Mitochondrial respiration after sepsis and prolonged hypoxia

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Taylor, David E., Stephen P. Kantrow, and Claude A. Piantadosi. Mitochondrial respiration after sepsis and prolonged hypoxia, Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L139–L144, 1998.—Recently, marked oxygen dependence of respiration by isolated mitochondria after exposure to prolonged hypoxia has been described. Because mitochondrial oxygen-dependent respiration could significantly influence oxygen consumption during critical illness, we sought to confirm the oxygen-dependent behavior of mitochondria. We hypothesized that mitochondria isolated during sepsis would exhibit increased oxygen dependence. We isolated rat liver mitochondria 16 h after cecal ligation and puncture and found a 30–40% greater oxygen uptake compared with control rats under state 3 conditions. Mitochondria incubated in deoxygenated buffer were studied for oxygen dependence at 10-min intervals for 90 min. Mitochondrial respiration after reoxygenation was 10% lower when mitochondria were reoxygenated at 15–25 Torr versus high (90–100 Torr) and low (10–15 Torr) versus intermediate (40–45 Torr) oxygen tension. Oxygen consumption with ascorbate+phenazine ethosulfate was 20% lower at low versus high oxygen tension. No increase in oxygen dependence was observed during 1 h of hypoxic incubation. Our data indicate only a modest oxygen dependence of respiration between 10 and 100 Torr, which is similar for septic and control mitochondria. Additionally, oxygen dependence did not increase significantly during a 1-h hypoxic exposure for well-coupled mitochondrial preparations.

oxygen dependence; oxygen conformance

In severe sepsis, the ability of tissues to extract oxygen from the blood is impaired, and in animals, the critical oxygen delivery required to maintain global oxygen consumption is increased (22). Regional measurements of skeletal muscle and intestinal oxygen consumption demonstrate that tissues are unable to increase the uptake of oxygen from blood normally during septic shock, but the etiology of this defect in oxygen extraction is poorly understood (5). Two main possibilities have been proposed: abnormal regulation of blood flow and abnormal oxygen utilization by the cell. Abnormal vasoregulation may result in a mismatching of oxygen delivery to consumption so that some tissue beds remain hypoxic while venous oxygen content remains high (26). The alternative explanation is that a decrease in oxygen extraction by tissues is due to altered cellular oxygen metabolism. The primary sites of cellular energy generation (in the form of ATP) and oxygen consumption in the cell are the mitochondria. The function of these organelles can be assessed by determining the maximal rate of electron transport and by determining the coupling between electron transport and ATP production (7). Isolated mitochondria from liver in peritonitis models of sepsis respire more briskly when supplemented with carbon substrates and may show high degrees of respiratory control (13, 15, 23).

Numerous studies of mitochondria isolated from healthy animals have found that oxygen consumption is largely independent of ambient oxygen tension until PO2 reaches 1 Torr or less (30). Recently, Chandel et al. (8) and Schumacker et al. (25) reported that hepatocytes exposed to moderate hypoxia (~20 Torr for 16 h) and mitochondria exposed to severe hypoxia (~2 Torr for 1 h) reversibly decrease oxygen consumption by as much as 40% at a PO2 of 20 Torr compared with 100 Torr. Subsequent work from the same investigators suggested that the inhibition of respiration occurs at the final electron acceptor cytochrome-c oxidase. It was proposed that molecular oxygen binds to and regulates the activity of the oxidase (9, 10). These authors suggested that decreased oxygen consumption after prolonged exposure to hypoxia could represent an adaptive response by the enzyme when the oxygen supply is inadequate. In contrast, Poderoso et al. (23) studied mitochondrial respiration during acute exposure to high or low oxygen tensions and found no oxygen dependence in preparations from control or septic animals.

To some extent, local hypoperfusion and tissue hypoxia are likely to occur during sepsis; therefore, the onset of oxygen-dependent mitochondrial function in a physiological range of oxygen tensions in vivo could contribute to a decreased oxygen extraction. The present study was carried out to test the hypothesis that sustained hypoxia recruits oxygen dependence of mitochondrial respiration in organelles isolated from the livers of septic rats.

MATERIALS AND METHODS

Animal protocol. Male Sprague-Dawley rats weighing 300–400 g were used for all studies. Animals were arbitrarily assigned to control or experimental groups. Experimental animals underwent ligation and double puncture (18-gauge needle) of the cecum after anesthesia with diazepeam (1 mg/kg) and ketamine (25 mg/kg) (29). Control animals underwent anesthesia and a superficial abdominal incision. All animals were given postoperative fluids consisting of intravenous 6% hetastarch (1 ml/100 g) and a subcutaneous injection of 0.9% sodium chloride (6 ml/100 g). Experimental and control animals fasted overnight but were allowed water intake ad libitum. Sixteen hours after surgery, the animals were killed, and the livers were removed and placed in ice-cold isolation medium.
Mitochondrial isolation. Liver mitochondria were prepared by discontinuous Percoll gradient centrifugation with an adaptation of previously published methods (28, 29). The isolation buffer contained 0.32 M sucrose, 1 mM EDTA, 10 mM Tris·HCl (pH 7.4), and 5 mg/ml of BSA. The livers were minced with scissors and then homogenized with a glass Dounce homogenizer at a 5% (wt/vol) concentration in isolation buffer. After a 1:1 dilution with a 24% Percoll buffer solution, the resulting homogenate (12% Percoll) was layered onto discontinuous density gradients (26% over 40% Percoll) and centrifuged at 30,700 g for 20 min at 4°C in a Sorvall RC-5 centrifuge with an SS-34 rotor (DuPont, Wilmington, DE). After centrifugation, the mitochondria-rich band at the interface between the 26 and 40% Percoll layers was collected. This fraction was pooled and diluted 1:4 with isolation buffer, and the resulting suspension was centrifuged at 16,700 g for 20 min. The supernatant was discarded, and the pellet was resuspended in isolation buffer followed by centrifugation at 7,300 g for 10 min. The final mitochondrial pellet was gently resuspended to a final volume of 2.5 ml and placed on ice. Protein content was determined by the bicinchoninic acid method with BSA as a standard.

Hypoxic mitochondria. Mitochondria were incubated at 25°C under hypoxic conditions (<2 Torr) in a 250-ml flask (Technoe) stirred at 60 rpm. Before addition of the mitochondria to the incubation flask, 125 ml of mitochondrial respiration buffer were equilibrated with nitrogen gas for 20 min. The mitochondria (0.05–0.15 mg protein/ml) were added to the stirred buffer, and a 5-ml aliquot was taken every 10 min during the incubation. Each aliquot was immediately transferred in a glass syringe and divided into two 2.4-ml nitrogen-flushed respiration chambers for oxygen consumption measurements. Hypoxic conditions (<2 Torr) in the stirred incubation flask were maintained for at least 1 h to determine the effect of oxygen concentration on mitochondria isolated from septic and control rats.

Mitochondrial respiration. Oxygen consumption at 25°C was measured polarographically in two 2.4-ml water-jacketed glass chambers equipped with miniature Clark electrodes (model 730, Diamond General, Ann Arbor, MI). Polarographic data were recorded on a Fisher Recordall (series 5000) chart recorder. The oxygen content of the respiration buffer equilibrated with air was assumed to be 230 nmol oxygen/ml at 25°C. Mitochondrial (Sims) respiration buffer contained 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 5 mM Tris phosphate (pH 7.4), 10 mM Tris·HCl (pH 7.4), and 0.05 mM EDTA. Sucrose buffer contained 250 mM sucrose, 25 mM HEPES (pH 7.4), 5 mM KH2PO4, and 2 mM EGTA. For measurement of respiration by fresh mitochondria, 0.75–1.25 mg of mitochondrial protein were added to the respiration buffer in respirometers equilibrated with 0 vs. 7% oxygen (0–50 Torr) for measurements at low versus intermediate oxygen tension or with 0 vs. 14% (0–100 Torr) for measurements at low versus high oxygen tension. Hypoxic mitochondrial samples were transferred in a glass syringe to the respirometers, and a 150-µl aliquot of buffer saturated with 21 or 100% oxygen was added for determinations at low (10–15 Torr) versus intermediate (45–50 Torr) oxygen tension. For measurements at low (15–25 Torr) versus high (90–100 Torr) oxygen tension, a 350-µl aliquot of buffer saturated with 14 or 100% oxygen was added to each respirometer (Fig. 1). After ~3 min to allow for equilibration of the added oxygen, deoxygenated respiratory substrates were added to each respiration chamber. Measurements at high and low PO2 were made simultaneously in different respirometers, and the substrates were added at the following final concentrations: 5 mM succinate, 2.5 mM malate, 2 mM glutamate, 1 mM ADP, 2 mM ascorbate, and 0.4 mM N,N,N’,N’-tetramethyl-p-phenylenediamine (TMPD). Respiratory control ratios (RCRs; state 3/state 4) were determined after inhibition of mitochondrial ATPase with 0 µM oligomycin.

Chemicals. All chemicals were of analytic grade or higher and were obtained from Sigma or Mallinckrodt. All gases were premixed and contained 0–100% oxygen with the balance nitrogen.
RESULTS

Effect of sepsis on mitochondrial respiration. Oxygen consumption by freshly isolated mitochondria supplemented with malate+glutamate and ADP (state 3) was increased by 30–40% during sepsis, whereas state 4 respiration did not differ for the two groups (P = 0.01; Table 1). There was also a trend toward an increased RCR, an index of coupling of electron transport to oxidative phosphorylation, for mitochondria isolated during sepsis (P = 0.06). Oxygen consumption by septic mitochondria supplemented with succinate, ADP, and oxygen after incubation in hypoxic buffer was increased 30–40% compared with control mitochondria (114 ± 10 vs. 86 ± 8 nmol·min⁻¹·mg protein⁻¹; P = 0.02).

Effect of hypoxia on mitochondrial respiration. To choose an optimal buffer for hypoxic incubations, mitochondrial RCRs were determined during hypoxic incubation in both Sims respiration buffer and the sucrose-HEPES buffer used by Chandel et al. (8). In Sims respiration buffer, the RCR determined in the presence of 1 mM ADP decreased by ∼20–30% during the first 60 min of the hypoxic incubation for control and sepsis samples. In the sucrose-HEPES buffer, the RCR fell to a value of 1 (completely uncoupled respiration) within 20–40 min. Therefore, Sims respiration buffer was used for the hypoxic incubations to assess oxygen dependence of respiration in this study.

When mitochondria were incubated in hypoxic buffer (<2 Torr), state 3 succinate-dependent oxygen consumption after reoxygenation was stable over 60 min for the control samples but deteriorated slightly over time for the septic samples. Control mitochondrial oxygen consumption at 60 min was 95 ± 9% of the value at time 0, whereas septic mitochondrial oxygen consumption declined to 79 ± 2% (P < 0.04 compared with control samples). By 90 min, oxygen consumption had decreased to 76 and 56% of the initial rate for control and septic mitochondria, respectively. These effects were not reversible when the Po2 in the respirometer was increased. Because of this time-dependent decrease in respiration, only measurements collected during the first 60 min of incubation were used to assess oxygen dependence. The oxygen consumption rate with ascorbate+TMPD to supply electrons to cytochrome-c oxidase did not decrease during the hypoxic incubation for the control or sepsis samples (data not shown).

Effect of hypoxia on mitochondrial oxygen dependence. After mitochondria were incubated under hypoxic conditions (<2 Torr), they were reoxygenated, and state 3 succinate-dependent mitochondrial respiration was determined simultaneously at high and low Po2 values. The measured Po2 values at the onset of respiration were 95 ± 3 and 21 ± 2 Torr, respectively. The respiration rate was minimally dependent on Po2, with a decrease in oxygen consumption of ∼10% at the lower Po2 (Fig. 1). This oxygen dependence of respiration was apparent within 10 min and did not increase during a 1-h hypoxic incubation (Fig. 2). No difference in the degree of oxygen dependence was observed between mitochondria isolated from control and septic rats. Similarly, measurements comparing state 3 succinate-dependent respiration at 46 ± 1 and 13 ± 1 Torr demonstrated very limited oxygen dependence (respiration ∼10% lower at the low Po2), which occurred with 10 min. No increase in Po2 dependence was found after the hypoxic incubation, and control and septic mitochondria showed identical responses. Ascorbate+TMPD-dependent oxygen consumption was measured at high (91 ± 1 Torr) and low (21 ± 1 Torr) oxygen tensions, and an oxygen dependence of ∼20% was observed throughout the 1-h hypoxic incubation. Again, no difference was observed between control and septic mitochondria (Fig. 3).

DISCUSSION

Mitochondria isolated by density gradient centrifugation from rat liver during sepsis appear to differ significantly from control mitochondria in respiratory function. Several previous reports (13, 15, 23) have found increased utilization of substrate and/or greater degrees of respiratory coupling in fresh samples from septic animals, and our findings are consistent with these observations. Although the etiology of this altered respiratory performance remains unknown, it is possible that the isolation procedure yields a different population of mitochondria from septic compared with control animals (1). Alternatively, higher maximum respiration of intact mitochondria could represent a homeostatic response to cellular stress during sepsis. For example, the increase in ADP-stimulated (state 3) respiration could reflect an increase in ADP transport into the mitochondrion during sepsis to meet increased metabolic demands.

Because some recent reports (8, 25) suggested that prolonged (1–4 h) exposure of mitochondria to hypoxia can increase the dependence of respiration on oxygen concentration, we characterized the response of control and septic mitochondria to prolonged severe hypoxia in vitro. After exposure of mitochondria to a gently stirred hypoxic buffer, state 3 respiration at any Po2 (high or low) remained stable for control mitochondria and declined slightly for septic mitochondria over 1 h. The maximal rate of respiration also decreased substantially by 90 min. Transport of ADP into mitochondria during state 3 respiration may require normal function.

Table 1. Respiration of mitochondria after isolation from control and septic rats

<table>
<thead>
<tr>
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<th>Control (n = 3)</th>
<th>Sepsis (n = 3)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 3</td>
<td>78 ± 10</td>
<td>108 ± 6</td>
<td>0.01</td>
</tr>
<tr>
<td>State 4</td>
<td>16 ± 1</td>
<td>16 ± 1</td>
<td>NS</td>
</tr>
<tr>
<td>RCR</td>
<td>5.0 ± 0.9</td>
<td>6.6 ± 0.5</td>
<td>0.06</td>
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Values are means ± SD in nmol·min⁻¹·mg protein⁻¹; n, no. of samples. RCR, respiratory control ratio; NS, not significant. State 3 and state 4 malate + glutamate-dependent respiration were measured with fresh mitochondria in buffer equilibrated with room air (duplicate measurements).
of the outer mitochondrial membrane (24), and this membrane may sustain mechanical, proteolytic, or oxidative damage during the hypoxic incubation. Although prolonged incubation of mitochondria in hypoxic buffer has important limitations, including damage to the organelles, the respiratory characteristics of mitochondria incubated in Sims respiration buffer remained quite stable for at least 1 h.

We designed our experiments with careful attention to preventing artifactual changes in the measured respiratory rate. We controlled for the effects of hypoxic incubation on respiratory coupling and minimized the inadvertent introduction of oxygen during the addition of ADP and substrates. In the respirometers, we recorded the $P_O_2$ of the hypoxic aliquots of mitochondria and continuously recorded $P_O_2$ during and after the addition of oxygen and deoxygenated substrates. Steady-state conditions were confirmed before oxygen consumption was determined. These precautions should have allowed us to detect the development of $P_O_2$-dependent mitochondrial respiration over time given the small standard error (~3%) of these measurements.

Reversible oxygen conformance in cells has been defined as a temporary decrease in oxygen consumption at oxygen tensions above the concentration that limits mitochondrial consumption of oxygen. Several variables could contribute to such a phenomenon, including intracellular diffusion gradients for oxygen (17, 19). Both heterogeneous metabolism and mitochondrial clustering may contribute to an apparent $P_{SO_2}$ of isolated hepatocytes for oxygen of ~3–6 Torr, which is substantially higher than the Michaelis-Menton constant ($K_m$) of isolated mitochondria of ~1 Torr (6, 16).

Although it is logical that some cellular oxygen gradient would occur in intact cells, not all investigators agree that large gradients in oxygen tension exist from cytosol to mitochondria (11). Potential experimental problems and theoretical considerations have been used to dispute the existence of large intracellular gradients. In intact liver, the apparent $K_m$ for oxygen may be significantly higher than that in isolated mitochondria, in part because concentration-activity profiles of oxygen-dependent enzymes in periportal (high oxygen tension) or pericentral (low oxygen tension) areas contribute to the observed oxygen dependence (27). Additionally, nonmitochondrial oxidases in the liver contribute to the total cellular oxygen consumption (21). These enzymes may have a $K_m$ for oxygen as
high as 75 Torr and could be inhibited by modest decreases in tissue P O₂ (17, 27).

Our studies do not support the concept that oxygen consumption of liver mitochondria contributes to a significant decrease in oxygen consumption by hepatocytes incubated at low (~20 Torr) oxygen tension. Although our experimental design differed from that of Chandel et al. (8), our end points were the same. Hence the reasons for our conflicting results remain unclear. Other mechanisms for downregulation of cellular oxygen consumption during hypoxia in mammalian cells have been described, including regulation of mitochondrial electron transport by nitric oxide. The inhibition of respiration by NO may occur at cytochrome-c oxidase, and the degree of inhibition may be inversely related to oxygen tension (2, 3, 12). This relationship could regulate mitochondrial respiration at oxygen tensions substantially higher than the mitochondrial Kₘ for oxygen. Our study, however, has not explored such mechanisms in this animal model of sepsis.

Other potential mechanisms to downregulate oxygen metabolism also exist in the cell. Hepatocytes of anoxia-tolerant aquatic turtles balance suppression of ATP consumption and production and maintain high-energy phosphate levels by downregulating energy demands via channel arrest, inhibition of protein translation, and extensively buffered anaerobic glycolysis (4, 20). These responses have been proposed to be under the control of a heme sensor activated by decreases in oxygen tension (14). Such responses have not been described for rat hepatocytes, which are relatively intolerant of anoxia and become more susceptible to injury (e.g., due to oxidative stress) during hypoxia (18).

In summary, isolated rat liver mitochondria exhibit more robust respiration after sepsis induced by cecal ligation and puncture compared with control mitochondria. During prolonged in vitro incubation under hypoxic conditions, respiration of mitochondria from septic rats deteriorates more quickly compared with control mitochondria. Mild dependence of mitochondrial respiration on oxygen tension (~10%) was apparent almost immediately and was very modest with succinate as the substrate; the observed P O₂ dependence was slightly greater (~20%) when ascorbate–TMPD was used as the substrate. Mitochondria from control and septic rats did not show increased oxygen dependence of respiration between 10 and 95 Torr over the course of 60-min exposures to hypoxia. Alterations in mitochondrial respiration at very low oxygen tensions (~<5 Torr) as a result of exposure to hypoxia have not been excluded as a cause for P O₂-dependent behavior of oxygen consumption in sepsis.

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