Mitochondrial adaptations to steatohepatitis induced by a methionine-and choline-deficient diet

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Mitochondrial adaptations to steatohepatitis induced by a methionine- and choline-deficient diet (MCDD) model of steatohepatitis were studied in rats. Liver mitochondria from MCDD rats exhibited a higher rate of oxidative phosphorylation with various substrates, a rise in cytochrome oxidase (COX) activity, and an increased content in cytochrome aa3. This higher oxidative activity was associated with a low efficiency of the oxidative phosphorylation (ATP/O, i.e., number of ATP synthesized/natom O consumed). Addition of a low concentration of cyanide, a COX inhibitor, restored the efficiency of mitochondria from MCDD rats back to the control level. Furthermore, the relation between respiratory rate and protractive force (in the nonphosphorylating state) was shifted to the left in mitochondria from MCDD rats, with or without cyanide. These results indicated that, in MCDD rats, mitochondrial ATP synthesis efficiency was decreased in relation to both proton pump slipping at the COX level and increased proton leak although the relative contribution of each phenomenon could not be discriminated. MCDD mitochondria also showed a low reactive oxygen species production and a high lipid oxidation potential. We conclude that, in MCDD-fed rats, liver mitochondria exhibit an energy wastage that may contribute to limit steatosis and oxidative stress in this model of steatohepatitis.

nonalcoholic steatohepatitis; mitochondria; uncoupling

NONALCOHOLIC STEATOHEPATITIS (NASH) is a disease of emerging importance, associated with obesity and insulin resistance and is now considered as a common cause of chronic liver disease in the United States (3, 11, 25). There is growing evidence that obesity-related insulin resistance in skeletal muscles and adipose tissue causes hepatic lipid accumulation that is harmful to the liver. Although the reasons for the deleterious effects of fat remain unclear, steatosis has been associated with lipid peroxidation, increased tumor necrosis factor (TNF)-α expression, and finally mitochondrial dysfunctions in the successive steps of the pathogenesis of NASH (2, 39). It now appears that mitochondrial dysfunction may play a crucial role in the genesis of NASH, since mitochondria are involved in fatty acid β-oxidation and are the most important source of cellular reactive oxygen species (ROS; see Ref. 23). However, long-term consequences of steatohepatitis on mitochondrial bioenergetics are poorly documented. In patients with NASH, liver mitochondria show ultrastructural abnormalities and impaired function (9, 49). Nevertheless, because of the difficulty in obtaining sufficient tissues from liver biopsies of patients with NASH, only a few studies have directly addressed the activity and control of mitochondrial complexes of the respiratory chain, and those studies remained superficial.

To overcome the difficulty in obtaining human tissue, rodent models of steatohepatitis have been developed. Nevertheless, current models do not really reflect the human pathology linked to abdominal obesity and/or insulin resistance or are not physiological. One of the most-used models involves rats fed a methionine- and choline-deficient diet (MCDD; see Refs. 22 and 24). The lack of methionine reduces glutathione synthesis and impairs the antioxidant defense against free radical attacks. In addition, the choline deficiency impairs lipid exportation, since choline is required to form the phosphatidylcholine of very low density lipoprotein (VLDL) particles, which transport fat from liver to peripheral tissues. When the supply of choline is inadequate, VLDL cannot be synthesized, leading to a reduction in fatty acid exportation and fat accumulates in liver (27). MCDD does not perfectly mimic the pathogenesis of human NASH that is associated with obesity and insulin resistance, but the advantage of this diet (22) is that it induces progressive fibrosing steatohepatitis that is histologically similar to human NASH and thus appears as a suitable model for studying the consequences of an established steatohepatitis on liver function. A number of studies have used MCDD-fed rats to investigate the mechanism of inflammation (51, 54), which couples steatosis and oxidative stress, but none has addressed the consequences of such diet on mitochondrial bioenergetics and potential adaptations to this steatohepatitis model.
E111

MITOCHONDRIAL ADAPTATIONS TO STEATOHEPATITIS

Our aim was to investigate the bioenergetics of liver mitochondria in a dietary model of steatohepatitis in rodents. The effects of steatohepatitis induced by a long-term (6-wk) MCDD on energy metabolism of liver mitochondria and the modifications of oxidative phosphorylation were investigated in vitro.

MATERIALS AND METHODS

Animals and Diet

Our study was performed following the recommendations provided by the European Convention for the protection of Vertebrate Animals used for Experimental and Scientific purposes (Council of Europe No. 123, Strasbourg, 1985). Laboratory staff and rooms were accredited by the French ministry of agriculture (no. 692660602). Male Wistar rats, which were purchased from Charles River Laboratories (France), were housed four per cage under a 12:12-h light-dark cycle. Animals were allowed to acclimatize to their new conditions for 1 wk before the beginning of the study. From the age of 9 wk until 15 wk, rats were separated in two different groups and received either a control diet (n = 12) consisting of the usual pellet rat Chow A04 (Scientific Animal Food Engineering) or a high-fat (25% energy derived from fat) MCDD (n = 12) provided in small pellets from ICN Pharmaceuticals (no. 960439) during 6 wk. At the beginning of the study, rats from the two groups had the same weight (control: 292 ± 3 g and MCDD 293 ± 4 g). Rats had free access to food and water and were weighed every week for the duration of the study. Food intake was monitored daily. The choice of the diet and the duration of the study were based on previous studies (24, 51). For the different experiments, one control rat and one MCDD rat were studied each day.

Tissue Preparation and Biochemical Analysis

At the end of the study, animals were killed, and a small piece of liver was removed and fixed in 4% formaldehyde for histological assessment. Blood, liver, and white adipose tissue (retroperitoneal + epididymal) samples were also collected, weighed, and frozen in liquid nitrogen for later analysis. The rest of the liver was used for mitochondria isolation.

Histopathology. Histological analyses were performed in the gastroenterology service of Caen hospital, France. The liver specimens were fixed in 10% buffered formalin for 24–48 h. They were then embedded in paraffin, cut at 5 µm, and routinely stained with hematoxylin–eosin and reticulin. Severity of steatosis was evaluated using the percentage of macrovesicular fat within hepatocytes, i.e., mild (5–30%), moderate (30–60%), and severe (>60%; see Ref. 46). Necroinflammation was histologically quantified by counting inflammatory foci.

Biochemical analysis. A small piece of liver (50 mg) was hydrolyzed in ethanolic KOH for measurement of triacylglycerol (TG) content in lipid extracts (18) by a colorimetric method using a commercial kit (Biemerieux). Blood was collected from the inferior vena cava, plasma was prepared, and aliquots were stored at −80°C until analysis. TG, alanine transaminase (ALT), and aspartate transaminase (AST) were measured from plasma and assayed spectrophotometrically using a commercial kit (TG: ref 61236; ALT: ref 63312; AST: ref 63212; Biomerieux).

Mitochondria Preparation and Oxygen Consumption

Mitochondria were isolated from liver and prepared in a medium containing (in mM) 250 sucrose, 2 KH2PO4, 1 EGTA, and 20 Tris·HCl (pH 7.2). Liver mitochondria were prepared according to standard differential centrifugation procedures (12). Mitochondrial protein content was determined by the biuret method (20), with BSA as standard. For oxygen consumption measurement, mitochondria were incubated at a final concentration of 1 mg/ml in an oxygraph cuvette with a Clark electrode. All experiments, except for ROS production (30°C), were done at 37°C in the following buffer (in mM): 125 KCl, 1 EGTA, 2 KH2PO4, and 20 Tris·HCl (pH 7.2). The control (nonphosphorylating) state of respiration was initiated by the addition of different substrates (6.25 mM glutamate/1.25 mM malate or 5 mM succinate/0.5 mM malate + 1.25 µM rotenone or 100 µM octanoyl-carnitine). State 3 (phosphorylating respiration) was obtained after the addition of 1 mM ADP. Oligomycin (1.25 µg/ml proteins), a specific inhibitor of the mitochondrial ATP synthase, was then added to the mitochondrial suspension to determine the nonphosphorylating respiratory rate (state 4). The efficiency of the mitochondrial oxidative phosphorylation was assessed by the state 3-to-state 4 ratio, which measures the degree of control imposed on oxidation by phosphorylation (respiratory control ratio, RCR), in the absence of any kinetic control upstream from phosphorylation processes. All experiments were done in the presence of free fatty acid (FFA)-BSA to rid the medium of FFA that might induce an uncoupling of the oxidative phosphorylation. A titration of mitochondrial respiration with FFA-BSA was done in both groups. The minimal concentration of FFA-BSA leading to optimal RCR was determined at a final concentration of 3 mg/ml (0.3% wt/vol; data not shown).

Cytochrome c oxidase (COX) activity was measured in the buffer used for the respiration assay supplemented with 5 mM ascorbate, 1–2 mM N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD), 3.6 µM myxothiazol, and 75 µM 2,4-dinitrophenol (DNP).

The yield of oxidative phosphorylation, assessed by ATP/O ratio, was determined from the ATP synthesis rate (JATP) vs. respiratory rate (J0) with an ADP-regenerating system, based on hexokinase plus glucose. Rat liver mitochondria (1 mg/ml) were suspended in the respiration buffer, added with 20 mM glucose, 1 mM MgCl2 and 125 µM ATP. Oxygen consumption, in the presence of 5 mM succinate/0.5 mM malate plus 1.25 µM rotenone, and ATP synthesis were titrated by addition of increasing concentrations of hexokinase (0.2–2 U/ml). Mitochondrial suspension (300 µl) was withdrawn from the cuvette, quenched in HClO4 (4% vol/vol final concentration), and neutralized with 2 M KOH/0.3 M MOPS for enzymatic measures. Production of ATP was determined by monitoring glucose 6-phosphate formation (4).

Measurement of Proton Conductance in Liver Mitochondria

The respiration rate of mitochondria, in the presence of oligomycin to inhibit ATP synthesis, is proportional to the loss of energy across the mitochondrial inner membrane. The kinetic response of the proton conductance to its driving force (protonmotive force; Δp) can therefore be estimated as the relation between respiration rate and mitochondrial membrane potential if one considers the H+/O ratio to remain constant (29).

Measurement of ΔΨ with TPMP+ electrode. Respiration rate and membrane potential were determined simultaneously by using electrodes sensitive to oxygen and to the potential-dependent triphenylmethylenephosphonium cation (TPMP+). Mitochondria (1 mg/ml) were incubated at 37°C in the respiration buffer containing 5 µM rotenone, 1 µg/ml oligomycin, and 65 ng/ml nigericin (to collapse the pH difference across the mitochondrial inner membrane). The electrode was calibrated with sequential TPMP+ additions, up to 2 µM, and 4 mM succinate was added to start the reaction. Respiration and potential were gradually inhibited through successive steady states by additions of malonate up to 3 mM. At the end of each run, 0.4 µM p-trifluoromethoxyphenyldiazore was added to dissipate the membrane potential and release all TPMP+ in the buffer, allowing correction for any small electrode drift. The TPMP+ binding correction factor was previously assessed as being 0.38 (15).

Determination of matrix volume, ΔΨ, and ΔpH with labeled probes. Mitochondria (4 mg/ml) were incubated at 37°C in the same respiration buffer containing 8 mM succinate and 1 µg/ml oligomycin. Matrix volume was determined by using [3H]water and inner membrane impermeable [14C]mannitol, and ΔΨ and ΔpH by distribution

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and \(^{14}C\)TPMP and \(^{3}H\)acetate, respectively. After equilibration (3 min), mitochondria were pelleted by rapid centrifugation (12,000 g; 30 s), and, within 10 s, 100 µl of the supernatant were removed and placed in a vial containing 4 ml of a liquid scintillation cocktail (ULTIMA GOLD; Perkin-Elmer), and the remainder was aspirated. The pellet was resuspended in 300 µl of 10% HClO\(_4\) (vol/vol) and centrifuged (12,000 g; 30 s), and 260 µl of the supernatant were treated as above (15).

Respiration rates (state 4 and 3), ATP/O ratio, and ∆P were also measured in the presence of a specific inhibitor of COX, sodium cyanide (NaCN) added to the MCDD group. A titration with small amounts of NaCN was done to determine the concentration required to raise the respiration rate of MCDD mitochondria to the control level (12–17 µM).

**Mitochondrial \(H_2O_2\) Production**

The rate of released mitochondrial \(H_2O_2\) was measured at 30°C by monitoring the linear increase in fluorescence (\(\lambda_{ex} = 312\) nm and \(\lambda_{em} = 420\) nm) resulting from the oxidation of Homovanillic acid (HVA) by \(H_2O_2\) in the presence of horseradish peroxidase (HRP) with an SFM-25 fluorometer (Kontron Instruments, Dardilly, France). Reaction conditions were 0.5 mg of mitochondrial protein/ml, 6 U/ml HRP, 0.1 mM HVA, 5 mM succinate, and 0.3% FFA-BSA (wt/vol) in the respiration buffer. Known concentrations of \(H_2O_2\) were used to establish the standard concentration curve.

**Mitochondrial Cytochrome Content**

The different cytochromes of the mitochondrial respiratory chain were measured by dual-wavelength spectrophotometry at 30°C by comparing the spectra of fully oxidized vs. fully reduced cytochromes (55). In each of the two cuvettes, 5 mg of mitochondrial protein, 1 ml of 0.1 M sodium phosphate, pH 7.4, and 0.01% (vol/vol) Triton X-100 were added. In the “oxidized” cuvette, 10 µl of ferricyanide (0.5 M) were added while a few grains of sodium hydrosulfite were added in the “reduced” cuvette. The components were mixed, and the spectrums of both cuvettes were recorded. A typical difference between the “reduced” and “oxidized” cuvette was obtained. Wavelength pairs and absorbance coefficient used were: cytochrome \(a_3\) (550–540 nm) \(\varepsilon = 18\) M\(^{-1}\)cm\(^{-1}\), cytochrome \(b\) (563–575 nm) \(\varepsilon = 18\) M\(^{-1}\)cm\(^{-1}\) and cytochrome \(a + a_1\) (605–630 nm) \(\varepsilon = 24\) M\(^{-1}\)cm\(^{-1}\).

**Antioxidant Enzyme Activities**

A portion of frozen liver was homogenized with a potter Elvehjem, at 4°C, in a buffer containing (in mM) 100 KH\(_2\)PO\(_4\), 1 dithiothreitol, and 2 EDTA, pH 7.4. After centrifugation (3,000 g for 5 min), the supernatant was used for enzymatic assays. Superoxide dismutase (SOD) activity was assayed by monitoring the rate of acetylated cytochrome \(c\) reduction by superoxide radicals generated by the xanthine-xanthine oxidase system (16). One activity unit of SOD was defined as the amount of enzyme that inhibited the rate of acetylated cytochrome \(c\) reduction by 50%. To distinguish manganese SOD, exclusively located in the mitochondrial matrix, from copper/zinc SOD, which is primarily located in the cytosol, SOD activity was determined after incubation with NaCN (1 mM). At this concentration, cyanide inhibited the copper/zinc isofrom of the enzyme but did not affect the manganese isofrom (16). The assay for total activity of glutathione peroxidase (Gpx) coupled the reduction of cumene hydroperoxide to the oxidation of NADPH by glutathione reductase, and this coupled reaction was monitored at 340 nm (53). The activity of catalase (CAT) was determined by the method of Aebi (1). This technique uses the first-order rate constant of the decomposition of H\(_2O_2\) by tissue CAT at 20°C. One unit of CAT activity was calculated by using \(k = (2.3/dt)/\log A_1/A_2\), where \(k\) is CAT activity, \(dt\) is change in time, \(A_1\) is initial absorbance, and \(A_2\) is final absorbance. All enzyme activities are expressed as units per milligram protein.

**Citrate Synthase Activity**

Citrate synthase activity was used as a mitochondrial enzymatic marker. Liver homogenates were added to a buffer with 100 mM Tris-HCl, 1 mM 5,5'-dithiobis-2-nitrobenzoic acid, 3 mM acetyl-CoA, pH 8, containing 0.2% (vol/vol) Triton X-100. The reaction was started by the addition of 1 mM oxaloacetate, and the initial rate was measured following the decrease of absorbance at 420 nm (50). All enzyme activities are expressed as units per minute per milligram protein. No significant variation in the activity of citrate synthase was found between preparations from control and MCDD livers (53 ± 4 vs. 49 ± 2 U min\(^{-1}\) mg protein\(^{-1}\)).

**RNA Extraction and RT-PCR**

Total RNA was extracted from liver samples (80 mg) of five control and five MCDD-fed rats using TRIzol (Invitrogen, Cergy Pontoise, France). The relative abundance of target mRNA for interleukin-6 (IL-6), TNF-α, and TNF receptor 1 (TNF-R) was measured by semiquantitative RT-PCR using cyclophilin as reference. Total RNA (1 µg) was reverse-transcribed for 1 h at 42°C using Moloney murine leukemia virus reverse transcriptase (Promega, Charbonnières, France) and oligo(dT) primer following the manufacturer’s protocol. PCR reactions were carried out with 2.5 µl of cDNA as template for EurobioTaq DNA polymerase (Eurobio, Courtaboeuf, France) as described by the manufacturer. PCR was performed for 23, 36, 32, and 27 cycles with primers specific of cyclophilin, IL-6, TNF-α, and TNF-R. Each cycle consisted in 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and the complete sequence was followed by a final elongation step of 10 min at 72°C. PCR primer pairs were designed to be functionally active at 60°C annealing temperature. Products were analyzed on 1.5% agarose gels prestained with ethidium bromide. For quantitation of relative bands intensities, pictures were taken with a Camera DC120 (Kodak), and the ratio of each target was determined with Kodak Digital Science 1D 2.0 (Kodak Scientific Imaging System). The extent of target gene expression was given as a relative unit, which was defined as the quotient of target gene expression vs. cyclophilin gene expression.

**Statistical Analysis**

All data were expressed as mean values ± SE. Statistical comparisons were done using ANOVA followed by Fischer’s test to determine difference between the standard and MCDD group. Correlations were assessed by linear regression.

**RESULTS**

**Effects of MCDD on Rat Physiological Characteristics**

After 6 wk of diet, MCDD-fed rats weighed less than controls (Table 1). Most of the weight loss occurred during the first 2 wk of diet (data not shown). Such a loss of weight is a common feature of the MCDD (24, 51). The estimated daily caloric intake per rat followed the same profile as body weight (Table 1). However, when expressed per 100 g body wt, the caloric intake was identical for the two groups (16–17 kcal/100 g day\(^{-1}\)). Apart from weight loss, the general condition of the animals under MCDD remained satisfactory as described by Kirsch et al. (24).

To understand the difference in body mass between the two groups, different tissues were taken and weighed, and, to take into account those differences in body mass, tissue mass was expressed per 100 g of body mass. MCDD-fed rats showed a 60% reduction in white adipose tissue relative mass compared with the control group, whereas liver relative mass was in-
creased by 34% (Table 1). Six weeks of diet affected lipid distribution. Indeed, although peripheral fat depots were reduced, hepatic TG content (Table 1) was increased markedly in MCDD rats, i.e., 476 ± 14 vs. 6.4 ± 0.5 μmol/g in MCDD and control rats, respectively (P < 0.001). In contrast, plasma TG content was reduced significantly in MCDD rats, i.e., 0.3 ± 0.1 vs. 2 ± 0.3 μmol/g (P < 0.001, Table 1). Macroscopically, the liver from MCDD rats appeared pale and fatty compared with controls. Histological examination (Fig. 1) revealed a severe macrovesicular steatosis, affecting >60% of MCDD rat hepatocytes, and inflammatory sites with the presence of microgranulomas of macrophages. Necrosis was a minor feature while fibrosis was absent.

Semiquantitative RT-PCR was used to investigate the expression in liver of a number of genes known to be upregulated during inflammation. The relative abundance of mRNA encoding IL-6, TNF-α, and TNF-R was higher, i.e., +437, +212, and +46%, respectively (P < 0.01), in MCDD than in control rat liver (Fig. 2). This finding confirmed the results of our histological study and the occurrence of inflammation in MCDD rat liver. Moreover, plasma ALT and AST levels, the markers of liver cell damage, were significantly higher in MCDD than in control rats (Table 1).
it is well known that a proton leak effect induced by a protonophore addition increases the respiratory rate in response to a decrease in $\Delta p$ (6, 28). Some degree of redox slipping can therefore not be excluded.

COX, the last component of the respiratory chain, is well recognized as a controlling step in nonphosphorylating oxygen consumption (40) and as a site of “energy wastage” by redox slipping (10, 35). Maximal mitochondrial COX activity was evaluated by an uncoupled respiratory rate (+DNP) with TMPD-ascorbate, an electron donor to COX, as substrate. In MCDD rat mitochondria, maximal COX activity was higher than in controls, i.e., +54% (Table 2), which was consistent with a higher content in cytochrome $aa_3$ (+49%; $P < 0.05$; Table 4). From our finding, it is clear that the modification in mitochondria oxidative phosphorylation is linked to an increase in the COX content in MCDD mitochondria. This finding and various data from the literature support a relation between COX content (33) and the altered efficiency of the respiratory chain in MCDD mitochondria.

### Table 2. Oxygen consumption from isolated liver mitochondria

<table>
<thead>
<tr>
<th></th>
<th>Glutamate/Malate</th>
<th>Succinate/Malate</th>
<th>TMPD/Ascorbate + DNP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State 4</td>
<td>State 3</td>
<td>RCR</td>
</tr>
<tr>
<td>Control</td>
<td>9±1</td>
<td>75±7</td>
<td>8.6±1.0</td>
</tr>
<tr>
<td>MCDD</td>
<td>14±1*</td>
<td>108±9*</td>
<td>7.9±0.5</td>
</tr>
</tbody>
</table>

Data are means ± SE, $n = 10$ for oxygen consumption and $n = 6$ for TMPD/ascorbate. Rat liver mitochondria (1 mg/ml) isolated from control or MCDD animals were incubated at 37°C in a respiratory medium, and oxygen consumption was measured with glutamate/malate (6.25/1.25 mM) or succinate/malate (5/0.5 mM) plus 1.25 μM rotenone and 1.25 μg/mg protein oligomycin (state 4), as described in MATERIALS AND METHODS. State 3 respirations were obtained in absence of oligomycin after addition of 1 mM ADP (phosphorylating respiration). The maximal activity of the cytochrome oxidase was assessed by addition of $N,N,N,N'$-tetramethyl-1-p-phenylenediamine (TMPD, 1 mM) plus ascorbate (5 mM) plus myxothiazol (3.6 μM) plus 2,4-dinitrophenol (DNP, 75 μM). RCR, respiratory control ratio. *$P < 0.01$ vs. controls.
To further investigate the role of COX activity in the increased state 4 respiratory rate of the MCDD mitochondria, we measured the ATP/O ratio (Fig. 4A) in the presence of NaCN (12–17 μM), a specific inhibitor of COX. With a quantity of NaCN sufficient to bring the respiratory rate of MCDD mitochondria up to the control level, the relation between ATP synthesis and oxygen consumption (ATP/O) was measured in rat liver mitochondria (1 mg/ml) suspended in the following buffer (in mM): 125 KCl, 1 EGTA, 2 KH₂PO₄, and 20 Tris-HCl (pH 7.2–7.3) supplemented with 5 mM succinate, 0.5 mM malate, 1.25 μM rotenone, 20 mM glucose, 125 μM ATP, and 1 mM MgCl₂. Oxygen consumption and ATP synthesis were titrated by addition of increasing concentrations of hexokinase (0.2–2 U/ml). ATP production was monitored by glucose 6-phosphate formation, which was measured enzymatically. ATP synthesis were titrated by addition of increasing concentrations of hexokinase (0.2–2 U/ml). ATP production was monitored by glucose 6-phosphate formation, which was measured enzymatically. ATP production was monitored by glucose 6-phosphate formation, which was measured enzymatically.

Table 3. Matrix volume and protonmotive force in isolated mitochondria of control and MCDD rats

<table>
<thead>
<tr>
<th>Matrix Volume, μl/mg protein</th>
<th>Δψ, mV</th>
<th>ΔpH, mV</th>
<th>Δp with TPMP⁺ Electrode, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.30±0.04</td>
<td>156±7</td>
<td>48±1</td>
</tr>
<tr>
<td>MCDD</td>
<td>0.33±0.03</td>
<td>156±6</td>
<td>49±1</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE; n = 5 rats in each group. Rat liver mitochondria isolated from control or MCDD animals were incubated at 37°C in a respiratory medium supplemented with 8 mM succinate plus 5 μM rotenone. Protonmotive force (Δp) measurements were performed either with triphenylmethylphosphonium (TPMP⁺) or with labeled probes (Δψ and ΔpH). Matrix space was determined by using [³H]water and [¹⁴C]mannitol, Δψ and ΔpH by distribution of [¹⁴C]TPMP⁺ and [³H]acetate.

Functional Consequences of the MCDD-induced Mitochondrial Uncoupling

To investigate the potential consequences of the alteration of mitochondrial energetics induced by MCDD, we investigated the ability of MCDD mitochondria to use lipids as substrates. With octanoyl-carnitine (100 μM), both state 4 (+40%; P < 0.01) and state 3 (+37%; P < 0.01) respiratory rates were increased in MCDD rat mitochondria compared with control mitochondria (Table 5).

Mitochondria are well recognized as one of the sites of ROS production. This production depends on many factors such as respiratory rate, redox status of the respiratory complexes, and membrane potential (26). We therefore studied the consequences of the MCDD-induced loss in mitochondria efficiency on H₂O₂ production. When expressed in picomoles H₂O₂ per minute per milligram mitochondrial proteins (Table 5), H₂O₂ production was higher in control mitochondria than in those isolated from MCDD rats (+42%). H₂O₂ production and O₂ consumption were measured in the same conditions (buffer, substrate concentration, temperature) that allowed the calculation of the fraction of O₂ turned into H₂O₂ instead of being reduced into water. H₂O₂ release per oxygen consumed was 42% lower in the MCDD group than in controls (Table 5). In this experiment, ROS measurement reflected the balance between mitochondrial H₂O₂ production and elimination by antioxidan systems. That the activities of GPx and mitochondrial SOD were either lowered or unchanged in MCDD rats (Table
5) ruled out the possibility that the drop in H2O2 production measured in MCDD mitochondria was associated with a more effective antioxidant system. The decreased mitochondrial H2O2 production in MCDD rats could rather be linked to the diminished mitochondrial efficiency due to the uncoupling of the respiratory chain.

**DISCUSSION**

The present study has established that, in rats fed a MCDD showing typical hepatic lesions of steatohepatitis, the bioenergetics of liver mitochondria was markedly affected, leading to increased respiratory rate with various substrates and decreased efficiency of the respiratory chain within mitochondria and an increased respiratory activity when steatohepatitis is established for a sufficiently long period of time.

The present results showed for the first time that the increased oxidative activity of MCDD-fed rat liver mitochondria was associated with decreased oxidative phosphorylation yield. Different mechanisms could contribute to this modulation of oxygen consumption: an increase in mitochondrial quantity and/or a change in the cytochrome content of the respiratory chain. In this study, MCDD livers present no mitochondrial proliferation (unchanged citrate synthase activity), but results clearly show a drop in mitochondrial oxidative phosphorylation yield, with a parallel shift to the right hand of the ATP/O relation in liver of MCDD-fed rat mitochondria. The control mitochondria efficiency was recovered in MCDD mitochondria with a small quantity of NaCN, suggesting an implication of the COX in this energy wastage. In the same time, in both groups (control and MCDD), the relation between J02 and ΔΨ was associated with decreased oxidative phosphorylation yield.

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5. Table 5. Liver fatty acid oxidation and antioxidant enzyme activities

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MCDD</th>
</tr>
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<tbody>
<tr>
<td>Octanoyl-carnitine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>State 4</td>
<td>10±1</td>
<td>14±1*</td>
</tr>
<tr>
<td>State 3</td>
<td>35±2</td>
<td>48±3*</td>
</tr>
<tr>
<td>H2O2 production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pmol H2O2/mg protein</td>
<td>138±22</td>
<td>97±9*</td>
</tr>
<tr>
<td>pmol H2O2/natom O^-</td>
<td>12±1</td>
<td>7±1*</td>
</tr>
<tr>
<td>CAT</td>
<td>0.55±0.01</td>
<td>0.33±0.02*</td>
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<tr>
<td>GPx</td>
<td>0.29±0.01</td>
<td>0.13±0.01*</td>
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<tr>
<td>SOD</td>
<td></td>
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</tr>
<tr>
<td>Mitochondrial</td>
<td>6.0±0.4</td>
<td>5.8±0.5</td>
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<tr>
<td>Cytosolic</td>
<td>19.6±1.7</td>
<td>12.4±1.4*</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE. SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase. *P < 0.05 vs. controls.
discriminate which of the redox slipping and/or proton leak is implied in this oxidative phosphorylation variation, we can assume the strong implication of the COX in the modification of the mitochondrial oxidative phosphorylation yield in MCDD rats.

Whatever the mechanism (redox slipping or proton leak) involved in energetic wastage, it is clear from this study that the increase in COX content plays a key role in the adaptative process developed in MCDD mitochondria. Indeed, 1) both COX activity and cytochrome aa₃ amount were increased by 54% and 2) the addition of a small amount of NaN₃ on MCDD mitochondria restored both control state 4 respiratory rate and oxidative phosphorylation yield. These observations in MCDD rats were supported by previous studies reporting a relation between a modified oxidative capacity and a change in mitochondrial efficiency (17, 43, 44). Indeed, when plotting the values of mitochondria nonphosphorylating respiratory rate (liver or heart), reflecting energy wastage, against the amount of cytochrome aa₃, a component of COX, obtained in the present study and in previous studies using different models [hypothyroidism, polyunsaturated fatty acid deficiency, alcohol ingestion (30, 32, 37, 43)], we found a close linear correlation ($r^2 = 0.89; P < 0.0001$, Fig. 5). Such relation supports a causal relation between COX content and the yield of oxidative phosphorylation already proposed by other authors.

However, it is important to emphasize that the Δp in state 4 was not completely restored in MCDD mitochondria treated with NaN₃, indicating some contribution of a proton leak. This last point was reinforced by a previous study showing a slight but significant increase in uncoupling protein (UCP) 2 mRNA in MCDD rat liver (51). Indeed, UCPs form a subfamily within the mitochondrial anion carrier protein family, which resides in the inner mitochondrial membrane and catalyzes a proton conductance that dissipates the proton electrochemical gradient built up by the respiratory chain and then partially uncouples electron transport from ATP synthesis. In our study, no significant change in UCP2 mRNA was observed (data not shown). Another factor that could contribute to proton leak was the mitochondrial membrane composition. Indeed, many studies demonstrated that a modification in phospholipids can lead to increased membrane fluidity (8, 45) and mitochondrial enzyme activity (37, 38).

Our data showed a decreased H₂O₂ production in the MCDD liver mitochondria, whereas antioxidant enzyme activities (SOD, CAT, GPx) in liver homogenate were either decreased or not changed in MCDD rats compared with controls. Given that mitochondria are the most important source of ROS (23), this finding somehow contradicts the general idea that, with the establishment of steatosis, hepatocytes and their mitochondria face a new environment rich in lipids, a situation favoring increased oxidative stress. Such results may indicate that, in our rodent model of steatohepatitis, adaptive mechanisms have developed within liver mitochondria to limit oxidative stress and its deleterious consequences. We have identified in MCDD rats two adaptative mechanisms leading to a decrease in ROS production by mitochondria. First, the increase in cytochrome aa₃ and c decreases the kinetics constraint for electron transport trough complex I and III. As a consequence, the redox state of the electron carriers decreases and thus the probability to generate superoxides decreases (36). Second, it is well known that increased redox slipping at the COX level of the respiratory chain and proton leak across the inner mitochondrial membrane also contribute to limited ROS production (7). These two mechanisms may also have beneficial effect on the fatty liver of MCDD rats. Indeed, both mechanisms would contribute to a stimulation of the mitochondrial oxygen consumption and a drop in the NADH/NAD ratio, two phenomena known to activate the β-oxidation of lipids (19). Hence, the higher capacity of MCDD rat liver mitochondria to oxidize octanoyl-carnitine (present study) may indicate an increased capacity of the liver to use fatty acids as respiratory substrates. This higher capacity to oxidize lipids was previously described by Rizki (48) on mice receiving MCDD. This would in turn help fatty liver of long-term MCDD rats to limit steatosis.

In conclusion, after 6 wk of MCDD, rats develop a steatohepatitis with steatosis and inflammation. Our results provide evidence that, in MCDD rats, adaptations of mitochondrial bioenergetics occur in liver. Hepatic mitochondria exhibit a small decrease in efficiency of energy transfer and an increase in the COX content. Consequently, both a decrease in H₂O₂ production and a stimulation of oxygen consumption with fatty acids as respiratory substrates were observed. Even if the MCDD model presents steatosis and inflammation, it does not perfectly reflect human pathology. Indeed, human NASH is linked to obesity and insulin resistance, features that are not found in MCDD rats. It appears that it remains necessary to find a more physiological model that reflects human NASH pathology and the associated factors, such as obesity, dyslipidemia, and

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**Fig. 5.** Correlation between cytochrome aa₃ content and state 4 $J_0$, in mitochondria. Oxygen consumption rates measured in the presence of oligomycin were collected from different studies using different models of altered hepatic function (hypothyroidism, polyunsaturated fatty acid deficiency, alcohol ingestion; see Refs. 32, 43, 44) or altered heart function (hypothyroidism; see Refs. 30 and 37) and plotted with data obtained in the present study. Respiratory chain cytochrome aa₃ concentration was measured as described in MATERIALS AND METHODS and expressed as percentage of controls. The correlation was assessed by a linear regression with $r^2 = 0.89; P < 0.0001$. 

AJP-Endocrinol Metab • VOL 294 • JANUARY 2008 • www.ajpendo.org
insulin resistance. Nevertheless, it results from this study that mitochondria could become a new therapeutic target of steatohepatitis since mitochondrial modifications could limit oxidative stress and lipid accumulation.

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