Mitochondrial functions and content of microsomal and mitochondrial cytochromes in human cirrhosis

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Summary

1. Mitochondria and microsomal fractions have been isolated from liver biopsies from patients with alcoholic cirrhosis, cryptogenic cirrhosis or chronic aggressive hepatitis.
2. Cirrhotic livers yielded fewer mitochondria than normal liver.
3. The most significant change was a decrease in mitochondrial respiratory control. Cirrhotic microsomal fractions had a 50% diminution in cytochrome \( b_5 \) and cytochrome \( P-450 \) content.
4. The two patients with chronic aggressive hepatitis showed similar mitochondrial and microsomal changes.

Key words: cirrhosis, cytochromes, microsomes, mitochondria.

Abbreviations: ADP, ATP, adenosine di- and tri-phosphate; EDTA, ethylenediaminetetra-acetate; EGTA, ethanedioxybis(ethylamine) tetra-acetate; NADH, reduced form of nicotinamide adenine dinucleotide.

Introduction

To our knowledge, only two previous reports have appeared in the literature concerning mitochondrial function in human cirrhosis. Pagliaro, Notabartolo, Maminò & Miguéco (1963) reported that mitochondria, isolated from cirrhotic livers, had a reduced ability to synthesize ATP and Benga & Muresan (1974) reported an increase in mitochondrial adenosine triphosphatase activity in freshly isolated mitochondria from cirrhotic liver. These studies, and the report of Cederbaum, Lieber & Rubin (1974) on the damage of coupling site I in the hepatic mitochondria of rats fed ethanol over a period, prompted us to carry out a systematic study of mitochondria isolated from liver biopsies of patients with alcoholic cirrhosis, who were undergoing abdominal surgery for other purposes. The hepatic microsomal cytochrome content in two cases of chronic active hepatitis and two cases of cryptogenic cirrhosis has also been studied.

Our results suggest that in cirrhosis there is a decrease in respiratory control in hepatic mitochondria and a decrease in microsomal cytochrome \( P-450 \) and cytochrome \( b_5 \). The significance of these findings to the pathogenesis and pathophysiology of cirrhosis deserves further study.

Material and methods

Biopsy specimens

Samples of liver were obtained by biopsy from patients undergoing surgery for abdominal diseases (duodenal or gastric ulcers or cholecystitis) and from patients with liver disease having a portacaval shunt operation.

The six control liver biopsies were from men aged 25, 34, 42, 50, 60 and 75 years. The patients
gave written consent for the liver biopsy before surgery and the Ethics Committee of the Hospital authorized the study.

Morphine was given as a pre-operative sedative and the anaesthetic agents were sodium hexobarbital, nitrous oxide and halothane; in addition curare was administered. Immediately after the abdomen was opened two catgut strings were stitched around an area at the edge of the left lobe of the liver. A triangular piece of liver tissue weighing 0.5–1.0 g was removed and, by pulling the catgut strings, a safe suture was then obtained with no bleeding or bile leakage. A small portion of each sample was separated for histological diagnosis and the remaining piece was immediately immersed in an ice-cold medium (pH 7.2) composed of sucrose (75 mmol/l), mannitol (225 mmol/l), EDTA (100 mmol/l) and bovine serum albumin (3 g/l) and transported immediately to the laboratory for isolation of mitochondria.

A total of 18 patients were biopsied: six with normal livers, eight with alcoholic cirrhosis, two with cryptogenic cirrhosis and two with chronic aggressive hepatitis. The patients with alcoholic cirrhosis had not ingested alcohol in the 2 months before surgery and had no histological evidence of acute alcoholic liver injury.

Normal subjects were those with no symptoms or signs of liver involvement, with normal liver histology and with normal liver function as indicated by these criteria: serum bilirubin 17 mmol/l, serum thymol test 4 units, serum glutamate-oxaloacetate transaminase 17 i.u./l. The differential diagnosis of liver disease was established by clinical, histological and biochemical investigations.

Preparation of mitochondria and microsomal fraction

All procedures were carried out at 1–2°C and as quickly as possible. The tissue was first sliced into pieces approximately 1 mm thick, which were then washed twice with medium (see below) to eliminate haemoglobin. The minced tissue was then homogenized in a bladed Sorvall homogenizer at 2000 rev./min for 5 s followed by two or three strokes at 1000 rev./min in a Potter homogenizer with a tight-fitting Teflon pestle. The homogenate was centrifuged for 10 min at 600 g and the resulting supernatant solution was further centrifuged for 15 min at 8500 g. The mitochondrial pellets were re-suspended very carefully, and washed twice in the same medium without EDTA, and finally resuspended in the sucrose solution at an approximate concentration of 20 mg/ml.

Normal livers yielded an average of 40 ± 3.4 mg of mitochondrial protein/g of liver. The purity of the mitochondrial fractions was checked by electron microscopy. The mitochondrial supernatant was centrifuged again at 15 000 g for 15 min to eliminate the light mitochondrial fraction. This procedure was followed by centrifugation for 1 h at 100 000 g with the production of a microsomal pellet. The microsomal pellet was washed twice with KCl (150 mmol/l) and once with sucrose (250 mmol/l) and was finally resuspended in the sucrose solution at an approximate concentration of 20 mg/ml.

Analytical methods for mitochondrial and microsomal suspensions

Duplicate assays for each liver sample were made. Mitochondrial oxygen uptake was measured with a Clark-type oxygen electrode. Mitochondrial respiratory control ratio and mitochondrial ADP/O ratio were determined as described by Estabrook (1967) with an oxygen electrode; in pathological cases with low respiratory control, it was necessary to use a recorder with a low 'noise' and long recording time (B. Chance, unpublished work). The mitochondrial respiratory control ratio was obtained in the presence of excess of substrate as the ratio of mitochondrial respiration in state 3 (presence of ADP) to that in state 4 (absence of ADP). Thus raised values for respiratory control indicate that the mitochondrial respiration is stimulated by ADP. The ADP/O ratio is equivalent to P/O ratio and represents the number of molecules of ADP phosphorylated to ATP by the mitochondria per atom of oxygen consumed. The assay medium (pH 7.3 at 22°C) was composed of KCl (20 mmol/l), Tris/Pi (5 mmol/l), MgCl2 (7 mmol/l), Tris/HCl (10 mmol/l) and sucrose (250 mmol/l).

The respiratory carriers were estimated by differential spectrophotometry in a Perkin–Elmer Spectrophotometer 356, as described by Chance & Williams (1956). To minimize the contamination with haemoglobin, the mitochondria were washed twice with Locke’s solution. The
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Pyridine nucleotides, flavoproteins and cytochrome b were determined from the difference spectra of aerobic mitochondria, with and without antimycin. The absorption coefficients and wavelength pairs were respectively: cytochrome a, 91 cm\(^{-1}\) mmol\(^{-1}\), 445 and 465 nm; cytochrome a, 16-5 cm\(^{-1}\) mmol\(^{-1}\), 605 and 630 nm; cytochrome c (+c\(_1\)), 19-0 cm\(^{-1}\) mmol\(^{-1}\), 550 and 540 nm; cytochrome b, 17-9 cm\(^{-1}\) mmol\(^{-1}\), 562 and 575 nm; flavoproteins, 11-0 cm\(^{-1}\) mmol\(^{-1}\), 465 and 500 nm; pyridine nucleotides, 6-0 cm\(^{-1}\) mmol\(^{-1}\), 340 and 374 nm (Chance & Williams, 1956). The concentration of protein in the suspensions used in measurements of absorbance was between 1 and 2 mg/ml. Microsomal cytochrome b\(_5\) and cytochrome P-450 were determined as described by Hildebrandt, Remmer & Estabrook (1968) and Omura & Sato (1964), with the use of dithionite and carbon monoxide. The coefficients of absorbance were 20 and 91 cm\(^{-1}\) mmol\(^{-1}\) respectively.

The protein was determined by the biuret reaction with crystalline bovine serum albumin as standard.

Results

Figs. 1, 2 and 3 illustrate the value of each variable for the mitochondria measured in the individual case of cirrhosis or precirrhotic disease in comparison with the average control value. The values of respiratory control ratio, ADP/O ratio, rates of respiration with different substrates were similar for all the control subjects, regardless of age. However, in control patients only, there was a significant elevation of cytochrome c (+c\(_1\)) with increasing age, as has been reported by Ozawa, Kitamura, Mizukami, Yamaoka, Kamano, Takeda, Takasan & Honjo (1972).

A major difference between normal and cirrhotic livers was in the recovery of mitochondrial protein. Normal livers yielded an average of 40 ± 4 (sd) mg of mitochondrial protein/g of tissue and the cirrhotic livers gave from 18 to 22 ± 1.5 mg/g. Fig. 1 shows the respiratory control ratios and the ADP/O ratio with different substrates. There was a substantial (P < 0.005) decrease in the mitochondrial respiratory control ratio with glutamate plus malate or
succinate as substrate for all pathological livers. The decrease in respiratory control was, in many cases, due to both the high rate of respiration in the absence of ADP (state 4) (denominator of the ratio) and to a diminished rate of respiration in the presence of ADP (Fig. 2) (numerator of the ratio). The ADP/O ratio both with glutamate plus malate and with succinate was slightly decreased in all but three patients with liver disease compared with the control subjects. However, the subjective element involved in assessing the ADP/O ratio with low respiratory control precludes any quantitative conclusion.

The respiration of the mitochondria with different substrates in the presence or absence of ADP is depicted in Fig. 2. There was a great variation in the hepatic mitochondrial respiration of the various patients with liver disease and at least four alcoholic cirrhotic patients had a decreased respiration with all substrates. Those cases with a normal value for respiration in state 3 had a low respiratory control (Fig. 1), due to the fact that they had a high respiration without ADP (state 4). The stimulation achieved by the addition of ADP was especially low in all the patients with alcoholic cirrhosis.
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**Fig. 3.** Concentrations of respiratory carriers in hepatic mitochondria from patients with liver disease. For explanation of abbreviations see Fig. 1.

**Fig. 4.** Comparison of microsomal spectra from normal human liver microsomes and microsomes isolated from one case of alcoholic cirrhosis. The peaks of cytochrome P-450 and cytochrome b₅ in normal microsomes are twice those of cirrhotic microsomes. The peak of cytochrome P-450 is detected either in CO-reduced minus oxidized spectra (continuous line) or in CO-reduced minus reduced spectra (broken line). The peak of cytochrome b₅ has been detected in reduced minus oxidized spectra. The concentration of microsomal protein was 1 mg/ml (P-450) and 0.5 mg/ml (b₅). Units of absorbance are indicated by values between vertical arrows.
In Fig. 3, the quantities of mitochondrial respiratory electron carriers are represented. The values for normal mitochondria were obtained from the control patients in the 42–75 years age-group. There is a great variability in the content of respiratory carriers in the pathological groups. There are some with a similar or higher content of respiratory carriers than the normal mitochondria and there are others which exhibit a decrease in the content of cytochromes. The most notable difference is the decrease in cytochrome $a_2$ in three of the six cases of alcoholic cirrhosis. There does not seem to be a relation between rates of respiration in the different cirrhotic patients and their liver cytochrome content. Although some of the alcoholic cirrhotic patients who showed low ascorbate respiration also had a low cytochrome $a_2$ content, there were other cases with low ascorbate respiration who had only minor decreases or even raised cytochromes.

Fig. 4 compares the spectra of normal liver microsomal fractions with those from a case of alcoholic cirrhosis. A decrease in the content of both cytochrome $P-450$ and $b_5$ is clearly demonstrated in the cirrhotic patient. In Fig. 5, the content of cytochromes $b_5$ and $P-450$ in all pathological cases is compared with the average control value. There is a clear diminution in cytochromes $b_5$ ($P<0.005$) and $P-450$ ($P<0.005$) in cases of chronic aggressive hepatitis and of cryptogenic cirrhosis for $P-450$ as well as alcoholic cirrhosis. The mean decrease of about 50% is in agreement with the data of Schoene, Fleischmann, Remmer & Oldershhausen (1972).

Discussion

Our mitochondrial preparations appear to be better preserved than those previously reported in the literature, since, in the control subjects respiratory control ratios ranging from 7 to 9 with NADH-linked substrates were obtained, in contrast to the values of less than 4 which were obtained by Bjorntrop, Bjorkerud & Scherten (1965), Benga, Muresan, Hadarnav & Dancea (1972) and Ozawa et al. (1972). The respiratory rates with ADP, oxidative phosphorylation rates and phosphorylation coupling of our preparations were similar to the preparations of Bjorntrop et al. (1965) and Benga et al. (1972). However, the amount of respiratory carriers (mean values) present were about half that found by Ozawa et al. (1972). This apparent discrepancy may be due to the different method used by these authors to measure the mitochondrial protein and also to the different concentration of mitochondria used for their spectra. The relative amounts of all respiratory carriers to cytochrome $a_2$ in our preparations, were very similar to the data of Ozawa et al. (1972).

From our results it can be concluded that cirrhotic livers yield less mitochondria than normal livers and that these mitochondria were
functionally different. In almost all cases the cirrhotic mitochondria exhibit a decreased respiratory control, which is caused mainly by an increase in the respiration uncoupled to ADP (state 4 respiration). In several instances there is also a slight decrease in the cytochrome a content. The data on respiratory rates and other cytochromes show great variability, which precludes any conclusions being drawn.

The decreased yield of mitochondria from cirrhotic livers could possibly be related to poor recovery due to different centrifugal behaviour of the organelles from these livers, but this seems unlikely. We believe that the decrease in the yield of mitochondria is caused by the low proportion of liver cells for each gram of tissue present in the cirrhotic samples, due to abnormal amounts of connective tissue.

The most consistent alteration in the property of cirrhotic mitochondria is the decrease in respiratory control. It is possible that this finding does not reflect the situation in vivo but merely indicates that mitochondria from cirrhotic livers are more fragile and hence more susceptible to damage during isolation; this argument has been previously put forward to explain the functional alterations of tumour mitochondria (Aisenberg, 1967). We do not favour such a possibility because the method of isolation was gentle, as shown by the high respiratory control obtained in normal livers.

The functional alteration of the cirrhotic mitochondria is characterized by a low respiratory control, which coexists with a normal or slightly altered ADP/O ratio. Such a state has been described first in the so-called 'mitochondrial myopathies' (Luft, Ekkos & Palmieri, 1962; Hulsman, Bethlem & Meiger, 1967; Vjingaarden, Bethlem & Meiger, 1967; Gimeno, Trueba, Blanco & Gosálvez, 1973). Loosely coupled mitochondria have a low respiration coupled to the synthesis of ATP, but this is coupled with normal efficiency or almost normal efficiency.

The measurement of ADP/O ratios in mitochondria with low respiratory control is difficult, but by using a low-'noise' recorder and long recording times, in our hands this has proved to be valid (Gimeno et al., 1973). It is very difficult to determine whether the biochemical alterations observed in mitochondria from cirrhotic livers are secondary or primary to the cirrhosis. Although chronic aggressive hepatitis also displays mitochondrial alterations similar to cirrhosis and this fact would be in favour of a primary alteration, the paucity of the cases studied precludes any conclusion. The mitochondrial respiratory control is perhaps the most sensitive index of the structural and functional integrity of mitochondria (Lehninger, 1964). Cederbaum et al. (1974) reported a malfunction of phosphorylation site 1 in mitochondria from rats fed chronically with ethanol. Both studies could be interpreted as implying that the mitochondrial alterations play a role in pathogenesis of alcoholic cirrhosis. However, similar mitochondrial alterations were observed in cryptogenetic cirrhosis and chronic aggressive hepatitis, which are both unrelated to the ingestion of alcohol. To help clarify the role of mitochondrial alteration in the pathogenesis and pathophysiology of cirrhosis, further studies on a sequence of pre-cirrhotic states would be necessary. It is very possible that fatty acids or Ca$^{2+}$ would play a role in loosening respiratory control of mitochondria from cirrhotic cells, but albumin or EGTA does not restore the control to higher values.

The decreases in cytochrome $b_{5}$ and cytochrome P-450 in cirrhotic microsomes are highly significant ($P<0.005$). Our results corroborate the data of Schoene et al. (1972) on cytochrome P-450 and point out that there is also a substantial decrease in cytochrome $b_{5}$. The decrease in microsomal cytochromes was already quite marked in the cases of chronic aggressive hepatitis, implying that this phenomenon is readily detected even in pre-cirrhotic states.

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References


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