Different effects of endotoxic shock on the respiratory function of liver and heart mitochondria in rats

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Kozlov, Andrey V., Katrin Staniek, Susanne Haindl, Christina Piskernik, Wolfgang Öhlinger, Lars Gille, Hans Nohl, Soheyl Bahrami, and Heinz Redl. Different effects of endotoxic shock on the respiratory function of liver and heart mitochondria in rats. Am J Physiol Gastrointest Liver Physiol 290: G543–G549, 2006; doi:10.1152/ajpgi.00331.2005.—This study was designed to clarify whether mitochondrial function/dysfunction and reactive oxygen species (ROS) production have a temporal relationship with organ failure during endotoxic shock. Adult male Sprague-Dawley rats were divided into three groups receiving 1) isotonic saline (control group, n = 16); 2) 8 mg/kg lipopolysaccharide (LPS; n = 8); or 3) 20 mg/kg LPS (n = 8) intraperitoneally under short anesthesia with 3.5% of isoflurane. After 16 h, animals were killed to analyze plasma, rat liver mitochondria (RLM), and rat heart mitochondria (RHM). In accordance with plasma analysis, LPS-treated rats were divided into “responders” and “nonresponders” with high and low levels of alanine aminotransferase and creatinine, respectively. RHM from responders had significantly higher respiratory activity in state 3, suggesting a decreased rate of ATP synthesis. In contrast, RLM from responders had significantly higher respiratory activity in state 3 than both nonresponders and the control group. This increase was accompanied by a decrease in phosphate-to-oxygen ratio values, which was not observed in RHM. ROS generation determined with a spin probe, 1-hydroxy-3-carboxypropylidene, neither revealed a difference in RHM between LPS and control groups nor between responders and nonresponders. In contrast, RLM isolated from responders showed a marked increase in ROS production compared with both the control group and nonresponders. Our data demonstrate that 1) RHM and RLM respond to endotoxic shock in a different manner, decreasing and increasing respiratory activity, respectively, and 2) there is a temporal relationship between ROS production in RLM (but not in RHM) and tissue damage in rats subjected to LPS shock.

lipopolysaccharide; reactive oxygen species; organ failure; electron paramagnetic resonance; spin trap

MULTIPLE ORGAN DYSFUNCTION and fatal multiple organ failure are caused by sepsis and other life-threatening diseases. Alterations of oxygen metabolism during critical illness have been well documented and are characterized by impaired tissue oxygen extraction and “pathological” consequences of altered oxygen consumption (13, 24, 33). A decrease in oxygen extraction by tissues should be due to altered cellular oxygen metabolism. Over 90% of cellular oxygen is consumed through mitochondrial oxidative phosphorylation of ADP. Cellular ATP is produced mainly by this process, although smaller amounts are generated via glycolysis. Therefore, an imbalance in ATP synthesis can be a reason for organ failure. Another important consequence of altered cellular oxygen metabolism reported during sepsis is increased generation of reactive oxygen species (ROS). There are two main sources of ROS: 1) immune cells generating ROS in the extracellular space and 2) mitochondria generating ROS inside cells.

Although mitochondrial function during sepsis and endotoxic shock has been studied extensively, the results are contradictory. Whereas some investigators have failed to find any abnormality in mitochondrial function in septic/endotoxic shock (11, 21, 31), others have found that mitochondria isolated from septic/endotoxic tissues have reduced state 3 activity and ATP synthesis activity (6, 7, 14, 20). Moreover, some investigators have found that mitochondrial function is improved under septic conditions (19, 30, 32), at least during the early phase of shock (19). Analysis of the literature has shown that mitochondrial function during septic/endotoxic shock depends on 1) the source of mitochondrial 2) the experimental induction of septic/endotoxic shock, and 3) the phase of illness. Mitochondria obtained from different organs show different susceptibilities to septic/endotoxic shock (for a review, see Ref. 33).

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There is ample evidence suggesting that ROS are produced in excess during sepsis and that they are involved in cellular damage within vital organs (5, 22, 23, 31). These conclusions have mainly been drawn from the imbalance of antioxidant enzyme activities (22) during sepsis and the beneficial effects of antioxidants (23). Only one group (31) has attempted to directly confirm the increase in ROS in liver mitochondria by measuring $H_2O_2$, a direct product of $O_2$ dismutation, and $\cdot OH$, a product of the interaction between $H_2O_2$ and iron ions (31). However, they have not shown a connection between mitochondrial function and organ failure. Whether radical production is elevated only in liver mitochondria or in other organs as well remains unclear.

Another point that has not yet been addressed is the underlying mechanism of increased free radical production. It has been suggested that mitochondrial electron transport is inhibited during the acute phase of sepsis, thereby contributing to inefficient oxygen utilization and the attendant increase in ROS production (5). Another hypothesis recently published suggests that organ failure is a potentially protective, reactive mechanism involving an activation of mitochondrial metabolism (26). In contrast to the previous study, the latter suggests that an increase in ROS production could be due to increased mitochondrial electron flow, reflected by increased mitochondrial transmembrane potential (17, 18).

Our recent study (15) demonstrated an increase in ROS levels in vivo during endotoxic shock in rats. This increase was significant only in 3 of 10 tissues: those of the heart, liver, and lung (15). These data suggest that mitochondrial ROS formation contributes to increased ROS levels in the heart, liver, and lung. However, the pathophysiological role of increased mitochondrial ROS production remains unclear.

The aim of this study was to clarify whether heart and liver mitochondria respond to endotoxic shock in a similar manner and, if so, whether these changes coincide with tissue damage.

**MATERIALS AND METHODS**

**Animals.** The study was approved by the local Committee on Animal Experiments of Vienna, Austria, and all experiments were performed under conditions described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 86-23, Revised 1985). Adult male Sprague-Dawley rats weighing 280 ± 21 g (Animal Research Laboratories, Himberg, Austria) were divided into three groups: a control group receiving saline intraperitoneally ($n = 16$), a group receiving 8 mg lipopolysaccharide (LPS)/kg ip (E. coli 026:B6, n = 8, Difco; Detroit, MI), and a group receiving 20 mg LPS/kg ip ($n = 8$). One control and one LPS-treated rat were killed per experimental day 16 h after the intraperitoneal injection, and blood was collected in heparinized tubes for biochemical analysis. The liver and heart were quickly extracted and stored in ice-cold preparation buffer.

**Histological investigation.** Liver tissue was fixed in 10% formaldehyde solution for 1–2 wk. The paraffin blocks and later sections were prepared. Sections were stained with hematoxylin and eosin. Some congestion, distension of Disse’s spaces, local necrosis, and cellular infiltration were seen under the light microscope.

**Preparation of mitochondria.** Rat heart mitochondria (RHM) and rat liver mitochondria (RLM) were prepared as described previously (27) and stored at 0°C for 4–5 h in a buffer containing 0.25 M sucrose, 10 mM Tris·HCl, 0.5 mM EDTA (pH 7.2), and 0.5 g/l essentially fatty acid-free BSA.

**Mitochondrial function and ROS generation.** Respiratory parameters of mitochondria isolated from control and LPS-treated rats were determined with a Clark-type oxygen electrode. RHM (0.5 and 0.25 mg/ml for glutamate/malate and succinate respiration, respectively) and RLM (0.5 mg/ml) were incubated in a buffer consisting of 105 mM KCl, 20 mM Tris·HCl, 1 mM diethylenetriaminepentaaetic acid, 5 mM KH$_2$PO$_4$, and 1 mg/ml fatty acid-free BSA (pH 7.4, 25°C). State 4 respiration was stimulated either by the addition of 5 mM glutamate plus 5 mM malate or 10 mM succinate in the presence of rotenone (1 μg/ml). The latter was used to prevent electron transport to and from complex I. The transition to state 3 respiration was induced by the addition of 200 μM ADP. The rate of ROS generation in mitochondria isolated from control and LPS-treated rats was detected in the presence of 250 μM of 1-hydroxy-3-carboxypropyrolidine (CPH). It has been shown that CPH reacts with superoxide radical and peroxynitrite (8, 9). Mitochondria (0.55 mg/ml) were incubated for 20 min at 22 ± 1.5°C under state 4 conditions. The incubation buffer was identical to the buffer used for the determination of respiratory parameters. Oxygen diffusion was facilitated using a shaking table to provide gentle mixing of the mitochondrial suspension with air.

**Electron paramagnetic resonance measurements.** Electron paramagnetic resonance (EPR) spectra were recorded at 22°C with a Bruker EMX EPR spectrometer (BioSpin; Rheinstetten/Karlsruhe, Germany) using a flat cell FZK 200 (Magnetech; Berlin, Germany). The general settings were as follows: microwave frequency, 9.777 GHz; modulation frequency, 100 kHz; modulation amplitude, 2 G; gain, 5 × 10$^5$; microwave power, 1 mW; time constant, 40 ms; and sweep time, 41.9 s. Upon reaction with ROS, CPH is transformed into a stable 3-carboxypropyrol radical. Therefore, standard solutions of 3-carboxypropyrol were used to quantify mitochondrial ROS levels.

**Detection of cytochrome levels in mitochondrial suspensions.** The concentrations of mitochondrial cytochromes in RLM preparations were determined by optical redox-difference spectroscopy using a DW-2000 dual-wavelength photometer (SLM AMINCO; Rochester, NY). Each 2 mg of mitochondrial protein were resuspended in 1 ml of oxygenated preparation buffer, and a spectrum in the range of 400–650 nm was recorded as a reference. Upon the addition of 10 mM dithionite as a reductant for cytochromes, the redox-difference spectrum was obtained in the same spectral range. The completeness of the reduction was verified by recording a second redox-difference spectrum after a further addition of 10 mM dithionite. Spectra were subjected to a baseline correction, and cytochrome concentrations were calculated by a system of linear equations according to Williams et al. (36) using our own software (DW-2000 Browser). Measurements were performed in triplicate for each RLM preparation, and the mean values obtained were used for the comparison between control and LPS-treated animals.

**Blood parameters.** Plasma levels of alanine aminotransferase (ALT) and creatinine were measured as described previously (10). Briefly, 1 ml of heparinized blood (10 IU/ml) was centrifuged at 1,000 g for 10 min, and plasma was frozen until use. ALT and creatinine were analyzed with a Cobas Faran analyzer using reagents from Roche Diagnostic.

**Statistics.** All data are presented as means ± SE. Statistical analysis was performed by one-way ANOVA followed by a post hoc test for the least-significant difference. Significance was based on a value of $P < 0.05$. Calculations were made with SPSS 11.5 software for Windows (SPSS) and MS Excel (Microsoft).

**RESULTS**

**Organ damage.** On the basis of the plasma levels of ALT and creatinine, individual responses of rats subjected to LPS-induced endotoxic shock showed a strong variation. Therefore, rats receiving LPS were divided in two groups in accordance with plasma ALT and creatinine (Fig. 1) but not in accordance with the LPS dose administered. The highest plasma concen-
tration of ALT in the control group (93 IU/l) was set as the limiting value for LPS-treated rats, defining “nonresponders” as ALT < 93 IU/l (LPS1 group) and “responders” as ALT > 93 IU/l (LPS2 group). Consequently, both LPS groups received endotoxin, but only the LPS2 group responded with organ damage. After this redistribution, the LPS1 group included five animals treated with 8 mg LPS/kg and one animal treated with 20 mg LPS/kg, whereas the LPS2 group included seven animals treated with 20 mg LPS/kg and three animals treated with 8 mg LPS/kg.

Histological examination of the liver. Plasma levels of ALT and creatinine were in accordance with histological findings. Focal necroses were found in responders only. Cellular infiltration was found in both the LPS1 and LPS2 groups (Fig. 2). All other histological parameters were similar in all three groups.

Function of RHM. Four parameters [oxygen consumption in state 4 and 3, the phosphate-to-oxygen ratio (P/O), and ROS generation] were compared in RHM obtained from the three groups. In the presence of glutamate/malate, oxygen consumption in state 3 respiration was lower in RHM from the LPS2 group compared with the control group (Fig. 3). In contrast, respiratory parameters of succinate-respiring RHM were not significantly influenced by LPS treatment. There was no difference between responders (LPS2) and nonresponders (LPS1) independently of the substrates used for respiration. Furthermore, there was no difference in ROS generation in RHM between the three groups (Fig. 3).

Function of RLM. In contrast to RHM, liver mitochondria isolated from LPS-treated rats showed an increase in state 3 respiration with both succinate and glutamate/malate (Fig. 4). At the same time, P/O values were decreased in responders (LPS2; Fig. 4). Oxygen consumption rates in state 4 respiration were neither influenced by LPS treatment in RLM (Fig. 4) nor in RHM (Fig. 3). ROS generation in RLM was increased in the LPS2 group but not in the LPS1 group compared with the control group.

Mortality 16 hours after LPS treatment

Fig. 2. Liver histology and survivability of rats. Light microscopy with hematoxylin and eosin staining is shown. Top and bottom: low and high magnification, respectively. C, congestion; SD, distension of Disse’s spaces; N, necrosis; CI, cellular infiltration (polymorphonuclear neutrophils).
A particularly high difference in ROS levels was observed when glutamate/malate was used as the substrate (Fig. 4).

Mitochondrial content in preparations. To prove whether the composition of mitochondrial preparations was affected by LPS or not, the contents of the four most important carriers of the respiratory chain (cytochromes $a$, $b$, $c$, and $c_1$) were compared between mitochondrial preparations in the three groups studied. No significant difference was found among the

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Fig. 3. Respiratory parameters of rat heart mitochondria isolated from the control, LPS1, and LPS2 groups. A: oxygen uptake in state 4; B: oxygen uptake in state 3; C: inorganic phosphate-to-oxygen ratio (P/O) values; D: ROS generation. All parameters were determined with glutamate/malate, substrates of complex I, and succinate, a substrate of complex II. $$##P < 0.01$$ compared with the control group.

Fig. 4. Respiratory parameters of rat liver mitochondria (RLM) isolated from the control, LPS1, and LPS2 groups. A: oxygen uptake in state 4; B: oxygen uptake in state 3; C: P/O values; D: ROS generation. All parameters were determined with glutamate/malate, substrates of complex I, and succinate, a substrate of complex II. $$##P < 0.01$$, $$###P < 0.001$$ compared with the control group; *$P < 0.05$ and **$P < 0.01$, compared with the LPS group.
groups (Fig. 5), suggesting that no severe disintegration of mitochondrial electron carriers takes place during endotoxic shock.

DISCUSSION

Organ damage versus LPS dose and RLM versus RHm. Endotoxic shock is known to trigger many signaling mechanisms. Each of these signaling mechanisms does not necessarily lead to organ damage and dysfunction. The organs most susceptible to endotoxic shock are the heart, liver, kidney, and lung (1, 4, 35). To gain insight into the relationship between mitochondrial function and tissue dysfunction, rats in this study were challenged with two different doses of LPS (8 mg/kg, a sublethal dose, and 20 mg/kg, lethal dose with mortality of 25% after 16 h). LPS-treated rats were divided into groups in accordance with organ damage (estimated as blood levels of ALT and creatinine and histological examination) not in accordance with the LPS dose administered. This was done to determine the contribution of mitochondrial dysfunction to the pathogenesis of organ failure as the end point of endotoxic shock. RHm and RLM isolated from the same animal were studied to determine whether mitochondrial dysfunction could be a universal mechanism of tissue damage involving mitochondria.

Respiration versus ROS in RHm. In RHm, we clearly saw that mitochondrial function was affected by endotoxin treatment. The respiration rate in state 3 and respiratory control (not shown) were decreased, whereas P/O values were unchanged, in the presence of glutamate/malate (substrates for mitochondrial complex I). In contrast, succinate-stimulated respiration (substrate for complex II) was not influenced. Accordingly, ATP synthesis rates, which can be calculated from the product of oxygen consumption rates during state 3 and P/O values, were reduced in RHm supplemented with complex I substrates but not in succinate-respiring RHm. However, this finding is not due to damage of the inner mitochondrial membrane (uncoupling), because there was no increased oxygen consumption under state 4 conditions. Assuming that the decreased state 3 respiration could be due to excessive ROS production leading to oxidative modification of proteins in complex I, we measured ROS production in RHm. However, we did not find any significant increase in ROS release of RHm from LPS-treated animals compared with the control group independent of the respiratory substrates used. Consequently, it is likely that there is a ROS-independent mechanism affecting mitochondrial function in RHm.

Respiration versus ROS in RLM. In contrast to RHm, RLM had a significantly increased respiration rate in state 3 in the LPS2 group with both glutamate/malate or succinate. Unexpectedly, increased state 3 respiration was accompanied by decreased P/O values, indicating that the efficiency of oxidative phosphorylation was reduced. Although the exact mechanism remains unclear at this point, there are two possible explanations for this phenomenon: 1) an increase in state 3 respiration and a decrease in P/O are coming from two different populations of mitochondria or 2) there is an additional mechanism of oxygen consumption not related to ATP synthesis in state 3.

Mitochondrial ROS generation was measured to find out whether increased oxygen consumption could be attributed to excessive ROS production. ROS production was significantly increased in glutamate/malate-respiring RLM as well as in succinate-respiring RLM isolated from LPS-treated rats with severe organ damage compared with control rats. This increase in ROS levels is probably not due to a decrease in the antioxidant capacity of mitochondria, because previous reports have shown an increase in SOD activity in liver tissue (2), and particularly in Mn-SOD typical for mitochondria (12, 16), in response to endotoxic shock.

In highly coupled mitochondria, the leakage of protons through the inner mitochondrial membrane is relatively small, so that a high transmembrane potential can be established. An increase in transmembrane potential in mitochondria has been shown to cause increased ROS production (17, 18), indicating that part of the electrons deviates from the respiratory chain to cause a one-electron reduction of oxygen, yielding ROS instead of participating in ATP synthesis. A possible explanation as to why mitochondria isolated from LPS-treated animals have increased ROS production simultaneously with increased respiratory activity comes from studies (17, 18) demonstrating that an increase in transmembrane potential in mitochondria results in an increase of ROS production. A recent study by Starkov and Fiskum (28) suggests that ROS production by mitochondria oxidizing physiological NADH-dependent substrates is regulated by membrane potential. Therefore, mitochondria having higher respiratory activity are expected to have higher membrane potential and consequently higher ROS production. On the other hand, this supports our data, because we found the highest increase in ROS generation in mitochondria results from studies (17, 18) demonstrating that an increase in transmembrane potential in mitochondria results in an increase of ROS production. On the other hand, the data presented in Fig. 4 show that there is no temporal relationship between the increased respiration rate in state 3 and ROS production in the LPS1 group. Therefore, the exact mechanism leading to increased ROS formation still needs to be clarified.

Independently of the mechanism leading to increased ROS formation in RLM, we clearly found a temporal relationship between increased mitochondrial ROS production and liver damage. This finding suggests the involvement of mitochondrial ROS generation in endotoxin-induced liver damage.

Recently, Suliman and co-workers (29) disclosed a new aspect of ROS in endotoxic shock. They demonstrated a duality of ROS effect in the cardiac response to LPS in which oxidative mitochondrial damage is opposed by oxidant-
duced stimulation of biogenesis in terms of mitochondrial DNA recovery (29).

Molecular mechanisms underlying endotoxin-induced changes in RLM. The changes observed in RLM can result from decreased liver perfusion and consequent hypoxia, as reflected by our model (10), or from partial inhibition of respiration by ROS and reactive nitrogen species (3). An important issue is whether the changes in mitochondrial respiration are the result or cause of free radical generation. Many authors have reported that free radical attack results in damage of mitochondrial membranes accompanied by leakage of protons (uncoupling), increased oxygen consumption in state 4, and decreasing efficiency of ATP synthesis. In contrast to those authors, we found that the state 4 respiration was not affected and the rates in state 3 were even higher than in control rats. Thus the changes we found in liver mitochondria are probably not the result of free radical attack. Our data are in line with a hypothesis recently published by Singer et al. (26), suggesting that the changes in mitochondria are primarily a defense reaction similar to that induced by thyroid hormones. It has also been suggested (by Singer et al. and others) that mitochondria respond to endotoxemia in a biphasic manner, with an initial increase in respiration followed by a decline. The duration of both phases has not yet been clarified, particularly for our model. In accordance with Singer et al.’s hypothesis, we observed the first phase in our experiments, whereas a decline in mitochondrial function (second phase) appears to cause death quickly and cannot be easily detected. These different phases of endotoxic shock may explain the controversial results obtained from different mitochondrial studies, ranging from impaired (6, 7) to unchanged (11, 31) and even to improved mitochondrial function (32).

There is also another explanation for the stimulation of high respiratory activity of mitochondria by endotoxin. It is possible that 16 h are sufficient to account for removal of malfunctioning mitochondria (i.e., autophagy) and cells (necrosis or apoptosis) such that the most damaged mitochondria are cleared from the organs or even that new mitochondria are produced soon after LPS treatment (29).

Purity of RLM. The changes in parameters normalized to protein concentration described above, e.g., state 4, state 3, and ROS generation, could be due to LPS-induced modification in the composition of mitochondrial preparation. Because oxygen consumption and ROS formation originates from the inner mitochondrial membrane, levels of the most important carriers of the respiratory chain (cytochromes a, b, c, and c1) were determined. Because no difference was found among the groups studied, the changes in mitochondrial parameters are related to their affected function and not to a modified composition of mitochondrial preparations.

In our study, we focused on the function of the main mitochondrial fraction. There is another mitochondrial fraction, however, the so-called light mitochondrial fraction, which has been found to behave differently in apoptotic muscle (34). We did not study this light fraction because it is strongly contaminated by peroxisomes and lysosomes, particularly in the liver. However, we performed preliminary experiments in the liver homogenate and in mitochondria separated from the same liver homogenate and did not find a significant difference in P/O values and respiratory control, a ratio between oxygen consumption in state 3 to state 4 (data not presented).

In summary, our data demonstrate that 1) RHM and RLM respond to endotoxic shock differently, decreasing and increasing respiratory activity, respectively; and 2) there is a temporal relationship between ROS production in RLM (but not in RHM) and tissue damage in rats subjected to LPS shock.

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