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Dietary fatty acids modulate liver mitochondrial cardiolipin content and its fatty acid composition in rats with non alcoholic fatty liver disease

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Abstract No data are reported on changes in mitochondrial membrane phospholipids in non-alcoholic fatty liver disease. We determined the content of mitochondrial membrane phospholipids from rats with non alcoholic liver steatosis, with a particular attention for cardiolipin (CL) content and its fatty acid composition, and their relation with the activity of the mitochondrial respiratory chain complexes. Different dietary fatty acid patterns leading to steatosis were explored. With high-fat diet, moderate macrosteatosis was observed and the liver mitochondrial phospholipid class distribution and CL fatty acids composition were modified. Indeed, both CL content and its C18:2n-6 content were increased with liver steatosis. Moreover, mitochondrial ATP synthase activity was positively correlated to the total CL content in liver phospholipid and to CL C18:2n-6 content while other complexes activity were negatively correlated to total CL content and/or CL C18:2n-6 content of liver mitochondria. The lard-rich diet increased liver CL synthase gene expression while the fish oil-rich diet increased the (n-3) polyunsaturated fatty acids content in CL. Thus, the diet may be a significant determinant of both the phospholipid class content and the fatty acid composition of liver mitochondrial membrane, and the activities of some of the respiratory chain complex enzymes may be influenced by dietary lipid amount in particular via modification of the CL content and fatty acid composition in phospholipid.

Keywords Mitochondria · Phospholipids · Cardiolipin · Membrane · Steatosis · Rats · High lipid diet · Complex activity

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Introduction

The incidence of obesity and insulin resistance is increasing rapidly in wealthy societies, and has become a major public health problem (Barness et al. 2007; Chiang et al. 2011). These are major risk factors for non-alcoholic fatty liver disease (NAFLD), the most common liver disorder worldwide (Chiang et al. 2011; Erickson 2009). The first stage of NAFLD is the accumulation of lipids in the hepatocytes (steatosis); lipid accumulation in the liver can occur from *de novo* lipogenesis, altered fatty acid oxidation and/or circulating fatty acids released from adipose tissue or from the diet (Gentile et al. 2011). Oxidative stress, mitochondrial dysfunction and insulin resistance may lead to large hepatocyte injury, inflammation and fibrosis, that represent the second stage of NAFLD (steatohepatitis/cirrhosis). As mitochondria are both a site for fat metabolism and the main

source of ROS/RNS in hepatocytes, mitochondrial dysfunction is postulated to play a central role in this process (Mantena et al. 2008).

Fatty acid composition of membrane lipids greatly influences membrane functions (Hulbert et al. 2005) and changes in the fatty acid composition of the diet can affect the fatty acid composition of membrane lipids. In mitochondria, phospholipid composition varies little among different cells, suggesting a great adaptation capacity for major changes in lipid intake (Osman et al. 2011). Cardiolipin (CL), a specific phospholipid of the mitochondrial inner membrane, is required for the optimal activity of numerous key mitochondrial enzymes and proteins involved in cellular energy metabolism, such as cytochrome c oxydase, ATP synthase or ADP/ATP carrier (Chicco and Sparagna 2007). Because of its central role in mitochondrial bioenergetics, especially those related to oxidative phosphorylation and coupled respiration, alterations of CL content and/or acyl chain composition may result in mitochondrial dysfunction, alterations in energy metabolism and ROS production (Paradies et al. 2010). Indeed, alterations in the content and/or composition of CL have been well documented in multiple tissues and in a variety of pathological settings associated with mitochondrial dysfunction, such as aging, heart failure and diabetes (Chicco and Sparagna 2007; Han et al. 2007). However, to our knowledge, no data were reported on changes in mitochondria phospholipids, particularly CL, in NAFLD, except in choline-deficient rats in whom a decrease of CL content was described (Petrosillo et al. 2007).

The present study was thus designed to determine the effect of different patterns of dietary lipids on the content of mitochondrial membrane phospholipids from rats with non alcoholic fatty liver, with a particular attention for CL content and its fatty acids composition. As not only the quantity but also the quality of fatty acids in the diet may influence the phospholipid content and fatty acid composition, different dietary fatty acid patterns were explored. Moreover, mitochondrial respiratory chain activities were also investigated and the impact of modulation of CL content and fatty acid composition on these activities was explored.

Materials and methods

Animals and diets

Forty eight male WISTAR rats (Charles River, L'Arbresle, France) aged 6 weeks were used in this study. Rats were housed under conditions of constant temperature (20–22 °C), humidity (45–50 %) and a standard dark cycle (20.00–08.00 h). The rats were randomized into six groups of 8 animals and fed for 12 week one of the following semi-purified diets: basal diet with 5 % or 30 % fat, lard-rich diet with 5 % or 30 % fat and fish oil-rich diet with 5 % or 30 % fat. The detailed composition of these six diets is given in Table 1. The detailed fatty acid composition of these diets is given in a supplemental table 1. Rats were given free access to distilled water and food. Body growth and diet consumption were determined weekly, and energy intake was calculated. Our institution guidelines for the care and use of laboratory animals were observed and all experimental procedures were approved by the local ethical committee in Montpellier, France (Reference CEEA-LR-11009).

Sampling and routine biochemical analysis

Blood was obtained from 16 h fasted rats anaesthetised with pentobarbital (Ceva Santé Animale, Libourne, Fr) by puncture in the abdominal vein with a heparinized syringe (Sodium heparinate, Panpharma SA Fougères, Fr). Blood was then distributed into a heparinized tube (6–8 ml) and centrifuged at 1,000 g for 10 min at 4 °C, and plasma was collected and stored at −80 °C until analysis. Liver was quickly removed, rinsed with 0.9 % NaCl solution and frozen in liquid nitrogen and kept at −80 °C.

Plasma glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride and free fatty acid concentrations were measured by enzymatic techniques (Konelab, Thermo Electron Corp., Vantaa, Finland). Plasma ALAT (alanine aminotransferase) and gamma-GT (gamma-glutamyl transferase) were determined using standard methods on Cobas MIRA (RocheDiagnostics, Basel, Switzerland) automated analyzer. Plasma insulin and leptin levels were quantified with ELISA kits (Linco Research, St Charles, Missouri, USA). Protein level in tissue homogenate was measured by Bradford's technique (Bradford 1976).

Oral glucose tolerance test (OGTT)

After 16 h of starvation, a 40 % glucose solution was given orally to the animals (2 g/kg). Blood was collected through a small puncture on the tail immediately prior to the gavage, as well as 10, 20, 30, 45, 60, 90, 120 and 180 min afterward. At each time point, glucose was measured using a glucometer (AccuChek Active, Roche Diagnostics, USA). The AUC values were calculated by the trapezium method (Jenkins et al. 1985).

Histological analysis

For microscopic studies, liver samples were fixed in 10 % neutral buffered formalin and embedded in paraffin. Serial tissue sections (5 μm) were processed. Liver injury, such as steatosis, portal inflammatory infiltrate and fibrosis was

Table 1 Macronutrient diet composition

	5 % fat diets			30 % fat diets		
	Basal diet	Lard-rich diet	Fish-oil rich diet	Basal diet	Lard-rich diet	Fish-oil rich diet
Energy, Kcal/g (KJ/g)	3.87 (16.2)	3.87 (16.2)	3.87 (16.2)	5.12 (21.4)	5.12 (21.4)	5.12 (21.4)
Casein, g	200	200	200	200	200	200
Cornstarch, g	420	420	420	254	254	254
Maltodextrin, g	131.5	131.5	131.5	84	84	84
Sucrose, g	100	100	100	63.5	63.5	63.5
Lard, g	20	45	17.5	120	270	105
Sunflower oil, g	12.5	5	10	75	30	60
Olive oil, g	12.5	0	10	75	0	60
Colza oil, g	5	0	2.5	30	0	15
Tuna oil[a], g	0	0	6	0	0	36
Sardine oil[b], g	0	0	4	0	0	24
Cellulose, g	50	50	50	50	50	50
Mineral mix (AIN-93)[c],g	35	35	35	35	35	35
Vitamin mix (AIN-93)[c], g	10	10	10	10	10	10
L-Cystine, g	3	3	3	3	3	3
Choline Chloride, g	0.5	0.5	0.5	0.5	0.5	0.5

[a] Tuna oil (OMEGAVIE® Tuna oil 25 % DHA, purchased from Polaris, France)

[b] Sardine oil (OMEGAVIE® 1812 TG purchased from Polaris, France)

[c] AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. Reeves PG et al. J Nutr. 1993 Nov;123(11):1939–51

evaluated by histological examination after hematoxylin and eosin staining.

Liver mRNA expression

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR) was used to measure cardiolipin synthase (CLSyn) mRNA expression in liver. Total RNA was extracted with Trizol reagent (Invitrogen Life Technologies, Cergy Pontoise, France). Reverse transcription reaction was performed with 5 μg total RNA. cDNA was synthesized with the use of SuperScript II Reverse Transcriptase for first strand cDNA synthesis (Invitrogen Life Technologies, Cergy Pontoise, France) and Oligo (dT) primers. The mRNA expressions of CLSyn were determined by RT-qPCR. RT-qPCR analysis was performed using IQ™ SYBR Green Supermix (Biorad, Hercules, CA, USA) with a MiniOpticon detection system (Biorad, Hercules, CA, USA). Results were normalized with the gene encoding 18S used as the reference. The primer sequences used for real-time RT-PCR are for CLSyn : forward TGG ATG GAT TTA TTG CTC GAA A; reverse TGG GAC TGG AAT AAG ATC TGC AT and for 18S :forward GAG GTG AAA TTC TTG GAC CGG; reverse CGA ACC TCC GAC TTT CGT TCT. After normalization by 18S, results were expressed as percent of control as means ± SD.

Liver lipid extraction and analysis

Liver samples were homogenized in NaCl (9 g/L) and Triton X-100 (0.1 %), using an UltraTurax homogenizer and lipids were extracted from the liver homogenate using the method of Folch et al. (Folch et al. 1957).

Free fatty acids, triglycerides and total cholesterol were quantified on the liver homogenate by enzymatic colorimetric methods (Wako-NEFA-C kit, Oxoid, Dardilly, France; Cholesterol RTU kit, Biomerieux, Lyon, France; TG PAP kit, Biomerieux, Lyon, France) whereas phosphorus was quantified on the chloroform/methanol homogenate in order to determine total phospholipid quantity as previously described (Bartlett 1959).

Mitochondria isolation

Mitochondria were isolated from frozen liver samples by differential centrifugation as described (Frezza et al. 2007). Briefly, a sample of about 4 g of liver was homogenized on ice in a ratio of 1 g wet tissue for 10 volumes of sucrose buffer (Sucrose 0.25 M, Tris base 10 mM, EDTA 0.5 mM, pH 7.5) using a Polytron homogeniser. The homogenate was centrifuged (refrigerated BECKMAN, Rotor JA17) at 1,000 g for 10 min. The pellet was discarded and the supernatant was centrifuged at 10,000 g for 10 min. The obtained

mitochondrial pellet was suspended in 2 ml of sucrose buffer. As the mitochondrial suspension may be contaminated by microsomal or other organelles membranes (Green et al. 1968), isolated mitochondria were purified as described with some modifications (Lansman et al. 1981). The mitochondrial suspension was layered over two performed layers of special discontinuous gradients of sucrose in 30 ml centrifuge tubes (UltraClear, Beckman). The bottom layer in the discontinuous gradient consisted of 10 ml of 1.75 M sucrose (sucrose 1.75 M, Tris Base 10 mM and Na_2EDTA 5 mM) and the upper layer was formed by 20 ml of 1.0 M sucrose (sucrose 1.0 M, Tris Base 10 mM and Na_2EDTA 5 mM). Centrifugation (BECKMAN COULTER, Rotor SW32) of the gradient was at 110 000 g for 30 min, in order to produce two distinct bands of material. Using a 10-ml syringe, the fraction accumulating near the interface of the lower two layers was collected and transferred to a 50 ml tube in order to extract total lipids of mitochondria. This fraction is therefore considered to be enriched in mitochondria. The efficiency of mitochondrial purification conditions was evaluated by following the activity of cytochrome c oxidase (complex IV) and citrate synthase (data not shown), and by determining the total phospholipid quantity in this fraction. All steps and centrifugations for mitochondrial preparation were performed on ice or at 4 °C.

Mitochondria lipid extraction

Total lipids for thin layer chromatography (TLC) were extracted from the mitochondrial suspension with chloroform/methanol 2:1 (v/v) according to Folch et al. (Folch et al. 1957) in the presence of butylated hydroxytoluen (BHT; 50 mg/L). Total phosphorus was quantified on the chloroform/methanol homogenate in order to determine total phospholipids quantity as previously described (Bartlett 1959).

The chloroform/methanol lipid extract was used for polar lipids separation in order to analyze the fatty acid composition of each lipid class by gas chromatography after transesterification.

Mitochondrial phospholipid analysis (content and fatty acids composition)

Mitochondrial lipid extracts were applied onto silica gel plates (60A°, 0.25 mm 20*20 cm, Whatmann) treated with 3 % boric acid, using an automated sampler (Camag automatic TLC sampler, Linomat 4). Phospholipids and neutral lipids were separated using methanol/acetic acid/pentane/chloroform (20/15/30/40, by volume) as the solvent. Spots were visualized by spraying the plates with 2′,7′-dichlorofluorescein and viewing under ultraviolet light. Different classes of phospholipids (SM, LPC, PC, PE, PI, PS, CL, PA, PG), and neutral lipids were identified by comparing their mobilities to authentic standards. Appropriate areas were scrapped off for phospholipids quantification and fatty acid determination.

For determination of different phospholipid concentrations, silica-gel scrapings from the TLC plates were transferred to boiling tubes and the phospholipids were mineralized with sulfuric acid (H_2SO_4). The phosphorus was measured as previously described (Bartlett 1959). The values were corrected for gel blanks which were treated similarly. Recovery of phospholipids phosphorus from the chromatograms averaged 80–90 %.

For fatty acid analysis of phospholipids, the content of the silica-gel scrapings were transmethylated with 3 mL of KOH/MeOH in pyrex tubes, after the addition of an internal standard solution (providing 2.6 mg triheptadecanoylglycerol/ml), to prepare the methyl esters of fatty acid (FAME). The tubes were sealed and incubated at room temperature for 10 min. The FAME were extracted into n-hexane, taken to dryness under N_2 and redissolved into isooctane. Individual FAME were identified according to the retention times of standards by gas liquid chromatography (FOCUS GC, Thermo Electron Corporation), with a VARIAN Cpsil88 capillary column [50mx0.25 mm idx0.25 μm df], an AS-3000 autosampler and flame-ionization detector [FID] (Thermo Electron Corporation). The FAME were quantified using the chromatographic peak area according to the internal standard (IS) method.

Determination of unsaturation index, delta9- and delta5- desaturases activity indices

The unsaturation index (UI) for each fatty acid within a phospholipid was calculated by multiplying the number of double bonds in each fatty acid by the mol percentage of that fatty acid. The UI for each phospholipid was generated by summing the individual fatty acid unsaturation indices.

Delta9-desaturase activity, enzyme that converts stearic acid (C18:0) and palmitic acid (C16:0) to oleic acid (C18:1n-9) and palmitoleic acid (C16:1n-7) respectively, was estimated by the ratio [C16:1n-7/C16:0] or [C18:1n-9/C18:0]. A decrease of this ratio in each of the lipid classes can be related to a decrease of the delta9-desaturase activity and vice versa (Biggemann et al. 1988).

Delta5-desaturase activity, enzyme that converts dihomo-γ-linoleic acid (C20:3n-6) to arachidonic acid (C20:4n-6), was estimated by the ratio [C20:4n-6/C20:3n-6] (Biggemann et al. 1988).

Measurement of mitochondrial respiratory complex activities

Citrate synthase activity was measured according to Srere (Srere 1969): the activity of the enzyme is measured by following the color of 5-thio-2-nitrobenzoic acid, which is

generated from 5,5′-dithiobis-2-nitrobenzoic acid present in the reaction of citrate synthesis, and caused by the deacetylation of acetyl-CoA. The different mitochondrial respiratory complex activities were determined as previously described (Feillet-Coudray et al. 2009). Complex I activity was measured spectrophotometrically at 600 nm during 45 sec by following the reduction of 2,6-dichloroindophenol by electrons accepted from decylubiquinol, itself reduced after oxidation of NADH by complex I (Janssen et al. 2007). Complex II activity was measured spectrophotometrically at 600 nm by following the reduction of 2,6-dichloroindophenol by the succinate during 120 sec. Complex II+III activities were measured spectrophotometrically by following the oxidation of oxidation of cytochrome c at 550 nm during 90 sec (Rustin et al. 1994). Cytochrome c oxidase (Complex IV) activity was measured spectrophotometrically by following the oxidation of reduced cytochrome c at 550 nm during 30 sec (Wharton and Tzagoloff 1967).

ATP synthase activity (complex V) was determined spectrophotometrically as described by Teodoro et al. (Teodoro et al. 2008) with minor modifications. Briefly, the reaction was carried out at 37 °C, in 0.5 mL reaction medium (125 mM sucrose, 65 mM KCl, 2.5 mM MgCl2 and 5 mM Hepes, pH 7.4). After the addition of freeze-thawed mitochondria (0.25 mg), the reaction was initiated by adding 2 mM Mg^{2+}-ATP, in the presence or absence of oligomycin (1 μg/mg protein). After 10 min, 0.25 mL of 40 % trichloroacetic acid was added to stop the reaction. Samples were centrifuged for 5 min at 3,000 g and 0.5 mL of ammonium molybdate plus 0.5 mL H_2O were then added to 0.25 mL of supernatant. Absorbance at 660 nm was measured to determine the released inorganic phosphate. ATPase activity was calculated as the difference in absorbance in the presence and absence of oligomycin.

Statistical analysis

Results were expressed as means±SD. Statistical analysis were based on two-way ANOVA followed by Fisher multiple comparisons test. Correlations were performed with the Spearman method. The limit of statistical significance was set at $P<0.05$. Statistical analyses were performed using the StatView program (SAS Institute, Cary, NC, USA).

Results

Characteristics of the rats

Weight gain remained statistically unchanged among the six experimental groups although a high tendency to increase with the 30 % lard-rich diet has been noted (Table 2). Indeed, when the three 30 % fat diet groups were analyzed separately, weight gain was significantly increased in the lard-rich diet group compared to basal diet and fish-oil rich diet (one way ANOVA, $p<0.05$). The dietary intake during the experiment was significantly decreased in the 30 % fat diet groups while energy intake was significantly increased. The dietary intake and energy intake were significantly increased with the 30 % lard-rich diet in comparison to 30 % basal diet and 30 % fish oil-rich diet groups.

Plasma glucose and leptin levels were increased with the 30 % lard-rich diet in comparison to 30 % basal diet and 30 % fish oil-rich diet groups (Table 2). The area under the curve of the oral glucose tolerance test was significantly increased with the 30 % fat diet in comparison to the 5 % fat diet and in particular with the 30 % lard-rich diet in comparison to 30 % basal diet and 30 % fish oil-rich diet groups. Moreover, plasma triglycerides and total cholesterol levels were significantly decreased in rats fed the 30 % fat diet. These parameters were significantly decreased with the 30 % fish oil-rich diet in comparison to 30 % basal diet and 30 % lard-rich diet groups. HDL/LDL ratio was significantly increased with the fish oil-rich diet in comparison to basal diet and lard-rich diet groups. Free fatty acid levels were not significantly different among groups. ALAT was not modified whatever the group and gamma-GT were only moderately increased with the 30 % fat diet, reflecting moderate liver injury.

The liver histological evaluation of hepatic steatosis showed that rats fed the 5 % basal diet and the 5 % lard-rich diet had a microvacuolar steatosis with few hepatocytes displaying intracytoplasmic droplets (Fig. 1a, b). Liver section from rats fed the 5 % fish-oil rich diet showed also a few steatotic macrovacuolar with simple bulky fat vacuoles that distend the hepatocyte and push the nucleus (Fig. 1c). On the other hand, histological studies on the liver section from rats fed the 30 % fat diets showed high-grade macrovacuolar steatosis in the three 30 % fat diet groups (Fig. 1d–f).

Liver free fatty acids, triglyceride and total cholesterol levels were significantly increased with the 30 % fat diets. Moreover, total cholesterol levels were significantly increased with the 30 % fish oil-rich diet in comparison to 30 % basal diet and 30 % lard-rich diet groups. Liver phospholipid levels were not modified whatever the diet (Table 3).

Effect of the diet on mitochondrial membrane phospholipid content

Total phospholipid content in the rat liver mitochondria was not modified whatever the diet (Table 4). The content of the two major phospholipids in total phospholipids (%), phosphatidylcholine (PC) and phosphatidylethanolamine (PE), was respectively not modified and significantly decreased in mitochondria of the rats fed the 30 % fat diets. The content of minor phospholipids (%) as phosphatidylinositol (PI), phosphatidylserine (PS) and lysophosphatidylcholine (LPC) was

Table 2 Body weight and weight gain, dietary and energy intakes and plasma glucose, lipids, insulin and leptin levels

	5 % fat diets			30 % fat diets			*P* values		
	Basal diet	Lard-rich diet	Fish-oil rich diet	Basal diet	Lard-rich diet	Fish-oil rich diet	Fat quantity	Fat quality	Interact
Weight gain (g)	310±50	318±43	331±17	322±46	382±52	328±55	NS	NS	NS
Final body weight (g)	476±47	488±54	502±21	490±45	544±56	491±58	NS	NS	NS
Dietary intake (g/d)	19.3±0.8	20.0±1.6	18.7±0.2	15.2±0.5[a]	17.9±0.7 [b]	15.3±1.0 [a]	$p<0.001$	$p<0.001$	$p<0.05$
Energy intake (kJ/d)	312±13	323±26	303±3	326±11 [a]	383±16 [b]	326±22 [a]	$p<0.001$	$p<0.001$	$p<0.001$
Glucose (mmol/L)	9.66±1.14	9.21±0.99	9.70±0.93	8.61±0.604 [a]	10.0±1.26 [b]	8.73±0.69 [a]	NS	NS	$p<0.05$
Insulin (μg/L)	1.80±0.632	3.00±1.14	4.25±0.28	3.25±2.79	6.62±4.30	4.23±2.73	$p<0.05$	NS	$p<0.05$
Leptin (μg/L)	22.6 ±+ 10.7	26.3±13.0	29.7±10.4	28.2±13.9 [a]	44.9±20.4 [b]	22.9±10.0 [a]	NS	NS	NS
OGTT (AUC)	13237±2480	11940±3127	10521±2883	12434±2192 [a]	16609±4137 [b]	11733±1319 [a]	$p<0.05$	$p<0.05$	$p<0.05$
Free fatty acids (mmol/L)	0.497±0.129	0.519±0.160	0.532±0.142	0.476±0.158	0.532±0.210	0.485±0.067	NS	NS	NS
Cholesterol (mmol/L)	1.58±0.25	1.82±0.37	1.53±0.27	1.56±0.13 [a]	1.61±0.41 [a]	0.95±0.17 [b]	$p<0.01$	$p<0.001$	NS
HDL-C/LDL-C	2.32±1.05	1.92±0.98	3.61±2.47	1.02±0.16	1.66±0.60	3.01±1.76	NS	$p<0.01$	$p<0.05$
Triglycerides (mmol/L)	1.32±0.32	1.49±0.32	1.77±0.44	0.95±0.28 [a]	1.17±0.40 [a]	0.62±0.07 [b]	$p<0.001$	NS	$p<0.05$
ALAT U/I	28.5±7.9	20.9±2.3	24.2±5.6	28.7±7.1	35.7±46.6	33.0±12.3	NS	NS	NS
Gamma-GT U/I	0.250±0.463	nd	nd	0.571±0.787	0.667±0.516	1.37±1.506	$p<0.01$	NS	NS

Results are mean ± SD. nd: non detectable

Two-way analysis of variance (ANOVA) was used, followed by Fisher multiple comparisons test. Significance was set at $p<0.05$. When there was interaction, a one way-ANOVA followed by Fisher multiple comparisons test was conducted on 5 % diet groups and on 30 % diet group distinctly; data not sharing the same letter are significantly different. *OGTT* oral glucose tolerance test; *ALAT* alanine transaminase; *Gamma-GT* gamma glutamyl transferase

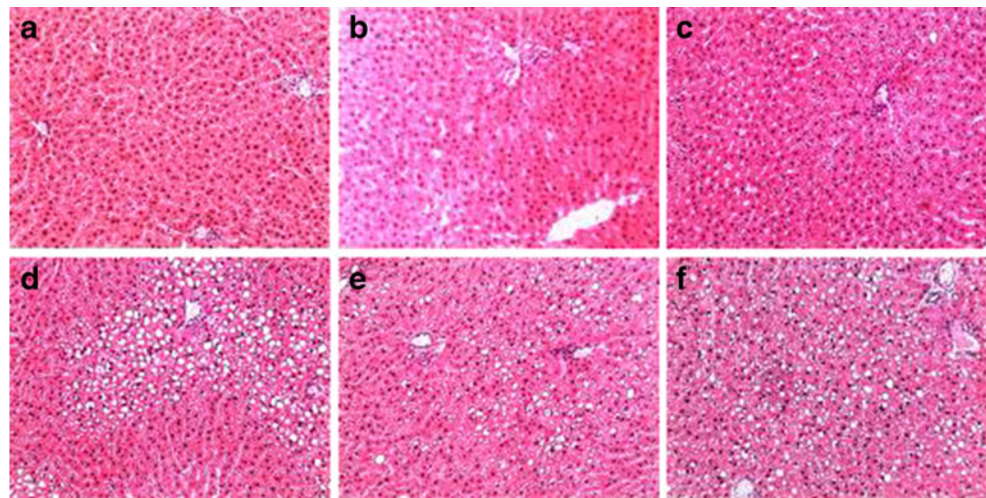

Fig. 1 Liver histology after hematoxylin/eosin staining of liver sections from a representative rat of each group (**a** basal diet 5 %, **b** lard-rich diet 5 %, **c** fish oil -rich diet 5 %, **d** basal diet 30 %, **e** lard- rich diet 30 %, **f** fish oil -rich diet 30 %) (original magnification × 100)

not modified, while CL and sphingomyelin (SM) content (%) was significantly increased in rats fed the 30 % fat diets.

The quality of dietary fatty acids in the diet had no effect on total phospholipid content and on PC%, PI%, PS% or LPC%, while it significantly affected PE%, CL% and SM%. Indeed, CL content was significantly decreased with the 5 % lard-rich diet and 5 % fish oil-rich diet and it was also significantly decreased with the 30 % fish oil-rich diet. SM content was significantly decreased with the fish oil-rich diet in comparison to basal diet and lard-rich diet. In contrary, PE was significantly increased with the fish oil-rich diet in comparison to basal diet and lard-rich diet.

CLsyn gene expression was not modified with the 30 % fat diets in comparison to the 5 % fat diets. However, it was significantly increased in the lard-rich diet in comparison to the basal diet and fish oil-rich diet (Fig. 2a) and this effect was particularly marked with the 30 % fat diet. Moreover, the CL content in phospholipids was positively correlated with the gene expression of CLsyn (Fig. 2b).

Effects of the diet on mitochondrial CL fatty acid composition

Effect of dietary fat quantity

The fatty acid composition of CL was modified in rats fed the 30 % fat diet in comparison to rats fed the 5 % fat diets (Table 5), except regarding saturated fatty acids (SFA). The proportions of total monounsaturated fatty acids (MUFA) in CL was 50 % decreased in rats fed the 30 % fat diets. In addition, the polyunsaturated fatty acids (PUFA) proportions significantly increased to more than 20 %, in particular

Table 3 Liver characteristics

	5 % fat diets			30 % fat diets			*P* values		
	Basal diet	Lard-rich diet	Fish-oil rich diet	Basal diet	Lard-rich diet	Fish-oil rich diet	Fat quantity	Fat quality	Interact
Liver weight (g)	10.5±1.4	10.6±1.1	11.2±0.9	11.0±1.6	12.0±2.0	12.2±2.0	$p<0.05$	NS	NS
Free fatty acids (μmol/g wet weight)	9.58±1.15	8.84±1.07	9.22±2.39	19.0±8.1	19.0±8.15	21.6±10.9	$p<0.001$	NS	NS
Cholesterol (μmol/g wet weight)	5.65±1.55	5.48±1.6	7.35±1.70	11.0±2.2 [a]	9.16±1.49 [a]	15.6±3.2 [b]	$p<0.001$	$p<0.001$	$p<0.05$
Triglycerides (μmol/g wet weight)	28.5±4.7	25.0±5.9	35.7±9.1	59.6±19.1	62.0±17.9	64.1±21.8	$p<0.001$	NS	NS
Phospholipids (μmol/g wet weight)	42.2±3.7	43.9±1.6	44.3±2.6	43.2±2.8	44.4±4.5	45.9±3.2	NS	NS	NS

Results are mean ± SD. Two-way analysis of variance (ANOVA) was used, followed by Fisher multiple comparisons test. Significance was set at $p<0.05$. When there was interaction, a one way-ANOVA followed by Fisher multiple comparisons test was conducted on 5 % diet groups and on 30 % diet group distinctly; data not sharing the same letter are significantly different.

Table 4 Phospholipid composition of liver mitochondrial membrane

	5 % fat diets			30 % fat diets			*P* values		
	Basal diet	Lard-rich diet	Fish-oil rich diet	Basal diet	Lard-rich diet	Fish-oil rich diet	Fat quantity	Fat quality	Interact
Total Phospholipid (nmol/mg prot)	250±42	252±36	255±22	256±39	249±16	243±29	NS	NS	NS
PC (%)	49.7±2.2	51.7±2.0	51.7±1.7	51.7±2.7	50.1±2.1	50.9±1.6	NS	NS	NS
PE (%)	29.4±2.7	29.3±1.3	30.3±1.3	27.0±1.3	28.1±1.7	29.6±1.0	$p<0.01$	$p<0.05$	NS
PI (%)	6.95±0.94	5.89±0.55	5.99±0.57	6.89±1.11	6.58±1.46	6.38±0.75	NS	NS	NS
CL (%)	4.56±0.60 [a]	3.90±0.43 [b]	3.56±0.22 [b]	4.54±0.69 [a]	4.86±0.72 [a]	3.85±0.53 [b]	$p<0.05$	$p<0.001$	$p<0.05$
SM (%)	3.86±0.31	3.70±0.50	3.24±0.36	4.19±0.65	4.40±0.69	3.91±0.51	$p<0.001$	$p<0.05$	NS
PS (%)	3.14±0.45	3.08±0.46	2.84±0.37	3.18±0.61	3.30±0.38	3.03±0.62	NS	NS	NS
LPC (%)	2.41± 0.46	2.44±0.37	2.35±0.32	2.49±0.33	2.68±0.51	2.40±0.46	NS	NS	NS

Results are mean ± SD

(%): percentages in phosphorus of total phospholipids

Two-way analysis of variance (ANOVA) was used, followed by Fisher multiple comparisons test. Significance was set at $p<0.05$. When there was interaction, a one way-ANOVA followed by Fisher multiple comparisons test was conducted on 5 % diet groups and on 30 % diet groups distinctly; data not sharing the same letter are significantly different

PC phosphatidylcholine; *PE* phosphatidylethanolamine; *PI* phosphatidylinositol; *CL* cardiolipin; *SM* sphyngomyelin; *PS* phosphatidylserine; *LPC*, lysophosphatidylcholine

C18:2 n-6, in rats fed the 30 % fat diets in comparison to 5 % fat diet fed rats. The ratio of n-6/n-3 fatty acids in liver mitochondrial CL of rats fed 30 % fat diets was higher than for rats fed the 5 % fat diets.

Effect of dietary fat quality

Feeding the lard-rich diet and the fish oil-rich diet altered the fatty acid composition of CL in comparison to basal diet fed rats, and the effect was more pronounced with the fish oil diet (Table 5). MUFA content was increased with the fish oil-rich diet in comparison to basal diet, both at 5 % and 30 % of fat in the diet, while the effect was only significant at 5 % for the lard-rich diet. Moreover, total PUFA was significantly decreased with the fish oil-rich diet in comparison to the basal diet. Total (n-6) were significantly lower in rats fed the fish oil-rich diet in comparison to basal diet while total (n-3) were significantly higher in rats fed the 30 % fish oil-fat diet in comparison to the 30 % basal diet and the 30 % lard-rich diet. No modification was observed on the major fatty acid C18:2n-6 whatever the diet. There was a significant decrease in the proportions of C20:4n-6 in rats fed the fish oil-rich diet in comparison to basal diet both at 5 % and 30 %. As for quantity, the amount of saturated fatty acids remained unchanged in the CL fraction

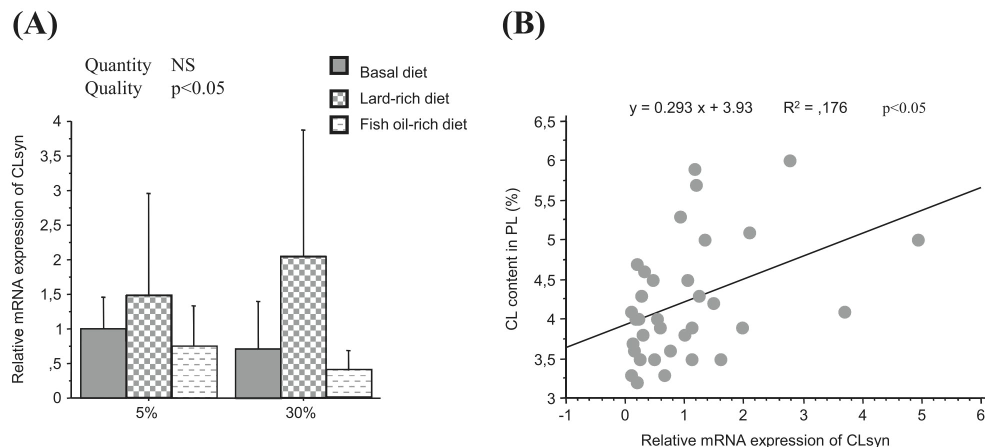

Fig. 2 **a** Real-time quantitative PCR analysis of mRNA expression of cardiolipin synthase (CLsyn). Values are expressed as mean ± SD (n=5–7). Two-way analysis of variance (ANOVA) was used, followed by Fisher multiple comparisons test. Significance was set at $p<0.05$. **b** Correlation between CLsyn gene expression in liver and total CL content in liver PL. Spearman correlation, significantly different $p<0.05$

Table 5 Effect of diet on fatty acid composition of cardiolipin from rat liver mitochondrial membrane

	5 % fat diets			30 % fat diets			*P* values		
	Basal diet	Lard-rich diet	Fish-oil rich diet	Basal diet	Lard-rich diet	Fish-oil rich diet	Fat quantity	Fat quality	Interact
Total SFA	10.6±3.5	8.61±2.56	7.97±1.60	8.55±2.9	7.91±2.63	8.39±3.12	NS	NS	NS
16:0	4.80±0.96	4.04±0.96	4.56±0.74	4.08±0.95	4.06±0.97	4.74±1.32	NS	NS	NS
18:0	5.79±2.34	4.57±1.78	3.41±1.11	4.47±2.09	3.85±1.72	3.65±1.82	NS	NS	NS
Total MUFA	27.4±2.9 [a]	33.7±3.8 [b]	34.5±2.1 [b]	14.7±1.2 [a]	15.2±1.4 [ab]	16.5±1.2 [b]	<0.001	<0.001	<0.01
16:1 n-7	10.9±2.0 [a]	14.3±2.2 [b]	14.4±2.1 [b]	0.828±0.139 [a]	1.11±0.34 [b]	0.796±0.278 [a]	<0.001	<0.01	<0.01
18:1 n-9	6.74±0.82 [a]	7.91±1.27 [b]	8.00±0.92 [b]	5.29±0.70 [a]	4.79±0.63 [a]	6.45±0.96 [b]	<0.001	<0.01	<0.05
18:1 n-7	9.73±1.00 [a]	11.6±0.9 [b]	12.1±1.1 [b]	8.62±0.86	9.25±0.86	9.31±0.99	<0.001	<0.001	<0.05
Total PUFA	62.0±2.4	57.7±4.0	57.5±2.8	76.7±3.6	76.9±2.7	75.1±3.7	<0.001	<0.05	NS
18:2 n-6	52.4±3.3	48.3±4.9	51.1±3.1	71.3±3.4	70.7±2.7	69.5±4.2	<0.001	NS	NS
20:3 n-6	4.07±0.65 [a]	5.49±1.21 [b]	3.40±0.33 [a]	1.88±0.37 [a]	2.64±0.52 [b]	1.90±0.54 [a]	<0.001	<0.0001	<0.05
20:4 n-6	3.35±1.34 [a]	2.26±0.54 [b]	1.21±0.42 [c]	1.58±0.21 [a]	1.75±0.20 [a]	1.27±0.36 [b]	<0.001	<0.0001	<0.01
20:5 n-3	0.172±0.157	0.042±0.077	0.334±0.315	nd	nd	0.360±0.119	NS	<0.0001	NS
22:5 n-3	0.056±0.109	0.055±0.103	0.071±0.132	nd	nd	0.095±0.162	NS	NS	NS
22:6 n-3	1.81±0.64	1.39±0.25	1.40±0.45	0.610±0.191 [a]	0.665±0.296 [a]	1.34±0.59 [b]	<0.001	NS	<0.01
Total(n-6)	60.0±2.6	56.2±4.2	55.7±2.8	76.1±3.5	76.3±2.7	73.2±4.2	<0.001	<0.05	NS
Total(n-3)	2.05±0.88	1.49±0.36	1.81±0.49	0.664±0.206 [a]	0.665±0.296 [a]	1.81±0.75 [b]	<0.001	<0.01	<0.01
n-6/n-3	33.3±11.5	40.1±11.6	33.0±8.9	123±34 [a]	140±69 [a]	49.1±25.7 [b]	<0.001	<0.001	<0.01
SFA/MUFA[§]	0.400±0.171	0.261±0.101	0.231±0.047	0.579±0.196	0.530±0.208	0.505±0.181	<0.001	NS	NS
PUFA/SFA[#]	6.44±2.17	7.56±3.60	7.54±1.87	10.4±5.0	10.8±3.9	10.1±3.7	<0.001	NS	NS
n-3/PUFA[&]	0.033±0.015	0.026±0.007	0.031±0.009	0.009±0.002 [a]	0.009±0.004 [a]	0.024±0.011 [b]	<0.001	<0.01	<0.05
UI[$]	170±5 [a]	165±5 [ab]	162±4 [b]	175±6	177±5	177±6	<0.001	NS	<0.05
Δ9-desaturase									
16:1n-7/16:0	2.47±0.98 [a]	3.75±1.17 [b]	3.20±0.53 [ab]	0.214±0.064 [ab]	0.293±0.129 [a]	0.173±0.053 [b]	<0.001	<0.05	<0.05
18:1n-9/18:0	1.32±0.48	2.10±1.17	2.61±0.97	1.51±0.87	1.45±0.60	2.09±0.86	NS	<0.05	NS
Δ5-desaturase									
20:4n-6/20:3n-6	0.884±0.524 [a]	0.429±0.137 [b]	0.363±0.149 [b]	0.868±0.197	0.688±0.169	0.729±0.378	<0.05	<0.01	NS

[$] *BD* basal diet; *LD* lard-rich diet; *FOD* fish-oil rich diet

Major fatty acids are represented and results are expressed as percentage of fatty acids in cardiolipin

Results are mean ± SD

Two-way analysis of variance (ANOVA) was used, followed by Fisher multiple comparisons test. Significance was set at $p<0.05$. When there was interaction, a one way-ANOVA followed by Fisher multiple comparisons test was conducted on 5 % diet groups and on 30 % diet group distinctly; data not sharing the same letter are significantly different

SFA saturated fatty acids; *MUFA* monounsaturated fatty acids; *PUFA* polyunsaturated fatty acids

[§] SFA/MUFA: SFA=%C16:0+%C18:0 and MUFA=%9c 16:1+%9c 18:1+%11c 18:1

[#] PUFA/SFA: SFA=%C16:0+%C18:0 and PUFA=%all cis PUFA

[&] n-3/PUFA: Total (n-3)/all PUFA

[$] *UI* unsaturation index = (%monoenoic*1)+(%dienoic*2)+(%trienoic*3)+(%tetraenoic*4)+(%pentaenoic*5)+(%hexaenoic*6)

regardless of quality of dietary lipids. The fatty acid composition of others phospholipids (PC, PE, PI and PS) are given in supplemental data (supplemental tables 2, 3, 4 and 5).

Fatty acid desaturase activities and unsaturation indices (UI) of CL

The ratio [C16:1n-7/C16:0] in the CL fraction were decreased in rats fed the 30 % fat diets in comparison to the 5 % diet, probably reflecting a decrease of the delta9-desaturase activity (Table 5). Moreover, the ratio of [C18:1n-9/C18:0] was increased with the fish oil-rich diet in comparison to basal diet in CL. UI of CL was increased in the 30 % fat diet in comparison to 5 %. The UI of CL was not modified with the type of fatty acid in the diet (Table 5).

Mitochondrial respiratory chain complex activities and correlations with CL content and fatty acid composition

Citrate synthase activity in liver mitochondria was significantly increased with the 30 % fat diets, and complex I, II and II+III activities were significantly decreased, while COX and ATP synthase activities remained unchanged (Table 6). Moreover, citrate synthase activity was significantly increased with the lard-rich diet in comparison to basal diet. Complex I activity was significantly decreased with the fish oil-rich diet and COX activity was significantly increased with the lard-rich diet in comparison to others diet.

Complex II+III activity was negatively correlated and ATP synthase activity was positively correlated with the CL content of liver phospholipids (Fig. 3), while complexes I, II and COX activities were not correlated. ATP synthase activity was positively correlated to the C18:2n-6 CL content (r=0.298, p<0.05) while complexes I, II, II+III and COX activities were negatively correlated (r=−0.356, p<0.05, r=−0.428, p<0.01; r=−0.55, p<0.001 and r=−0.361, p<0.05 respectively).

Discussion

Description of the model of hepatic steatosis

The present study was performed on a model of rats with liver macrosteatosis induced by high-fat (30 %) diets and associated with moderate liver injury, but without insulin resistance except with the 30 % lard-rich diet. These characteristics support the recent suggestion that hepatic steatosis is not necessarily associated with insulin resistance and that the accumulation of lipids in the liver may precede the state of insulin resistance (Postic and Girard 2008). It seems that the accumulation of lipids in tissues with the lard-rich diet was more deleterious than with basal diet or fish oil-rich diet as insulin resistance was observed only with the 30 % lard-rich diet. This result is not surprising as it is known that saturated fat significantly increase insulin resistance (Assy et al. 2009).

Modification of mitochondrial phospholipids class distribution

In accordance with a previous report (Colbeau et al. 1971), we observed that PC and PE were the two main phospholipids (about 40 % and 30 % respectively) in mitochondrial membranes while CL and PI accounted together for about 10–15 %. To compare, in liver plasma membranes, 55 % of the total lipids extracted are phospholipids, the three major components of phospholipids being PC, PE and SM (more than 70 % of total phospholipids) (Ray et al. 1969).

The distribution of phospholipid classes was altered in the 30 % fat diet-fed rats, with globally lower PE and higher CL and SM percentage. In contrast, it was previously reported no significant variations in the percentage of the main phospholipids of liver mitochondria from rats fed a diet rich in saturated fatty acids or a diet rich in n-3 fatty acids (Giudetti et al. 2003). In spite of its low percentage

Table 6 Mitochondrial respiratory chain complex activities (mIU/mg mitochondrial protein)

	5 % fat diets			30 % fat diets			*P* values		
	Basal diet	Lard-rich diet	Fish-oil rich diet	Basal diet	Lard-rich diet	Fish-oil rich diet	Fat quantity	Fat quality	Interact
Citrate synthase	205±19	224±16	210±14	216±28	248±40	241±22	p<0.01	p<0.05	NS
Complex I	237±24	237±32	196±32	195±29	199±41	163±39	p<0.001	p<0.01	NS
Complex II	296±74	296±71	296±51	213±79	220±70	192±48	p<0.001	NS	NS
Complex II+III	22.5±7.6	25.4±8.9	23.7±6.7	15.9±4.2	20.0±5.2	19.2±5.2	p<0.01	NS	NS
COX	710±149	780±110	704±87	668±79	817±172	538±64	NS	p<0.01	NS
ATP synthase	130±24	138±24	132±23	144±44	159±25	130±31	NS	NS	NS

Results are mean ± SD

Two-way analysis of variance (ANOVA) was used, followed by Fisher multiple comparisons test. Significance was set at p<0.05

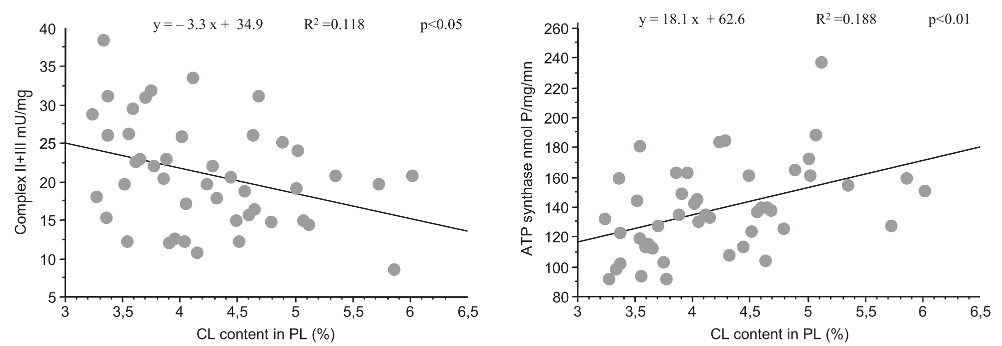

Fig. 3 Correlation between total CL content in liver PL (%) and complex II+III and ATP synthase activities in liver mitochondria. Spearman correlation, significantly different $p<0.05$

compared to PE and PC, CL plays a very important role as a result of its interaction with numerous mitochondrial proteins, and small changes in CL may affect membrane protein functions (Stefanyk et al. 2010). Indeed, CL has been proposed to regulate the induction of the mitochondrial permeability transition pore (Ferreira et al. 2003) and its content is inversely correlated with mitochondrial proton leak (Hoch 1998). In a previous publication, we described increased mitochondrial membrane potential in liver of rats fed the 30 % lipid diets (Aoun et al. 2011) and these results may be explained by the higher content of CL with the 30 % fat diets. Decreased CL levels or its abnormal composition have been linked to common diseases such as diabetes and heart failure (Houtkooper and Vaz 2008; Saini-Chohan et al. 2009), and decreased CL content was observed in a choline-deficient model of NASH (Petrosillo et al. 2007). However, in our model of liver steatosis induced by high fat diets, CL percent was rather increased (Aoun et al. 2011).

Although CL plays a key role in many mitochondrial functions, recent evidence suggests that PE should be another key mitochondrial phospholipid (Gohil and Greenberg 2009) and reduction of both mitochondrial PE and CL levels were reported to result in abnormal mitochondrial morphology (Osman et al. 2011). In this study, we observed decreased level of PE but increased level of CL with the 30 % fat diet, and it would have been interesting to evaluate the consequences on the mitochondrial morphology. Some work demonstrated that membranes rich in PE are less fluid compared to membranes rich in PC (Ladbrooke and Chapman 1969), and in accordance, the percent of PE was decreased in rats fed the 30 % fat diets in this study while our previous results demonstrated that the fluidity of mitochondria was increased in rats fed the 30 % fat diets (Aoun et al. 2011).

The quality of dietary lipids influenced the relative distribution of phospholipid classes. In fact, feeding the fish oil-rich diet decreased SM content and increased PE content. As SM preferentially binds to saturated and monounsaturated fatty acids (O'Brien and Rouser 1964), the decrease in SM content with the fish oil-rich diet may be explained by the fact that this diet provided high levels of n-3 PUFA. Moreover, CL content was decreased with the fish oil-rich diet. When CL content decreases, mitochondrial oxidative phosphorylation is often defective (Li et al. 2010). In accordance, complex I activity was altered in mitochondria from rats fed the fish-oil rich diet. CL is synthesized from phosphatidic acid (Schlame et al. 2000) and the conversion of phosphatidyl glycerol to "nascent" CL, the last step of its biosynthesis, is catalyzed by the CL synthase. The selective decrease of CL content in mitochondria of rats fed the fish oil-rich diet could result from impaired function of the CL synthase (Taylor et al. 2002). For example, in heart failure, alteration in CL synthase gene expression and activity was observed while CL content decreased in the heart (Saini-Chohan et al. 2009). Therefore, in order to understand the CL content changes, we studied CL synthase gene expression. Our results showed that CL synthase gene expression was not significantly modified with the fish-oil rich diet. Decreased bioavailability of CL precursors (Cheng and Hatch 1995; Okumura et al. 1991) or enhanced CL degradation (hydrolysis by phospholipase A2) (Nakahara et al. 1992) with the fish oil-rich diet might better explain the lower CL content with the fish oil-rich diet. On the other hand, CL synthase gene expression was significantly higher with the lard-rich diet, in particular with the 30 % fat diet, and the CL content in phospholipids was significantly and positively correlated to CL synthase gene expression.

Modification of the CL fatty acid composition with diet fatty acids

The fatty acid composition of CL is highly specific, being predominantly comprised of 18-carbon unsaturated acyl chains (Hoch 1992). An 18:2-rich CL profile, with four acyl chains (Schlame et al. 1993), is particularly evident in the mammalian heart and liver where 18:2 constitutes more than 50 % CL acyl chains and tetralinoleoyl CL (L_4CL) is the most abundant specie (Schlame et al. 1993; Schlame and Otten 1991). In mammalian mitochondria, it is well known that the optimal functions of many mitochondrial proteins depend on the association of these proteins with specific L_4CL molecular species profiles (Han et al. 2007). In

consequence, CL compositional changes may result in mitochondrial protein dysfunctions. Our results showed that the 30 % fat diet intake modified the fatty acid composition of hepatic mitochondrial CL in rats in comparison to rats fed the 5 % lipid diets, in particular leading to a 40 % increase in C18:2n-6 and a 50 % decrease in total MUFA. In cardiac CL, a paradoxal loss of C18:2n-6 fraction in rats fed a 20 % sunflower oil diet (rich in C18:2n-6) has been shown (Ghosh et al. 2006), but CL fatty acid composition had not been determined in liver.

While the lard-rich diet did not affect strongly the composition of CL fatty acids, the main effect of fish oil-rich diet, essentially in rats fed the 30 % fat diets, was a decrease in n-6 PUFA which was counterbalanced by an increase in the levels of n-3 PUFA and total MUFA. Similar results were observed in mitochondrial CL from liver, heart and brain from rats fed high lipid fish-oil diet (Power et al. 1994; Yamaoka et al. 1988; Hong et al. 2002). In particular, there was a significant decrease in the proportions of C20:4n-6 in rats fed the fish oil-rich diet in comparison to basal diet, and the fact that n-3 PUFAs are inhibitors of delta-6 desaturase might explain this decrease (Brenner 1981). Physiologically, these findings are relevant because changes in CL fatty acyl composition may influence the activity of associated inner mitochondrial membrane enzymes and result in mitochondrial dysfunction. However, we did not observe any modifications of the content of the major fatty acid C18:2n-6 in CL with the type of fatty acids in the diet, in contrast to Yamaoka S et al. (Yamaoka et al. 1988) who reported remarkable differences in C18:2n-6 CL content of liver from rats fed a corn oil or a fish oil; but their measurements were made on the total liver extract and not on isolated mitochondria.

Mitochondrial respiratory complex activities and CL content/fatty acid composition

CL plays an important role in mitochondrial bioenergetics, optimizing the activity of the respiratory chain complexes (Schlame et al. 2000). CL is specifically required for the electron transfer in complex I and III of the mitochondrial respiratory chain. Complex II from *Escherichia Coli* has been shown to be associated with two acyl chains of one CL molecule (Yankovskaya et al. 2003). CL is tightly bound to the cytochrome c oxidase complex (McMillin and Dowhan 2002) and ATP synthase (Eble et al. 1990), and is essential for the stability of the quaternary protein structure of the ADP-ATP carrier (Schlame 2008). In addition, the ability of CL to interact with proteins seems to play a major role in the formation of respiratory supercomplexes (Claypool 2009). In accordance, we observed that ATP synthase activity was positively correlated to the total CL content (% of phospholipids) and to the C18:2n-6 content of CL. Surprisingly, complex II+III was negatively correlated to the total CL content (% of phospholipids). This result was unexpected as it was reported that changes in complex III activity were quantitatively related to changes in the CL content (Petrosillo et al. 2003). Moreover, complexes I, II, II+III and COX activities were negatively correlated to the C18:2n-6 content of CL. Polyunsaturated fatty acids are particularly susceptible to ROS attack because of the presence of double bonds (Paradies et al. 2000), thus impairment of mitochondrial complexes activity with high-fat diet, despite higher CL content but with higher CL unsaturation indices, may be due to ROS-induced CL damage. In fact, several studies indicate that peroxidized CL is unable to support the reconstituted activity of mitochondrial respiratory enzymes (Chicco and Sparagna 2007). In accordance, a decrease in COX activity was previously reported in liver mitochondrial membranes containing high levels of long-chain polyunsaturated fatty acids (Barzanti et al. 1994).

In conclusion**,** the present study showed that dietary lipid intake may be a significant determinant of the CL content and fatty acid composition of the liver mitochondrial membrane in hepatic steatosis. CL content and CL C18:2n-6 level were increased in rats fed the high fat diets and correlated to the activity of the ATP synthase. Moreover, feeding the fish oil-rich diet increased the (n-3) PUFA content of CL and decreased complex I activity. The lard-rich diet did not affect strongly the fatty acids composition of CL, even if it increased CL synthase gene expression. In the future, it would be interesting to evaluate CL content and fatty acid composition in steatohepatitis since a direct relationship between CL loss and cytochrome c release from mitochondria was identified as an initial step in the pathway to apoptosis, which may lead to liver injury (McMillin and Dowhan 2002).

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Conflict of interest None.

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