Defective oxidative metabolism of rat liver mitochondria in hemorrhagic and endotoxin shock

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The work to be presented in this paper was designed to study the effect of hemorrhagic and endotoxin shock on mitochondrial metabolism.

Defective oxidative metabolism of rat liver mitochondria in hemorrhagic and endotoxin shock. Am. J. Physiol. 220(2): 571-577. 1971.—Rat liver mitochondrial functions were studied after hemorrhagic hypovolemia and Escherichia coli endotoxin shock. The respiratory activity in State 4 was increased indicating normal utilization of substrates and oxygen, but loose coupling. The electron-transfer reactions induced by substrates were unaltered. The respiratory rates in State 3, however, were inhibited in the presence of any of the substrates used, succinate, glutamate-malate, or a-ketoglutarate. Due to increased State 4 and decreased State 3 rates, the respiratory control ratios dropped from control values of 6-8 to about 2. The ADP utilization rate decreased parallel with the respiratory rates. Also, an addition of ADP to mitochondria prepared at final stages of shock failed to induce steady-state changes of the cytochromes. The mitochondrial uncoupler-sensitive ATPase activity was inhibited as well by 70%. All these alterations occurred both in hemorrhagic and endotoxin shock. The only significant difference was the dependence of the alterations on the length of the shock. The data suggest that the mitochondrial defect caused by shock is in the adenine nucleotide translocase and/or utilization mechanism in connection with the fault in ATPase activity.

mitochondrial oxidative phosphorylation; electron transfer; respiratory enzymes; mitochondrial ATPase; E. coli endotoxin; subcellular response in shock

Elevations of the concentration of serum lactate, the end product of anaerobic metabolism in septic shock (14, 17), suggest that glucose metabolism passes freely through the glycolytic Embden-Meyerhof pathway to lactate, but that the end product of glycolysis is not effectively removed or utilized in the cell. If the latter is the case, the fault along the metabolic pathway could be on the oxygen side of the pyruvate-lactate pair, and thus in the oxidative metabolism, which takes place in mitochondria. The same conclusion could be reached on the basis of decreased total oxygen consumption (4, 14) and low tissue adenosine triphosphate (ATP) levels (18) in septic shock. Our earlier work on the inhibition of mitochondrial functions due to the binding of Escherichia coli endotoxin to normal intact mitochondria in vitro suggests that the oxidative metabolism could be defective in septic shock (9, 10). The work to be presented in this paper was designed to study the effect of hemorrhagic and endotoxin shock on mitochondrial metabolism.

The alterations common to both types of shock were found to be: 1) inhibition of mitochondrial adenosine triphosphatase (ATPase) activity; 2) uncoupling of respiration; 3) inhibition of adenosine diphosphate (ADP)-activated respiration in the presence of succinate or pyridine nucleotide-linked substrates, glutamate-malate or a-ketoglutarate; and 4) inhibition of ADP utilization.

MATERIALS AND METHODS

In all experiments male Sprague-Dawley rats of 180- to 290-g weight were used.

Hemorrhagic shock was induced in rats, anesthetized with pentobarbital (Nembutal), by a modified Wiggers procedure, as reported elsewhere (1). The animals were bled through a cannula in the femoral artery into a reservoir, until the blood pressure reached 30 mm Hg. The second cannula in the femoral arteries was connected via a strain-gauge transducer to a Beckman type R Dynograph for continuous monitoring of blood pressure and heart rate. The pressure was kept constant throughout the experiment by controlling the amount of blood in the reservoir. The rectal temperature was also recorded.

Endotoxin shock was induced in anesthetized rats by an intraperitoneal injection of E. coli lipopolysaccharide B (Difco Laboratories). An LD50 dose, 4-5 mg of endotoxin per 100 g body wt, was used. In parallel experiments on anesthetized and cannulated animals, the pathophysiologic pattern of endotoxin shock in rats was studied by recording blood pressure, heart rate, and rectal temperature as described above in connection with hemorrhagic shock.

Mitochondrial preparations. Livers of the rats were removed from 30 min to 6 hr after the onset of either hemorrhagic or endotoxin shock, and placed in ice cold mannitol-sucrose solution (0.225 M mannitol, 0.075 M sucrose) for use in mitochondrial preparation. The mitochondria were isolated immediately, in the presence of 100 µM ethylenediaminetetraacetate (EDTA), by a standard technique (3). The two final washings of the mitochondrial pellet were performed in the absence of EDTA, since EDTA was not always desirable in the mitochondrial suspensions used for the assays of the respiratory activity. The isolated mitochondria were used within 6 hr after isolation. All the assays were performed at 25 C.

Assays of the respiratory activity. Mitochondrial respiratory activity was assayed in a Clark oxygen electrode in a
closed, stirred cuvette, in air-saturated medium, using either succinate as a substrate in a rotenone-blocked system, or the pyridine nucleotide-linked substrates, glutamate-malate, or α-ketoglutarate. Each substrate was added at a final concentration of 8 to 10 mM. The reaction medium consisted of 0.225 M mannitol, 0.075 M sucrose, and 20 mM Tris Cl buffer, pH 7.4 (MST Cl), or 10 mM Tris Cl plus 10 mM Tri-phosphate buffer, pH 7.4 (MST Pi). State 4 (in the presence of added substrate) and State 3 (substrate + ADP) rates were computed, as well as the respiratory control ratios (RCR).

Mitochondrial electron transfer. The electron-transfer reactions were studied by measuring the redox changes of the respiratory chain components in an Aminco Chance dual-wavelength spectrophotometer at appropriate wavelengths: cytochrome b at 430-410 nm or 560-575 nm, cytochrome c at 550-540 nm, cytochrome c1 at 555-540 nm, cytochrome a + a3 at 445-455 nm, and flavoproteins at 465-510 nm, according to the method of Chance (2). During these experiments the swelling of the mitochondria was checked by calibrating the optical density of the samples before and after each experiment. The rates of ADP utilization were calculated from cytochrome b measurements.

Both MST-Cl and MST-Pi were used as reaction media in the optical measurements.

Adenosine triphosphatase activity. The mitochondrial uncoupler-activated, oligomycin sensitive ATPase activity was measured with a recording pH meter at high sensitivity equipped with a 5-mv potentiometric recorder, according to the method of Nishimura and Chance (13), with MST-Cl as a reaction medium. The endogenous respiration was inhibited by rotenone.

Succinate- and DPNH-cytochrome c reductase activities. The succinate-cytochrome c reductase activity was measured in the presence of 1 mM KCN and 1 μM added cytochrome c (Sigma, type III) in sonicated liver mitochondrial particles according to the method of King (8) and the DPNH-cytochrome c reductase, similarly in sonicated mitochondria, in the presence of 2 mM azide and 7 μM added cytochrome c, according to Hafci and Rieske (5). Both measurements were done in a dual-wavelength spectrophotometer at 550-540 nm.

Magnesium ion concentrations. The concentrations of endogenous Mg++ ions were determined in the atomic absorption spectrophotometer using known standards for Mg++ ions.

Protein concentrations were determined by the Biuret method.

RESULTS

Uncoupling effect of shock. The respiratory activity of the mitochondria was studied, using succinate or pyridine nucleotide linked substrates as electron donors, in the presence and absence of ADP. With increasing time, after the onset of hypovolemia or the administration of endotoxin, the oxygen-uptake rates during State 4 respiration (excess substrate, no added ADP) increased, an indication of poorly controlled respiration or uncoupling of respiration from phosphorylation. Parallel with the increase in State 4 rates there was a decrease in State 3 rates (substrate + ADP). These two factors depress the respiratory control ratios (ratio of State 3 rate to State 4 rate). Comparison of the respiratory control ratios after endotoxin and hemorrhagic shock, using succinate or glutamate-malate as substrates, is shown in Fig. 1. The respiratory control ratios used as controls (at zero time) are slightly different in
endotoxin and hemorrhagic shock due to the small depression caused by Nembutal anesthesia, which was used only in hemorrhagic shock experiments. Initially, the respiratory control ratios remain constant and even increase slightly. A decline is apparent after 1 hr of hemorrhagic shock and after 2 hr of endotoxin shock; thereafter, the fall, in both types, is linear with time. Particularly in endotoxin shock is the decline very abrupt: about 50% decrease within 60 min. By 5–6 hr, the ratios have fallen to about the same final level in both types of shock, utilizing succinate, glutamate-malate, or α-ketoglutarate as substrates. The final point, with succinate as substrate in endotoxin shock, is 27% of control; in hemorrhagic shock it is 33%. With glutamate malate as substrates, the final points are 33 and 52% of control, respectively. These data indicate that the mitochondria isolated from rat livers after shock, either endotoxin or hemorrhagic, are heavily uncoupled.

**Inhibition of State 3 respiration.** The drop in respiratory control ratios is not completely attributable to the uncoupling effect of shock. Inhibition of respiratory activity, per se, is also a contributing factor. The inhibition cannot be detected during State 4 respiration. However, State 3 rates, which under normal conditions are nearly maximal even in the absence of uncoupling, show the inhibition clearly. Table 1 presents the data. The endotoxin-treated rats in group I show a decrease in respiratory rates, calculated as nanomoles of oxygen per minute per milligram of mitochondrial protein, of about 30–50%, 200 min after endotoxin administration. In hemorrhagic shock, the drop is about 40–50% with all substrates except α-ketoglutarate. In the absence of Mg++ the latter falls to 35% of control values, a value reached in endotoxin shock at about 300 min.

**Effect of shock on α-ketoglutarate oxidation.** Figure 2 shows an oxygen-electrode recording of the respiratory activity, with α-ketoglutarate as substrate, in a preparation made at the 40% blood-uptake point, 4 hr after hemorrhage. The trace on the left shows a declining rate of respiration about 0.5 min after the addition of α-ketoglutarate. Adenosine diphosphate does not stimulate the respiration. This experiment gives a value of 1 for the respiratory control ratio and indicates a strongly inhibited rate of respiration. The trace on the right is a similar experiment in the presence of added Mg++ ions. In this case the respiration proceeds

**TABLE 1. RLM\textsubscript{w}, State 3 rates**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Succinate, anoles</th>
<th>Glutamate + Malate, anoles</th>
<th>α-Ketoglutarate, anoles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O\textsubscript{2} min\textsuperscript{−1} mg\textsuperscript{−1}</td>
<td>O\textsubscript{2} min\textsuperscript{−1} mg\textsuperscript{−1}</td>
<td>O\textsubscript{2} min\textsuperscript{−1} mg\textsuperscript{−1}</td>
</tr>
<tr>
<td>Fasted, normal</td>
<td>46 + Mg\textsuperscript{++}</td>
<td>36 + Mg\textsuperscript{++}</td>
<td>32 + Mg\textsuperscript{++}</td>
</tr>
<tr>
<td>Fasted + endotoxin</td>
<td>27 + Mg\textsuperscript{++}</td>
<td>25 + Mg\textsuperscript{++}</td>
<td>19 + Mg\textsuperscript{++}</td>
</tr>
<tr>
<td>200 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasted, nembutal</td>
<td>46 + Mg\textsuperscript{++}</td>
<td>34 + Mg\textsuperscript{++}</td>
<td>32 + Mg\textsuperscript{++}</td>
</tr>
<tr>
<td>Fasted + hemorrhage</td>
<td>24 + Mg\textsuperscript{++}</td>
<td>21 + Mg\textsuperscript{++}</td>
<td>15 + Mg\textsuperscript{++}</td>
</tr>
</tbody>
</table>

Oxygen uptake rates measured from rat liver mitochondria (RLM\textsubscript{w}) isolated from control rats or from rats 200 min after hemorrhage or endotoxin injection. RLM\textsubscript{w} were suspended in MST-P\textsubscript{1} medium at 1 mg protein/ml in the presence of 8 mM substrates and 280–530 μM ADP, in the absence and presence of 1.3 mM MgCl\textsubscript{2}.

**FIG. 2.** Oxygen-electrode recording showing O\textsubscript{2} uptake in rat liver mitochondria after 4–5 hr of hemorrhagic hypovolemia. Mitochondria were suspended at 2.7 mg protein/ml in MST-P\textsubscript{1} medium. Trace on left shows respiratory activity after an addition of 8 mM α-ketoglutarate (α-kg) and 530 μM ADP. Trace on right is a similar experiment in the presence of 1.3 mM added MgCl\textsubscript{2}.
with the initial rate, and ADP stimulates the respiration about 3 times, giving a respiratory control ratio of 3.

Inhibition of State 3 rates, at various stages of hemorrhagic shock with α-ketoglutarate as substrate, is shown in Fig. 3. In the absence of added Mg++ ions (closed circles) there is a linear drop with time from 17 to 2.2 nmol oxygen/min/merg of mitochondrial protein, an inhibition of 87%.

Addition of Mg++ (open circles) to the mitochondrial suspensions from animals at the later stages of shock increase the respiratory rates considerably; the final point of inhibition in the presence of added Mg++ is only 50%. Thus, the inhibition of α-ketoglutarate oxidation is of the same magnitude as the inhibition of glutamate-malate oxidation. The fact that the inhibition is greater in the absence of added Mg++ suggests that the mitochondria are lacking endogenous Mg++ under these conditions. This was also shown by the determinations of the Mg++ content of the mitochondria by atomic-absorption techniques. In isolated control mitochondria, the average endogenous Mg++ content was 30 nmol Mg++/mg of mitochondrial protein. In hemorrhagic shock preparations, with respiratory control ratios of about 2 with α-ketoglutarate as substrate, the Mg++ content dropped to about 18 nmol Mg++/mg of protein, which is a decrease of about 40%. Similar results were obtained in endotoxin shock experiments. The α-ketoglutarate dehydrogenase enzyme complex is Mg++ dependent. In the absence of Mg++, the oxidation of α-ketoglutarate does not occur in mitochondria.

Adenosine diphosphate response. The mitochondrial electron-transfer reaction was followed by measuring the response of the individual cytochromes to additions of substrate and ADP. In Fig. 4 the response of cytochrome b is shown. The top trace is a control experiment done with normal, intact rat liver mitochondria. Rotenone was used to block respiration by endogenous pyridine nucleotide-linked substrates. The addition of rotenone causes oxidation of cytochrome b. The subsequent addition of succinate initiates the electron transfer from succinate to oxygen, and causes reduction of cytochrome b, which is indicated by the downward deflection of the top trace. Addition of 330 µM ADP after succinate causes a change in the steady-state level of cytochrome b to a more oxidized state. When the added ADP has all been utilized, cytochrome b returns back to its original reduced state. The cyclic response to ADP can be repeated as long as there is oxygen available.

The bottom trace of Fig. 4 shows the same sequence of responses in mitochondria after endotoxin shock. The succinate response is similar to the control, which indicates that the electron transfer induced by succinate proceeds normally. However, the addition of ADP does not under these conditions induce any steady state change of cytochrome b. At earlier stages of both endotoxin and hemorrhagic shock the ADP response can still be recorded. However, it is smaller than the response in control mitochondria. Also, the ADP cycle time increases towards later stages of shock.

From the length of the ADP cycle on cytochrome b, the time for the utilization of the added ADP can be measured and the rate of ADP utilization calculated as nanomoles of ADP utilized per minute per milligram of mitochondrial protein. In Fig. 5 the ADP utilization rates at various stages of endotoxin shock are correlated to the respiratory control ratios of the same preparations. Also the oxygen-uptake rates of these preparations are plotted on the same graph. The top trace of Fig. 5 shows the State 3 oxygen-uptake rates; the bottom trace shows the difference between the State 3 and State 4 rates, that is, the increase in the oxygen uptake due to activation of

![Fig. 3. Effect of hemorrhagic shock on α-ketoglutarate oxidation. Experiments similar to the one described in Fig. 2 were done on mitochondria isolated at various times after hemorrhage (time in minutes) and respiratory rates in State 3 in presence of 0 mM α-ketoglutarate and 330 µM ADP are plotted against time. Straight line (closed circles) indicates results in absence of added Mg++ in reaction mixture; graph with open circles indicates results in presence of 1.3 mM MgCl₂.](http://ajplegacy.physiology.org/)

![Fig. 4. Effect of endotoxin shock on mitochondrial electron transfer. Cytochrome b was measured at 430–410 nm. Upward deflection of traces indicates oxidation of cytochrome b. Top trace is a control experiment in normal mitochondria, the lower trace in mitochondria isolated 4.5 hr after endotoxin injection. All additions are indicated on diagram. Rat liver mitochondria were suspended at 2.7 mg protein/ml in MST-P₁ medium in presence of 1.7 mM MgCl₂ in both experiments.](http://ajplegacy.physiology.org/)
Phosphorylation. The middle trace is the measure of the ADP utilization rate. Besides the fact that there is a decline in all these three parameters with declining respiratory control, all three traces show an initial increase with decreasing respiratory control. This indicates that in shock the first drop in respiratory control, which in the case of succinate is from 6 or 5 to 4, is due to increased State 4 respiration and not due to decreased State 3 respiration or ADP utilization. Thus, in the beginning stages of shock, the respiratory activity in both State 4 and State 3 increases as well as ADP utilization. Only after this initial rise, which occurs within the first 2 hr after endotoxin injection, ADP utilization and oxygen uptake drop linearly with time and decreasing respiratory control. At the point where the respiratory control ratio is 1, ADP is not utilized (middle trace) and does not stimulate respiration (bottom trace, Fig. 5). However, the mitochondria still respire with the State 4 rate as shown by the plateau in the top trace at 50 natsoms oxygen/min per mg of mitochondrial protein.

Inhibition of mitochondrial ATPase activity. In the experiments of Fig. 6 the uncoupler-sensitive ATPase activity was measured by measuring the rate of hydrogen ion production due to ATP hydrolysis. The reaction was initiated in a mitochondrial suspension after an addition of 1.9 mM ATP by varying concentrations of uncoupling agent carbonyl cyanide p-trifluoro-methoxyphenylhydrazone (FCCP). The maximum activity in each preparation was used for the graph shown. The concentration of FCCP needed for maximal activity varied from 0.1 to 0.4 \( \mu \text{m} \).

Figure 6 shows the ATPase activity as \( \Delta [\text{H}^+] \) in nanomoles of \( \text{H}^+ \) per minute per milligram of mitochondrial protein against time of the preparation in minutes after the onset of bleeding or the endotoxin injection. The graphs show that the ATPase activity drops at 4 to 5 hr to about 30% of the control values found in normal mitochondria. The extent of the fall in activity is similar in endotoxin and hemorrhagic shock. Inhibition of ATPase activity in endotoxin shock lags about 1 hr behind the chronologic pattern seen in hemorrhagic shock. This time difference is probably due to the time needed for absorption of the endotoxin from the intraperitoneal space at high enough concentration to cause metabolic changes in the liver.

Mitochondrial ATPase is Mg\(^{2+}\) dependent. However, addition of Mg\(^{2+}\) to the mitochondrial suspensions during the experiments did not improve the ATPase activity after shock.

The assays of ATPase activity were also done in broken mitochondria thus eliminating the permeability barrier to adenine nucleotides. The inhibition of ATPase activity measured under these conditions was the same as in intact mitochondria.

Succinate- and DPNH-cytochrome c reductase activity in shock. Both succinate- and DPNH-cytochrome c reductase activities were measured in sonicated mitochondrial membranes in 0.1 \( \text{m} \) phosphate buffer, as described above. Both activities were found to be unaltered in shock, thus indicating intactness of the electron transfer from succinate or pyridine nucleotides to cytochrome c.

DISCUSSION

Earlier reports (6, 12, 15, 16) have appeared suggesting alterations of mitochondrial functions in the shock state.
It has been suggested that liver homogenates from rats after *E. coli* endotoxin injection consume more oxygen than controls (12). Recently it was shown that after late hemorrhagic shock the respiratory control ratios and ADP/oxygen ratios in rat livers were depressed, if α-ketoglutarate was used as substrate (15), while the respiratory activity with other substrates, such as succinate, stayed unaltered. Other workers, however, found inhibition of succinate oxidation together with uncoupling (16). Also, reports have appeared suggesting unaltered mitochondrial function in shock (6).

The present data provide evidence for profound alterations of mitochondrial energy-linked functions, both in hemorrhagic and endotoxin shock. Increase in mitochondrial respiratory rates during State 4 indicates uncoupling of mitochondrial respiration. This occurs with all substrates, succinate as well as pyridine nucleotide-linked substrates. Thus, the uncoupling induced by shock is general, involving all three phosphorylation sites. The uncoupling phenomenon appears equal in endotoxin and hemorrhagic shock.

Inhibition of State 3 rates with all studied substrates, succinate, glutamate-oxalacetate, and α-ketoglutarate, was found both in endotoxin and hemorrhagic shock. Since the State 4 rates are increased concomitantly, the electron-transfer reaction, induced by addition of substrates, proceeds with normal speed, and the *Km* for substrates is not altered, the inhibition of State 3 cannot be due to decreased substrate permeability or utilization. On the basis of our data, it is obvious that the reason for the inhibition at State 3 has to do with adenine nucleotide permeability and/or utilization. The decline in ADP utilization rate in shock together with the lack of ADP-stimulated cytochrome response and respiration, strongly supports this conclusion. Thus the adenine nucleotide translocase mechanism could be defective.

Another reason for the decline in ADP utilization could be the profound inhibition of ATPase activity. The increase in ATP/ADP ratio in mitochondria could cause a product inhibition of ATP formation and thus an inhibition of ADP utilization. The cause might also be decreased adenine nucleotide permeability which then would result in ATPase inhibition. This, however, is unlikely, because ATPase was inhibited in broken mitochondria as well, where the permeability barrier had been eliminated. Of course, the two factors, the inhibition of ATPase activity and the inhibition of adenine nucleotide utilization and/or translocation, can occur in parallel.

Based on the experiments with α-ketoglutarate oxidation, and the measurements of Mg**++** content of the mitochondria, we can conclude that the mitochondria in shock lose the ability to keep their bound Mg**++**. The Mg**++** deficiency, solely, causes a significant inhibition of α-ketoglutarate oxidation. The function of the enzyme, the α-ketoglutarate dehydrogenase complex, per se, is clearly intact. The fact that mitochondria in shock lose their bound Mg**++** is a sign of impaired membrane structure reflected in morphologic swelling of mitochondria in situ. We have verified this complementary finding with electron micrographs of liver tissue from animals subjected to the same shock preparations (R. R. White, unpublished observations).

Almost identical alterations were found both in endotoxin and hemorrhagic shock. The only significant difference between the two types is the time dependence of the changes. During the 1st hr of the endotoxin experiment, the mitochondria are tightly coupled, and the respiratory and phosphorylative activities increase. This initial rise in mitochondrial activity is a characteristic initial response to endotoxin administration. After this lag period with increased activity, the decline occurs very rapidly; the main portion of impairment occurs about 2-3 times as fast as in hemorrhagic cases.

What causes the alterations in mitochondrial function in shock? Since the alterations are so similar in hemorrhagic and endotoxin shock, one might postulate a common etiology. This, of course, could be anoxia. The fundamental mechanism by which anoxia could cause these changes, is not clear. Considering the whole cell, one contributing possibility could be increased lysosomal activity (7, 19). The released lysosomal enzymes might destroy the structure of the mitochondrial membrane and, thereby, its function (11).

If there is any direct effect of endotoxin on the mitochondrial functions in the cell in vivo, is not evident on the basis of the present data. Our earlier work on the direct effects of endotoxin on mitochondrial functions in vitro indicate close similarities to the changes in shock. However, there is one important difference: the inhibition of succinate oxidation per se seen in vitro (9) was not seen in shock. This suggests that in vivo we might not be dealing with effects of endotoxin directly on the mitochondrial membranes. It is also questionable if the cell membrane would be permeable to the whole endotoxin molecule. This argument is based on our experiments on liver slices in vitro where the endotoxin had no effect on the cellular respiration. Whatever the underlying mechanism, it is clear that the alterations in mitochondrial energy-linked functions, to the extent reported here, necessarily are connected to a disturbance in the integrity of the mitochondrial membrane.

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