Survival in Critical Illness Is Associated with Early Activation of Mitochondrial Biogenesis

Jane E. Carre1, Jean-Christophe Orban1,2, Lorenza Re1,3, Karen Felsmann4, Michael Bauer5, Hagir B. Suliman6, Claude A. Piantadosi6, Terry M. Mayhew7, Patrick Breen1, Martin Stotz1, and Mervyn Singer1

1Bloombsury Institute for Intensive Care Medicine, Department of Medicine and Wolfson Institute of Biomedical Research, University College London, London, United Kingdom; 2Service de Réanimation, Faculté de Medicine and CHU de Nice, Hôpital Saint-Roch, Nice, France; 3Dipartimento di Anestesia e Rianimazione, Azienda Ospedaliero-Universitaria Ospedali Riuniti Umberto I-Lancisi-Salesi, Ancona, Italy; 4SIRS-Lab GmbH, Jena, Germany; 5Clinic for Anesthesiology and Intensive Care Therapy, Friedrich-Schiller-University Jena, University Hospital, Jena, Germany; 6Department of Medicine, Duke University Medical Center, Durham, North Carolina; and 7School of Biomedical Sciences, Queen’s Medical Centre, University of Nottingham, Nottingham, United Kingdom

Rationale: We previously reported outcome-associated decreases in muscle energetic status and mitochondrial dysfunction in septic patients with multiorgan failure. We postulate that survivors have a greater ability to maintain or recover normal mitochondrial functionality.

Objectives: To determine whether mitochondrial biogenesis, the process promoting mitochondrial capacity, is affected in critically ill patients.

Methods: Muscle biopsies were taken from 16 critically ill patients recently admitted to intensive care (average 1–2 d) and from 10 healthy, age-matched patients undergoing elective hip surgery.

Measurements and Main Results: Survival, mitochondrial morphology, mitochondrial protein content and enzyme activity, mitochondrial biogenesis factor mRNA, microarray analysis, and phosphorylated (energy) metabolites were determined. Ten of 16 critically ill patients survived intensive care. Mitochondrial size increased with worsening outcome, suggestive of swelling. Respiratory protein subunits and transcripts were depleted in critically ill patients and to a greater extent in nonsurvivors. The mRNA content of peroxisome proliferator-activated receptor γ coactivator 1-α (transcriptional coactivator of mitochondrial biogenesis) was only elevated in some proliferator-activated receptor γ coactivator 1-α (PGC-1α), nuclear respiratory factor-1 (NRF-1), and mitochondrial transcription factor-A (TFAM) (10, 11). Expression of mitochondrially localized proteins is regulated by nuclear and mitochondrial genomes and regulation of mitochondrial content and morphology (10, 11). Expression of mitochondrial mRNA content. In surviving patients, these changes appear to be counteracted by early activation of the mitochondrial biogenesis (restorative) response. An early response to maintain mitochondrial functional capacity may thus be crucial to balance mitochondrial protein turnover, maintain cellular energetic status, and promote organ recovery in critical illness.

Scientific Knowledge on the Subject

An inability to use oxygen at the cellular level has been suggested in multiple organ failure. Mitochondria use oxygen to provide energy for cellular processes. Dysfunction of these organelles has been identified in critical illness, leading to the hypothesis that cellular energetic failure may contribute to organ failure.

What This Study Adds to the Field

Our data suggest the early decrease in functional capacity of mitochondria seen in the muscle of critically ill patients is associated with decreases in mitochondrial respiratory protein content. In surviving patients, these changes appear to be counteracted by early activation of the mitochondrial biogenesis (restorative) response. An early response to maintain mitochondrial functional capacity may thus be crucial to balance mitochondrial protein turnover, maintain cellular energetic status, and promote organ recovery in critical illness.

Multiple organ failure (MOF) after sepsis and other acute inflammatory insults is a significant cause of mortality and morbidity (1). MOF is associated with overwhelming systemic inflammation with immune, metabolic, endocrine, and cardio-

vascular dysfunction (2, 3). Although failure to restore compromised oxygen delivery in shock states is a major contributor, an inability to use cellular oxygen at the mitochondrial level (cytopathic dysoxia) is also suggested to play a significant role (4–6). Recovery would therefore be contingent on adequate early maintenance, or subsequent restoration, of mitochondrial function to meet metabolic energy demands and to fulfill other roles including calcium homeostasis, maintenance of cellular redox state, and cell signaling (7).

We previously reported an association between decreasing skeletal muscle mitochondrial functionality and illness severity in patients with septic shock and MOF within 24 hours of intensive care admission (8). Markers of oxidative and nitrosative stress also correlated with decreased activity of respiratory Complex I, a mitochondrial respiratory enzyme susceptible to inhibition by reactive nitrogen species (9). ATP content was significantly lower in nonsurvivors compared with eventual survivors at this early point in their critical illness.

Mitochondrial biogenesis involves coordination of expression, import, and assembly of mitochondrial proteins from nuclear and mitochondrial genomes and regulation of mitochondrial content and morphology (10, 11). Expression of mitochondrialy localized proteins is regulated by nuclear and mitochondrial factors, including peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α), nuclear respiratory factor-1 (NRF-1), and mitochondrial transcription factor-A (TFAM) (10, 11).
12, 13). PGC-1α seems to be particularly important in global regulation of oxidative metabolism (14) and is itself regulated at multiple levels (e.g., by the nutrient/energy sensing pathways AMP-activated protein kinase, sirtuins and mechanistic target of rapamycin, and the inflammation-linked pathways p38 MAP kinase and NO synthase/guanylate cyclase) in response to exercise/immobility, cytokines, hormones, nitric oxide, and oxygen availability (13). Because these factors are implicated in sepsis and MOF (2), altered mitochondrial biogenesis represents a strong candidate mechanism by which mitochondrial function and its ability to recover is affected in critical illness. Because recent laboratory data support this hypothesis (15, 16), we investigated biogenesis responses in muscle biopsies sampled from critically ill patients soon after their admission to intensive care. Some of this work has been reported in abstract form at the European Society of Intensive Care Medicine Annual Congress (17, 18).

**METHODS**

**Patients**

The study was approved by the UCL/UCLH Ethics Committee. Patients with recent-onset critical illness and MOF were recruited from the intensive care unit (ICU). Patients with long-term hospitalization, cachexia, severe coagulopathy (platelet count <30 × 10^9/L or international normalized ratio >2), or immunosuppression (e.g., post-chemotherapy) were excluded. Patients (or next-of-kin) were asked for informed consent (or agreement) before enrollment. Retrospective consent to use their data was obtained from patients who regained mental competency. The control group consisted of otherwise healthy patients undergoing elective total hip replacement surgery for degenerative arthropathy.

**Procedures**

Full details appear in the online supplement. Briefly, soon after intensive care admission (on average 1–2 d) (Table 1), a vastus lateralis muscle biopsy (100–250 mg total) was performed as described (8). Tissue was freeze-quenched in liquid nitrogen and stored at −80°C until batch analysis. Some fresh tissue was fixed for transmission electron microscopy. Blood was sampled immediately before the biopsy; serum was separated and stored at −80°C for later analysis. In orthopedic patients, biopsies were taken from the vastus lateralis through the operation site at the beginning of surgery and processed as described above.

**Analyses**

Samples were analyzed in a blinded fashion. Nucleotides and creatine compounds were quantified by reverse-phase HPLC of neutralized perchloric acid extracts (19). Relative content of mitochondrial proteins was determined in tissue extracts by immunoblotting, using primary antibodies for Complex I (NDUFA9, NDUFB8) and Complex IV (COX1, COX2, COX4) subunits (Mitosciences, Eugene, Oregon) and manganese superoxide dismutase (MnSOD) (SOD2: Abcam, Cambridge, UK). Data were normalized to Coomassie blue total protein stain and referenced to an internal standard (a control patient sample). Data were analyzed for statistical significance across groups using nonparametric Kruskal-Wallis testing, with Mann-Whitney U post hoc analysis. P values less than 0.05 were considered statistically significant; for clarity, P values less than 0.1 are shown in the figures. Correlations were calculated by determining Spearman’s rank correlation coefficient (r_s).

**Statistical Analysis**

Due to limitations in sample size, not all analyses could be performed on every patient. Data were analyzed for statistical significance across groups using nonparametric Kruskal-Wallis testing, with Mann-Whitney U post hoc analysis. P values less than 0.05 were considered statistically significant; for clarity, P values less than 0.1 are shown in the figures. Correlations were calculated by determining Spearman’s rank correlation coefficient (r_s).

**RESULTS**

**Patients**

Sixteen patients with MOF were recruited, 10 of whom survived. Ten age-matched patients undergoing elective hip surgery served as control subjects. Overall patient demographics are shown in Table 1; individual personal data, clinical characteristics, and interventions are presented in Table E1 in the online supplement.
online supplement. Briefly, all 16 critically ill patients were receiving antibiotics plus sedation for mechanical ventilation, and 11 required vasoactive drug administration for circulatory support at the time of biopsy. Insulin was given where needed to maintain glycemic control. Mean arterial blood pressure and catecholamine requirements were similar in critically ill survivors and nonsurvivors. No patient was moribund at the time of biopsy. Clinical severity, as determined by the SOFA Score (24), was similar on ICU admission but higher at the time of biopsy (average 1–2 d after ICU admission) in eventual nonsurvivors \( (P = 0.045) \). No complications occurred from the biopsy procedure.

Morphology

Muscle morphology in critically ill patients differed qualitatively from control subjects, with regional sarcromere disruption, including loss of integrity of A, I, and M bands; greater filament separation; and poorer Z line registration (Figure 1). There was also mild swelling of mitochondria. In muscle tissue from control subjects, mitochondria accounted for 3.2% (coefficient of variation [CV], 30%) of muscle volume, and this fraction was similar in survivors and nonsurvivors. In muscles tissue from control subjects, \( S_V \) was 0.51 \( \mu \text{m}^3/\mu \text{m}^4 \) (CV, 89%); this declined by 39% in survivors \( (P = 0.013) \) and by 53% in nonsurvivors \( (P = 0.028) \). Similarly, the S/V ratio fell by 28% in survivors \( (P = 0.075) \) and by 52% in nonsurvivors \( (P = 0.007) \). Changes in \( S_V \) and S/V ratios imply changes in mitochondrial dimensions; for circular cylinders, these equate to diameters of 0.24 \( \mu \text{m} \) (CV, 14%) in control subjects, 0.40 \( \mu \text{m} \) (CV, 53%) in survivors, and 0.56 \( \mu \text{m} \) (CV 38%) in nonsurvivors.

Mitochondrial Respiratory Chain Complex Protein Subunits and Activities

Mitochondrial-encoded (COX1 and COX2) and nuclear-encoded (NDUFA9, NDUFB8, and COX4) Complex I and Complex IV respiratory chain protein subunits were decreased in critically ill patients compared with control subjects (Figure 2; Figure E1a in the online supplement). Eventual nonsurvivors showed an overall trend toward lower subunit content. Complex I activity followed a similar pattern, decreasing progressively with worsening outcome. Complex I activity was proportional to that of citrate synthase, a reference mitochondrial enzyme. Thus, the ratio of Complex I to citrate synthase activity remained overall unchanged \( (P = 0.47) \) because citrate synthase activity was 40% lower in nonsurvivors compared with survivors \( (P = 0.022) \). Complex IV activity also correlated with that of citrate synthase; protein-normalized activities of both enzymes were overall maintained or elevated in eventual survivors versus nonsurvivors. The cause of the disparity between subunit content and activity of Complex IV in critically ill patients is unknown but may involve regulatory mechanisms such as subunit phosphorylation (25) and differential isoform expression (26, 27) that, in sepsis, have received little attention to date.

Transcriptomics

Transcriptomic analysis of skeletal muscle detected a total of 23,876 bead-types, of which 875 met the \( P \) value and fold-change thresholds for differential gene expression between survivors and nonsurvivors (Figure 3A; Table E2). Molecular pathways most significantly represented among these altered transcripts were mitochondrial respiration and dysfunction and inflammatory signaling pathways (Table E3).

Expression patterns for mitochondrial respiratory complex subunit (OXPHOS) transcripts were examined. Figure 3B shows heatmaps depicting normalized expression values for 127 detected nuclear-encoded subunits of the five respiratory complexes. Despite some interpatient variation within groups, an overall decrease in OXPHOS transcript abundance was seen in critically ill patients, particularly in Complexes I and V. This trend became clearer when the mean fold-change of the 26 OXPHOS transcripts (eight from Complex I, two from Complex II, three each from Complexes III and IV, and 10 from Complex V) meeting significance criteria was evaluated (Figure 3C). For nonsurvivors versus control subjects, all 26 transcripts showed a mean fold-decrease of at least 1.5 compared with only 10 of the transcripts for survivors. OXPHOS transcripts were more abundant in survivors versus nonsurvivors.

Mitochondrial Biogenesis and Oxidative Stress Factors

Transcript abundance of key factors involved in mitochondrial biogenesis was also measured by quantitative real-time RT-PCR (Figure 4A). Compared with control subjects, mRNA levels of PGC-1\( \alpha \) were elevated on average by 2.5-fold in survivors, whereas transcript abundance in nonsurvivors was unchanged. A similar trend was observed for NRF-1. Besides serving as a coactivator for NRF-1, PGC-1\( \alpha \) promotes expression of NRF-1 mRNA (28). Positive correlation was seen between transcript levels of these two nuclear factors \( (r^2 = 0.576; P = 0.005; \text{Fig E2}) \). Microarray analysis revealed that,

**Figure 1.** Ultrastructural features of muscles and their mitochondria. (A) Control muscle showing well-aligned sarcomeres with well-defined A, I, and M bands and Z lines. Clusters of dense mitochondria are seen between fibers. (B and C) Muscle from a sepsis survivor showing focal breakdown of muscle morphology with loss of sarcomere structural integrity and swollen or damaged mitochondria. (D and E) Muscle from a nonsurvivor showing that mitochondrial changes affect the subsarcolemmal and interfiber regions. Bar = 2 \( \mu \text{m} \) in all cases.
relative to control subjects, transcript levels of PGC-1α (PPARGC1A) were 8.8-fold lower in nonsurvivors (P = 0.006) but were unchanged in survivors. Besides regulating expression of OXPHOS subunits, PGC-1α is a coactivator of gene expression for some components of the mitochondrial oxidative stress response (e.g., MnSOD) (29). Consistent with the above findings, skeletal muscle MnSOD protein (Figure 4B; Figure E1B) was significantly (2.5-fold) elevated in survivors but was unchanged in nonsurvivors. PGC-1α transcript and MnSOD protein were positively correlated across the three patient groups (Figure 4C).
B. 18S rRNA; median, 25th and 75th percentile and range for biopsies

...protein manganese superoxide dismutase (MnSOD). (A) transcription factor-A (TFAM) and the mitochondrial oxidative stress...nonsurvivors (Carre´, Orban, Re, and PGC-1...program of mitochondrial biogenesis in survivors. First, tran...

Figure 4. Changes in critical illness of the mitochondrial biogenesis factors peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α), nuclear respiratory factor-1 (NRF-1), and mitochondrial transcription factor-Α (TFAM) and the mitochondrial oxidative stress protein manganese superoxide dismutase (MnSOD). (A) Transcript abundance for PGC-1α, NRF-1, and TFAM, normalized to content of 18S RNA; median, 25th and 75th percentile and range for biopsies from 10 control patients, 9 survivors, 3 nonsurvivors. (B) Semiquantitative analysis of MnSOD (SOD2) protein from immunoblots, expressed semiquantitatively as pixel density relative to an internal standard, normalized to total protein stain. Median plus 25th and 75th percentile and range for biopsies from 10 control patients, 10 survivors, and 6 nonsurvivors. (C) Positive association between MnSOD protein and PGC-1α mRNA. Control (open circles), survivors (gray diamonds), nonsurvivors (closed squares).

Phosphorylated Metabolites

As we reported previously (8), ATP content was significantly higher in survivors compared with nonsurvivors (Figure E3). The phosphocreatineATP and phosphocreatine/creatinine ratios were decreased in survivors relative to the other groups. Total creatine (phosphocreatine + creatine) content was similar across groups.

DISCUSSION

Despite the clinically heterogeneous nature of our patients, skeletal muscle mitochondrial capacity was decreased soon after intensive care admission, and to a greater extent in those who subsequently died. Although mitochondrial volume fraction of muscle was similar across groups, the outer membrane Sn decreased and mitochondria were more swollen in critically ill patients, more so in nonsurvivors. Together, these data are consistent with our previous findings of outcome-related differences in mitochondrial function in human and experimental septic shock in muscle and liver (8, 15, 30). An important new observation is that this early decrease in functional capacity is associated with global decreases in mitochondrial respiratory protein and transcript content, which, in the survivor group, appears to be counteracted by early activation of mitochondrial biogenesis and oxidative stress response.

Likely mechanisms underlying the decline in mitochondrial capacity in severe sepsis and other systemic inflammatory critical illnesses include direct oxidative/nitrosative inhibition of respiratory enzyme complexes, oxygen supply limitation from concurrent circulatory perturbations, hormonal disturbances, decreased mitochondrial gene transcription, or increased mitochondrial degradation through specific action of mitochondrial proteases or globally through autophagy (6, 31, 32). Substrate limitation or decreased coupling efficiency of oxidative phosphorylation could also affect mitochondrial function in critical illness (5).

Complex I is particularly susceptible to inhibition by S-nitrosation (33). In the current study, Complex I activity decreased in critically ill patients when normalized to tissue protein content, was proportional to citrate synthase activity, and was associated with Complex I protein subunit depletion. Although not directly measured, these data imply protein turnover rather than posttranslational modification of Complex I as a likely mechanism of decreased activity. Increased mitochondrial protein degradation has been inferred in muscle taken from patients with sepsis (34); mitochondrial protein depletion was observed despite unchanged protein synthesis rates. A morphological study of postmortem liver sections from patients with sepsis indicated that hepatocyte autophagic vacuolization increased during sepsis compared with control subjects and was associated with signs of mitochondrial injury (although the control samples were taken from living patients) (31). Similar findings were made in livers of septic mice (31, 32) or rats (35).

The perturbations in mitochondrial shape, including an increase in mean diameter, are consistent with localized mitochondrial swelling (36). The fractional volume of muscle occupied by mitochondria was constant across groups. However, because muscle atrophy is a feature of sepsis and critical illness (37), a constant volume fraction would represent a decrease in total mitochondrial volume if the volume of vastus lateralis muscle declined in critically ill patients. Equally, the number of mitochondrial genomes in a given volume of muscle may be decreased as the same volume fraction of muscle is occupied by larger (swollen) mitochondria.

Mitochondrial biogenesis represents an important mechanism through which regulation of mitochondrial capacity can occur during MOF and its recovery phase. Indeed, in a long-term murine peritonitis model, we demonstrated activation of mitochondrial biogenesis programs (demonstrated by transcript levels of PGC-1α, NRF-1, and TFAM) that preceded recovery of mitochondrial function, metabolic rate, and physiologic and biochemical organ function (15). Exercise and endotoxin exposure activate muscle PGC-1α by protein phosphorylation. This modification increases PGC-1α protein stability, promotes its nuclear translocation, and precedes increases in its mRNA abundance (38–40). In the present study, attempts to measure PGC-1α protein content or its cellular location were hampered by the low relative abundance of the protein precluding detection in these tissue extracts. Nevertheless, several lines of evidence do suggest an early activation of the transcriptional program of mitochondrial biogenesis in survivors. First, transcript levels of the biogenesis effector NRF-1 were positively correlated with those of its major coactivator, PGC-1α. Second, the decrease in relative abundance of respiratory chain subunit transcripts occurred to a lesser extent in survivors than nonsurvivors. Third, protein content of the respiratory complex subunits declined to a lesser extent in survivors, whereas MnSOD, transcription of which can be coactivated by PGC-1α (29), was significantly elevated in the survivor group alone.

A recent study (34) comparing ICU and elective surgical patients (biopsied at variable times between Days 1 and 42)
reported unaltered skeletal muscle transcripts for mitochondrial or nuclear genes encoding for mitochondrial-related enzymes. Although PGC-1α and NRF-1 transcripts were unchanged, evidence of mitochondrial biogenesis was suggested by increased expression of NRF-2a/GABP and nuclear-encoded mtDNA-regulating factors (TFAM, TFB1M, TFB2M). Differences with our findings likely reflect disparities in biopsy timing and illness severity because our patient cohort was sampled earlier and was more severely ill. These authors did not examine survivors and nonsurvivors separately.

We found no association between transcript levels of mitochondrial biogenesis markers and age, timing of biopsy (Figure E2C), or clinical measures of severity (e.g., SOFA score, catecholamine requirements) (data not shown). However, similar to our previous findings (8), ATP content was negatively associated with catecholamine requirements (an indicator of shock severity); a similar trend was seen for respiratory protein subunits and MnSOD (data not shown). Using serum levels of the acute-phase cytokine IL-6 as a marker of the degree of inflammation (41), MnSOD and ATP content were depressed at high IL-6 levels, although the relationship between inflammation and PGC-1α mRNA was less apparent (Figure E4). Systemic inflammation causes muscle wasting (42), and the qualitative differences seen in fine structure morphology are consistent with some degree of atrophy during early critical illness. Any causal relationship between mitochondrial perturbations and muscle atrophy in cachetic states is unknown.

Interventions performed in intensive care may affect mitochondrial biogenesis or functional capacity (43, 44). These include bacteriostatic antibiotics (45–47), catecholamines (48), corticosteroids (49), thyroid analogs (50), and nutrition (51). Similarly, complications of critical illness, including hyperglycemia (51), immobility, and mechanical ventilation (52), may compromise mitochondrial function. The sample size and heterogeneity of our patient population precludes definitive conclusions being drawn on the influence of these iatrogenic factors.

Muscle mitochondrial ATP production in health is primarily regulated in response to ATP demand. Decreases in mitochondrial capacity typically become evident as dysfunction only when ATP demand outstrips supply (53). The depressed phosphocreatine/creatine and phosphocreatine/ATP ratios in survivors suggest an increase in ATP consumption, although the pH sensitivity of these ratios is a potential confounder. An early biogenesis response to maintain mitochondrial function, as seen in survivors, may partly enable increased ATP demands to be met by balancing mitochondrial protein turnover.

Limitations of this study include the relatively small group sizes and heterogeneity in clinical characteristics. Recruitment was problematic because of underlying patient factors that excluded enrolment (e.g., prolonged illness before ICU admission, obvious cachexia, severe coagulopathy) or next-of-kin reluctance to agree to their relatives’ participation in this nontherapeutic study. Genome-wide expression analyses generating false discovery rate based upon random chance, particularly when using a small sample size and multiple (i.e., thousands of) gene transcript measurements. However, pathway analysis assessing changes in related groups of transcripts (e.g., those relating to oxidative phosphorylation) increases confidence that our findings are biologically significant rather than due to random chance. Additionally, the multifaceted approach used in this study strongly supports the biological significance of our findings because similar trends in muscle mitochondrial capacity between patient groups were seen at the transcript, protein, and enzyme activity level (Figures 2–3) as well as in functional measures, including ATP and phosphocreatine (Fig. E3).

In summary, we found that survival in critical illness was associated with early activation of mitochondrial biogenesis and the oxidative stress response that may serve to counteract depletion of mitochondrial transcripts and proteins, enabling ATP demands to be met. Failure (or delay) to activate mitochondrial biogenesis early in critical illness (due to illness severity, drug administration, or patient phenotypes or genotypes) may increase susceptibility to oxidative and nitrosative mitochondrial damage, exacerbate differences in clearance of damaged organelles, and lead to a net decrease in mitochondrial content with cellular energetic failure. Such an impairment may affect the ability to recover function. Although associative in nature, we hope this work will stimulate mechanistic investigations aimed toward a new therapeutic approach for critical illness, namely active methods to support mitochondrial biogenesis (54). Attention should also be paid to the impact of current therapies that inhibit mitochondrial biogenesis, notably bacteriostatic antibiotics.

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**References**

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