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Diverse and tissue specific mitochondrial respiratory response in a mouse model of sepsis-induced multiple organ failure

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Abstract

Mitochondrial function is thought to play a role in sepsis-induced multiple organ failure. However, the temporal and organ specific alterations in mitochondrial function has yet to be fully elucidated. Many studies show reduced phosphorylating capacity while others have indicated that mitochondrial respiration is enhanced. The objective of the study was to evaluate the temporal dynamics of brain and liver mitochondrial function in a mouse model of sepsis.

Sepsis was induced by cecal ligation and puncture. Controls were sham operated. Using high-resolution respirometry, brain and liver homogenates from 31 C57BL/6 mice were analyzed at either 6 hours or 24 hours. ROS-production was simultaneously measured in brain samples using fluorometry.

Septic brain tissue exhibited an early increased uncoupling of respiration. Temporal changes between the two time points were diminutive and no difference in ROS-production was detected.

Liver homogenate from the septic mice displayed a significant increase of the respiratory control ratio at 6 hours. In the 24-hour group, the rate of maximal oxidative phosphorylation, as well as LEAK respiration, was significantly increased compared to controls and the resultant respiratory control ratio was also significantly increased. Maximal Protonophore-induced respiratory (uncoupled) capacity was similar between the two treatment groups.

The present study suggests a diverse and tissue specific mitochondrial respiratory response to sepsis. The brain displayed an early impaired mitochondrial respiratory efficiency. In the liver the primary finding was a substantial activation of the maximal phosphorylating capacity.

Keywords: rodent; brain; liver; respirometry; fluorometry

Introduction

The pathogenesis of sepsis-induced multiple organ failure (MOF) is complex and our understanding of the pathophysiology is incomplete. There is an ongoing controversy regarding O₂ delivery in MOF and hypo-perfusion of the organs certainly plays a role. However, it has been argued that perhaps the problem does not only relate to O₂ delivery and tissue hypoxia. Tissue oxygen levels can apparently be normal or even higher than normal in organ failure and it has been shown that failing organs show limited cell death and failed organs have the potential for recovery (1-3). Elevated tissue oxygenation may compound organ failure due to the production of reactive oxygen species (ROS). The majority of the oxygen delivered to cells is consumed by mitochondria for oxidative phosphorylation and mitochondrial respiratory dysfunction has been suggested to play a role in the pathogenesis of sepsis-induced multiple organ failure, although the precise pathological mechanisms are not clear (4).

One proposed theory is that of cytopathic hypoxia, which suggests that mitochondrial dysfunction is caused by an inability to utilize available oxygen (5). However, the composed data on the subject is seemingly contradictory. Studies have indicated altered mitochondrial respiratory function in human muscle tissue as well as in diverse animal models examining various tissues and different tissues seem to respond differently to sepsis (6-10). Many studies show reduced phosphorylating capacity (11) whereas other studies have indicated enhanced mitochondrial respiration in sepsis (12, 13). Further, as a framework for this study, it has previously been shown in a rodent model, that sepsis can induce increased uncoupling of the oxidative phosphorylation system in brain homogenates (14, 15).

The somewhat contradictory results obtained not only seem to depend on the model used but also on the specific organ from which the mitochondria are obtained as well as the studied time-point during the disease progression. The pathogenesis is multifaceted and

sepsis is clearly a dynamic process. Mitochondrial biogenesis occurs together with the respiratory alterations and we have previously demonstrated, in human platelets, that an early increase in uncoupling is followed by a gradual increase in respiration during the first week of sepsis and that the increase was most pronounced in non-survivors, likely correlated to the severity of the septic insult (13).

The aim of the present study was to evaluate the mitochondrial respiratory function in two different organs commonly affected in sepsis and the subsequent multiple organ failure, the brain and the liver. By also analyzing two different time-points we wanted to evaluate the temporal dynamics of mitochondrial function. In addition, the tissue generation of ROS was evaluated in the brain tissues simultaneously to respiratory measurements.

Materials and Methods

Chemicals used were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) if not stated otherwise.

Animals and study design

The ethical committee of animal experiments at Tokyo Medical University (H-24013) approved the study. Animals were kept under standard conditions with *ad libitum* access to food and water and maintained at a 12-hour day and night cycle. The population consisted of 31 pathogen free 8 weeks old male C57BL/6 mice. Sepsis was induced by cecal ligation and puncture (CLP) as previously described, but with a few alterations (16). In short, the mice were anesthetized using continuous inhalation of isoflurane. Laparotomy was performed with a 10 mm incision. After the cecum was exposed using blunt anatomical forceps, 75% of the cecum was ligated right below the ileocecal valve. Before perforation, feces was gently relocated towards the distal cecum. The cecum was perforated by a single through and

through (mesenteric to antimesenteric) puncture using a 21 G needle at half way from tip to ligation. A small droplet of feces was then gently squeezed through the punctures. The cecum was then returned to the peritoneal cavity with careful attention so that feces did not contaminate the margins of the abdominal and skin wound. The muscle and skin incisions were closed using 4-0 interrupted sutures. This method represents a high-grade sepsis with a 100% mortality in 72 hours (16). The animals were resuscitated directly post-operatively by 1.0 ml subcutaneous injection of saline (37 °C). Controls were sham operated and underwent the same procedure except the ligation and the puncture of the cecum.

Termination and preparation of homogenates

The mice were selected at a predetermined time point, regardless of symptoms, at either 6 hours (\pm 15 min) or 24 hours (\pm 30 min) after surgery. The mice were video filmed for later unbiased evaluation of clinical status. The clinical parameters assessed included: convulsions, loss of balance, loss of gripping reflex, paralysis, ruffled fur and a score was assigned from the clinical assessment table (Table 1). The mice were subsequently terminated by cervical dislocation. Body temp was measured directly after cervical dislocation using a digital rectal probe (BWT-100, Bio Research Center CO, Ltd, Japan) and blood glucose was measured (Terumo Finetouch, Japan). The organs for analysis were harvested, beginning with the brain, by rapid but gentle dissection and then immediately transferred into ice-cold buffer solution (320 mM Sucrose, 2 mM EGTA, 10 mM Trizma base, pH 7.4). The tissues were weighed and manually homogenized in a 5 ml Potter-Elvehjem teflon-glass homogenizer to a concentration of 1 mg wet weight tissue / 10 μ l MiR05 buffer (0.5 mM EGTA, 3 mM MgCl₂, 60 mM K-lactobionate, 20 mM Taurine, 10 mM KH₂P0₄, 20 mM HEPES, 110 mM Sucrose, 1 g/l BSA, pH 7.1). The entire procedure was performed on ice and all buffer solutions were ice-cold.

High-resolution respirometry

Mitochondrial respiration was measured using a high-resolution Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). Mitochondrial respiration was corrected for instrumental oxygen flux, measured separately for automatic correction. Calibration was performed daily with air saturated MiliQ water. An oxygen solubility factor relative to H₂O was set to 0.92 for MiR05. Experiments were carried out at a controlled constant temperature of 37 °C. Experiments were started by the addition of 22 µl of brain homogenate (2.2 mg wet weight brain tissue) into chamber prefilled with MiR05 to a final concentration in the closed chamber of 1 mg/ml. Oxygen consumption and oxygen flux was monitored and recorded in real-time using DatLab 5.1 software (Oroboros Instruments, Innsbruck, Austria).

Fluorometric measurements of ROS

Measurement of ROS-production in brain homogenates was carried out using the add-on module O2k-Flourescence LED2 (Oroboros instruments, Innsbruck, Austria) to allow simultaneous measurement of ROS-production and mitochondrial respiration. ROS-production was detected using the Amplex Red (N-acetyl-3,7 dihydroxyphenoxazine) hydrogen peroxide (H₂O₂) assay. In the presence of horseradish peroxidase, Amplex Red reacts with H₂O₂ to produce the fluorescent compound resorufin. The addition of superoxide dismutase ensures that all superoxide is converted to H₂O₂.

Amplex red (5 µM), horseradish peroxidase (1 U/ml) and Superoxide dismutase (10 U/ml) was added to the chamber prior to adding homogenates. Calibration of fluorometric signal was conducted prior to each measurement by the addition of 100 nM H₂O₂. Hydrogen peroxide for calibration was diluted daily from 1M stock solution. Measurements of ROS was not performed in liver homogenates due to the high content of endogenous cytosolic

antioxidants that interfere with the Amplex red assay and the measurement of H_2O_2 production.

Protocol for assessment of mitochondrial respiratory function

A substrate, uncoupler and inhibitor titration (SUIT) protocol with consecutive additions was utilized as previously described, but with a few modifications (17). By using complex-specific substrates and inhibitors it is possible to examine the respiratory capacities with electron flow through both Complex I (CI) and Complex II (CII) as well as the convergent electron input through the Q-junction (CI + II). The maximal phosphorylating and non-phosphorylating respiration were measured as stimulated by combined NADH-linked substrates and succinate. Digitonin was added to brain homogenates for permeabilization of the synaptosomes. The homogenates were then allowed to stabilize at basal respiration without exogenous substrates in MiR05 when calibration of the fluorometric signal was conducted in the brain homogenate. The remaining protocol was identical for both brain and liver homogenates as follows. Malate (5 mM) and pyruvate (5 mM) was added followed by ADP (1 mM) and glutamate (5 mM) attaining oxidative phosphorylation (OXPHOS) capacity supported through CI (OXPHOS_{CI}), driven by the NADH-related substrates. OXPHOS respiration is an ATP-producing state where the electron flow is controlled by the ATP-synthase. Subsequently, succinate (20 mM) was added stimulating maximal OXPHOS capacity by convergent input through CI and CII (OXPHOS_{CI+II}). Oxidative phosphorylation was inhibited by oligomycin, an ATP-synthase inhibitor, inducing LEAK respiration state (LEAK_{CI+II}), i.e. mitochondrial respiration independent of ATP-production. LEAK respiration reveals the respiration related to electron flow through the electron transport system (ETS) necessary to uphold the membrane potential compensating for leakage of protons independent of the ATP-synthase. Maximal convergent respiratory capacity of the

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ETS (ETS_{CI+II}) was then evaluated by titrating the protonophore, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) until no further increase in respiration was detected. ETS_{CI+II} reveals respiration related to electron flow, through both CI and CII, when it is not restricted by the ATP-synthase. To measure the ETS capacity supported by succinate alone through CII (ETS_{CII}) rotenone was added to inhibit CI. The complete electron flow through the ETS was inhibited by the addition of the complex III (CIII) inhibitor antimycin-A (1 µg/ml) revealing the residual oxygen consumption not related to the ETS. This value was subtracted from the different respiratory states in the final analysis. Finally, an addition was made of ascorbate and N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride (TMPD), which is an artificial substrate for reducing cytochrome c, to measure the activity of cytochrome c oxidase (CIV). Sodium-azide was subsequently added for evaluation of the level of auto-oxidation which was subtracted from the TMPD value. Respiratory control ratios were calculated for OXPHOS_{CI}, the convergent OXPHOS_{CI+II}, and max ETS by dividing each separate rate with LEAK respiration rate. Control ratios were also calculated for ETS divided by OXPHOS_{CI} and OXPHOS_{CI+II}. The duration of the complete titration protocol was approximately 1 hour.

Measurements of citrate synthase

Citrate Synthase activity was used as a marker for mitochondrial content (18). After sonication of samples, citrate synthase activity was measured in a spectrophotometric plate-reader using a commercially available kit according to the manufacturer's instructions (Citrate Synthase Assay Kit, CS0720, Sigma).

Statistical analysis

The data from two control mice in the original design of 33 mice were excluded altogether due to evident laboratory error related to contamination. One analysis of liver tissue from a 24-hour control was removed due to technical laboratory error related to substrate titration. Statistical analysis was carried out using PRISM 6.0 software (GraphPad Software Inc., La Jolla, CA, USA). To calculate if the differences in separate respiratory states between the treatment groups were statistically significant Student's *t*-test was used. The respiratory control ratios, clinical scores and temperatures were not considered to be normally distributed and a Mann-Whitney non-parametric test was used. Differences were considered significant where $P < 0.05$.

Results

Clinical signs of sepsis

The CLP-treated mice developed clinical signs of sepsis and lowered body temperatures compared to controls. The median temperature dropped from 36.8° to 31.7° ($p < 0.001$) in the 6-hour group and from 37.6° to 30.8° ($p < 0.01$) in the 24-hour group. The median clinical score went from 0 in both controls to 2 in the 6-hour group ($p < 0.001$) and to 3 in the 24-hour group ($p < 0.01$). Clinical parameters are summarized in table 2.

Mitochondrial function in brain homogenates following sepsis

No statistically significant differences were detected in citrate synthase activity between the groups (data not shown). The respiratory capacities of OXPHOS_{CI}, OXPHOS_{CI + CII}, LEAK_{CI} + CII, ETS_{CI + CII}, ETS_{CII} and CIV were evaluated for each treatment group as illustrated in table 3A. All respiratory data was normalized to wet weight of tissue.

As shown in figure 1A the respiratory control ratio (RCR) of OXPHOS_{CI+II} and LEAK_{CI + CII} was significantly ($P < 0.05$) decreased in the septic mice in the 6-hour group

compared to controls. This was mainly due to an underlying increase in the oligomycin-induced LEAK_{CI + CII} state in septic mice compared to controls ($P < 0.05$) (Table 3A).

At the 24-hour time point there was a non-significant decreased respiratory control ratio of OXPHOS_{CI+II} and LEAK_{CI+II} ($P = 0.055$) in the septic mice. (Figure 1B).

OXPHOS_{CI}, OXPHOS_{CI + CII} and LEAK_{CI+II} separately all showed similar trends at both time points of decreased oxidative phosphorylation and increased LEAK respiration. ETS_{CI + CII}, ETS_{CII} and CIV did not differ significantly between septic mice and controls at each separate time-point. FCCP titration (ETS_{CI + CII}) did not increase respiration further compared to OXPHOS (Table 3A).

The maximal phosphorylating capacity as a ratio of maximal uncoupled ETS-capacity (OXPHOS_{CI+II} / ETS_{CI+II}) was just above 1.0 for both groups at both time-points (Table 3 A), suggesting that the respiratory capacity was not restricted by the ATP-synthase. Despite being similar at 6-hours the median difference of the ratio (control 1.06; sepsis 1.03, i.e. 2.8 % decrease) was statistically significant. (Figure 1C, D).

ROS-production was in control mice significantly altered at different respiratory states and inhibiting the ATP-synthase increased ROS-production dramatically. However, there were no differences in ROS-production between septic mice and controls at either time-point, neither were there any differences in ROS-production between the time-points of the two septic groups (Figure 2).

Mitochondrial function in liver homogenates following sepsis

The liver samples displayed no statistically significant changes in citrate synthase activity between the groups (data not shown). The respiratory capacities of OXPHOS_{CI}, OXPHOS_{CI + CII}, LEAK_{CI + CII}, ETS_{CI + CII}, ETS_{CII} and CIV were evaluated for each treatment group as illustrated in table 3B. All respiratory data presented was normalized to wet weight.

Contrary to the brain, the RCR of OXPHOS_{CI+ CII} and LEAK_{CI+ CII} was increased ($P < 0.001$) at 6 hours (Figure 3A). In the 6-hour group there was also a general trend towards lower rates of respiration in the septic mice for all respiration states except OXPHOS_{CI+ CII} (e.g. $P = 0.054$ for OXPHOS_{CI}). LEAK state respiration also tended to be, differing to the brain, lower in septic mice ($P = 0.11$) at 6 hours.

At 24 hours the RCR of OXPHOS_{CI+ CII} and LEAK was also increased ($P < 0.05$) as shown in figure 3B. The respiration associated with oxidative phosphorylation using CI (OXPHOS_{CI}) and CI+II substrates (OXPHOS_{CI+ CII}) in liver from septic mice was dramatically increased compared to controls at 24 hours (Table 3B) ($P < 0.01$ and $P < 0.0001$ respectively). There was also an increased LEAK in the septic mice ($P < 0.001$) compared to control at 24 hours (Table 3B).

The maximal uncoupled ETS capacity (ETS_{CI+ CII}) was comparable between control and septic mice at both time-points. When comparing the phosphorylating capacity as a ratio of maximal uncoupled ETS-capacity (OXPHOS_{CI+II} / ETS_{CI+II}), at 6 hours the septic mice exhibited a trend towards an increased ratio ($P = 0.072$) (Figure 3C) and at 24 hours the median ratio of the controls was 0.44 whereas the septic mice had a median ratio of 0.75 ($P < 0.001$) (Figure 3D). The respiratory capacity was thus to a lesser extent restricted by the phosphorylating capacity in the septic mice.

Discussion

The present study indicates a diverse and tissue specific mitochondrial respiratory response to sepsis-induced multiple organ failure. The brain tissue displayed a slightly impaired mitochondrial respiratory efficiency already at an early stage of sepsis, but the temporal dynamics of the brain mitochondrial function were diminutive. The most interesting finding is that there seems to be an activation of the phosphorylating capacity in the liver by the

septic insult, most evidently, after 24 hours. The maximal uncoupled ETS-capacity was comparable between the two groups, whereas the insult dramatically increased the phosphorylating capacity in the septic mice. It is not the difference between the treatment groups in the same tissue that is the main finding, a difference that indeed is quite small with significant overlap in the data. The main finding is the fact that the tissues respond very differently to the septic insult. We therefore argue that the mitochondrial respiratory response in sepsis is clearly organ specific and that we need to move beyond a simplistic view of a mitochondrial dysfunction in sepsis when directing further preclinical research towards the clinical setting.

In this study we chose to analyze homogenates instead of isolated mitochondria in order to reveal possible short acting factors affecting mitochondrial respiration. In isolated mitochondria it is also possible that defective mitochondria are to a higher extent removed in the isolation process and the process may also affect the structure and function of the mitochondria (19). A previous study of mitochondrial respiratory function in both brain and liver found that homogenates were comparable to that of isolated mitochondria (20). In brain homogenates formation of synaptosomes takes place. For substrate access to mitochondria trapped within synaptosomes digitonin was used to permeate the plasma membrane. However it has been demonstrated that digitonin produces swelling and cytochrome c release in brain mitochondria (21). Therefore careful titration of digitonin was conducted before setting the protocol to find the appropriate dose. Exogenously administered cytochrome c did not induce any significant effect on respiration with the digitonin dose used in the present study, indicating intact integrity of the outer mitochondrial membrane.

Brain and liver specifically were selected for both methodological and clinical purposes. Brain mitochondrial respiratory alterations have previously been shown in a rodent model using similar techniques and could therefore be used as a benchmark (14, 15).

Furthermore, our group recently published that sepsis alters cerebral redox status (22). Both organs are also unquestionably of key relevance to MOF. Due to technical aspects of the Oroboros Oxygraph-2k, which is limited to only two chambers per machine, it is not possible to measure additional organs when using fresh tissue. Previous experience in our group, as well as pilots specifically for this project, has indicated that it is not feasible to store tissue for sequential analysis. Another advantage of analyzing brain and liver specifically, in contrast to most other tissues, is that almost identical methods and protocols for tissue preparation and analysis could be used and therefore decreasing the influence of confounding factors.

In a protocol with consecutive titrations the order of additions may affect the absolute values of the respiratory states obtained. However, by measuring these different respiratory states, it is possible to determine more specifically how and where the mitochondrial respiration is effected. The protocol used was designed to include evaluation of maximal OXPHOS and ETS capacities, and complex I, II and IV function, as well as LEAK respiration.

The present data indicated that there was no difference in ROS-production in the brain between the groups. A previous study indicated lower ROS-production in brain from septic mice (14). Higher LEAK will most likely also mean a lower proton motive force, which could decrease ROS-production. A strength in the present study is that ROS-production measurements were done simultaneously to the respiratory measurements in the same sample. ROS-production was therefore also measured during respiration with electron flow through both CI as well as the convergent electron input through the Q-junction (CI + II).

Increased LEAK will be detrimental to ATP-production and at a certain level the cell will no longer be able to uphold its energy demand. But perhaps increased LEAK respiration should not necessarily be viewed as a pathological mechanism but rather as an adaptive and protective metabolic response to conceivably decrease ROS-production and increase heat-

production (23).

The increased phosphorylating capacity seen in the liver seems to be adaptive to the insult and is possibly caused by increased levels of catecholamines. A previous study showed that norepinephrine increased the activity of hepatic succinate dehydrogenase (24) and in another study epinephrine has been shown to enhance the activity of mitochondrial phosphorylation and ATP production, increasing oxygen consumption (25). There is however apparently conflicting data in the literature, using isolated swine liver mitochondria it has also been shown that catecholamines impair the efficiency of mitochondrial complex I respiration *in vitro* (26). Regardless of mechanism, the organ specific response with increased oxidative phosphorylation in the liver is noteworthy and is also supported by previous research. In an equivalent study but with heart and liver mitochondria, in rats that responded to endotoxin, the heart mitochondria showed decreased oxidative activity whereas the liver mitochondria showed increased oxidative phosphorylation compared to both non-responders and controls (27).

The two separate groups of controls of liver at 6 and 24 hours respectively, displayed some differences. All 6-hour mice were consistently done at a different time during the day then the 24-hour mice, although the septic and control mice at each time point were performed at the same time during the day. The differences observed in the controls could be due to post-translational circadian and nocturnal metabolic changes. Changes in rodent mitochondrial respiratory function due to nocturnal changes have been known for a long time, in one study the rate of succinate oxidation in liver mitochondria from rats was 40 percent greater at night (28). One study indicated that the activity oscillations of succinic dehydrogenase showed a tendency to 24-hour rhythmicity (29). In another study they found that darkness induces a more efficient phosphorylation, as indicated by a higher ratio of ATP synthase/ATPase activity (30). Another pivotal study on circadian changes in metabolism

revealed the influence of clock-driven acetylation on multiple mitochondrial proteins involved in metabolic pathways in both glycolysis and the citric acid cycle demonstrating how the circadian clock can regulate the metabolic state of the cell (31). One important finding of this study is that the circadian alterations seem to affect primarily the ATP synthase, as ETS capacity was similar, but not OXPHOS capacity. These alterations could perhaps be modulated by PGC-1 β , as in one study using PGC-1 β KO mice an altered expression in a number of nuclear-encoded genes governing mitochondrial and metabolic functions was detected in both brain and liver. The PGC-1 β KO mice showed decreased activity during the dark cycle and also had altered thermoregulation and developed abnormal hypothermia when exposed to cold compared to wild type, perhaps because of an inability to increase uncoupling. Interestingly when involuntarily subjected to exercise on a treadmill they showed equivalent endurance (32). The animals in this study had *ad libitum* access to food and water, which could also have implications for liver mitochondrial function (33). These changes are perhaps not surprising as the liver plays an important role as a metabolic pacemaker, changing full body metabolism back and forth, from anabolic to catabolic states in an oscillatory manner. For these reasons we chose to only compare the data to each respective control.

The cecal ligation and puncture (CLP) method to induce sepsis in rodents is widely used and is considered to be the gold standard, corresponding to a bowel perforation with a subsequent peritonitis and polymicrobial sepsis. Small alterations in needle size, number of punctures etc. will drastically change mortality rates. The precise procedure used was chosen to avoid unwanted selection between the groups due to mortality, but still ensuring that the animals would also be severely affected at the 24-hour endpoint (16, 34). The effects of anesthetics are a major concern of mitochondrial research as studies have shown interference with mitochondrial function (35). For ethical and practical reasons anesthetics must be used

for CLP procedure. In the present study continuous gas anesthesia using isoflurane was chosen. Also isoflurane may interfere with mitochondrial respiration by for example opening the mitochondrial permeability transition pore (mPTP) (36). This is one of the reasons why it was important to use separate controls for each time point as the effects of isoflurane could possibly be short acting.

Care need to be taken when extrapolating the findings of altered respiratory function in this mouse model to humans in a clinical setting. Mice do not respond to infection with a rise in temperature but with a decreased body temperature. Septic mice in general had higher LEAK than controls and this could be an adaptive response to low body temperature, with an up regulation of uncoupling proteins to produce more heat. Therefore, interpreting the data related to uncoupling (LEAK) and the hypothermic response is challenging. For obvious reasons obtaining brain and liver samples from septic patients are not feasible. But nonetheless, animal models can indeed only complement human data and the present data needs to be viewed in the context of previous human data.

Concerning the controversy regarding O₂ delivery in MOF, the present study does not resolve this issue. Preferably venous O₂ saturation and lactate, for example, should have been measured to provide basic information related to oxygen delivery. The lack of this data, including other functional parameters, is a limitation. However, one can also argue that this type of data would not be sufficient to fully answer a question related to O₂ delivery. Venous O₂ saturation and lactate does not necessarily reflect the tissue-oxygenation and microcirculation of the organs. An increase in lactate could reflect a mitochondrial inhibition and an increased anaerobic glycolysis.

The theory one embraces regarding mitochondrial respiration in sepsis will have implications on which strategy one should pursue for pharmaceutical development. As indicated by the results in this study the target needs to be organ specific. One strategy could

be to find methods to support and restore function by optimized and specific substrate supply and essentially increasing respiration and the phosphorylating capacity (37). Another strategy could be to help protect the mitochondria and a possible adaptive metabolic change during the insult. Sepsis is a dynamic process and perhaps a combination of strategies is best suited during the initial development and hopefully later recovery from sepsis. Nonetheless, the mitochondria seem to be affected and therefore might be a suitable pharmaceutical target for the treatment of sepsis.

In conclusion the present study suggests a diverse and tissue specific mitochondrial respiratory response to sepsis-induced multiple organ failure. The brain displayed an early impaired mitochondrial respiratory efficiency. Interestingly, as a possible response to an increased metabolic demand, the liver increased the phosphorylating capacity as a response to the septic insult. The liver also displayed increased uncoupling of oxidative phosphorylation at 24 hours.

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1. Brain mitochondrial function. (A) Brain - 6 hours. The respiratory control ratio (RCR) at 6 hours of $\text{OXPHOS}_{\text{CI+II}} / \text{LEAK}_{\text{CI+II}}$ was significantly decreased ($P < 0.05$) in septic mice compared to controls. **(B) Brain - 24 hours.** At 24 hours there was a similar trend that however did not reach significance ($P = 0.055$). $\text{OXPHOS}_{\text{CI+II}}$ was measured using the NADH-linked substrates malate, pyruvate and glutamate as well as CII substrate succinate. $\text{LEAK}_{\text{CI+II}}$ respiration, i.e. mitochondrial respiration independent of ATP-production, was induced by the ATP-synthase inhibitor oligomycin. **(C) Brain - 6 hours.** At 6 hours the $\text{OXPHOS}_{\text{CI+II}} / \text{ETS}_{\text{CI+II}}$ ratio was significantly decreased, but both groups displayed ratios just above 1.0. **(D) Brain - 24 hours.** At 24 hours there was no statistical difference in

OXPHOS_{CI+II} / ETS_{CI+II} ratio and likewise both groups displayed ratios just above 1.0, indicating that the respiratory capacity was not restricted by the phosphorylating capacity. The maximal uncoupled ETS capacity (ETS_{CI + CII}) was induced by FCCP titration. Line at median. * = $P < 0.05$.

2. ROS-production in brain homogenates

Fluorescent measurement of ROS-production using the Amplex Red assay. H₂O₂ levels measured in the presence of superoxide dismutase that ensures that all superoxide is converted to H₂O₂. Comparing ROS-production under convergent CI + CII respiration and LEAK respiration state. Inhibiting ATP-synthase increases ROS-production dramatically as expected. No statistical significant differences between the groups. Data presented as pmol H₂O₂ / (s*mg).

3. Liver mitochondrial function. (A) Liver – 6 Hours. The respiratory control ratio (RCR) at 6 hours of OXPHOS_{CI+II} / LEAK_{CI + CII} was significantly increased ($P < 0.001$) in septic mice compared to controls. **(B) Liver – 24 Hours.** At 24 hours there was likewise an increase compared to controls ($P < 0.05$). OXPHOS_{CI+II} was measured using the NADH-linked substrates malate, pyruvate and glutamate as well as CII substrate succinate. LEAK_{CI+II} respiration, i.e. mitochondrial respiration independent of ATP-production, was induced by the ATP-synthase inhibitor oligomycin. **(C) Liver – 6 Hours.** At 6 hours the OXPHOS_{CI+II} / ETS_{CI+II} ratio displayed a trend towards increased phosphorylating capacity ($P = 0.072$). **(D) Liver – 24 Hours.** At 24 hours the OXPHOS_{CI+II} / ETS_{CI+II} ratio was dramatically increased ($P < 0.001$) with a median of 0.75 in septic mice and 0.44 in the controls. The maximal uncoupled ETS capacity (ETS_{CI + CII}) was induced by FCCP titration. Line at median. * = $P < 0.05$. *** = $P < 0.001$.

Figure 1a

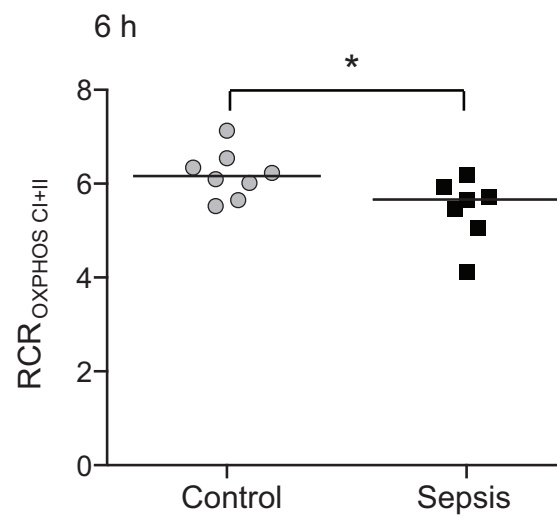
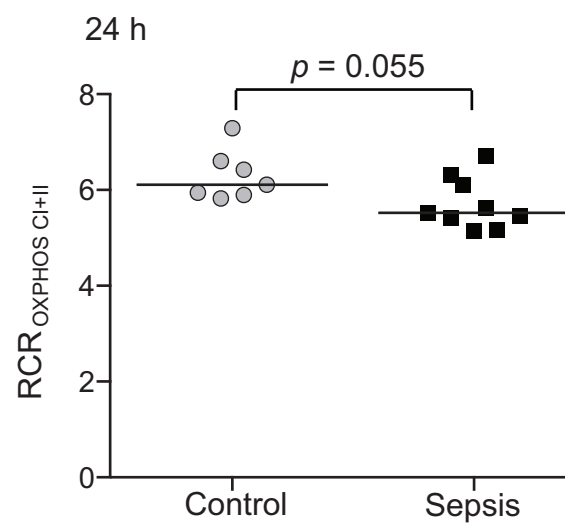


Figure 1b



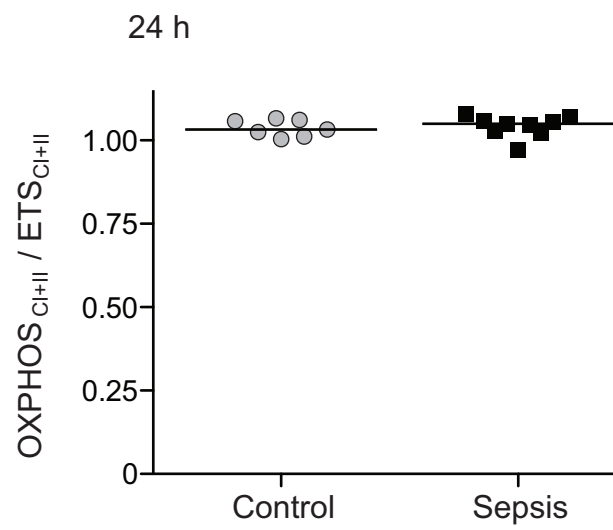
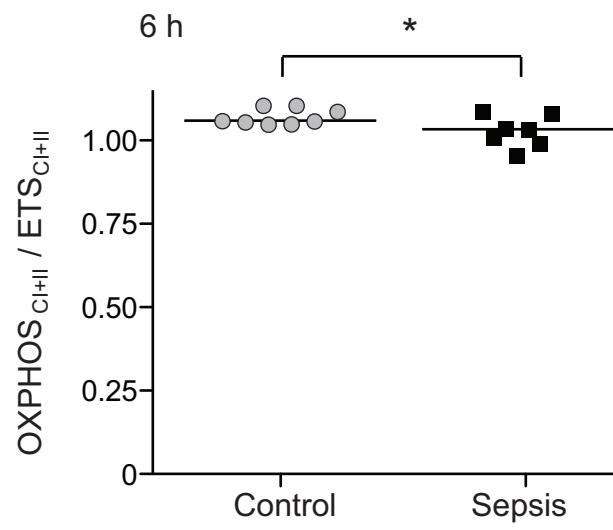


Figure 2

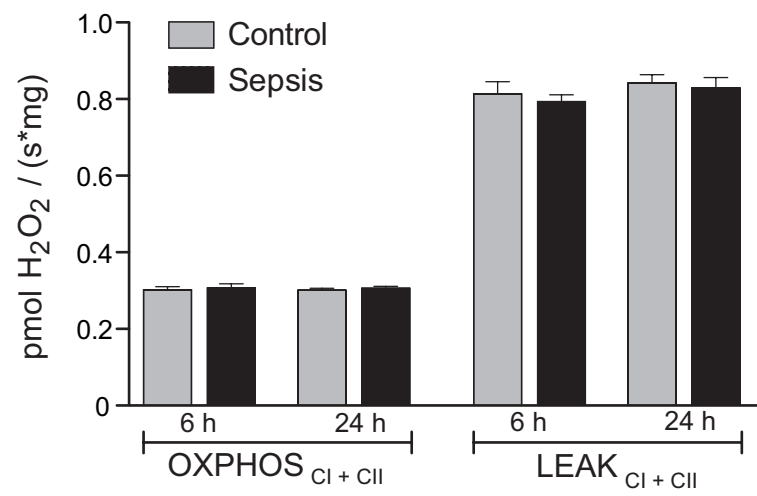


Figure 3a

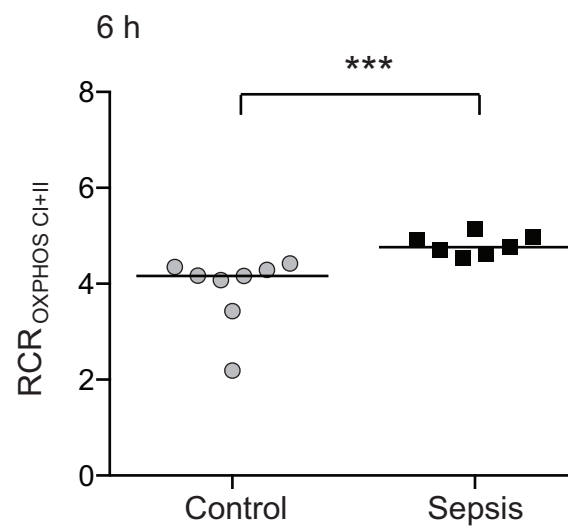


Figure 3b

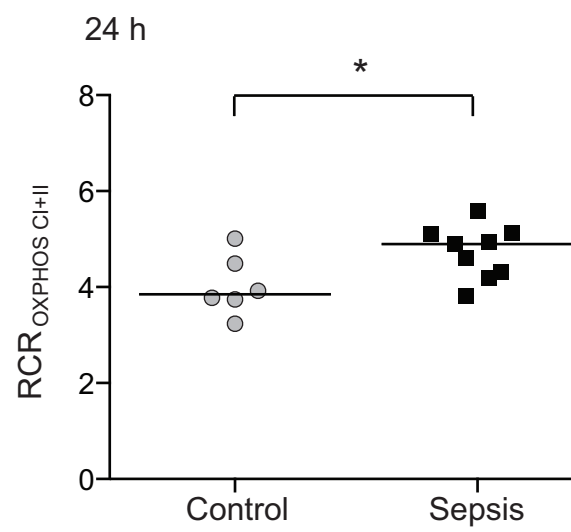


Figure 3c

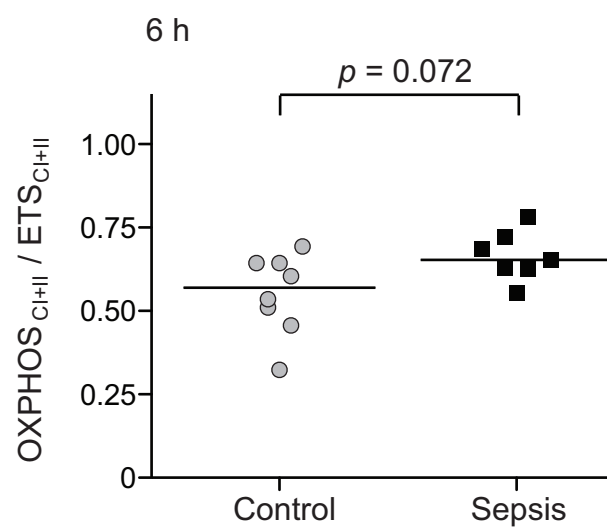


Figure 3d

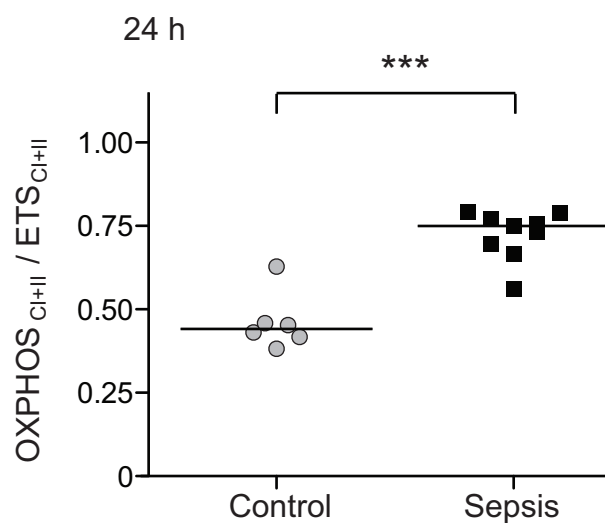


Table 1. Clinical scoring criteria

Score	Observation
0	No discernible clinical signs
1	Hunched back, slightly ruffled fur
2	Very ruffled fur, reduced rate of movement, developing motor impairments
3	Very ruffled fur, impaired balance/coordination, severe motor impairments such as ataxia, hemiplegia and paraplegia, convulsions, fitting
4	Very little movement, convulsions, fitting
5	Loss of consciousness/coma

Table 2. Clinical characteristics at termination

	6 hours		24 hours	
	Control (n = 8)	Sepsis (n = 7)	Control (n = 7)	Sepsis (n = 9)
Clinical score	0 (0-1)	2 (2-3)	0 (0)	3 (1-4)
Blood glucose	8.65 mmol/L (7.90-9.00)	9.30 mmol/L (6.80-10.2)	9.50 mmol/L (7.90-9.70)	5.30 mmol/L (4.15-5.65)
Temperature	36,8° (36.2-37.4)	31,7° (29.6-33.1)	37,6° (36.9-38.0)	30,8° (26.4-36.4)

2. Clinical score presented as median (range). Blood glucose and temperature presented as median (IQR).

Table 3A. Brain homogenates - mitochondrial respiration

Respiratory parameters	6 hours		24 hours	
	Control (n = 8)	Sepsis (n = 7)	Control (n = 7)	Sepsis (n = 9)
OXPHOS _{CI}	87.74 ± 10.3	76.88 ± 15.1	95.61 ± 9.72	84.37 ± 13.0
OXPHOS _{CI + CII}	152.1 ± 10.9	147.6 ± 14.1	158.5 ± 9.81	150.7 ± 15.4
LEAK _{CI + CII}	24.64 ± 1.84	27.29 ± 2.56*	25.24 ± 1.67	26.40 ± 2.17
ETS _{CI + CII}	142.1 ± 12.0	143.6 ± 12.5	152.9 ± 9.80	144.7 ± 15.0
ETS _{CII}	74.83 ± 4.59	76.88 ± 7.39	75.20 ± 3.87	75.08 ± 6.49
CIV	240.6 ± 23.4	256.1 ± 22.5	246.1 ± 15.6	250.8 ± 25.1
RCR _{OXPHOS CI}	3.64 (3.12-3.74)	3.04 (2.25-3.42)*	3.57 (3.38-4.21)	3.04 (2.85-3.74)*
RCR _{OXPHOS CI+II}	6.16 (5.74-6.49)	5.66 (5.05-5.94)*	6.11 (5.90-6.60)	5.53 (5.29-6.21)
OXPHOS _{CI + CII} / ETS _{CI + CII}	1.06 (1.05-1.10)	1.03 (0.99-1.08)*	1.03 (1.01-1.06)	1.05 (1.02-1.06)

3A. Values presented as mean ± SD. Ratios presented as median (IQR). OXPHOS respiration associated with ATP synthesis by oxidative phosphorylation, ETS respiration associated with maximal protonophore stimulated flux through the electron transport system, LEAK idle respiration without ATP-synthase activity, RCR - respiratory control ratio. CI - complex I, CII - complex II, CIV - complex IV. Statistical comparison to control at the same time-point.

* = $p < 0.05$

Table 3B. Liver homogenates - mitochondrial respiration

Respiratory parameters	6 hours		24 hours	
	Control (n = 8)	Sepsis (n = 7)	Control (n = 6)	Sepsis (n = 9)
OXPHOS _{CI}	65.13 ± 11.7	53.17 ± 9.90	36.93 ± 3.15	56.86 ± 12.2*
OXPHOS _{CI + CII}	157.6 ± 39.2	174.8 ± 17.1	112.6 ± 20.2	180.6 ± 21.4*
LEAK _{CI + CII}	40.45 ± 5.53	36.34 ± 3.30	28.04 ± 3.14	38.47 ± 5.46*
ETS _{CI + CII}	284.4 ± 21.6	264.1 ± 22.1	244.6 ± 13.3	249.8 ± 17.2
ETS _{CII}	251.7 ± 30.1	236.6 ± 9.41	228.2 ± 12.9	227.4 ± 15.4
CIV	390.0 ± 36.8	388.8 ± 34.7	359.5 ± 25.6	353.1 ± 34.5
RCR _{OXPHOS CI}	1.71 (1.32-1.85)	1.54 (1.41-1.68)	1.28 (1.20-1.43)	1.39 (1.24-1.76)
RCR _{OXPHOS CI+II}	4.17 (3.59-4.33)	4.76 (4.63-4.98)*	3.85 (3.62-4.62)	4.90 (4.25-5.12)*
OXPHOS _{CI + CII} / ETS _{CI + CII}	0.57 (0.47-0.64)	0.65 (0.63-0.72)	0.44 (0.41-0.50)	0.75 (0.68-0.78)*

3B. Values presented as mean ± SD. Ratios presented as median (IQR). OXPHOS respiration associated with ATP synthesis by oxidative phosphorylation, ETS respiration associated with

maximal protonophore stimulated flux through the electron transport system, LEAK idle respiration without ATP-synthase activity, RCR - respiratory control ratio. CI - complex I, CII - complex II, CIV - complex IV. Statistical comparison to control at the same time-point.

* = $p < 0.05$