Mitochondrial Function of Rat Liver in Biliary Obstruction

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KOYAMA, K., ITO, K., OUCHI, K. and SATO, T. Mitochondrial Function of Rat Liver in Biliary Obstruction. Tohoku J. exp. Med., 1980, 131 (1), 59-69 — An investigation was performed of the liver mitochondrial respiratory function in rats after 1, 3 and 6 weeks of biliary obstruction and the following results were obtained: 1) Various parameters of liver mitochondrial respiratory function, such as respiratory control ratio, ADP/0₂ consumption in state 3 respiration and adenosine triphosphate synthesis were found to decrease with prolongation of biliary obstruction. 2) The mitochondrial respiratory enzymes, cytochrome a (+a₃) and cytochrome c(+c₁) showed that both decreased in contents with prolongation of obstruction, particularly the latter. 3) The activation ratio of ATPase (latent ATPase/dinitrophenol stimulated ATPase) was increased after long term biliary obstruction, which was thought to indicate the severe damage of mitochondrial membrane. 4) Investigation of the respiratory function with the various respiratory substrates showed that the locations of mitochondrial respiratory inhibition in obstructive jaundice are at sites 1 and 2, which is the same as the situation seen in nonspecific damage of mitochondria. 5) There was a high degree of mitochondrial respiratory disturbance by bile acids, particularly CDCA, which is thought to be one of the causal factors of liver dysfunction in obstructive jaundice. 6) Mitochondrial respiratory function was markedly disturbed in hypotension, and the degree of which correlated with length of time of biliary obstruction. — liver mitochondria; obstructive jaundice; cytochrome; bile acids; ATP

In patients with obstructive jaundice due to malignant tumors of the pancreato-biliary tract, the high incidences of operative death and complications caused by various liver disorders are observed occasionally. We have studied on the pathophysiology of these liver damages from various view points (Koyama et al. 1975a, b, 1979; Yamauchi et al. 1976). In the present study the respiratory functions of mitochondria in the liver of rats with obstructive jaundice will be described.

MATERIALS AND METHODS

Preparation of the experimental animals

The common bili duct of male Wistar rats weighing approximately 200 g was ligated and cut off to produce obstructive jaundice and the animals were used for experiments.

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The 5th report of a series of studies on obstructive jaundice.
1, 3 or 6 weeks later. Following exanguination by cardiac puncture under ether anesthesia, the liver was excised and 10% homogenate was prepared with cold 0.25 M sucrose (pH 7.4). Mitochondrial fraction was separated following Hogeboom and Schneider’s method (Hogeboom and Schneider 1948) and was resuspended at a protein concentration of 20 mg/ml.

Measurement of mitochondrial respiratory function

Mitochondrial respiratory function was measured polarographically with the oxygen consumption meter (Yanaco, Japan) adding approximately 200 μl of mitochondrial suspension to the following reaction mixture; 0.125 M sucrose, 0.3 M KCl, 0.1 M MgCl₂, 0.05 M EDTA 2Na, 0.05 M Tris-HCl (pH 7.4), and 0.3 M phosphate buffer (Hagihara 1961). The respiratory substrate was primarily succinate (5~10 μmoles), but α-ketoglutarate or ascorbate+tetramethyl phenylene diamine (TMPD) were also used for comparison. In each sample, 250~300 nmoles of adenosine diphosphate (ADP) were added to gain oxidative phosphorylation. In this way, the oxygen consumption curve was recorded from State 1 to State 5 and respiratory control ratio (RC), oxygen consumption in each state, ADP/O ratio (P/O) and adenosine triphosphate (ATP) synthesis were calculated following the method of Chance and Williams (1956). After completion of the reaction, the protein content of the mitochondrial suspension in the reaction cells was determined using Lowry’s method (Lowry et al. 1951).

Measurement of the content of the respiratory enzymes

The amounts of mitochondrial cytochrome a (cyt a) and cyt c were measured by the difference spectrum method (Chance and Williams 1955) using a split beam spectrophotometer (Hitachi Model 124). Content of cyt a (+a₃) was calculated by the following formulas and corrected by the mitochondrial protein concentration.

\[
\text{cyt a} \ (\pm a_3) = \frac{\Delta a_1 - \Delta a_2}{16.5} \text{ (mmoles/ml)}
\]

\[
\text{cyt c} \ (\pm c_1) = \frac{\Delta c_1 - \Delta c_2}{19.0} \text{ (mmoles/ml)}
\]

where \(\Delta a_1, \Delta a_2\) are the differences of optical density at 605 nm and 630 nm respectively, while \(\Delta c_1, \Delta c_2\) are the differences at 550 nm and 500 nm respectively. The constants, 16.5 and 19.0, are the extinction coefficients of cyt a (+a₃) and cyt c(+c₁), respectively.

Assay of ATPase activity

Following addition of 20 μl of mitochondrial suspension to 1 ml of reaction mixture comprised of 0.25 M sucrose, 0.01 M KCl, 0.2 mM EDTA, 0.01 M Tris-HCl (pH 7.4) and 3 mM ATP at 25°C, the reaction was brought to a halt after 10 min with 8% perchloric acid (PCA) and the inorganic phosphorus was measured. The latent ATPase activity was defined as the amount of increase of inorganic phosphorus per min per mg mitochondrial protein during the reaction. The dinitrophenol (DNP) stimulated ATPase was measured by addition of 2×10⁻⁵ M DNP to the above-mentioned reaction solution (Cooper and Lehninger 1957). The ratio of the activity of latent to DNP stimulated ATPase was calculated.

Investigation of the inhibition of bile acids on mitochondrial respiration

In order to clarify the mechanism of the disturbance in mitochondrial respiration by bile acids, the respiratory function was measured in control rat liver mitochondria to which cholic acid (CA), glyco-CA, tauro-CA, chenodeoxycholic acid (CDCA), glyco-CDCA or tauro-CDCA (Sigma Co., over 98% purity) was added. That is, by adding each of bile acids in various concentrations to the reaction cells and preparing the final concentration in the cells to 5~2000 μM, mitochondrial respiratory function was measured as described above.
Investigation of the influences of exanguination and hypotension on mitochondrial respiration

Twenty ml/kg of blood were withdrawn from the carotid artery in both control and biliary obstruction rats and after a blood pressure of 50~60 mmHg had been maintained for 2 hr, the mitochondrial respiratory function of the liver was investigated as described above.

RESULTS

Mitochondrial respiratory function of the liver in obstructive jaundice

The parameters of mitochondrial respiratory function are shown in Table 1. In Fig. 1, the values have been expressed in terms of the percent of the values obtained in normal animals. These results are outlined below.

Respiratory control ratio (RC)

In comparison with the control group, the group with biliary obstruction showed significant decreases of RC ($p<0.005$) with prolonged obstruction (50% of control values after 3 weeks and 40% after 6 weeks).

Oxygen consumption in State 3 ($S_3$)

Significant decreases ($p<0.005$) were found in State 3 oxygen consumption in rats after 3 and 6 weeks of obstruction in comparison with control value.

P/O ratio

In contrast to the control animals, P/O ratio of the group with biliary obstruction showed significant decreases ($p<0.005$).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (9)</th>
<th>1 week (7)</th>
<th>3 weeks (9)</th>
<th>6 weeks (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC</td>
<td>4.4±0.5</td>
<td>3.0±0.4$t$</td>
<td>2.1±0.6$t$</td>
<td>1.6±0.3$t$</td>
</tr>
<tr>
<td>$S_3$ (nAtoms/mg protein/min)</td>
<td>117.2±18.2</td>
<td>102.8±23.2</td>
<td>64.2±26.9$t$</td>
<td>50.3±16.4$t$</td>
</tr>
<tr>
<td>P/O</td>
<td>2.2±0.2</td>
<td>1.7±0.3$t$</td>
<td>1.4±0.3$t$</td>
<td>1.1±0.3$t$</td>
</tr>
<tr>
<td>ATP synthesis (nmole/mg protein/min)</td>
<td>220.3±51.0</td>
<td>177.3±63.0</td>
<td>89.7±41.7$t$</td>
<td>58.0±24.0$t$</td>
</tr>
<tr>
<td>$\text{TN}_p^a$ (^-sec)</td>
<td>76.1±31.8</td>
<td>97.3±38.9</td>
<td>43.7±9.9$t$</td>
<td>33.2±9.9*$</td>
</tr>
<tr>
<td>cyt a(+a$_4$)</td>
<td>17.0±1.5</td>
<td>14.0±3.1</td>
<td>12.8±2.8$t$</td>
<td>13.3±1.4$t$</td>
</tr>
<tr>
<td>(1×10$^{-2}$ nmole/mg protein)</td>
<td>28.2±3.5</td>
<td>20.2±3.6$t$</td>
<td>15.4±3.0$t$</td>
<td>14.0±2.8$t$</td>
</tr>
</tbody>
</table>

Values are mean±s.d. Figures in parentheses show the number of experimental animals. Significant differences from the control value are indicated with $^*$ ($p<0.01$) or $^t$ ($p<0.005$). RC is the abbreviation of respiration control ratio and $S_3$ is that of oxygen consumption in state 3. ($\text{TN}_p^a$), the abbreviation of turnover number of phosphorylation with cyt a (+a$_4$), is calculated as $4\times$ATP synthesis (moles/mg/sec)/cyt a (+a$_4$) (moles/mg).
Fig. 1. Percent changes of various respiratory parameters of rat liver mitochondria in biliary obstruction. Means of 9 (control), 7 (1 week), 9 (3 weeks) and 6 (6 weeks) animals. o-o, RC; Δ-Δ, S2; x-x, P/O; o-•-•, ATP synth.; Δ-Δ, cyt c (+c1); x-•-•, cyt a (+a3); •-•-•, (TNp)a.

ATP synthesis

In ATP synthesis, comparing with the control, 60% reduction was observed after 3 weeks of obstruction and it was 73% after 6 weeks.

Respiratory enzyme content

Cyt a (+a3) values significantly decreased in animals with biliary obstruction after 3 weeks (76% of control values) and 6 weeks (78% of control values) (p <0.05). Cyt c(+c1) values decreased to 50% of control values after 6 weeks obstruction.

Turnover number of phosphorylation

Turnover number of phosphorylation, [(TNp)a] (Ozawa et al. 1972), calculated from the volume of ATP synthesis and cyt a (+a3) content, as described above, increased slightly after 1 week obstruction. After 3 and 6 weeks, however, the turnover number had decreased to 58% and 45% of control values, respectively.

Changes of the mitochondrial respiratory parameters in several different respiratory substrates

The mitochondrial respiratory function was also investigated for other substrates, α-ketoglutarate and ascorbate (+TMPD) in the same manner as for succinate. Taking the control values for each substrate as 100%, the mitochondrial respiratory parameters after 3 and 6 weeks of biliary obstruction were as shown in Fig. 2. α-Ketoglutarate as substrate showed similar tendencies to succinate with regard to RC and ATP production. In contrast, when ascorbate was used as a
substance, respiratory parameters of mitochondria showed almost no decrease; rather, slight increases were seen in the P/O ratio and ATP synthesis.

**ATPase activity of the mitochondria**

In Fig. 3, the values for latent ATPase, DNP stimulated ATPase of the mitochondria and their ratio are represented. DNP stimulated ATPase activity with obstruction for 3 weeks was similar to control values, but decreased to 50% after 6 weeks. There were significant increases \( p<0.005 \) in latent ATPase after 1 and 3 weeks obstruction, but values were reduced to control values after 6 weeks. The ratio of latent ATPase to DNP stimulated ATPase was significantly increased in animals with biliary obstruction.

**Effects of bile acids on mitochondrial respiration**

Taking the control values without addition of bile acids as 100%, the effects of various bile acids on mitochondrial respiratory parameters were shown in Fig. 4. The respiratory inhibitions by CDCA were very intense; even at a concentration of 5 \( \mu \text{M} \), RC values were found to be 80% of control; and at 200 \( \mu \text{M} \) no oxidative phosphorylation was found. Similar tendencies were seen with regard to \( S_3 \), P/O and ATP synthesis. The effects of glyco-CDCA and tauro-CDCA on respiratory
Fig. 3. ATPase activity of rat liver mitochondria in biliary obstruction. A (o-o), DNP stimulated ATPase; B (Δ-Δ), latent ATPase. Significant differences from the control value are indicated with * (p<0.01) or † (p<0.005). Figures in parentheses show the number of animals.

Fig. 4. Effects of various bile acids on mitochondrial respiratory parameters. Data show percentage of control values obtained without addition of bile acids. Each symbol shows a mean of three experiments.
+x-+, CA; Δ-Δ, glyco CA; △-△, tauro CA; v-v, CDCA; o-o, glyco CDCA; ●-●, tauro CDCA.
function were smaller than that of free CDCA. The effects of CA as well as conjugated CDCA on the other hand were considerably less severe than those of free CDCA. The effects of conjugated CA were weaker; even at a concentration of 1000 μM, RC, S₃ and ATP synthesis were maintained at 50% of control values, and oxidative phosphorylation was still apparent at 2000 μM. Almost identical mitochondrial respiratory disturbances were obtained between taurine conjugation and glycine conjugation.

**Effects of hypotension induced by exanguination on liver mitochondrial respiratory function**

Fig. 5 shows the effects of exanguination on mitochondrial respiratory parameters as compared to controls. In various periods of biliary obstruction, decreases in RC and P/O values due to hypotension were demonstrated even in the group without biliary obstruction. Marked decreases were evident after 3 and 6 weeks of biliary obstruction and one third of rats with 6 weeks obstruction died while undergoing exanguination.

![Graph showing effects of exanguination on mitochondrial respiratory parameters](image)

Fig. 5. Effects of exanguination on mitochondrial respiratory parameters in rat liver in biliary obstruction.
- •-○: Biliary obstruction only. Numbers of animals were as shown in Table 1.
- •-●: Biliary obstruction with exanguination. Four animals were used in each group.

Significant differences from the control value are indicated with * (p<0.01) or † (p< 0.005).
DISCUSSION

The prognosis of patients with obstructive jaundice due to tumors of pancreatobiliary tract is not so good, for which one of the reasons is thought to be the deterioration of liver functions (Maki et al. 1969). We have reported previously that the ability to metabolize short chain fatty acids (Koyama et al. 1975b) and ammonia (Koyama et al. 1979), which are causative factors of hepatic failure, decreases in the rat with obstructive jaundice. It is presumed that such a condition is fundamentally due to the severe disturbance of liver mitochondrial function. Ozawa et al. (1972) reported the changes of the various respiratory parameters of the liver mitochondria in the early stage of biliary obstruction in rabbits. They found that ATP production transiently increased in 3 hr of biliary obstruction, reduced in 6 hr, and reached to minimum level in 24 hr, maintaining plateau level for 7 days.

We selected the animal model of biliary obstruction in a period of 1 to 6 weeks because of the similarity to the clinical setting with reference to the histological findings of the liver. It was demonstrated that the rate of ATP synthesis decreased markedly in 3 and 6 weeks after biliary obstruction in comparison with that of 1 week obstruction; approximately 80% of the control value in a group of 1 week obstruction, 60% in 3 weeks obstruction, and below 30% in 6 weeks obstruction which histologically built up biliary cirrhosis. RC, P/O ratio and S3 also decreased each other with prolongation of biliary obstruction. Reductions in respiratory enzymes were relatively mild. Though cyt c (+c3) reduced clearly with prolongation of obstruction, the fall in cyt a(+a3) was slight without any difference between the 3 and 6 week groups. Ozawa et al. (1973) illustrated that in clinical cases of obstructive jaundice a marked decrease of cyt a(+a3) was indicative of a poor prognosis. They noted that cyt a(+a3) of $0.5 \times 10^{-10}$ moles/mg was the critical point, the value being 63% of their control values ($0.79 \pm 0.05 \times 10^{-10}$ moles/mg). The present experimental data have supported Ozawa's concept for critical point, because rats which had average cyt a(+a3) level greater than 75% of Ozawa's control value survived more than 6 weeks after biliary obstruction.

As Ozawa et al. (1972) mentioned, the absolute phosphorylation activity of mitochondria was referred to the content of cyt a (+a3) in the mitochondria, and it was thought to be better to represent the ATP synthesis as the turnover number of phosphorylation $[\text{TN}_p]_a$, four times the moles of ATP synthesis divided by the moles of cyt a(+a3) per second, rather than as the ATP synthesis per mitochondrial protein.

We found that the $[\text{TN}_p]_a$ increased compensatory in the group of 1 week biliary obstruction, but decreased to 60% and 45% after 3 and 6 weeks, respectively. However, the reduction rate of ATP synthesis per moles of cyt a(+a3), that is $[\text{TN}_p]_a$, was clearly smaller than that per weight of mitochondrial protein.

It is thought that these decreases in mitochondrial respiratory function in obstructive jaundice are due primarily to the bilirubin as an uncoupler of phosphorylation. Zetterström and Ernster (1956) investigated liver mitochondrial
respiration using a Warburg manometer and found that addition of $3 \times 10^{-4}$ M of bilirubin caused a nearly complete inhibition of phosphorylation, and a decrease by half in oxygen consumption. From the fact that addition of cyt c and diphosphopyridine nucleotide caused recovery of oxygen consumption, but the P/O ratio remained unchanged, they mentioned that the toxicity of bilirubin itself was the uncoupling effect reducing the P/O ratio. Mustafa et al. (1969) also found that the P/O ratio was 1.68 in the mitochondrial respiration using $\beta$-hydroxybutyrate as a substrate. By adding 1.5 $\mu$M of bilirubin, the P/O ratio fell to 0.98, and by adding 100 $\mu$M it fell to zero, while simultaneously there appeared irreversible swelling of the mitochondria. They reported also that at a concentration of 20 $\mu$M bilirubin in liver mitochondrial suspension, $S_3$ decreased but, on the contrary, $S_4$ increased and then RC ($=S_3/S_4$) showed marked decrease.

According to Greim et al. (1972) the concentration of intrahepatic bile acids, in contrast to the control value (169±34 nmoles/g liver; mean±s.d.), increased to 606±145 nmoles/g liver after 3 days of biliary obstruction and 866±147 nmoles/g liver after 8~10 days. It is well known that the microsomal function is disturbed by bile acids as well as bilirubin. Our results indicated that mitochondrial functions were also inhibited by bile acids, especially those of free type such as CDCA, and the toxicity of bile acids was markedly reduced following conjugation with glycine or taurine.

Although microsome, ATP, Mg++ and lysosome are necessary for the conjugation of bile acids, these factors decrease quantitatively and qualitatively in biliary obstruction, and then the conjugation of bile acids becomes incomplete. These situations with both increased concentration and incomplete conjugation of bile acids in the liver, are thought to be one of the important factors to inhibit the mitochondrial function in biliary obstruction.

Mitochondrial respiratory function was found to decrease with prolongation of biliary obstruction, whereas various liver function parameters showed maximal impaired values after 1~2 weeks of obstruction, after which there were no changes or even a improving tendency. Serum level of bilirubin, GOT, Al-p etc. do not parallel to the decrease in mitochondrial respiratory function. Therefore, the reduction of mitochondrial respiratory function in jaundiced liver, may not be identical to the respiratory disturbances which are resulted in the addition of bilirubin or bile acids to normal mitochondrial suspension. We recognized the significant swelling of mitochondria and its quantitative increment per unit volume of the liver tissue with prolongation of biliary obstruction on electron-microscopy (Yamauchi et al. 1976).

It is likely that the extent of mitochondrial respiratory inhibition is regulated not only by a concentration of inhibitory substances around the mitochondria but also by a period for which mitochondria exists with inhibitory substances.

It is, therefore, thought that organic alteration of mitochondria occurs and ATP synthesis in a mitochondria decreases under these situations for a long time. Shirakawa (1978) reported that the serum mitochondrial GOT (m-GOT) level of
rats increased after 2 weeks biliary obstruction, which indicated that the damage of mitochondria itself begun at that time and the reduction of mitochondrial respiration during 2 weeks after biliary obstruction was due mainly to the uncoupling action of increased bilirubin. Such hypothesis also finds a support in the results of our morphological and biochemical studies.

With regard to the disturbances of mitochondrial function caused by factors other than biliary obstruction, Caplan and Greenawalt (1968) have investigated the respiratory function of mitochondria which have succumbed to swelling and lysis induced by the treatment with distilled water. The result of the treatment of water was similar to those of prolonged biliary obstruction; parameters of mitochondrial function such as P/O, S₃ and ATPase deteriorated with the frequency of the treatment of water.

As shown by Caplan and Greenawalt (1968), damage of the membrane of mitochondria was manifested by the activation of ATPase. We found that the latent ATPase activity increased markedly in the early stage of biliary obstruction, but after 6 weeks, it had returned to control levels, seemingly indicating the recovery of mitochondrial membrane from the damage. However, the activity of DNP stimulated ATPase was found to be decreased and the activation ratio of ATPase markedly increased after prolonged periods of biliary obstruction which indicated that the damage to the mitochondria was lasting.

Caplan and Greenawalt (1968) also reported that the site 1 and 2 of mitochondrial respiratory chain were severely inhibited and only the site 3 was maintained within normal range in the water treated mitochondria.

In our investigation, when ascorbate was used as the substrate, there was a slight decrease in RC, but even after long term biliary obstruction, P/O, S₃ and ATP synthesis were nearly normal. In contrast, when α-ketoglutarate or succinate were used, obvious decrease of mitochondrial function was shown after the 3 weeks of biliary obstruction. These results suggested that there were inhibitions to the site 1 and site 2, and site 3 was not inhibited in the mitochondria in biliary obstruction similar to the water treated mitochondria. The mitochondria of the liver in biliary obstruction was in a state of dysfunction caused by a nonspecific damage of its membrane and by the uncoupling action of bilirubin and/or bile acids.

In the clinical cases of obstructive jaundice, it is not rare to fall into hepatic failure when severe hypotension is imposed by gastrointestinal bleeding from acute ulcer. Our results with hypotensive animal models with long term biliary obstruction demonstrated marked reduction of mitochondrial respiratory function. The mitochondrial function of 1 week biliary obstruction with hypotension corresponded to that of 3 weeks obstruction without hypotension and that of 3 weeks with hypotension did 6 weeks without. These accelerations of mitochondrial dysfunction are believed to be one of the important reasons to fall into the hepatic failure associated to gastrointestinal bleeding in patients with obstructive jaundice.
Mitochondrial Function in Biliary Obstruction

References


