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Mitochondrial dysfunction in rat with nonalcoholic fatty liver Involvement of complex I, reactive oxygen species and cardiolipin

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Abstract

Mitochondrial dysfunction and oxidative stress play a central role in the pathophysiology of nonalcoholic fatty liver disease (NAFLD). This study aimed to elucidate the mechanism(s) responsible for mitochondrial dysfunction in nonalcoholic fatty liver. Fatty liver was induced in rats with a choline-deficient (CD) diet for 30 days. We examined the effect of CD diet on various parameters related to mitochondrial function such as complex I activity, oxygen consumption, reactive oxygen species (ROS) generation and cardiolipin content and oxidation. The activity of complex I was reduced by 35% in mitochondria isolated from CD livers compared with the controls. These changes in complex I activity were associated with parallel changes in state 3 respiration. Hydrogen peroxide (H_2O_2) generation was significantly increased in mitochondria isolated from CD livers. The mitochondrial content of cardiolipin, a phospholipid required for optimal activity of complex I, decreased by 38% as function of CD diet, while there was a significantly increase in the level of peroxidized cardiolipin. The lower complex I activity in mitochondria from CD livers could be completely restored to the level of control livers by exogenously added cardiolipin. This effect of cardiolipin could not be replaced by other phospholipids nor by peroxidized cardiolipin. It is concluded that CD diet causes mitochondrial complex I dysfunction which can be attributed to ROS-induced cardiolipin oxidation. These findings provide new insights into the alterations underlying mitochondrial dysfunction in NAFLD.

Keywords: Nonalcoholic fatty liver; Mitochondria; Complex I; ROS; Cardiolipin

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) refers to the wide spectrum of liver damage that ranges from simple steatosis to steatohepatitis, advanced fibrosis and cirrhosis [1–3]. Several predisposing factors have been related to NAFLD such as obesity, diabetes, dyslipidemia, drugs and parenteral nutrition [4,5]. However, the pathogenesis of NAFLD and its progression to fibrosis and chronic liver disease remains still unknown. Dietary choline deficiency is a well-known experimental model to induce NAFLD in rats. [6–8]. Within few days of choline restriction triglycerides begin to accumulate inside the hepatocytes leading to a massive liver steatosis, predominantly macrovescicular [8–10].

There is accumulating evidence that mitochondrial dysfunction plays a key role in the physiopathology of NAFLD, although the mechanism(s) underlying this dysfunction are still unclear [2,11]. Mitochondrial dysfunction not only impairs fat homeostasis in liver but also leads to an overproduction of reactive oxygen species (ROS) which are considered an important factor in producing lethal hepatocyte injury associated with NAFLD [2,12]. Mitochondrial respiratory chain (mainly complexes I ad III) is an important subcellular source of ROS and hence a potential contributor to NAFLD [2]. Enhanced mitochondrial ROS formation associated with an impairment of mitochondrial respiratory chain has been demonstrated in rats fed a choline-deficient diet [13].

Peroxidation of membrane lipid components has been hypothesized to be a major mechanism of oxygen-free radical toxicity [14]. Cardiolipin, a phospholipid of unusual structure localized almost exclusively within the inner mitochondrial

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membrane (IMM), is an early target of oxygen-free radical attack, either because of its high content of unsatured fatty acids or because of its location in the IMM, near the site of ROS production, mainly at the level of complexes I and III of the mitochondrial respiratory chain [15,16]. Recent studies have demonstrated that cardiolipin plays an essential role in mitochondrial bioenergetics, optimizing the activity of key IMM proteins including several anion carriers and some electron transport complexes [17–19]. It seems likely that an enhanced ROS production may lead to cardiolipin oxidative damage and hence to loss of mitochondrial enzyme functions. Recent results from this and other laboratories have demonstrated that mitochondrial-mediated ROS generation affects the activity of the respiratory chain complexes via oxidative damage of cardiolipin [20–23]. These results have been useful to explain the molecular basis of the decline in respiratory chain activity observed in mitochondria isolated from animal under different physiopathological conditions, such as thyroid hormones status [24,25], aging [26–28] and ischemia/reperfusion [29–32] which are characterized by an increase in the basal rate of ROS production.

Complex I, also known as NADH–ubiquinone oxidoreductase, is a multisubunit integral membrane complex of the mitochondrial electron transport chain that catalyzes electron transfer from NADH to ubiquinone. The activity of this enzyme complex is considered the rate-limiting step for the mitochondrial respiratory chain and therefore an important factor in the regulation of oxidative phosphorylation. Complex I is also considered an important site of superoxide anion generation in mitochondria [15] and thus a potential source of ROS in fatty liver.

Results from different laboratories have shown that cardiolipin molecules are specifically required for functional activity of the mitochondrial complex I [33–37]. We also reported that mitochondrial-mediated ROS production affects the complex I activity through cardiolipin peroxidation in beef heart submitochondrial particles [22]. In addition, we demonstrated an impairment of mitochondrial complex I activity in ischemic/reperfused rat heart which was attributed to ROS-induced cardiolipin oxidative damage [32]. Reasoning that complex I is a major source of reactive oxygen species in liver mitochondria [15] and considering that phospholipid composition is altered significantly in choline-deficient mitochondria [38,39], we hypothesized that mitochondrial complex I might be altered during choline withdrawal, as a consequence of ROS-induced cardiolipin damage and this might result in mitochondrial dysfunction and subsequent hepathocyte injury in fatty liver. Here, we report that dietary choline restriction causes complex I dysfunction, cardiolipin oxidation and increased ROS generation in rat liver mitochondria. These results may provide new insights into the alterations that underlie the pathogenesis of mitochondrial dysfunction in NAFLD.

2. Experimental procedures

2.1. Animals and protocol

Male Wistar rats were used throughout these studies. Fatty liver was induced by feeding the animals a choline-deficient diet (for composition, see Ref. [40]) for 30 days. Control rats were kept on a standard diet containing adequate levels of choline. With this method, rats developed a massive fatty liver predominantly macrovescicular, without any inflammation and/or fibrosis [8,12]. Triglycerides represent the main component of the lipids accumulated in the cytoplasmatic vacuoles. Serum levels of triglycerides, in control and choline-deficient rats, were 172±15 and 394±28 mg% respectively. All procedures involving rats were conducted according to the guidelines for the care and the use of laboratory animals approved by our institutions.

2.2. Histological analysis

Specimens obtained from rat liver after 30 days of CD diet and from control rats were fixed in a mixture of 3% paraformaldehyde and 1% glutaraldehyde in 0.1 M PBS at pH 7.4 for 4 h at 4 °C. After an overnight wash in the same buffer, the specimens were dehydrated in ethanol and embedded in Epon resin (TAAB, Reading, England). Semithin sections (2 μm) were routinely stained with toluidine blue or with the PAS reaction to detect glycogen stores. All stainings were performed at 70 °C, without resin removal. Images were captured using an E600 photomicroscope equipped with a DMX 1200 digital camera (Nikon, Kawasaki, Japan).

2.3. Mitochondrial isolation

Liver mitochondria from control and CD diet rats were isolated in ice-cold medium containing sucrose 250 mM, Tris 10 mM EGTA 1 mM, BSA 0.1% pH 7.4, by differential centrifugation essentially as described in [41]. Mitochondrial pellet was resuspended in sucrose 250 mM, Tris 10 mM pH 7.4 and protein concentration was measured by the biuret method. The yield of mitochondrial proteins (mg/g liver wet weight) within these two groups of animals was consistent, suggesting minimal variations in the preparation of the mitochondrial fraction.

2.4. Citrate synthase activity

Citrate synthase activity was used as mitochondrial enzymatic marker. Mitochondrial protein 100 μg/ml, 0.3 mM acetyl CoA and 0.2 mM DTNB (5,5′-dithiobis-2-nitrobenzoic acid) were added to a 10 mM Tris–HCl buffer, pH 7.4, containing 0.2% (v/v) Triton X-100. The reaction was started by the addition of 0.5 mM oxalacetate and the initial rate was measured following the decrease of absorbance at 412 nm. No significant variation in the activity of citrate synthase was found between mitochondrial preparations from control and CD diet livers.

2.5. Mitochondrial oxygen consumption

Mitochondrial ADP-dependent state 3 respiration was measured polarographically with an oxygen electrode at 25 °C. Respiration was initiated by the addition of 5 mM glutamate + 5 mM malate. After 2 min, state 3 respiration was induced by the addition of 0.5 mM ADP. For uncoupled respiration 0.5 μM FCCP was added.

2.6. Complex I activity

The complex I (NADH–CoQ reductase) activity was measured in mitochondrial particles obtained by three cycles of freezing and thawing of 1 mg of rat liver mitochondria dissolved in 1 ml of 50 mM phosphate buffer pH 7.2. The assay mixture contained 3 mM sodium azide, 1.2 μM antimycin A, 50 μM decylubiquinone and 50 mM phosphate buffer pH 7.2. The mitochondrial sample (50 μg) was added to 3 ml of the assay mixture and the reaction was started by the addition of 60 μM NADH. The reaction was measured by following the decrease in absorbance of NADH at 340 nm with a diode array spectrophotometer. The activity was calculated using an extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH. The specific activity of the enzyme is expressed as nmol of NADH oxidized/min/mg of mitochondrial protein.

2.7. Analysis of phospholipids in mitochondrial membranes

Phospholipids were analyzed by high-pressure liquid chromatography (HPLC) using a Hewlett Packard series 1100 gradient liquid chromatograph.

Lipids from liver mitochondria were extracted with chloroform/methanol by the procedure of Bligh and Dyer [42]. Phospholipids were separated by an HPLC method with an Lichrosorb Si60 column (4.6×250 mM) and detected at 206 nm as previously described [43].

2.8. Preparation and analysis of peroxidized cardiolipin

Bovine heart cardiolipin was autoxidized overnight in a thin film at 37 °C [44,45]. Peroxidized cardiolipin was identified by the normal phase HPLC method described above, with UV detection at 235 nm, indicative of conjugated dienes [46].

2.9. Mitochondrial H_2O_2 production

The mitochondrial H_2O_2 formation, in state 4 respiration, was determined fluorometrically by the scopoletin-horseradish assay [47]. Rat liver mitochondria (0.5 mg/3ml) were suspended in 3 ml of a medium composed of sucrose 150 mM, KCl 50 mM Tris 10 mM, Pi 1 mM pH 7.4 supplemented with 1 μM horseradish peroxidase and 1 μM scopoletin. The amount of H_2O_2 produced was calculated by measuring the fluorescence changes upon addition of known amounts of H_2O_2.

2.10. Preparation of liposomes

Liposomes were prepared by sonicating 1.7 mg of phospholipids in 1 ml of incubation medium of 25 mM phosphate buffer pH 6.7 with the microtip probe of a Branson sonifier (mod. 250) at 40 W for six cycles of 2.5 min in a ice bath under N_2 stream.

2.11. Fusion of liposomes with mitochondria

The liposome–mitochondrial membrane fusion was carried out essentially as described by Hackenbrock and Chazotte [48] with some modifications [49]. Briefly, 1 mg of mitochondria was added to 1 ml of freshly sonicated liposomes (1.7 mg of phospholipids) in phosphate buffer pH 6.7 at 30 °C with constant stirring. After 40 min of incubation, phospholipid-enriched mitochondria were centrifuged at 10,000×*g* for 20 min in order to remove the phospholipid excess. The mitochondrial pellet was then washed and resuspended in 250 mM sucrose 10 mM Tris, pH 7.4.

2.12. Statistical analysis

Results are expressed as mean±S.E. Statistical significances were determined by the Student's *t* test.

3. Results

After 30 days of CD diet rat liver showed steatosis which was panlobular and mainly macrovesicular (Fig. 1). Most hepatocytes were characterized by a single large lipid droplet surrounded by a ring of cytoplasm. The nucleus was flattened and displaced peripherally. Some centrilobular hepatocytes contained numerous microvesicles that do not displaced the nucleus. Glycogen stores were scarce. Foci of necrosis, fibrosis as well as inflammatory infiltrate were not observed. The livers of control animals did not show histological alterations.

The activity of complex I was measured in liver mitochondria isolated from control rats and rats fed with a choline deficient (CD) diet. As shown in Fig. 2, the rotenone-inhibitable NADH oxidation rate was diminished by approximately 35% in mitochondria isolated from CD rats relative to mitochondria from normal rats. The same pattern of significant variation was obtained when the mitochondrial complex I activities were expressed as relative to citrate synthase.

Respiratory activities of liver mitochondria from control and CD animals, measured in the presence of glutamate and malate (i.e. complex I dependent substrates) and ADP to stimulate respiration are reported in Table 1. Mitochondria from CD rats exhibited a significant reduction in the rates of state 3 respiration, compared with control value, while state 4 rates of respiration were slightly increased. The respiratory control ratio (RCR) was also decreased in CD mitochondria due mainly to the decrease in state 3 rates of respiration. Rates of state 3 respiration were also measured in the presence of an uncoupler. Changes in the rates of uncoupled respiration in CD mitochondria were practically similar to those observed for ADP-dependent respiration. No significant changes in the ADP/O ratio were observed between control and CD mitochondria.

Cardiolipin has been shown to be specifically required for the optimal functioning of mitochondrial complex I [33–37]. Thus, it is possible that the ROS-induced oxidative damage to mitochondrial cardiolipin may be responsible, at least in part,

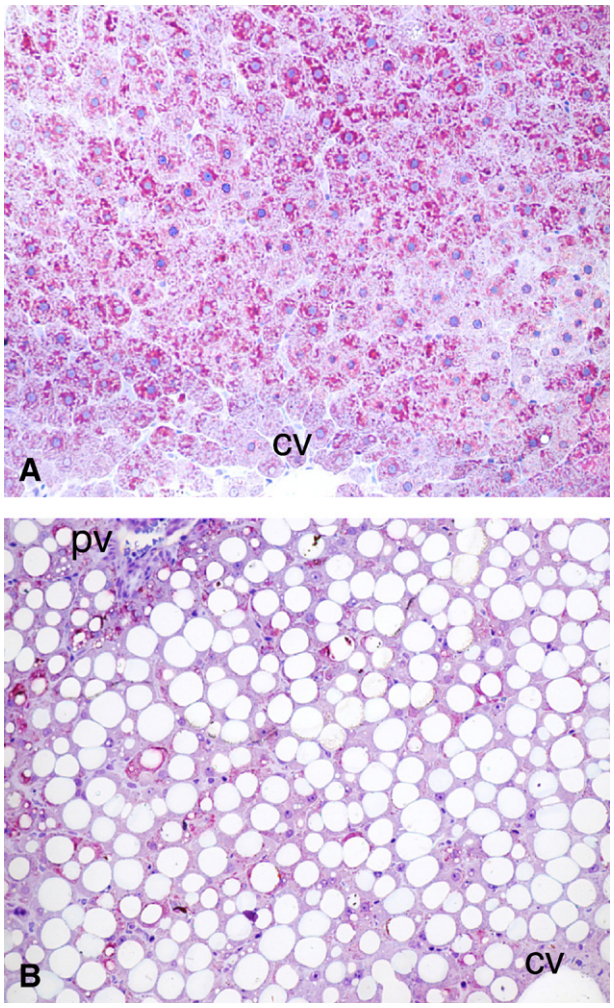

Fig. 1. Histological features of control (A) and choline-deficient rat livers (B). Rat liver fed 30 days with a choline-deficient diet shows severe fatty change evident in both periportal and centrilobular hepatocytes. Steatosis is mainly macrovesicular. Toluidine blue-PAS staining. Original magnification: 200×.

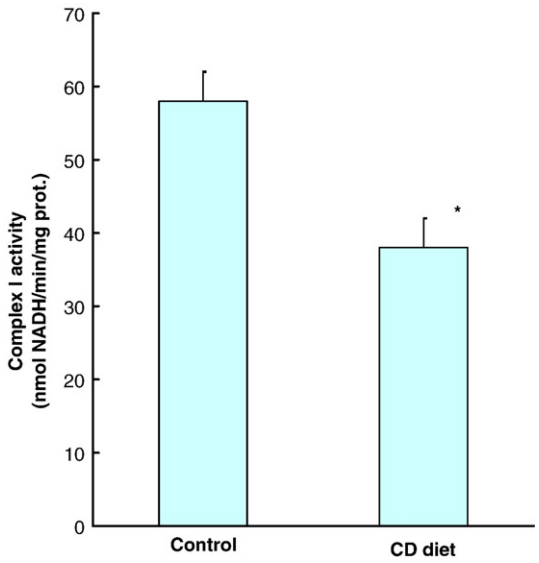

Fig. 2. Activity of complex I in liver mitochondria isolated from control and CD diet rats. The complex I activity was measured as described in Experimental procedures. Each value represents the mean ± S.E. of six separate experiments. *$P<0.05$ vs. control.

for the observed defect in complex I activity in CD rats. The content of cardiolipin and of the major classes of phospholipids was analyzed in liver mitochondrial preparations isolated from control and CD rats, by a very sensitive HPLC technique set up in our laboratory [43]. As illustrated in Table 2, alterations were found in the mitochondrial content of the various phospholipid classes, the most pronounced changes occurred in the negatively charged phospholipid cardiolipin, the level of which decreased by approximately 38% in mitochondrial preparations from CD livers with respect to controls.

To asses that the decrease in the cardiolipin content observed in mitochondria from CD animals could be due to ROS-induced cardiolipin peroxidation, the content of peroxidized cardiolipin was measured in mitochondrial preparations, by an HPLC method based on the absorbance at 235 nm, indicative of the formation of conjugated dienes [44,45]. As shown in Fig. 3 an increase of peroxidized cardiolipin was observed in mitochondria isolated from CD rats, relative to mitochondria from normal rats.

The changes in the cardiolipin content observed in mitochondria from CD animals paralleled the changes in complex I activity, this suggesting a possible involvement of cardiolipin in complex I dysfunction. To asses this more directly, we investigated whether addition of exogenous cardiolipin to mitochondria from CD animals was able to reverse the observed loss in complex I activity. As cardiolipin is poorly permeable to mitochondrial membranes, a previous reported method of fusion of vesicular lipids with mitochondrial membrane was used to enrich the IMM with cardiolipin [48,49]. Using this procedure, we studied the effect of fusion of mitochondria, isolated from control and CD livers, with liposomes composed of different phospholipids, such as cardiolipin, phosphatidylcholine and phosphatidylethanolamine on the activity of complex I. The results of these experiments are reported in Fig. 4. As shown above, mitochondria isolated from CD rats exhibited a 35% decline in complex I activity (see Fig. 1). This lower activity of complex I was almost completely restored to the level of control animals, following fusion of CD mitochondria with liposomes containing cardiolipin, whereas no restoration was obtained with other types of liposomes. Most notably, no restoration was

Table 1
Respiratory activities in mitochondria isolated from control and CD diet rats

Glutamate + malate	Respiratory activity (ng atoms O_2/min/mg proteins)	
	Control	CD diet
State 3	105 ± 6	75 ± 8*
State 4	20 ± 3	25 ± 4
RCR	5.25 ± 0.5	3.0 ± 0.4*
ADP/O	2.5 ± 0.2	2.4 ± 0.3
Uncoupled	110 ± 8	78 ± 8*

Mitochondrial respiratory activities were determined as described in the Experimental procedures.
* $P<0.05$ vs. control.

Table 2
Phospholipid composition in liver mitochondria isolated from control and CD diet rats

	Normal	CD diet
Cardiolipin	22.8 ± 1.3	14.3 ± 1.2*
Phosphatidylcholine	81.0 ± 2.5	56.7 ± 2.1*
Phosphatidylethanolamine	45.2 ± 2.0	33.0 ± 1.8*

For phospholipid extraction and analysis, see Experimental procedures. Values are expressed as nmol/mg protein. Each value represents the mean ± S.E. obtained from six different experiments.
*$P<0.05$.

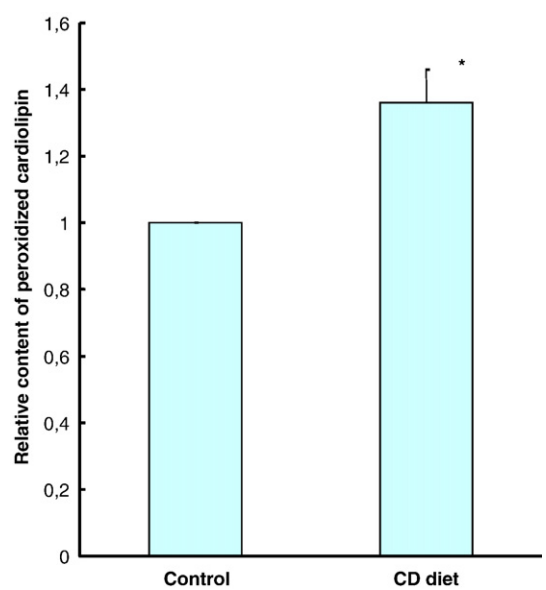

Fig. 3. Relative content of peroxidized cardiolipin in liver mitochondria isolated from control and CD diet rats. Mitochondrial content of peroxidized cardiolipin was determined by the HPLC technique described in the Experimental procedures. The content of peroxidized cardiolipin is expressed as peak area (at 235 nm) per milligram of phospholipids, and the peak area of the control is assumed as the unit. Each value represents the mean ± S.E. obtained from six different experiments. *$P<0.05$ vs. control.

obtained with liposomes containing peroxidized cardiolipin, this suggesting that integral molecules of cardiolipin are required for complex I reactivation.

It has been shown that the addition of respiratory substrates, glutamate+malate to aerobic mitochondria during NADH-stimulated state 4 respiration, generates increased amount of H_2O_2 which arises from superoxide anion formed at the level of complex I [15,50]. Accordingly, it might be expected that liver mitochondria from CD rats would generate more H_2O_2 than would normal mitochondria. Mitochondria from control and CD livers were investigated for their capacity to generate oxygen radicals in the presence of complex I substrates (glutamate+malate) in state 4 respiration. As illustrated in Fig. 5, the basal rate of H_2O_2 production was significantly enhanced in mitochondria from CD rats with respect to control animals.

4. Discussion

Dietary choline deficiency is a classical general model to induce fatty liver in rats [6,8]. The absence of choline interferes with the synthesis and/or secretion of very low-density lipoproteins and therefore blocks the transport of triglycerides outside of the hepatocytes leading to fatty liver in rats. Steatosis induced by the choline deficient diet presents close pathological and biochemical similarity with fatty liver in humans.

Reactive oxygen species have been implicated in the hepatic tissue injury associated to fatty liver [2,11,12]. Damaged mitochondrial respiratory chain is considered an important source of oxygen radicals and hence a potential contributor in the pathogenesis of NAFLD [13,51,52]. It is conceivable that mitochondrial-mediated ROS generation leads to primary reactions and damages in the immediate area surrounding where these ROS are produced, given that they are a highly reactive and short lived species. Therefore, as a major source of ROS production, mitochondria also could be the major target of ROS attack. This effect of ROS should be greatest at the level of mitochondrial membrane constituents including the complexes of the respiratory chain and phospholipid constituents rich in unsatured fatty acids, particularly cardiolipin.

In the present study we demonstrate that the activity of complex I is significantly diminished in liver mitochondria isolated from rats fed a choline-restricted diet with respect with control animals (Fig. 2). Complex I dysfunction has been already reported in mitochondria isolated from rats under dietary choline restriction [13], although the dietary paradigms used in this investigation may not be directly comparable to the CD diet used in the present study, as well as in patients with nonalcoholic

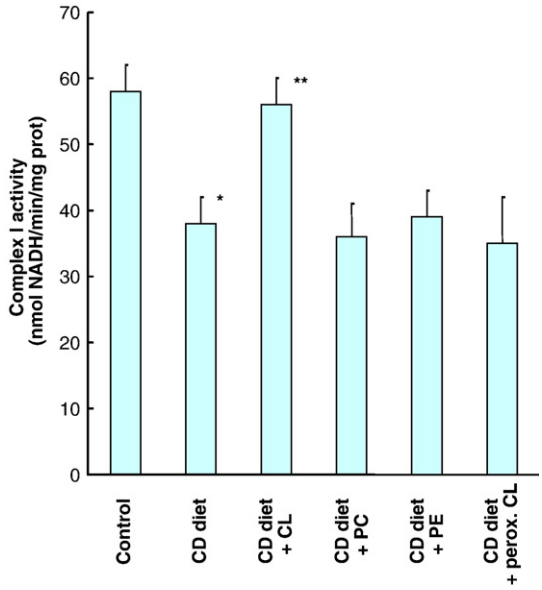

Fig. 4. Decreased complex I activity in liver mitochondria isolated from CD diet rats and specific reactivation by cardiolipin liposomes. The fusion of mitochondria with liposomes composed of PC/CL (phosphatidylcholine/cardiolipin) (4 :1 molar ratio), PC (phosphatidylcholine) and PC/PE (phosphatidylcholine/phosphatidylethanolamine) (1:1 molar ratio) liposomes was carried out as described in the Experimental procedures. Control and CD mitochondria were treated in the same manner as the liposome-treated mitochondria, but in the absence of liposomes. Each value represents the mean±S.E. of six separate experiments. *$P<0.05$ vs. control; **$P<0.05$ vs. CD diet.

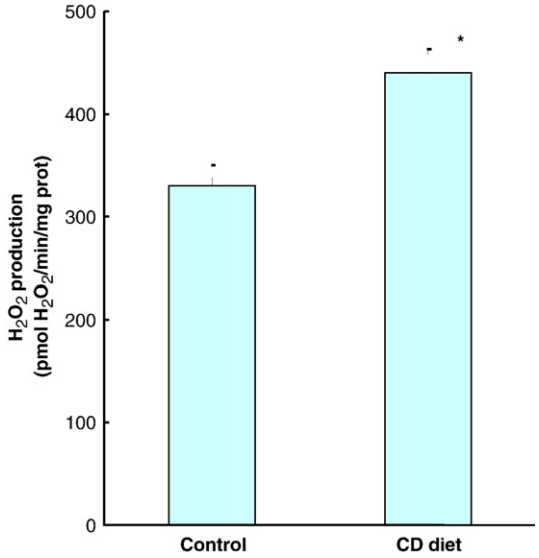

Fig. 5. H_2O_2 production in liver mitochondria isolated from control and CD diet rats. The H_2O_2 formation was induced by the addition of 5 mM glutamate+5 mM malate and measured as described in Experimental procedures. Each value represents the mean±S.E. of six different experiments. *$P<0.05$; vs. control.

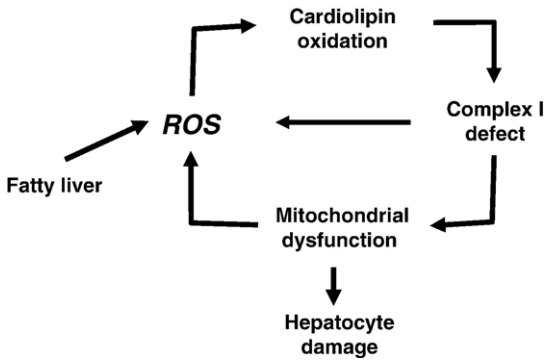

Fig. 6. Schematic diagram of the role of ROS and cardiolipin in mitochondrial complex I dysfunction and hepatocyte injury associated with fatty liver.

steatohepatitis [51]. The molecular mechanism underlying this defect in complex I has not been clearly identified.

Complex I is considered an important factor in the regulation of mitochondrial respiration. A decrease in mitochondrial complex I activity, as observed in liver mitochondria from CD diet fed rats, should be associated with a decline in mitochondrial respiration. The results reported in Table 1 demonstrate that mitochondria from CD livers exhibit lower rate of state 3 respiration compared with control animals. These changes in state 3 respiration are quantitatively related to changes in complex I activity, this suggesting that the lower complex I activity is probably the most important and rate-limiting step responsible for the alteration to the mitochondrial oxidative metabolism in fatty liver.

Complex I is considered a major source of oxygen radicals in mitochondria [15,53–55], although the mechanism responsible for this production is not well established. The lower activity of complex I can account for the enhanced production of H_2O_2 observed in mitochondria isolated from CD livers, supplemented with glutamate+malate (see Fig. 5). This finding is consistent with the results reported by others showing that dietary choline restriction causes complex I dysfunction and increased H_2O_2 generation in liver mitochondria [13].

Cardiolipin is emerging as an important factor in the regulation of mitochondrial bioenergetics in that it interacts with several vital IMM proteins, including anion carriers and respiratory chain complexes [17–19], even if its precise mechanism of action is still not well understood. It has been reported that cardiolipin is specifically required for electron transfer in complex I of the respiratory chain [33–37]. Additional evidence for the cardiolipin involvement for the complex I functioning comes from our recent finding showing that NAO (nonyl acridine orange), a compound that interacts specifically with cardiolipin, inactivates the complex I activity in SMP and that added cardiolipin fully prevented this inactivation [22].

The content of cardiolipin in the IMM may change either as a consequence of an alteration of one of the enzymatic steps involved in its biosynthetic process [56] or as a consequence of oxidative damage by ROS [20–22]. In fact, due to its high content of unsaturated fatty acids (90% represented by linoleic acid), cardiolipin molecules are particularly susceptible to peroxidative attack by ROS. Accordingly, our results demonstrate a pronounced loss in the cardiolipin content in liver mitochondria isolated from CD rats, associated with an increase in peroxidized cardiolipin content, compared with control animals (Table 2 and Fig. 3).

The changes in complex I activity observed in mitochondria from CD rats are associated to parallel changes in cardiolipin content. In addition, exogenously added cardiolipin to mitochondria from CD rats is able to almost completely restore the activity of this enzyme complex to control value (see Fig. 4). This effect of cardiolipin could not be replaced by other phospholipids nor by peroxidized cardiolipin. On this basis, it is reasonable to assume that the defect in complex I activity, observed in liver mitochondria from CD rats, can be mainly ascribed to an oxidative damage of cardiolipin molecules, which are required for the optimal functioning of this enzyme complex.

In conclusion, our results demonstrate a decline in the activity of complex I and in respiratory activity in liver mitochondria isolated from CD rats. The molecular basis of this decline seems to be, at least in part, a decrease in the mitochondrial content of cardiolipin, due to peroxidative attack of its unsaturated fatty acids by oxygen-free radicals. The activity of complex I is considered the rate-limiting step for the mitochondrial respiratory chain and therefore an important factor in the regulation of oxidative phosphorylation. This enzyme complex is also considered an important site of mitochondrial oxygen radical production. It is reasonable to assume that the impairment of complex I, observed in mitochondria from CD livers, attributable to ROS induced cardiolipin damage, may increase the electron leak from the electron transport chain, generating more superoxide radicals and perpetuating a cycle of oxygen radical-induced mitochondrial membrane damage which ultimately leads to hepatocyte injury (see Fig. 6). A likely initial source of ROS triggering this ROS-damaging cycle might be long-chain free fatty acids which accumulate in nonalcoholic fatty liver [2]. These molecules have been shown to promote ROS production in mitochondria, although the mechanism(s) underlying this effect is not fully understood [57–59]. Our results may prove useful in providing an explanation for some of the factors that lead to mitochondrial dysfunction and hence to the development of the main NAFLD lesions, as well as in providing valuable information for the development of appropriate treatment strategies.

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