DIETARY DOCOSAHEXAENOIC ACID AFFECTS THE ALTERATIONS INDUCED IN RAT CARDIAC MITOCHONDRIAL FUNCTION IN INSULIN DEFICIENCY AND INSULIN RESISTANCE.

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Running Title: N-3 PUFAs and diabetic rat cardiac mitochondria.

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Abstract

The effect of docosahexaenoic acid (DHA) intake on cardiac mitochondrial function was evaluated in permeabilized fibers in insulin deficiency and insulin-resistance in rats. The insulin deficient state was obtained by streptozotocin injection 2 months before investigations. Insulin resistance was obtained by feeding a 62% fructose diet for three months. DHA was incorporated in the diet to modify the fatty acid composition of cardiac membranes, including mitochondria. Insulin deficiency decreased mitochondrial creatine kinase (mi-CK) activity and mitochondrial sensitivity to ADP. DHA intake prevented these alterations. Moreover, the insulin deficient state significantly decreased n-3 polyunsaturated fatty acids (PUFA) and slightly increased n-6 PUFA in both cardiac and mitochondrial membranes, inducing a significant increase in n-6/n-3 ratio. DHA intake maintained high myocardial and mitochondrial DHA content. Insulin deficiency also decreased glutamate and palmitoylcarnitine-supported mitochondrial respiration, but DHA intake did not prevent these effects. In contrast, insulin resistance did not affect mi-CK activity or sensitivity to ADP. However, insulin resistance influenced the myocardial fatty acid composition with decreased n-6 and n-3 PUFA contents and increased monounsaturated fatty acid content. Only slight alterations were observed in mitochondrial fatty acid composition and they were corrected by DHA intake. Moreover, insulin resistance decreased the glutamate-supported respiration, and DHA intake did not influence this effect. In conclusion, the impairment of cardiac mitochondrial function was more pronounced in insulin deficient state than in insulin resistance. The modification of fatty acid composition of cardiac and mitochondrial membranes by DHA partially prevented the mitochondrial alterations induced in the two models.

Keywords: insulin deficiency, insulin resistance, mitochondrial respiration, DHA, myocardium.
**Introduction**

Diabetes is associated with high cardiovascular morbidity and mortality, and both insulin-deficient and insulin-resistant diabetes induce biochemical and physiological changes in the heart (15). Animal studies have demonstrated a number of diabetes-induced changes in the diabetic heart, including changes in mitochondrial function (14) and fatty acid composition of heart membrane phospholipids. In uncontrolled diabetes, myocardial glucose utilization is markedly reduced, causing fatty acids to support a greater portion of total energy metabolism (23). Moreover, when diabetic rat hearts are perfused in the absence of fatty acids, glucose oxidation represents less than 20% of the ATP synthesis (14) suggesting an alteration of the fatty acid uptake regulation in mitochondria. In the diabetic heart, the level of malonyl-CoA, the physiological inhibitor of carnitine palmitoyltransferase I (CPT I), seems to be reduced. In streptozotocin diabetic rats, the activity of acetyl-CoA carboxylase (ACC), which produces malonyl-CoA, was shown to decrease (14). In the same model, Sakamoto et al. (24) reported an increase in malonyl-CoA decarboxylase activity, due to an increased expression of the enzyme, contributing to the malonyl-CoA decrease. The high rates of fatty acid oxidation in the diabetic heart markedly decrease the glucose oxidation rates and increases the oxygen requirement. On the other hand, the diabetic heart is characterized by significant alterations of the fatty acid composition of heart membranes. Hu et al. have shown a substantial decrease in the relative level of arachidonic acid and an increase in linoleic acid in the major cardiac phospholipids, in streptozotocin diabetic rats (9). The activity of the Δ6-desaturase is impaired in the streptozotocin-induced diabetic rats (1), resulting in an increase in linoleic acid and a decrease in arachidonic acid. Moreover, the n-3 polyunsaturated fatty acid (PUFA) content of cardiac phospholipids is strongly decreased in the same model, although a dietary docosahexaenoic acid (DHA , 22:6 n-3) supplementation was able to maintain the cardiac DHA content (6). In contrast to the diabetic heart, little is known about the alterations of energy metabolism and cardiac membrane fatty acid composition that occur with insulin resistance. The aim of this study was to
compare the effects of streptozotocin induced diabetes and insulin resistance in rats on mitochondrial function and membrane fatty acid composition and to evaluate the influence of a DHA-enriched diet.

Material and methods

Animals and models

Insulin deficiency was induced in male Wistar rats (150-200 g) with streptozotocin (STZ; 55 mg/kg i.m., freshly dissolved in sodium citrate buffer 1 mM, pH 7.4). The Sham rats received citrate buffer. The development of the pathology was confirmed by determination of blood glucose concentration, one week after the injection. Any rats in which the hyperglycemia was not confirmed after one week were given a second dose of streptozotocin. The mean blood glucose concentration was 84.9 ± 2.3 mg/dl (n=15) and 266.1 ± 31.8 mg/dl (n=15) in the Sham group (Sh group) and the STZ-treated group (STZ group), respectively. All animals were individually housed for 8 weeks and weighed once a week. At the end of experiment, the fasting plasma insulin level was 66.2 ± 6.95 mUI/l and 17.5 ± 3.50 mUI/l in Sham and STZ-treated rats, respectively. Insulin resistance was induced in male Wistar rats (100-125 g) with a custom made fructose diet (HI group) as previously described (22). Non hyperinsulinemic rats (NHI group) were fed a standard diet contained starch and sucrose. The rats received these diets for 12 weeks and were weighed once a week. At the end of experiment, the fasting plasma insulin levels was 135.1 ± 11.35 mUI/l in HI rats.

Diets

The rats were fed ad libitum a semi-purified jellied diet (21) in accordance with the AIN-93 recommendations. The meal was prepared to form a jellied mass cut into cubes for feeding, stored at −20°C and fed daily to maintain moisture content and food intake. For the insulin deficiency model involving the Sham and STZ groups, we prepared two diets, which differed only in lipid composition. Different fats (80 g.kg⁻¹) were incorporated into a standard fabricated diet composed of starch (526.2 g.kg⁻¹), sucrose (100 g.kg⁻¹), cellulose (50 g.kg⁻¹), soy protein isolate (140 g.kg⁻¹),...
ICN 905456), L-cystine (1.8 g.kg⁻¹), gelatin to jellify (50 g.kg⁻¹), salt mixture (40 g.kg⁻¹, ICN 960401), vitamin mixture (10 g.kg⁻¹, ICN 960402) and choline bitartrate (2 g.kg⁻¹). For the dietary control groups Sh-Ctrl and STZ-Ctrl, the control lipid part was composed of 40 g.kg⁻¹ cocoa butter (CACAO BARRY, France) + 40 g.kg⁻¹ sunflower seed oil (FRUIDOR, France). For the DHA-enriched diet groups Sh-DHA and STZ-DHA, the lipid part was composed of 40 g.kg⁻¹ cocoa butter + 30.4 g.kg⁻¹ sunflower seed oil + 9.6 g.kg⁻¹ DHA (ROPUFA ‘60’, HOFFMANN-LAROCHE, Switzerland). For the insulin resistant model (NHI and HI rats), we prepared four diets which differed in their carbohydrate and lipid composition. The NHI-Ctrl group received the same diet as the Sh-Ctrl group, with carbohydrate comprised of starch (526.2 g.kg⁻¹) and sucrose (100 g.kg⁻¹) and lipid comprised of 40 g.kg⁻¹ cocoa butter + 40 g.kg⁻¹ sunflower seed oil. NHI-DHA rats received the same diet as Sh-DHA, composed of starch (526.2 g.kg⁻¹) and sucrose (100 g.kg⁻¹) as the carbohydrate 40 g.kg⁻¹ cocoa butter + 30.4 g.kg⁻¹ sunflower seed oil and 9.6 g.kg⁻¹ DHA as the lipid. For the HI groups, fructose (626.2 g.kg⁻¹) replaced starch and sucrose as the carbohydrate. For HI-Ctrl, the lipid comprised 40 g.kg⁻¹ cocoa butter + 40 g.kg⁻¹ sunflower seed oil and for HI-DHA, the lipid comprised 40 g.kg⁻¹ cocoa butter + 30.4 g.kg⁻¹ sunflower seed oil + 9.6 g.kg⁻¹ DHA. Independently of the carbohydrate composition, the fatty acid composition of the Ctrl and DHA diets was determined by gas chromatography, after lipid extraction. As shown in Table 1, the diets were quantitatively similar in their saturated, monounsaturated and polyunsaturated fatty acids but differed qualitatively in their polyunsaturated fatty acid composition.

**Biochemical investigations**

At the end of the diet period, the rats were anesthetized after 12-hour fast with pentobarbital (60 mg.kg⁻¹) and blood was collected for serum biochemistry. Plasma triglycerides were determined using the spectrophotometric TRIGLYCERIDES-INT kit (SIGMA, France). Insulin was determined by radioimmunoassay using the Insulin-CT 100 (CIS-BIO INTERNATIONAL, France) in vitro test and glucose was measured by the glucose oxidase method. The kidneys were collected and
weighted and the hearts were used for permeabilized muscle fiber investigations, isolated mitochondria investigations and cardiac fatty acid determination.

**Mitochondria functional properties**

The respiratory parameters of the mitochondria populations were investigated *in situ* in saponin-permeabilized fibers (31). Thin fiber bundles (100-250 µm in diameter) were excised from the subendocardial surface of the left ventricle. The bundles were incubated with intense shaking for 30 min in solution S supplemented with 50 µg/ml saponin to selectively destroy the integrity of the sarcolemma. The bundles were then transferred into solution R for 10 min to wash out adenine nucleotides and phosphocreatine. All procedures were carried out at 4 °C. The respiratory rates were determined with a Clark electrode (HANSATECH, EUROSEP INSTRUMENTS, France) in an oxygraphic cell containing 7-10 fibers bundles in 1 ml of solution R at 22°C with continuous stirring. After measurement, the bundles were removed and dried and the respiratory rates were expressed as millimoles of O₂.min⁻¹.g⁻¹(dry weight), on the basis of an oxygen solubility of 230 mmol of O₂/l. To measure the mitochondrial respiratory characteristics, cardiac fibers were exposed to increasing ADP concentrations with glutamate (5 mM) and malate (2 mM) in the absence or presence of creatine (20 mM). The ADP-stimulated respiration above basal oxygen consumption was plotted to determine Vmax and the apparent Michaëlis constant for ADP (Km). Km represents the mitochondrial sensitivity to ADP. The Km ratio in the absence and in the presence of creatine (Km-cr / Km+cr) was taken as an index of functional activity of mitochondrial creatine kinase (mCK). To evaluate the functional activity of PDH and beta-oxidation, basal and ADP-stimulated respiration (Vo and Vmax, respectively) were measured in the presence of malate (2mM) and either pyruvate (200 µM), or octanoate (100 µM), or palmitoylcarnitine (100 µM). For each substrate, mitochondrial oxidation/phosphorylation coupling was assessed through the value of the acceptor control ratio (ACR = Vmax/Vo).
Isolation of heart mitochondria

A fragment of the heart (400 mg) was placed immediately in ice-cold isolation medium and chopped with scissors. Trypsin 250 (DIFCO LABORATORIES) was added (0.125 mg/ml) and the samples were mixed thoroughly and left for 15 min on ice. The preparations were diluted twice with isolation medium containing trypsin inhibitor (0.65 mg/ml) and bovine serum albumin (1 mg/ml). The suspension was stirred and decanted and the supernatant was then homogenized carefully in a glass potter. The samples were centrifuged at 600 g for 10 min at 4°C. The supernatant was centrifuged again at 8000 g for 15 min at 4°C. The pellet was resuspended in isolation medium containing bovine serum albumin (1 mg/ml) and centrifuged again at 8000 g for 15 min at 4°C. This operation was repeated twice. The pellet was finally suspended in 1 ml of serum albumin-free isolation medium. The protein concentration was measured using the bicinchoninic acid protein assay kit (SIGMA, France).

Analysis of lipids

The lipids were analysed as previously described (22). Briefly, the lipids were extracted from heart or mitochondria according to Folch (7) in chloroform-methanol 2/1. For the heart, phospholipids (PL) were separated from non-phosphorous lipids (NPL) on silicic acid cartridges (11) and the fatty acids were trans-methylated with BF3-methanol (17). The methyl esters were analyzed by gas chromatography on a EC-WAX capillary column (0.32x30 m) with FID, using C17:0 as internal standard.

Solution and reagents

Solutions S and R both contained 10 mM EGTA-CaEGTA buffer (free Ca\(^{2+}\) concentration, 100nM), 1 mM free Mg\(^{2+}\), 20mM taurine, 0.5mM dithiothreitol, and 20 mM imidazole. The ionic strength was adjusted to 0.16 M by addition of potassium methanesulfonate. Solution S (pH 7.1) also contained MgATP (5 mM) and phosphocreatine (15 mM). Solution R (pH 7.1) contained 3 mM potassium phosphate and 2 mg/ml fatty acid-free bovine serum albumin instead of high energy
phosphates. Malate (2 mM) + either glutamate (5 mM) or pyruvate (200 µM) or octanoate (100 µM), or palmitoylcarnitine (100 µM) were used as respiration substrates. The isolation medium (pH 7.2), used for mitochondrial isolation contained 0.3 M sucrose, 10 mM Na-HEPES, 0.2 mM EDTA. All reagents were purchased from SIGMA.

Statistical analysis

Nonlinear fits to Michaëlis-Menten kinetics were computed by a nonlinear least square routine. The data were expressed as mean ± sem and submitted to analysis of variance (ANOVA) with two fixed factors among diet and diabetes. When significantly different, the means were further compared by the Neuman-Keuls test (3).

Results

Characteristics of the rats

The STZ treatment resulted in a diabetic state with polyuria, polydipsia and a polyphagia. The evolution of the body weight in the Sham and STZ groups is shown on figure 1A. From the first week after the injection of STZ, the body weight gain was significantly lower in STZ-Ctrl rats than in sham-Ctrl rats, in spite of polyphagia. This difference kept increasing throughout the experiment. The addition of DHA to the diet did not affect growth in the Sham group, but significantly limited the negative effect of STZ on weight gain. There was a significant cross-interaction between the pathology and the DHA diet from the sixth week. The values of blood glucose and triglycerides and the weight of heart and kidneys are shown in table 2. In both STZ-treated groups, the rats displayed significantly higher blood glucose levels and similar triglyceride levels. The DHA supplement reduced the mean blood glucose levels in both Sham and STZ groups. As compared with the Sh-Ctrl group, the kidney weight was significantly increased and the heart weight significantly decreased in the STZ-Ctrl group (+35% and −13%, respectively), but DHA did not influence these differences. Conversely, the rats of the STZ-Ctrl group displayed an increased heart/body weight
ratio, because of the reduced weight gain in the STZ-Ctrl group. For this parameter, the effect of DHA was significant, although it could be related to the positive effect of this diet on weight gain (see above). The time course of the body weights in the NHI and HI groups is shown in figure 1B. From the first week the body weight gain was significantly lower in HI rats than in NHI rats, independently of the diet. This difference remained constant throughout the experiment. The heart and kidney weights and blood levels of glucose and triglycerides are shown in Table 2. The heart weight was not affected by insulin resistance nor by dietary DHA. In contrast, the kidney weight increased moderately (+ 6%) in the HI-Ctrl group, but DHA had no effect on this parameter. The development of insulin resistance did not affect blood glucose levels but significantly increased plasma triglycerides. The DHA intake significantly decreased glycemia in both NHI and HI groups, but did not affect triglycerides.

**ADP-stimulated mitochondrial respiration**

In the presence of glutamate + malate, NADH-generating substrates, the basal oxygen consumption rate was accelerated by stepwise additions of ADP. Figure 2A shows the mean of oxygraphic traces of mitochondrial respiration in the presence of creatine, in sham-Ctrl, STZ-Ctrl and STZ-DHA groups. The curves show a decrease in mitochondria sensitivity to ADP in the STZ-Ctrl group, whereas for the STZ-DHA, the respiration rate curve was in-between the curve of the Sham and STZ-Ctrl rats, suggesting that the alteration in affinity for ADP induced by insulin-deficiency was limited by dietary DHA. The mean oxygraphic traces of mitochondrial respiration in the presence of creatine, in NHI-Ctrl, HI-Ctrl and HI-DHA groups is shown in figure 2B. The mean oxygen consumption curves of NHI-Ctrl and HI-Ctrl rats are exactly superimposed, suggesting that insulin resistance did not affect the mitochondrial affinity for ADP. The mean curve of the HI-DHA group was slightly shifted suggesting a moderate increase of ADP affinity by dietary DHA.

**Mitochondrial affinity for ADP**
From the oxygraphic traces, the respiration rate was calculated for each ADP concentration and plotted as a function of ADP concentration, and the Km for ADP was determined. Figure 3 shows the Km values for the STZ treated rats. Without creatine, the STZ treatment had no effect on Km (STZ-Ctrl vs Sh-Ctrl and STZ-DHA vs Sh-DHA). However, the DHA groups (Sh-DHA and STZ-DHA) showed an increased Km as compared to the control groups (Sh-Ctrl and STZ-Ctrl). The addition of creatine to the medium significantly decreased the Km for ADP due to ADP regeneration catalyzed by mitochondrial creatine kinase (CK). In the presence of creatine, the Km increased in the STZ-Ctrl group as compared to the Sh-Ctrl group, indicating a decrease of the mitochondrial sensitivity to ADP. However, the effect of dietary DHA was significantly different in the STZ-DHA group compared to the Sh-DHA group, demonstrated by an increased mitochondrial sensitivity to ADP in the STZ-DHA group. As shown in figure 2B, neither insulin resistance nor dietary DHA changed the ADP sensitivity of cardiac mitochondria. In the absence of creatine, the km values were 385 ± 36.8 µM and 392 ± 22.5 µM in the NHI group and the HI group, respectively. In the presence of creatine, the km values were 94 ± 12.3 µM and 99 ± 8.7 µM in the NHI group and the HI group, respectively (data not shown).

Mitochondrial CK functional activity

Figure 4 shows the Km ratio in the absence and in the presence of creatine (Km-cr / Km+cr), in the insulin deficient model. In the STZ-Ctrl group, the activity of mitochondrial CK was significantly decreased as compared with the Sh-Ctrl group. Dietary DHA had no effect on mitochondrial CK in the sham rats, but significantly increased the Km ratio in the STZ-DHA group. In contrast, as stated above for the mitochondrial affinity for ADP, neither insulin resistance nor dietary DHA affected mi-CK activity in either the NHI group (4.50 ± 0.90) or the HI group (4.05 ± 0.33).

Substrates used for mitochondrial respiration

To investigate the effect of fatty acid/carbohydrate balance on energy production, the mitochondrial respiration was measured in the presence of several substrates. Figure 5 shows the respiration rate
for each substrate, in the absence and the presence of 1 mM ADP (Vo and Vmax, respectively), in
the insulin deficiency model. For both Vo and Vmax, the STZ treatment induced a significant
decline in glutamate- and palmitoylcarnitine-supported respiration (figure 5A and 5D,
respectively). In contrast, the STZ treatment had no effect on the pyruvate and octanoate-supported
respiration (figure 5B and 5C, respectively). Irrespective of the substrate provided, dietary DHA did
not affect mitochondrial oxygen consumption nor was the acceptor control ratio altered by STZ
treatment or by dietary DHA (data not shown). The same parameters were investigated in insulin
resistance and figure 6 shows the Vo and Vmax respiration rates for each substrate. Neither insulin
resistance nor DHA intake affected the Vo respiration rate. In contrast, the Vmax of glutamate-
supported respiration (figure 6A) was significantly lower in the HI groups (both HI-Ctrl and HI-
DHA) than in the corresponding NHI groups. Again, dietary DHA had no effect. Pyruvate-
supported respiration slightly increased in the HI-Ctrl group compared to the NHI Ctrl group (figure
6B). But in the HI-DHA group, respiration was significantly decreased compared to NHI-DHA and
HI-Ctrl. The effect of dietary DHA in the HI group was opposite to that in the NHI group (figure
6B). The octanoate-supported respiration was not affected by hyperinsulinemia (HI-Ctrl vs NHI-
Ctrl, figure 6C), but in the HI-DHA group, the respiration was significantly decreased compared to
NHI-DHA and HI-Ctrl. The palmitoylcarnitine-supported respiration (figure 6D) was not affected
by hyperinsulinemia (HI-Ctrl group vs NHI-Ctrl group and HI-DHA group vs NHI-DHA group),
but in both the NHI-DHA and HI-DHA groups respiration was significantly increased compared to
the NHI-Ctrl and HI-Ctrl groups. Whatever the substrate, neither insulin resistance nor dietary DHA
affected the mitochondrial acceptor control ratio (data not shown).

Fatty acid composition

The fatty acid composition of cardiac membrane phospholipids and mitochondrial membranes in
STZ-treated rats is shown in table 3. The fatty acid composition was strongly affected by insulin
deficiency. Interestingly, the STZ-Ctrl group was characterized by an increase in n-6 PUFA and a
decrease in n-3 PUFA including eicosapentaenoic acid (EPA, 20:5 n-3), docosapentaenoic acid (DPA, 22:5 n-3) and DHA. Then, the n-6/n-3 ratio was strongly increased in the STZ-Ctrl group. As expected, DHA supplementation strongly altered the PUFA composition of cardiac phospholipids with a significant increase in n-3 PUFA including mainly DHA, but also EPA and DPA. The increase in n-3 PUFA was balanced by a significant decrease in arachidonic acid. Dietary DHA prevented the increase in the n-6/n-3 ratio in the STZ-treated rats. The ANOVA revealed a significant cross-interaction between STZ treatment and DHA supplementation. Moreover, as shown in table 3, the STZ treatment also strongly affected the fatty acid composition of cardiac mitochondrial membranes. The linoleic acid content significantly increased in STZ-Ctrl group whereas arachidonic acid content significantly decreased. Moreover, the n-6/n-3 ratio increased in the STZ-Ctrl group due to a significant decrease in n-3 PUFA. The DHA intake limited any further decrease of arachidonic acid content in STZ treated rats and prevented the increase of n-6/n-3 ratio. The fatty acid composition of phospholipids of heart membranes in insulin resistance model is shown in table 4. The fatty acid composition was weakly affected by insulin resistance. Interestingly, insulin resistance increased the saturated fatty acid and monounsaturated fatty acid content but decreased the n-6 PUFA content, mainly at the level of the arachidonic acid precursors, 20:2 n-6 and linoleic acid (18:2 n-6). Moreover, the n-3 PUFA content was not significantly affected. The DHA supplementation induced the same modifications in the fatty acid composition as those described above for the STZ groups. After three months, the development of insulin resistance did not significantly affect the cardiac phospholipid fatty acid composition although a significant cross-interaction between insulin resistance and dietary DHA was observed for some fatty acids. Insulin resistance weakly affected the fatty acid composition of mitochondrial phospholipids (Table 4). Although the n-6/n-3 ratio was slightly increased by insulin resistance, saturated, monounsaturated and PUFA were not significantly affected by the pathology. Moreover, the fatty acid alterations induced by DHA supplementation was roughly similar in mitochondrial
membranes and total cardiac phospholipids. The cross-interaction between insulin resistance and dietary DHA was significantly different only for the n-6/n-3 ratio.

**Discussion**

An imbalance between fatty acid and glucose utilization in energy synthesis is known to occur in the insulin deficient state. Several studies using isolated cardiac mitochondria (2; 6; 20; 25; 26; 28; 29) or permeabilized cardiac fibers (32) have shown that mitochondrial function is strongly affected in insulin deficiency. In this study, we evaluated the effects of STZ-induced insulin deficiency on mitochondrial function in cardiac permeabilized fibers. This animal model is known to develop many of the features reported in human subjects with uncontrolled diabetes mellitus, including hyperglycemia, polydipsia, polyuria and weight loss (30; 30). In this study, the rats treated with STZ developed hyperglycemia and hypoinsulinemia after two months, and the body weight gain was lower than in sham rats. In this experiment, dietary DHA elicited a slight reduction in glycemia, which was observed in the 2 models. A similar result was observed in a previous study using the same insulin-resistance model (22). This subject is not well documented in the literature and claims for specific investigations on a possible hypoglycemic effect of DHA. Moreover the insulin deficient rats developed renal hypertrophy and an increased heart weight / body weight ratio compared to sham rats. The functional state of the total cardiac mitochondrial population was investigated in saponin-permeabilized fibers. In this model of insulin deficiency, the mitochondrial sensitivity to ADP was slightly decreased in the presence of creatine. The activity of mitochondrial creatine kinase, which catalyzed the local ADP regeneration, was decreased. Using the same model, Savabi observed a decrease in mitochondrial creatine kinase activity and a decrease in the ability of creatine to stimulate oxidative phosphorylation (25). Another study reported a significant decrease in total creatine kinase activity and a diminution of the various creatine kinase isoenzymes in both atria and ventricles (26). In this study, dietary DHA compensated for the diminution of creatine kinase activity associated with insulin deficiency, suggesting a relationship between the decrease in
activity and the modifications of mitochondrial membrane fatty acid composition. In STZ-induced insulin deficiency, an increase in linoleic acid together with a decrease in arachidonic acid content of cardiac phospholipids was previously reported (9; 12). In this study the same effect was observed in both cardiac phospholipid fatty acid and mitochondrial membrane fatty acid composition. The decrease in long-chain PUFA and the linoleic acid accumulation, observed in both cardiac and mitochondrial membranes supports the previously reported decrease in Δ6 desaturase (5), the key enzyme in the conversion of linoleic acid to long chain PUFA. Moreover, the insulin deficient state decreased the membrane n-3 PUFA content (mainly DHA), balanced by an increased n-6 PUFA content, resulting in a significant increase in n-6/n-3 ratio. This model of insulin deficiency was reported to put myocardial n-3 fatty acid status at risk in the absence of dietary n-3 PUFA supplementation (16). Liautaud et al (1991) pointed out the poor capacity of the cardiac myocyte to achieve the last desaturation step of long chain PUFAs, from the close precursor DPA (22:5 n-3) to DHA (22:6 n-3) (13). The occurrence of a Δ4 desaturase is not clearly documented, and the conversion of DPA to DHA was shown to require 2 successive elongation steps, a Δ6 desaturation step, and one cycle of β-oxidation on a 24-carbone PUFA, which may be the inefficient step in the heart (33). This may explain why the insulin deficiency-induced Δ6 desaturase inhibition may contribute to hampered DHA incorporation in membrane lipids. The present study showed that dietary DHA can compensate for this trend by promoting increased incorporation of DHA in cardiac membranes, including mitochondria. The decrease in creatine kinase activity may be related to the alterations in mitochondrial membrane fatty acid composition observed in this study. This enzyme is located on the internal mitochondrial membrane surface, and the altered composition of this membrane may influence the membrane bound enzymes, perhaps resulting in altered creatine kinase activity and/or mitochondrial affinity for ADP. Interestingly, the two PUFA that were affected by insulin deficiency in this study are linoleic acid, which is the main component of
cardiolipin, a major mitochondrial phospholipid, and DHA, a fatty acid which affects oxygen consumption by the heart (19).

We investigated the mitochondrial utilization of different substrates in cardiac saponin-permeabilized fibers. In the presence of NADH-generating substrates (glutamate + malate), the insulin deficient state strongly decreased both basal and ADP-stimulated respiration, in accordance with the previous observations by Veksler et al. (32). Dietary DHA was unable to affect this respiration decrease. In the presence of pyruvate, insulin deficiency did not affect basal respiration but slightly decreased ADP-stimulated respiration. The pyruvate dehydrogenase and krebs cycle were not affected by the pathology because basal oxygen consumption was similar in insulin deficient rat mitochondria and sham rat mitochondria. The reduction of glutamate utilization in insulin deficient rat mitochondria could be explained by decreased glutamate uptake into mitochondria or by decreased glutamate dehydrogenase activity. In cardiac muscle, insulin deficiency increases the utilization of fatty acids for energy synthesis (14). We compared the ability of mitochondria to oxidize long-chain fatty acids that require carnitine for entry into mitochondria and short-chain fatty acids that do not require carnitine. Permeabilized fibers used in this study lost their cytoplasmic content, and it was necessary to use the acyl-carnitine form of the long-chain fatty acid. The respiration rate in the presence of short-chain fatty acid was not affected by insulin deficiency, a confirmation that the Krebs cycle was not impaired in insulin deficient state. Moreover, the beta-oxidation rate was not affected. Conversely, in the presence of long-chain fatty acyl carnitine, basal and ADP-stimulated respiration were decreased in insulin deficient mitochondria, but DHA supplementation failed to prevent this effect. In the conditions used here for mitochondrial respiration measurements, fatty acids and pyruvate were not in competition and the glucose/fatty acid balance for energy production, which decreases in insulin deficient state, was not determined. Moreover, irrespective of the substrate used, neither STZ-treatment nor DHA supplement affected the coupling between oxygen consumption and phosphorylation.
This nutritional model of insulin resistance is based on a diet in which fructose is the only carbohydrate supplied to the rats and they develop hyperinsulinemia and insulin resistance in a short time (10). In the present study, after three months of fructose diet, the rats exhibited hyperinsulinemia, normoglycaemia characteristic of insulin resistance and hypertriglyceridemia. Moreover, body weight decreased compared to control rats. The effect of fructose-enriched diet on the body weight is a matter of debate. Some authors observed a body weight increase after up to 8 weeks fructose diet (18; 22). Others reported a body weight decrease after 13 days (10), or six weeks of fructose diet (8) or no difference compared to the control rats after three months (27). Moreover, we observed renal hypertrophy but no difference in heart weight. DHA supplementation did not prevent these morphologic effects of the fructose diet. The functional state of the total cardiac mitochondria population was investigated in saponin-permeabilized fibers. The mitochondrial sensitivity for ADP was not influenced by either insulin resistance or dietary DHA, and the activity of creatine kinase was not altered. This small effect correlated with the slight effect of the fructose-diet on mitochondrial membrane fatty acids. Insulin resistance did not change the n-6 or n-3 PUFA content in mitochondrial membrane. Nevertheless, the n-6/n-3 ratio was slightly increased in insulin resistant rats and this effect was reversed and overcompensated by dietary DHA. More alterations were observed in cardiac phospholipid fatty acid composition, including a significant decrease in both n-6 and n-3 PUFA content. Interestingly, this decrease in PUFA was balanced by an increase in MUFA. The PUFA decrease was more pronounced for the n-3 than the n-6 series, producing a slight increase in the n-6/n-3 ratio. These results are in agreement with the previous observations by Rousseau et al. (22), using the same animal model. Moreover, dietary DHA compensated for all the changes in cardiac membrane n-3 PUFA composition induced by insulin resistance.

Regarding the use of various substrates, our results suggest that insulin resistance does not influence the mitochondrial main basal respiration pathways (pyruvate dehydrogenase, krebs cycle and beta-
oxidation). However, in ADP-stimulated respiration, the mitochondria of insulin resistant rat hearts showed a significant decrease in glutamate-supported oxygen consumption. This result suggests a deficiency of either glutamate dehydrogenase or mitochondrial glutamate uptake to supply complex I with glutamate when the respiration is stimulated by ADP. Dietary DHA was unable to affect this respiration decrease. The slight increase of pyruvate-supported respiration in insulin resistance could be explained by a weak increase in pyruvate dehydrogenase activity. Dietary DHA did not affect this pyruvate-supported respiration in non insulin resistant rats but totally compensated for the effects of insulin resistance. This discrepancy between the effect of DHA on basal function and pathological function was previously reported for cardiac cell electrophysiology. Although incorporation of DHA in cardiomyocytes membranes had no effect on action potential and contraction parameters in normoxia, it significantly improved the recovery of the same parameters after ischemia and reperfusion (4). Cardiomyocyte incorporation of DHA also affects active but not basal oxygen consumption in the intact working heart (19). Neither insulin resistance nor dietary DHA individually affected the respiration of octanoate, however the cross-interaction between the two factors was statistically significant, suggesting that DHA is only effective in insulin resistance, as it is for pyruvate. The observation that insulin resistance-induced alterations in pyruvate- and octanoate-supported respiration are fully compensated when the mitochondria membrane n-3 fatty acid composition is maintained supports the hypothesis that the metabolic changes arise as a secondary response to membrane alteration rather than from a direct effect of the pathology. On the other hand, in contrast to the three and eight carbon fatty acids (pyruvate, octanoate) the respiration rate in the presence of a long chain fatty acid (16 carbon palmitoyl carnitine) was affected by DHA supplementation in both sham control and hyperinsulinemic groups. Nevertheless, insulin resistance did not affect the coupling between oxygen consumption and phosphorylation, irrespective of the metabolic substrate.
In conclusion, the present study identified that mitochondrial function was altered in both insulin deficient and hyperinsulinemic insulin resistant states. Furthermore, while some alterations were similar in the two pathologies, mitochondrial function was affected more by insulin deficiency than by insulin resistance. We also observed similar modifications in both mitochondrial membrane and cardiac cell membrane phospholipid fatty acid composition, but the effects of insulin deficiency were more pronounced, and were associated with alterations in mitochondrial creatine kinase activity. However, the results of this study demonstrate that DHA supplementation during development of insulin deficiency or insulin resistance, has beneficial effects on mitochondrial function. DHA intake was able to prevent the decrease in function of mitochondrial membrane-bound systems, including mitochondrial creatine kinase activity and mitochondrial sensitivity to ADP.
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The authors would like to thank the Cacao Butter Company and Hoffman-LaRoche for the generous gift of pure cacao butter and DHA, respectively. The authors are indebted to Mrs. D. Anger (Hôpital Avicenne, Bobigny, FRANCE) and Pr D. Porquet (Hôpital Necker, Paris, FRANCE) for insulin determinations, to Pr P.L. McLennan (Dept. of Biomedical Science, Sidney, Australia) for editing the manuscript and to Mrs. A.M. Gueugneau, and Drs C. Héliès-Toussaint and S. Tardivel for experimental assistance.
Reference List


33. **Voss A, Reinhart M, Sankarappa S and Sprecher H.** The metabolism of 7,10,13,16,19-docosapentaenoic acid to 4,7,10,13,16,19-docosahexaenoic acid in rat liver is independent of a 4-desaturase.
Figure legends

Figure 1: Time course of the body weight (mean±s.e.m.) in the insulin-deficiency experiment (panel A) and insulin-resistance experiment (panel B). Results of the 2-way ANOVA in insert including STZ (or HI), and DHA as fixed factors (CI: cross interaction).

Figure 2: Time course of the oxygen consumption (mean±s.e.m.) – A- Sham and STZ-treated groups. The Sh-DHA curve was exactly superimposed on the Sh-Ctrl curve and was omitted for clarity – B- NHI and HI groups. The NHI-DHA curve was exactly superimposed on the NHI-Ctrl curve and was omitted for clarity.

Figure 3: Effect of STZ-treatment on the Michaelis constant for ADP (Km) in the absence and in the presence of creatine 20 mM (mean±s.e.m). sh-Ctrl: sham rats, control diet; STZ-Ctrl: STZ treated rats, control diet; Sh-DHA: sham rats, DHA diet; STZ-DHA: STZ treated rats, DHA diet; STZ effect: STZ-treated groups vs sham group; DHA effect: Control group vs DHA group; CI: cross interaction.

Figure 4: Effect of STZ-treatment on mitochondrial creatine kinase activity (mean±s.e.m). sh-Ctrl: sham rats, control diet; STZ-Ctrl: STZ treated rats, control diet; Sh-DHA: sham rats, DHA diet; STZ-DHA: STZ treated rats, DHA diet; STZ effect: STZ-treated groups vs sham group; DHA effect: Control group vs DHA group; CI: cross interaction.

Figure 5: Effect of STZ-treatment on the use of substrates in mitochondrial respiration (mean±s.e.m). A-Glutamate (5mM) + Malate (2mM) – B-Pyruvate (200µM) + Malate (2mM) – C-Octanoate (100µM) + Malate (2mM) – D-Palmitoylcarnitine (100µM) + Malate (2mM); sh-Ctrl: sham rats, control diet; STZ-Ctrl: STZ treated rats, control diet; Sh-DHA: sham rats, DHA diet; STZ-DHA: STZ treated rats, DHA diet; Vo: respiration rate without ADP; Vmax: respiration rate with ADP 1 mM; STZ effect: STZ-treated groups vs sham group; DHA effect: Control group vs DHA group; CI: cross interaction.
Figure 6: Effect of hyperinsulinemia on the use of substrates in mitochondrial respiration (mean±s.e.m). A- Glutamate (5mM) + Malate (2mM) – B-Pyruvate (200µM) + Malate (2mM) – C- Octanoate (100µM) + Malate (2mM) – D-Palmitoylcarnitine (100µM) + Malate (2mM); NHI-Ctrl : non hyperinsulinemic rats, control diet; HI-Ctrl : hyperinsulinemic rats, control diet; NHI-DHA : non hyperinsulinemic rats, DHA diet; HI-DHA : hyperinsulinemic rats DHA diet; V_o : respiration rate without ADP; V_max : respiration rate with ADP 1 mM; HI effect : hyperinsulinemic group vs non hyperinsulinemic group; DHA effect : Control group vs DHA group; CI : cross interaction.
Table 1: Fatty acid composition (as % of total fatty acid) as analyzed in the lipid blend of the 2 dietary groups

(2 blends were prepared for each group); nd: not detected

<table>
<thead>
<tr>
<th>Fatty acid</th>
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<th>DHA</th>
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</tr>
<tr>
<td>16:1 n-9</td>
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<tr>
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<td>18:1 n-9</td>
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<td>22.2</td>
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<tr>
<td>18:2 n-6</td>
<td>34.3</td>
<td>27.0</td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>nd</td>
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</tr>
<tr>
<td>22:5 n-3</td>
<td>nd</td>
<td>3.8</td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>nd</td>
<td>17.2</td>
</tr>
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<td>Minor SFA</td>
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<tr>
<td>Minor PUFA</td>
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<td>22.5</td>
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<tr>
<td>Total PUFA</td>
<td>36.8</td>
<td>51.1</td>
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Table 2: General characteristics STZ treated rats and hyperinsulinemic rats and their controls

STZ effect = difference between Sham and STZ groups, DHA effect = difference between Ctrl and DHA groups, HI effect = difference between NHI and HI groups, CI = cross-interaction between STZ treatment and dietary DHA, * = p<0.05, ** = p<0.01, *** = p<0.001, NS = not significant

<table>
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<th></th>
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<th>NHI groups</th>
<th>HI groups</th>
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<td>NHI-Ctrl(n=8)</td>
<td>NHI-DHA(n=8)</td>
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<td>±0.03</td>
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<td>±0.02</td>
<td>±0.03</td>
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<td>Kidneys weight (g)</td>
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<td>2.73</td>
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Note: NS = not significant
Table 3: Effect of STZ-induced diabetes on the fatty acid composition (as % of total fatty acid) of phospholipids of heart membranes and mitochondrial membranes; SFA. MUFA. PUFA = saturated. mono- and polyunsaturated fatty acids; nd = not detected. STZ effect = Sham group vs STZ group, DHA effect = Ctrl group vs DHA group, CI = cross interaction between STZ treatment and DHA intake, * = p<0.05, ** = p<0.01, *** = p<0.001, NS = not significant

<table>
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<tr>
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<th>Mitochondria</th>
<th>ANOVA mitochondria</th>
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<td>nd</td>
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<tr>
<td>22:5 n-3</td>
<td>0.7±0.1</td>
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Table 4: Effect of hyperinsulinemia on the fatty acid composition (as % of total fatty acid) of phospholipids of heart membranes and mitochondrial membranes.

SFA. MUFA. PUFA = saturated. mono- and polyunsaturated fatty acids; HI effect = NHI group vs HI group, DHA effect = Ctrl group vs DHA group, CI = cross interaction different between HI and DHA intake, * = p<0.05, ** = p<0.01, *** = p<0.001, NS = not significant

<table>
<thead>
<tr>
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<th>Mitochondria</th>
<th>ANOVA mitochondria</th>
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<td>HI DHA (n=8)</td>
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<td>22:6 n-3</td>
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<td>1.5±0.2</td>
<td>11.4±1.0</td>
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</table>
Figure 1

A

STZ effect  \( p < 0.001 \)
DHA effect  \( p < 0.05 \)
CI STZ/DHA  \( p < 0.05 \)

B

HI effect  \( p < 0.01 \)
DHA effect  NS
CI HI/DHA  NS
**Figure 2**

A

![Graph A](image)

B

![Graph B](image)
Figure 3

<table>
<thead>
<tr>
<th>Condition</th>
<th>Effect</th>
<th>p-value</th>
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<tr>
<td>STZ effect</td>
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<td></td>
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<tr>
<td>DHA effect</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CI STZ/DHA</td>
<td>p&lt;0.01</td>
<td></td>
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</tbody>
</table>

Sh-Ctrl
STZ-Ctrl
Sh-DHA
STZ-DHA

Km (µM)

without creatine
creatine 20mM
Figure 4

- STZ effect: NS
- DHA effect: NS
- CI STZ/DHA p<0.05
Figure 5

A

STZ effect  p<0.05
DHA effect  NS
CI STZ/DHA  NS

B

STZ effect  NS
DHA effect  NS
CI STZ/DHA  NS

C

STZ effect  NS
DHA effect  NS
CI STZ/DHA  NS

D

STZ effect  p<0.05
DHA effect  NS
CI STZ/DHA  NS

Vo  Vmax

Sh-Ctrl
STZ-Ctrl
Sh-DHA
STZ-DHA