Mechanisms of disease

Association between mitochondrial dysfunction and severity and outcome of septic shock

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Summary

Background Sepsis-induced multiple organ failure is the major cause of mortality and morbidity in critically ill patients. However, the precise mechanisms by which this dysfunction is caused remain to be elucidated. We and others have shown raised tissue oxygen tensions in septic animals and human beings, suggesting reduced ability of the organs to use oxygen. Because ATP production by mitochondrial oxidative phosphorylation accounts for more than 90% of total oxygen consumption, we postulated that mitochondrial dysfunction results in organ failure, possibly due to nitric oxide, which is known to inhibit mitochondrial respiration in vitro and is produced in excess in sepsis.

Methods We did skeletal muscle biopsies on 28 critically ill septic patients within 24 h of admission to intensive care, and on nine control patients undergoing elective hip surgery. The biopsy samples were analysed for respiratory-chain activity (complexes I–IV), ATP concentration, reduced glutathione (an intracellular antioxidant) concentration, and nitrite/nitrate concentrations (a marker of nitric oxide production).

Findings Skeletal muscle ATP concentrations were significantly lower in the 12 patients with sepsis who subsequently died than in the 16 septic patients who survived (p=0·0003) and in controls (p=0·05). Complex I activity had a significant inverse correlation with norepinephrine requirements (a proxy for shock severity, p=0·0003) and nitrite/nitrate concentrations (p=0·0004), and a significant positive correlation with concentrations of reduced glutathione (p=0·006) and ATP (p=0·03).

Interpretation In septic patients, we found an association between nitric oxide overproduction, antioxidant depletion, mitochondrial dysfunction, and decreased ATP concentrations that relate to organ failure and eventual outcome. These data implicate bioenergetic failure as an important patho-physiological mechanism underlying multiorgan dysfunction.

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Introduction

Sepsis is the systemic inflammatory response associated with an infectious insult. It is the leading cause of death in critically ill patients, and the predominant cause of multiple organ dysfunction.[1] However, precise mechanisms through which organ dysfunction develops remain unknown, as do reasons for its persistence long after cessation of the acute inflammatory phase. Although microvascular flow redistribution undoubtedly occurs,[2] we and others have shown increased tissue oxygen tension in the organs of animals and patients with sepsis.[3,4] This finding suggests that the predominant defect might lie in cellular oxygen use (tissue dysoxia) rather than in oxygen delivery per se.

Mitochondrial OXIDATIVE PHOSPHORYLATION is responsible for over 90% of total body oxygen consumption and ATP generation. The RESPIRATORY CHAIN (electron-transport chain) includes four individual enzyme complexes (I–IV). These enzyme complexes, notably NADH-ubiquinone oxidoreductase (complex I) and cytochrome C oxidase (complex IV), can be inhibited by reactive oxygen and nitrogen species such as nitric oxide.[5–7] These reactive species are produced in substantial excess during sepsis and are also generated by the mitochondria.[8] Complex I inhibition by nitric oxide is facilitated, in vitro, by depletion of the intracellular antioxidant reduced glutathione.[5,6] Concentrations of this antioxidant are also known to decrease in septic states.[9,10]

No study has yet addressed whether alterations in BIOENERGETIC STATUS in severe sepsis are associated with increased nitric oxide production, mitochondrial dysfunction, and antioxidant depletion, and whether these abnormalities relate to organ failure and to outcome. To address these questions, we undertook a systematic study of mitochondrial dysfunction in critically ill patients with sepsis admitted to intensive care.

Methods

Patients

After obtaining approval from the ethics committee of the University College London Hospitals National Health Service Trust, patients were recruited from the intensive-care unit or from the orthopaedic department. Patients (or their next-of-kin) were asked for informed consent (or agreement) before enrolment.

Patients with recent-onset severe sepsis or septic shock (as defined by standard criteria[11]) were enrolled. Those with severe coagulopathies (platelet count $<30\times10^9$/L or international normalised ratio >2), immunosuppression (endogenous or long-term chemotherapeutic), or both, were excluded. Routine physiological and biochemical variables were recorded, and scores on the simplified acute physiology score (SAPS II) and sequential organ failure assessment (SOFA) calculated. The control group consisted of otherwise healthy patients undergoing

GLOSSARY

BIOENERGETIC STATUS
Energy "stored" in a form (ATP) that is readily available for cellular metabolism.

OXIDATIVE PHOSPHORYLATION
The coupling of energy released from substrate oxidation by the respiratory chain to the synthesis of ATP.

RESPIRATORY CHAIN
Terminal pathway of oxidative phosphorylation, a series of mitochondrial oxidoreductive molecules including cytochromes responsible for the stepwise transfer of electrons from substrates to oxygen.

elective total hip replacement surgery for degenerative arthropathy.

Procedures

Within 24 h of patients' admission to the intensive-care unit, a vastus lateralis muscle biopsy was done with Henckel Tilley forceps via a small incision in the lateral thigh. This procedure is safe for critically ill patients.[12] Five to six biopsy samples (averaging 750 mg total) were frozen immediately in liquid nitrogen. In the controls, muscle biopsies were taken through the operation site from the vastus lateralis thigh muscle at the beginning of surgery and processed as above.

Mitochondrial complex activities were measured by well described spectrophotometric methods.[13] Tissue samples were homogenised on ice with a hand-held glass homogeniser, then underwent three episodes of rapid freeze-thawing to ensure cell lysis. Complex activities were measured as the inhibitor sensitive rates (rotenone for complex I and antimycin A for complexes II and III). To correct for mitochondrial enrichment (or depletion) within the sample, results were expressed as a ratio of citrate synthase activity.[14] Complex I activity was also measured after addition of reduced glutathione (homogenates were incubated in up to 5 mmol/L reduced glutathione for up to 40 min) to determine whether inhibition could be reversed.[5]

Concentrations of reduced glutathione were measured in muscle by reverse-phase high-performance liquid chromatography and electrochemical detection;[15] results were expressed as a ratio of total protein. Adenine nucleotide (ATP, ADP, and AMP), phosphocreatine, and creatine concentrations were measured in the muscle samples by reverse-phase high-performance liquid chromatography after freeze-drying;[16] results were expressed as nmol/mg of dry-weight tissue. Muscle nitrite/nitrate concentrations were measured by a modified Griess reaction as a marker of nitric oxide production.[17] These results were expressed as a ratio of total wet weight protein.

	Septic survivors (n=16)	Septic non-survivors (n=12)	Controls (n=9)
Age	65 (46–71)	64 (47–73)	70 (65–73)
Sex (male/female)	8/8	7/5	5/4
Weight (kg)	80 (58–90)	65 (59–73)	64 (57–68)
Source of sepsis			
Chest	4	6	..
Abdominal	6	6	..
Septicaemia	4	0	..
Other	2	0	..
Prior surgery	5	5	0
Comorbid factors			
Diabetes	2	1	0
Malignancy	0	1	0
1st 24 h SAPS II score	42 (31–50)	53 (37–79)	..
1st 24 h SOFA score	8 (7–13)	11 (8–14)	..
Vasoactive drug requirement (μg/min)	22·3 (7·2–35·7)	23 (19–28)	0
Arterial base deficit (mEq/L)	5·8 (1·7–7·8)	7·0 (2·7–10·1)	..
Blood glucose (mmol/L)	5·6 (5·2–8·2)	6·3 (3·9–8·8)	..
Oxygen delivery (mL/min)	1404 (1040–1634)	1132 (715–1404)	..
Cardiac output (L/min)	7·1 (4·9–9·3)	7·2 (6·0–8·6)	..
Mean blood pressure (mm Hg)	66 (60·5–70·0)	73 (66–80)	..
SVR (dyn.s.cm^{-5})	630 (339–699)	630 (449–691)	..
Length of ICU stay (days)	9·0 (4·0–18·5)	6·0 (4·0–8·8)	..

Data are numbers of patients or median (IQR). SAPS=simplified acute physiology score. SOFA=sequential organ failure assessment. SVR=systemic vascular resistance. ICU=intensive-care unit.

Table 1: **Demographic and clinical characteristics at the time of biopsy**

Statistical analysis

Data are expressed as median (IQR). Overall statistical significance between the groups was tested with a one-way ANOVA or Kruskal-Wallis test, depending on normality of distribution. Post-hoc analyses with correction for multiple comparisons were done with least significant difference or Dunnett tests, as appropriate. Correlations were calculated with a Spearman correlation coefficient (two-tailed test of significance).

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Of the 28 patients with sepsis, 16 survived the septic episode (survivors). Non-survivors died in the intensive-care unit, with a median stay of 6 days. All patients had increased cardiac outputs and all but two were requiring norepinephrine to maintain a mean blood pressure in excess of 60 mm Hg. All were mechanically ventilated and 12 underwent haemofiltration. For patients who did

	Septic survivors (A)	Septic non-survivors (B)	Controls (C)	p (A *vs* B *vs* C)
ATP (nmol/mg dry weight)	15·8 (12·1–18·6), n=12	7·6 (6·6–10·0), n=9	12·5 (9·7–13·7), n=8	0·001
ADP (nmol/mg dry weight)	2·3 (1·6–2·7), n=12	2·3, (1·5–5·4), n=9	2·6 (1·2–3·1), n=8	0·83
AMP (nmol/mg dry weight)	0·12 (0·06–0·23), n=12	0·44 (0·1–0·8), n=9	0·2 (0·09–0·4), n=8	0·22
ATP:ADP ratio	7·44 (5·2–8·7), n=12	4·39 (1·4–5·1), n=9	5·5 (4·1–8·0), n=8	0·02
Total adenine content (nmol/mg dry weight)	18·2 (14·0–22·0), n=12	12·5 (9·9–13·6), n=9	16·1 (13·4–16·5), n=8	0·02
Phosphocreatine (nmol/mg dry weight)	47·1 (47·1–65·3), n=11	36·1 (28·4–45·7), n=9	62·4 (56·8–63·9), n=8	0·02
Creatine (nmol/mg dry weight)	43·2 (36·3–50·3), n=11	41·5 (35·2–46·8), n=9	31·6 (28·3–43·0), n=8	0·24
Phosphocreatine:creatine ratio	1·28 (0·76–1·6), n=11	0·98 (0·84–1·1), n=9	1·88 (1·4–2·2), n=8	0·006

Data are median (IQR). n=number of patients on whom sample analysis was done.

Table 2: **Concentrations in skeletal muscle of ATP, ADP, AMP, phosphocreatine, and creatine**

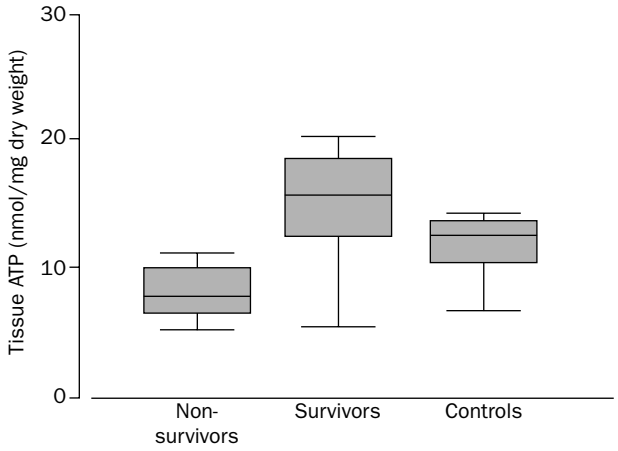

Figure 1: **Tissue ATP concentrations in patients with sepsis and in controls**
Horizontal lines=medians, boxes=quartiles, whiskers=ranges.

not respond to high-dose norepinephrine (seven eventual survivors and two non-survivors), a 16 mg dose of dexamethasone was given within 6 h of the biopsy. No significant difference was seen between septic survivors and non-survivors in terms of demographics, comorbid and other factors, cause of sepsis, severity of organ dysfunction, and physiological variables at the time of biopsy (table 1). All nine control patients undergoing elective total hip replacement surgery were discharged home after an uneventful postoperative course.

Owing to variation in the amount of tissue obtained, we were unable to do a full set of analyses on all patients. Muscle ATP concentrations were significantly lower in septic non-survivors than in either septic survivors (p=0·0003) or controls (p=0·05; table 2, figure 1). AMP concentrations were slightly higher in non-survivors than survivors, and the total adenine pool was significantly lower, suggesting increased ATP hydrolysis. Complex I activity was significantly lower in septic non-survivors than controls, and reduced glutathione concentrations were significantly lower in both septic groups (table 3). No reversibility of complex I inhibition was achieved by addition of exogenous glutathione. No difference between the groups was seen in the activities of complex II and III, whereas complex IV activity was significantly higher in septic non-survivors than controls. The phosphocreatine:creatine ratio was also lower in the septic patients than controls (table 2).

We found significant positive correlations between tissue concentrations of nitrite/nitrate and severity of disease (norepinephrine requirement and SAPS II) and between complex I activity and both ATP and reduced glutathione concentrations (figures 2 and 3). Significant negative correlations were seen between norepinephrine requirement and both complex I activity and ATP concentration (figure 2); and between nitrite/nitrate concentrations and both complex I activity and reduced

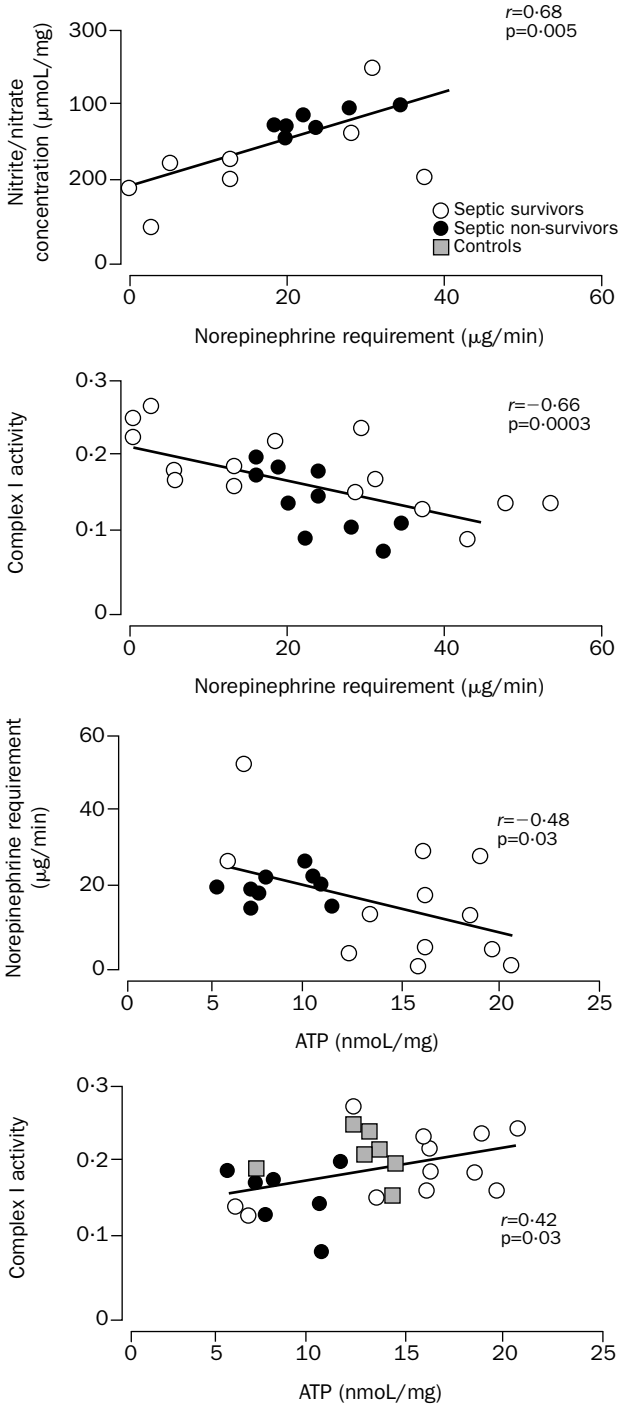

Figure 2: **Correlation between norepinephrine requirement, tissue nitrite/nitrate concentration, complex I activity, and ATP concentration**

	Septic survivors (A)	Septic non-survivors (B)	Controls (C)	p (A *vs* B *vs* C)
Complex I activity*	0·18 (0·14–0·23), n=16	0·15 (0·11–0·18), n=10	0·21 (0·18–0·24), n=8	0·03
Complex II and III activity*	0·13 (0·10–0·16), n=16	0·13 (0·12–0·15), n=12	0·12 (0·11–0·14), n=9	0·35
Complex IV activity*	0·014 (0·01–0·02), n=16	0·020 (0·013–0·02), n=12	0·011 (0·01–0·017), n=8	0·05
Reduced glutathione (nmol/mg total protein)	5·0 (3·5–5·8), n=13	3·9 (2·2–4·8), n=11	9·8 (8·4–11·0), n=8	0·0004
Nitrite/nitrate (μmol/mg total protein)	118 (99–159), n=8	176 (173–197), n=7	86 (45–103), n=6	0·001

*Expressed as a ratio of citrate synthase activity. Data are median (IQR). n=number of patients on whom sample analysis was done.

Table 3: **Activities of mitochondrial complexes and concentrations of reduced glutathione and nitrite/nitrate in skeletal muscle**

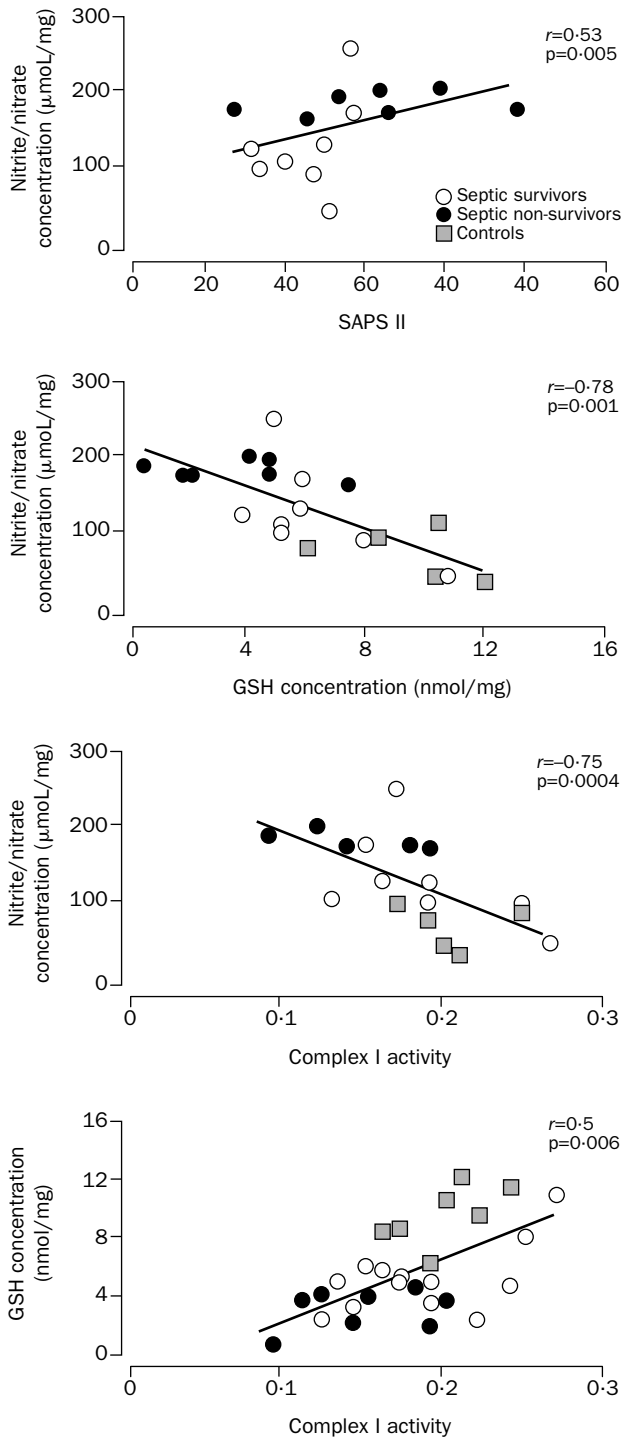

Figure 3: **Correlation between tissue nitrite/nitrate concentration, simplified acute physiology score (SAPS II), tissue reduced glutathione (GSH) concentration, and complex I activity**

glutathione concentration (figure 3). No correlation was found between complex II and III or complex IV activity and any other variable investigated.

Discussion

We have shown, in patients with sepsis and multiple organ failure, a relation between shock severity (as gauged by norepinephrine requirements to maintain an adequate blood pressure), mitochondrial dysfunction, ATP depletion, intracellular antioxidant (reduced glutathione) depletion, and nitric oxide production (as gauged by nitrite/nitrate concentrations) in skeletal muscle. Despite being unable to distinguish clinically between eventual survivors and non-survivors, significant differences were seen in muscle bioenergetic status taken within 24 h of intensive-care admission.

Although the number of patients is relatively low and multiple comparisons were made on the data, the findings are nevertheless in keeping with cellular, animal, and the sparse human data previously reported. Short-term (<6 h) laboratory models of sepsis have shown variable changes in mitochondrial respiration, whereas longer term models (>12 h) consistently reveal decreases in mitochondrial activity or ATP concentrations as the predominant finding.[18] ATP depletion[19,20] and inhibition of the mitochondrial respiratory-chain complexes have been shown separately in small case series of patients with sepsis.[21,22]

The respiratory chain is located in the mitochondrial inner membrane and consists of the four complexes plus specialised electron carriers. Passage of electrons down the chain creates a proton gradient across the inner membrane sufficient to drive ATP synthase (complex V) to phosphorylate ADP to ATP. These complexes can be inhibited by nitric oxide and its congeners,[5–7] which are produced in excess during sepsis.[23] Although studies of nitric oxide have focused heavily on its role in inducing vascular hyporeactivity, little attention has been paid to its role as a mitochondrial inhibitor.

Cell models have shown rapidly reversible inhibition of complex IV by nitric oxide competitive with oxygen tension.[7] Because nitric oxide is unstable, free nitric oxide concentrations that remain in the homogenates are unlikely to be high enough to allow observation of this reversible inhibition, especially at the much higher oxygen partial pressures (room air) under which the assay is done. However, the observed rise in complex IV activity was unexpected, although nitric oxide has been shown to induce the transcription of complex IV.[24]

Nitric oxide has a longer-lasting effect on complex I than on complex IV.[5,6] Our finding of low complex I activity in combination with raised nitrite/nitrate concentrations and low reduced glutathione concentrations are in keeping with these in-vitro data. However, unlike cell models,[5] we were unable to detect any reversibility on addition of exogenous glutathione. We presume that the lengthy course of sepsis and extended exposure to reactive nitrogen species results in irreversible inhibition or permanent damage to the enzyme complex.

We cannot exclude a norepinephrine effect on mitochondria, but we consider this possibility unlikely. Animal and cellular studies of sepsis show mitochondrial dysfunction in the absence of vasopressor agents,[18] whereas preliminary data (not shown) from four of our patients with cardiogenic shock who required similar doses of epinephrine revealed no fall in ATP compared with the controls.

Absolute muscle ATP concentrations and ATP:ADP ratios were significantly lower in non-surviving septic patients than survivors. The rise in AMP (and a lower adenine nucleotide pool) suggests an imbalance in ATP turnover, which could be a result of decreased ATP production, although an increase in ATP use might also be contributory. ATP turnover cannot be readily elucidated by an ex-vivo assay; however, cells must maintain steady-state ATP concentrations above those that trigger apoptosis or necrosis.[25]

Damage or inhibition of complex I could decrease mitochondrial capability to generate ATP. The 30% decrease seen in the present study is similar to that

associated with diseases known to be associated with mitochondrial dysfunction, such as Parkinson's disease.[26]

Two unresolved issues are the need to establish that mitochondrial dysfunction is causative rather than epiphenomenal, and to determine how relevant these findings in skeletal muscle are to other, more vital, organs such as liver and kidney. We are currently studying long-term laboratory models of sepsis that seek to address these questions. Nevertheless, our findings implicate bioenergetic failure as an important pathogenetic mechanism underlying sepsis-induced multiple organ failure that could offer new therapeutic avenues.

Contributors

All authors contributed to the interpretation of the results and writing of the paper; D Brealey did the biopsies and collected the clinical data; and D Brealey, M Brand, I Hargreaves, N A Davies, and R Smolenski did the biochemical assays.

Conflict of interest statement

None declared.

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