycles to mimic in vivo diaphragm muscle activity to assess muscle fatigue (59). To our knowledge, the present study is the first description of this functional assessment in a septic model. The plots show a characteristic three-phase response (39); the second phase, a quasiplateau phase of reduced function, involves recruitment of oxidative metabolism and ends with depletion of high-energy substrates and accumulation of phosphate and other end-products of metabolism (5). We found that this second phase was significantly shorter in septic mice, was affected by hypoxia (data not shown), but was independent of UCP3 protein expression. This finding supports the notion of linkage between disorders of oxidative metabolism and an inability to maintain high levels of energy substrate.

A significantly lower $\Delta\psi_m$ was seen in the diaphragmatic myocytes of septic mice. While previously shown in cells (1,
to our knowledge this is the first demonstration of altered Δψₘ in sepsis in a complex muscle preparation. The low Δψₘ was a consistent finding in the septic mice and suggests either a decreased rate of Δψₘ generation (e.g., because of a respiratory chain defect) or an increase in turnover of pathways that dissipate the gradient such as proton leak or ATP production (23). Blocking ATP synthase with oligomycin increased fluorescence and hence Δψₘ. This rise was significant in the septic group and suggests higher ATP turnover in these diaphragm muscle cells. This was an unexpected finding, since previous studies had shown lower respiratory complex activity and lower oxygen consumption in tissues and cells taken from septic animals and humans (13, 16, 20).

Our interrogation of kinetic pathways involved in the maintenance of Δψₘ implies that the mitochondrial pathway that generates Δψₘ through succinate oxidation was unaffected in this model of sepsis. Substrate oxidation rates of mitochondria isolated from starved sham mice were depressed at all values of Δψₘ compared with the other groups. This implies either an altered sensitivity of these mitochondria to FCCP and/or that metabolic adaptations in sepsis do not simply reflect a stressed nutritional state. Previous descriptions of mitochondrial function in sepsis suggest complex I dysfunction (12, 13). Whereas a full kinetic description of NADH-linked substrate oxidation kinetics was not attempted here because of technical constraints, neither state 3 nor state 4 respiration was significantly different between groups when the mitochondria were energized with complex 1 substrates pyruvate and malate.

Proton leak kinetics were unaffected by the septic insult, despite the rise in UCP3 protein levels in the septic mice, although these experiments were not repeated in the presence of free fatty acids or GDP (47). However, ATP turnover...
kinetics were significantly affected in the septic mice with lower oxygen consumption rates at given values of $\Delta \psi_m$ when mitochondria were phosphorylating ADP. Defects in ATP synthase in sepsis have been described previously and may explain these observations (16, 37, 43, 52). The higher ATP turnover in the diaphragms of septic mice suggested by the $\Delta \psi_m$ experiments, in conjunction with the altered ATP turnover kinetics seen in muscle mitochondria isolated from septic mice, may explain, at least in part, the earlier fatigue observed in the diaphragm muscle strip studies.

An issue to consider with the use of isolated mitochondria, especially in conditions such as sepsis, is that removal from their usual cellular environment takes away the impact of local regulatory processes and high levels of inflammatory mediators, and also exposes them to a supranormal oxygen milieu. Such regulatory processes include energy-consuming pathways, resulting in altered ATP demand, or pathways that impose control over substrate trafficking into the mitochondria and substrate oxidation, thereby regulating respiratory chain function and, ultimately, ATP synthesis (14). Nitric oxide, pyruvate dehydrogenase kinase-4 and hypoxia-inducible factor-1a are all implicated in sepsis (3, 12, 49); these likely continue to impose control over mitochondrial function in the intact cell and tissues. Following mitochondrial isolation and washing, and exposure to hyperoxic conditions, such control may be lost. A comparison of our findings using in situ and isolated mitochondria implies that sepsis modifies both local regulatory processes and the oxidative phosphorylation machinery itself. These functional changes were not associated with swollen or ruptured mitochondria in an examination of electron micrographs of diaphragm muscles from a limited number of sham and septic mice (data not shown). Although no formal morphological examination of these was carried out, no gross changes in the shape, size, or distribution of mitochondria were noted.

In summary, the long-term, resuscitated septic mouse model that we have described exhibits metabolic suppression, hypothermia, and decreased cardiovascular activity. Diaphragm muscle strips from septic mice exhibit increased fatigability ex vivo; this may be related in part to mitochondrial dysfunction. Despite increased UCP3 protein abundance in sepsis, kinetic analysis of isolated mitochondria from skeletal muscle revealed that proton leak kinetics were not different from sham controls, whereas the lack of UCP3 protein did not impact on mortality, physiological changes, or muscle functionality. However, ATP turnover kinetics were significantly altered, implying a defect with ATP synthesis at the level of the F$_0$F$_1$ synthase or an associated transport process.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: P.S.Z., J.E.C., N.P., N.A.C., M.R.D., and M.S. conceived and designed the experiments; P.S.Z. performed the experiments; P.S.Z. and N.P. analyzed the data; P.S.Z., J.E.C., N.P., N.A.C., M.R.D., and M.S. interpreted the data; P.S.Z. prepared figures; P.S.Z. drafted the manuscript; P.S.Z., J.E.C., N.P., N.A.C., M.R.D., and M.S. edited and revised the manuscript; P.S.Z., J.E.C., N.P., N.A.C., M.R.D., and M.S. approved final version of the manuscript.

REFERENCES


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