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MITOCHONDRIAL FUNCTION IN CARBON TETRACHLORIDE-INDUCED CIRRHOSIS IN THE RAT

QUALITATIVE AND QUANTITATIVE DEFECTS

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Abstract—Mitochondrial function is impaired in patients and experimental animals with liver cirrhosis. The relationship between mitochondrial impairment and severity of cirrhosis is unknown, however. We therefore characterized the severity of cirrhosis in rats with phenobarbital/CCl_4-induced cirrhosis by the aminopyrine breath test, a microsomal function test reflecting hepatocellular mass. Mitochondrial function was evaluated by measuring oxygen consumption, enzyme activities and ATP production in mitochondria isolated from cirrhotic (N = 8) and control livers (N = 4). Oxygen consumption and mitochondrial enzyme activities calculated per liver were significantly reduced in the presence of cirrhosis. This decrease corresponded to the loss of hepatocytes calculated from the reduction in aminopyrine breath test. The effect of atractylate, oligomycin and dinitrophenol on state 3 respiration was equal between the two groups. The respiratory control ratio was significantly reduced in mitochondria from cirrhotic livers with beta-hydroxybutyrate (4.01 ± 0.94 vs 5.45 ± 0.40), but not with succinate as substrate. The rate of ATP production was significantly decreased in mitochondria from cirrhotic rats for both substrates. In contrast, the static head (state 4) phosphate potential was fully developed after 10 min and was equal between the two groups. We conclude that cirrhosis of the liver leads to a loss of hepatocytes which is paralleled by reduced oxygen uptake and reduced mitochondrial enzyme activities.

Mitochondrial function has been shown to be disturbed in patients with cirrhosis of the liver [1] and in rats with thioacetamide [2] and CCl_4-induced liver cirrhosis [3, 4]. Similar changes of mitochondrial function have been described after chronic ethanol feeding [5–7]. Reduced respiratory control in mitochondria of cirrhotic rats suggested impaired coupling of oxygen consumption and ATP production [3]. Studies in rats fed chronically with ethanol provided evidence that coupling of oxidative phosphorylation at site I of the respiratory chain is more susceptible to damage than at sites II and III [5, 7]. Adenylate energy charge level, a measure of the energetic state of the cell, has been found to be decreased in livers from CCl_4-cirrhotic rats [3], indicating a reduced ATP production or an increased cytoplasmatic ATP metabolism.

Studies comparing the severity of CCl_4-induced cirrhosis with mitochondrial function are lacking so far. We have recently shown *N*-demethylation of aminopyrine as measured by a breath test to adequately reflect loss of hepatocellular mass in CCl_4-induced cirrhosis in the rat [8]. We therefore studied oxygen consumption, enzyme activities and ATP production in mitochondria isolated from cirrhotic and normal rats and compared it to the elimination rate constant of aminopyrine.

MATERIALS AND METHODS

Materials. Male Sprague–Dawley rats were obtained from the Süddeutsche Versuchstierfarm (Tuttlingen, F.R.G.). Di-^{14}C-methylaminoantipyrin was from New England Nuclear (Boston, MA). All enzymes used for determination of enzyme activities were from Boehringer Company (Mannheim, F.R.G.). All other reagents were analytical grade from different sources.

Induction and characterization of cirrhosis. Animals were kept on a standard rat chow and tap water *ad libitum* on a 12 hr light/dark cycle. Cirrhosis was induced by the method of McLean *et al.* [9], as described previously from our laboratory [8]. Treatment was ceased two weeks prior to the study. Control animals were kept under the same conditions without any treatment. Severity of cirrhosis was characterized by the aminopyrine breath test (ABT) [10] carried out as previously described [8]. Briefly, after injection of 0.5 μCi (0.3 mg) of di-^{14}C-methylaminoantipyrin i.p. breath samples were collected in 10 min intervals; ^{14}C-radioactivity was determined in a Packard Tri-Carb 2660 liquid scintillation counter using external standardization for quench correction. Results were analyzed on semi-log plots for breath ^{14}C-radioactivity vs time. The descending slope of the ABT gave the fractional elimination rate constant (ABT-k).

Preparation of mitochondria. Nonfasted animals were killed at 7.00 a.m. and the liver was quickly removed, weighed and put in 0° isolation medium. Mitochondria were isolated by differential centrifugation according to Johnson and Lardy [11] with the exception that the homogenizing medium contained 0.07 M sucrose and 0.25 M mannitol [12]. Washed mitochondria were suspended in the homogenizing medium at a concentration of 1 g original liver weight/ml. An aliquot of the resulting mitochondrial fraction was used immediately for the oxygen consumption studies and determination of phosphate potential. The rest of the suspension was

frozen at −20° for determination of enzyme activities. Aliquots of the homogenate were also stored at −20°.

Oxygen consumption. Mitochondria (approximately 5 mg protein) were incubated in open beakers at 37° and stirred. The incubation medium contained 40 μM potassium, 30 μM magnesium, 30 μM sulfate, 20 μM phosphate and 20 μM triethanolamine in a final volume of 3 ml sucrose 0.07 M and mannitol 0.25 M solution at pH 7.4. Oxygen consumption was determined by a Clark-type electrode (Yellow Springs Instruments, OH) as described elsewhere [13]. Substrates added were ADP (1 μM) and succinate (25 μM) or beta-hydroxybutyrate (25 μM). Inhibitors were added during state 3 respiration with beta-hydroxybutyrate as substrate (10 nM atractylate or 1 μg oligomycin); dinitrophenol (0.2 μM) was added after inhibition of state 3 respiration with oligomycin. Oxygen consumption, respiratory control and P/O ratio were calculated according to Estabrook [13]. ATP production velocity was calculated as the product of state 3 respiration and the corresponding P/O ratio. Oxygen consumption by the whole liver was calculated as the product of oxygen consumption per mg mitochondrial protein and the total amount of mitochondrial protein in the liver.

Phosphate potential. Phosphate potential was determined as described before [14]. Approximately 5 mg mitochondrial protein were incubated in open beakers at 37° in a water bath shaken at 100 strokes/min. The final volume of the incubation medium was 3 ml. The composition of the incubation medium was KCl 100 mM, glutamate 5 mM, potassium malate 5 mM, $MgCl_2$ 1 mM and potassium phosphate buffer 8 mM, pH 7.4. Adenine nucleotides were added up to a final concentration of 2 mM. One-millilitre samples were taken at 10 and 20 min and quickly deproteinized by addition of perchloric acid to a final concentration of 0.15 M. The extracts were neutralized with KOH and ATP, ADP and AMP were determined by enzymatic methods [15, 16]. Phosphate potential was calculated as described before [14] as

$$\Delta G_p = \Delta G_p^0 + RT \ln \frac{ATP}{ADP.P_i}$$

with $\Delta G_p^0 = 8.5$ kcal [14].

Enzyme activities. All enzyme activities were determined in the homogenate and the mitochondrial fraction using standard methods. Activity of lactate dehydrogenase (EC 1.1.1.27) and glutamate dehydrogenase (EC 1.4.1.3) was measured by a coupled enzyme assay [17, 18]. Activity of cytochrome *c* oxidase (EC 1.9.3.1) was determined spectrophotometrically according to Wharton and Tzagoloff [19]. Mitochondrial ATPase activity was determined in the presence of ouabain using a coupled enzyme reaction according to Scharschmidt *et al.* [20]. Activities of arylsulfatase (EC 3.1.6.1) and glucose-6 phosphatase (EC 3.1.3.9) were measured by colorimetric methods [21, 22]. Catalase (EC 1.11.1.6) was determined by a spectrophotometric method according to Aebi [23]. Results were expressed as μmol/min per whole liver or per mg protein with the exception of catalase, where the reaction constant of the first order kinetic is given. Protein concentration was determined by the method of Lowry *et al.* [24] using bovine serum albumin as a standard.

Statistical analysis. All results are expressed as mean ± 1 SD. Means were compared by an unpaired *t*-test, after having tested the equality of variances by an F-test. Regression analysis was performed by the method of least squares [25]. $P < 0.05$ was considered to be statistically significant.

RESULTS

All treated animals had cirrhosis by macro- and microscopic examination. They weighed less, body weight averaging 629 ± 74 and 789 ± 106 g ($P < 0.05$) in control and cirrhotic rats, respectively. The corresponding liver weights were not different statistically at 21.9 ± 3.3 and 26.6 ± 5.8 g. As previously described, ABT-k was decreased in the cirrhotic animals (1.025 ± 0.053 vs 0.678 ± 0.151 hr^{-1}; $P < 0.01$).

Total protein content of cirrhotic liver was reduced but not affected when expressed per g liver weight (Table 1). By contrast, total liver mitochondrial protein as well as mitochondrial protein expressed per g of liver were significantly reduced (Table 1). Mitochondrial protein averaged 12.2 ± 0.5 and 10.5 ± 2.5% of total protein (n.s.) in control and cirrhotic liver, respectively.

The specific activities of the mitochondrial enzymes are reported in Table 2. The specific activity of neither membrane enzymes (ATPase and cytochrome oxidase) nor matrix enzyme (glutamate dehydrogenase) was affected by cirrhosis. When multiplied by protein content, however, all three were significantly reduced in cirrhotic animals as expected (data not shown). Both relative enrichment (5.1 ± 0.8 vs 5.2 ± 2.3) and recovery (62 ± 8 vs 55 ± 30%) of ATPase were similar in control and cirrhotic animals. The figures were similar when calculated for cytochrome oxidase and glutamate dehydrogenase (data not shown).

Contamination by other organelles did not differ between the two groups (data not shown). Both, the cytosolic (lactate dehydrogenase) and the microsomal marker (glucose-6-phosphatase) were decreased to a similar extent in the mitochondrial fraction. The mitochondrial fractions of both groups were contaminated to a similar extent by peroxisomes and lysosomes.

Mitochondrial oxygen consumption, respiratory control, P/O ratios and ATP production velocities under the different experimental conditions are reported in Table 3. Mitochondrial oxygen consumption calculated for the whole liver was significantly reduced in the cirrhotic group for both substrates, except for beta-hydroxybutyrate during state 4 respiration. On average, oxygen consumption of the cirrhotic livers was about 60% of that of control livers. When oxygen consumption is expressed per mg protein, however, no differences could be detected between the two groups (data not shown). The respiratory control ratio was reduced by 26% ($P < 0.05$) in the cirrhotic group with beta-hydroxybutyrate but was not altered with succinate

Table 1. Protein content (mg) of liver homogenate and the mitochondrial fraction

	Control (N = 4)	Cirrhosis (N = 8)
Homogenate		
total	5730 ± 587	4196 ± 834*
g liver^{-1}	219 ± 30	191 ± 18
Mitochondrial fraction		
total	700 ± 73	440 ± 131*
g liver^{-1}	27 ± 3	20 ± 5†

* $P < 0.01$ compared to controls.
† $P < 0.05$ compared to controls.

Table 2. Specific activity (μmol · min^{-1} · mg protein^{-1}) of mitochondrial enzymes in liver homogenate and the mitochondrial fraction

	Homogenate		Mitochondrial fraction	
	Control (N = 4)	Cirrhosis (N = 8)	Control (N = 4)	Cirrhosis (N = 8)
ATPase	0.20 ± 0.04	0.16 ± 0.03	1.00 ± 0.12	0.81 ± 0.27
Cytochrome *c* oxidase	0.08 ± 0.01	0.08 ± 0.02	0.24 ± 0.04	0.20 ± 0.05
Glutamate dehydrogenase	0.50 ± 0.22	0.58 ± 0.12	1.29 ± 0.64	1.80 ± 0.80

There were no statistically significant differences between the two groups.

Table 3. Mitochondrial oxygen consumption, respiratory control ratio, P/O ratio and ATP production rate using beta-hydroxybutyrate (BOB) or succinate (SUC) as substrate

	Control (N = 4)	Cirrhosis (N = 8)
Oxygen consumption (μatoms/min, calculated for the whole liver)		
BOB State 3	54.3 ± 9.5	30.7 ± 16.2*
State 4	10.0 ± 1.8	7.2 ± 2.5
SUC State 3	148.5 ± 18.1	75.1 ± 44.5†
State 4	41.2 ± 7.4	21.8 ± 7.1†
Respiratory control ratio		
BOB	5.45 ± 0.40	4.01 ± 0.94*
SUC	3.65 ± 0.55	3.30 ± 0.91
P/O ratio (nmol ATP/atoms O_2)		
BOB	2.95 ± 0.26	2.44 ± 0.39*
SUC	1.62 ± 0.08	1.54 ± 0.11
ATP production rate (nmol ATP · min^{-1} · mg protein^{-1})		
BOB	227 ± 16	156 ± 34†
SUC	343 ± 15	250 ± 67*

* $P < 0.05$ compared to control.
† $P < 0.01$ compared to control.

as substrate. The P/O ratio (ATP produced/oxygen consumed during state 3) also was reduced by 17% ($P < 0.05$) when using β-hydroxybutyrate but not when using succinate as substrate. In contrast, ATP production rate was reduced for both substrates significantly (Table 3).

The effect of neither the inhibitors oligomycin and atractylate nor of dinitrophenol on mitochondrial oxygen consumption differed between the two groups. Atractylate inhibited state 3 respiration by 74 ± 2 and 72 ± 11% in control and cirrhotic animals, respectively. The corresponding figures for oligomycin were 78 ± 6 and 78 ± 9%. Dinitrophenol stimulated control and cirrhotic mitochondria 483 ± 29 and 473 ± 134%, respectively, in state 3.

Phosphate potential at static head (state 4) was fully developed after 10 min of incubation, averaging 13.6 ± 0.2 vs 13.8 ± 0.2 kcal in controls and cirrhotics, respectively (n.s.); the corresponding values at 20 min were 13.7 ± 0.2 vs 13.7 ± 0.2 kcal (n.s.).

There was a linear relationship between microsomal aminopyrine *N*-demethylation *in vivo* (ABT-k) and the mitochondrial ATP production rate for both substrates (Fig. 1). ABT-k was also positively correlated with ATPase and cytochrome *c* oxidase activities, two enzymes of the inner mitochondrial membrane, but not with glutamate dehydrogenase, a matrix enzyme (data not shown). In addition, there was a linear relationship between mitochondrial oxygen consumption per liver and the activity of ATPase (Fig. 2A) and cytochrome *c* oxidase (Fig. 2B), but not with glutamate dehydrogenase. No correlation could be found when the enzyme activities and oxygen consumption were expressed per mg mitochondrial protein (data not shown).

DISCUSSION

Our investigation presents evidence for both quantitative and qualitative changes of mitochondrial function in cirrhosis. Mitochondrial oxygen consumption and enzyme activities are reduced when calculated for the whole liver. The positive correlation between the elimination rate constant for aminopyrine metabolism (ABT-k) and mitochondrial oxygen consumption suggests that this

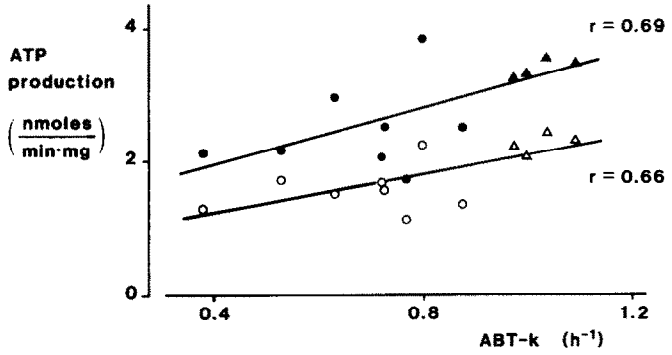

Fig. 1. Relationship between the elimination rate constant of aminopyrine metabolism (ABT-k) and mitochondrial ATP production rate in preparations from cirrhotic (○) and control livers (△). The regression equations were $y = 0.109 + 0.216x$ ($r = 0.69$, $P < 0.01$) and $y = 0.065 + 0.144x$ ($r = 0.66$, $P < 0.01$) for succinate (solid symbols) and beta-hydroxybutyrate (open symbols) as substrate, respectively.

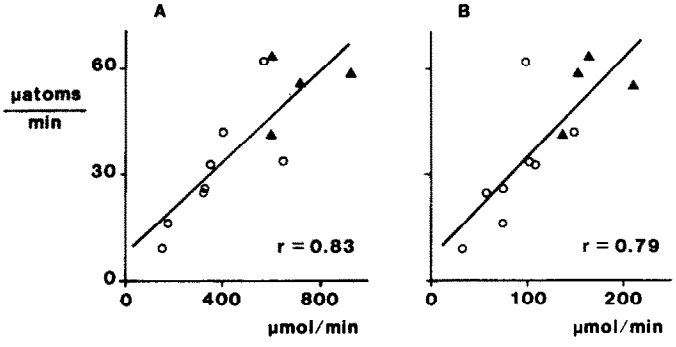

Fig. 2. Relationship between ATPase and cytochrome *c* oxidase activity and oxygen consumption in mitochondria isolated from cirrhotic (○) and control livers (▲). Both, enzyme activity and oxygen consumption are calculated for the whole liver. The regression equations are $y = 7.66 + 0.0644x$ ($r = 0.83$, $P < 0.001$) for ATPase (A) and $y = 6.42 + 0.25x$ ($r = 0.79$, $P < 0.001$) for cytochrome *c* oxidase (B). Within the cirrhotic group, the corresponding equations are $y = 3.90 + 0.0731x$ ($r = 0.78$, $P < 0.01$) and $y = 3.78 + 0.31x$ ($r = 0.68$, $P < 0.05$).

reduction is due to the loss of liver cell mass. By contrast, when oxygen consumption or enzyme activities were expressed per mg of mitochondrial protein, no difference between cirrhotic and control livers was evident, suggesting that the single mitochondrion is intact. Qualitative differences were observed when ATP production rate was measured; however, the phosphate potential at static head was not different between the two groups. Subtle differences in respiratory control and P/O ratio suggest a defect at coupling site I whereas lower sites are not affected.

Isolated mitochondria were of good quality and similar enrichment in both groups; using ATPase to calculate recovery, about 50% of mitochondria were recovered in our mitochondrial fraction (Table 1). A similar figure is obtained using protein recoveries reported in Table 1. Therefore, the noted differences are not due to a different behaviour of mitochondria of cirrhotic liver during the isolation procedure. In both groups, there was similar contamination by lysosomal and peroxisomal marker enzymes. In particular, the peroxisomal contamination raises the question of whether mitochondrial oxygen consumption has been overestimated. In the intact liver, peroxisomes consume about 10% of the total oxygen used by the organ [26]. In the present study, catalase was enriched in the mitochondrial fractions not as much as the mitochondrial enzymes, suggesting that peroxisomal contribution to mitochondrial oxygen consumption is less than 10%; moreover, quantitatively it was similar in both groups and cannot, therefore, explain any of the differences found.

Reduced mitochondrial oxygen consumption, enzyme activities and protein content calculated per liver are explained by the expected loss of hepatocytes: using morphometric analysis, we have recently shown a linear relationship between ABT-k and hepatocellular volume [8]. Using this linear relationship the observed reduction of the ABT-k corresponds to reduction of hepatocyte mass of 30–50%, which compares well to the observed reduction of mitochondrial enzyme activities and oxygen consumption of between 30 and 50%.

There was no difference between the two groups

following administration of the uncoupler dinitrophenol or the inhibitors oligomycin and atractylate. This further demonstrates the lack of qualitative changes of mitochondrial function when considering only oxygen consumption. By contrast, such qualitative changes were found regarding respiratory control ratio, P/O ratio and ATP production rate (Table 3). Respiratory control and P/O ratio were reduced in the cirrhotic group for beta-hydroxybutyrate but not for succinate indicating a damage at the coupling site I of the respiratory chain. A decrease in respiratory control has been reported in mitochondria isolated from rats with CCl_4-induced [3] and thioacetamide-induced cirrhosis [2] as well as in rats with chronic ethanol intoxication [5–7]. Results reported from patients with cirrhosis of the liver are contradictory, both reduced [1] and increased [27] respiratory control having been reported. A specific inhibition at coupling site I has been reported in chronically ethanol-fed rats only so far [5]. A possible explanation for the susceptibility of site I as compared to other sites is its higher lipophilicity [28], rendering it more susceptible to damage by organic solvents such as ethanol and CCl_4.

The possibility that alterations in membrane lipid composition are responsible in part for the observed alterations is supported by the finding that the activity of enzymes of the inner mitochondrial membrane (ATPase and cytochrome *c* oxidase), but not that of the matrix enzyme glutamate dehydrogenase is decreased (Table 2). Indeed, in thioacetamide-induced cirrhosis alterations of the lipid composition of the mitochondrial membranes have been described [2].

Mitochondrial ATP production rate was markedly reduced in the cirrhotic group for both substrates (Table 3). Both, a reduction of the P/O ratio and of ATP production rate has been reported in mitochondria from thioacetamide-induced cirrhotic rats [2] and from patients with cirrhosis of the liver [1]. By contrast, Uchida [27] found higher values in patients with cirrhosis of the liver and Jikko [3] reported the same for rats with CCl_4-induced cirrhosis. This apparent discrepancy presumably does not reflect differences in the models of cirrhosis employed but the fact that Jikko *et al.* studied rats which had been fasted overnight, whereas ours had free access to food. Fasting leads to important changes in the coupling of oxidative phosphorylation [29] and therefore could explain the difference.

Despite reduced ATP production rate in mitochondria from cirrhotic rats, phosphate potential at static head was not different between the two groups. Phosphate potential has been determined after an incubation period of 10 and 20 min, whereas the ATP production rate has been measured in a period of about 1 min after the addition of the substrates. Mitochondria isolated from cirrhotic livers therefore reach the same phosphate potential as those from control livers, but they do it at decreased velocity. A possible significance of the reduced ATP production rate is an insufficient response to a sudden demand of ATP, whereas longstanding energy requirements can be satisfied. This could explain the increased susceptibility of the cirrhotic liver to hypovolemic shock and anoxia. Our finding that mitochondria isolated from cirrhotic livers can reach the same phosphate potential as mitochondria from control livers are in contrast to the decrease of the energy charge level in the same model reported by Jikko *et al.* [3] in the perfused liver. This could reflect increased ATP metabolism in the whole organ which could not be detected in isolated mitochondria.

We conclude that in CCl_4-induced cirrhosis in the rat mitochondrial enzymes and mitochondrial oxygen consumption are reduced in parallel with the expected loss of hepatocytes. Under normal conditions, only quantitative changes, predicted by the loss of hepatocytes, are present. Under stress conditions with a highly increased demand of ATP, however, the observed qualitative differences could become significant and provide the explanation for the increased susceptibility of cirrhotic livers to hypovolemic and anoxemic stress.

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