Olive Oil Increases the Hepatic Triacylglycerol Content in Mice by a Distinct Influence on the Synthesis and Oxidation of Fatty Acids

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Received June 8, 2007; Accepted September 20, 2007; Online Publication, January 7, 2008

Diet supplementation with olive oil exerts beneficial effects on an organism, even if an increase in the level of hepatic lipids has been concomitantly observed. This study was therefore designed to investigate whether the stimulation of lipogenesis was responsible for the olive oil-induced hepatic fat accumulation. In mice fed for 8 weeks with an olive oil-enriched diet, an increase of about 2.6 fold in the level of liver triglycerides was found in comparison to animals fed with a corn oil-containing diet. Despite that, no increase in the activities of cytosolic lipogenic enzymes or of the mitochondrial tricarboxylate carrier was found; on the contrary, a decrease in the activity of carnitine palmitoyltransferase I was observed. This impairment of fatty acid oxidation, which was not apparent in corn oil-fed animals, may have had a role in the increase of hepatic lipid content found in the olive oil-fed mice.

Key words: fatty acid synthesis; fatty acid oxidation; mitochondrial citrate carrier; olive oil; corn oil

The fatty acid composition of the diet is an important factor capable of modulating the liver lipid metabolism. As has been widely reported, different fatty acids exert different effects on the transcription of specific genes involved in hepatic lipid metabolism. In recent years, the beneficial effects of the Mediterranean diet have been greatly publicized, and consequently olive oil is extensively used as a dietary fat. One of the most intriguing aspects regarding this oil is its effect on hepatic lipid metabolism. In some studies carried out on rodents, an olive oil-enriched diet induced fat accumulation in the liver. One of the most intriguing aspects regarding this oil is its effect on hepatic lipid metabolism. In some studies carried out on rodents, an olive oil-enriched diet induced fat accumulation in the liver.4–7 In further studies, the higher content of hepatic lipids, especially of triglycerides, found in rats receiving the olive oil diet was positively correlated with higher activities of the liver lipogenic enzymes.8,9 However, it is generally known that polyunsaturated fatty acids (PUFA) are the strongest down-regulators of hepatic lipogenesis, whereas saturated (SFA) and monounsaturated fatty acids (MUFA) have little or no effect on the synthesis of fatty acids.1,2,10–12

We investigated in this study the effect of two diets, one enriched with 7.5% olive oil and the other with 7.5% corn oil, on hepatic fatty acid synthesis and oxidation in mice. Besides the enzymes acetyl-CoA carboxylase (ACC) and fatty acid synthetase (FAS), which are both involved in the synthesis of fatty acids, and carnitine palmitoyltransferase I (CPT I), which is involved in fatty acid oxidation, we also concentrated our attention on a less investigated protein, the mitochondrial tricarboxylate or citrate carrier (CIC). This protein, which belongs to the large family of the inner membrane metabolite carriers,15 plays a key role in hepatic lipogenesis by transporting the molecule of citrate, which has been synthesized in the matrix, towards the cytosol where de novo fatty acid synthesis occurs. The activity of the CIC is therefore closely connected to that of the cytosolic lipogenic enzymes, ACC and FAS, to which the CIC physiologically supplies substrates. Furthermore, the molecule of citrate transported outside mitochondria by the CIC is also the positive allosteric modulator of ACC,16 the first cytosolic step of hepatic fatty acid synthesis. Previous studies have indicated that the activity of the mitochondrial CIC was down-regulated during starvation17 and by diets enriched in PUFA of the n-6 and n-3 series.18–21 On the contrary, a significant increase in the mitochondrial CIC activity has been found in mice fed with a diet containing 1% conjugated linoleic acid (CLA).22 In these studies, parallel changes in the activities of the mitochondrial CIC and of the cytosolic lipogenic enzymes have been found, thereby leading to the concept that the mitochondrial CIC may be considered as a good sensor of the changes occurring in hepatic lipogenesis.

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Abbreviations: ACC, acetyl-CoA carboxylase; 1,2,3-BTA, 1,2,3-benzenetricarboxylate; CIC, mitochondrial tricarboxylate or citrate carrier; CPT, carnitine palmitoyltransferase; FAS, fatty acid synthetase; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids
In mice fed with a diet enriched with 7.5% olive oil for different periods of time, ranging from 2 to 8 weeks, a significant increase in the level of hepatic lipids, and particularly triglycerides, was found in comparison to corn oil-fed animals. This increase in the level of hepatic triglycerides was statistically significant at any time of the dietary treatment. Despite this substantial change in the hepatic lipid profile, the activities of the mitochondrial CIC and of the cytosolic lipogenic enzymes were practically unaltered over time in the olive oil-treated mice. On the contrary, a slight, but significant decrease in the activity of the CPT I, the rate-limiting step for fatty acid oxidation, was found in the olive oil-treated mice with respect to the corn oil-fed animals.

Materials and Methods

Materials. The protein assay kit was purchased from Bio-Rad. Amberlite XAD-2, Dowex AG1-X8, Pipes, Triton X-100, Triton X-114, Sephadex G-75, 1,2,3-benzenetricarboxylate (1,2,3-BTA), cardiolipin, acetyl-CoA, phosphoenolpyruvate, ATP, NADH, NADPH, pyruvate kinase, lactate dehydrogenase, malonyl-CoA, DTNB, carnitine and palmitoyl-CoA were all from Sigma. [1,5-14C]citrate was from Amersham, and egg-yolk phospholipids were from Fluka. Virgin olive oil was from a local supplier, and corn oil was from Carapelli (Italy). Kits for the assay of triglycerides, total cholesterol and phospholipids were purchased from Futura System. All other reagents were of analytical grade.

Animals. Male ICR mice were obtained from Harlan at 5 weeks of age and housed individually at a temperature of 22 ± 1 °C. The mice were randomly divided into two groups of 25 animals each and fed ad libitum either with a standard diet supplemented with 7.5% corn oil or with the same diet supplemented with 7.5% olive oil. Table 1 reports the composition of both diets that were prepared each week and stored frozen until used. The body weight, liver weight and food intake were recorded throughout the study, ranging from 2 to 8 weeks. The study was carried out in accordance with local and national guidelines regarding animal experiments.

Citrate transport in mice liver mitochondria. Mice liver mitochondria were prepared using standard procedures. Freshly isolated mitochondria were resuspended in 100 mM KCl, 20 mM Hepes, 1 mM EGTA and 2 µg/ml of rotenone at pH 7.0, to a final concentration of about 5 mg of protein/ml before being loaded with L-malate as reported.17) The assay of citrate transport, which was carried out at 9 °C, was initiated by adding 0.5 mM [14C] citrate and stopped by 12.5 mM 1,2,3-BTA. Mice liver mitochondria were resolubilized at 18000 g for 10 min, washed and then extracted with 20% HClO4. The mixture was centrifuged, and the radioactivity present in the supernatant was counted by liquid scintillation.

Solubilization and reconstitution of the CIC into liposomes. Mice liver mitochondria were solubilized with a buffer containing 3% Triton X-100 (w/v), 20 mM Na2SO4, 1 mM EDTA and 10 mM Pipes at pH 7.0, to a final concentration of about 10 mg of protein/ml. After incubating for 10 min at 2 °C, the mixture was centrifuged at 25000 g for 20 min at 2 °C, thereby obtaining a mitochondrial extract.25) The mixture used for the reconstitution experiments contained 20 µl of this mitochondrial extract, 90 µl of 10% Triton X-114, 20 µl of 20 mg/ml of cardiolipin, 100 µl of 10% phospholipids in the form of sonicated liposomes, 70 µl of 100 mM Pipes (pH 7.0) and 35 µl of 200 µM citrate in a final volume of 700 µl. After vortexing, this mixture was passed 15 times through the same Amberlite XAD-2 column in order to obtain the proteoliposomes as previously described.24) The external citrate was removed by gel-filtration in a Sephadex G-75 column, and the proteoliposomes were then used for transport studies. The reaction, carried out at 25 °C, was initiated by the addition of 0.5 mM [14C] citrate and stopped by 20 mM 1,2,3-BTA. The radioactivity external to the proteoliposomes was removed by chromatography, whereas the internal radioactivity was measured by scintillation counting.

Assay of hepatic enzymes. Mice liver cytosol was obtained by centrifuging the post-mitochondrial supernatant at 20000 g for 20 min at 2 °C. The pellet was discarded and the supernatant was then centrifuged at 105000 g for 1 h. The activities of ACC and FAS were measured in the resulting cytosol as previously described.25,26) The assay of total CPT activity was carried out spectrophotometrically at 412 nm on mice liver mitochondria, essentially as described.27) CPT I activity was
calculated by subtracting the CPT activity that was insensitive to 100μM malonyl-CoA from the total CPT activity that was experimentally determined.

Assay of lipids. Total lipids were extracted from mice liver with a 1:1 mixture of chloroform and methanol as described. The extract was dried under a nitrogen flow and resuspended in 0.1% Triton X-100 as previously described, before carrying out individual assays of triglycerides, cholesterol and phospholipids by using commercial kits. Mice were starved overnight before their sacrifice for the determination of plasma lipids. Blood was collected and centrifuged to separate the plasma. Plasma triglycerides, cholesterol and phospholipids were then measured by using the commercial kits.

Other methods and statistical analysis. Protein was determined as described or by the Lowry method modified for the presence of Triton. Polyacrilamide gel electrophoresis was performed in the presence of 0.1% SDS (SDS–PAGE) according to standard procedures. The mitochondrial proteins that had been separated by SDS–PAGE were then transferred to a nitrocellulose membrane. This protein transfer was carried out for 1 h at 25 °C (250 mA). The nitrocellulose sheets were then treated for 1 h at 25 °C with an antiserum directed against the C-terminus of the rat liver citrate carrier added at a dilution of 1:3 × 10^3. After washing, the nitrocellulose sheets were incubated for 30 min at 25 °C with horseradish peroxidase-conjugated anti-rabbit Ig. The immunoreacted proteins were detected by the peroxidase reaction, using N, N’ Diamino Benzydine (DAB) and hydrogen peroxide.

Experimental data are presented as the mean ± SE. Student’s t-test was performed to detect significant differences between the control and the olive oil-treated animals. Differences were considered statistically significant at P < 0.05.

Results

Food intake, body weight and liver weight
The male mice were divided into two groups and fed on a standard diet supplemented with 7.5% corn oil or with 7.5% olive oil for different time periods. The olive oil-supplemented diet, as reported in Table 1, contained approximately twice as much oleic acid as the corn oil-enriched diet, which, in contrast, contained twice the amount of linoleic acid. The level of the remaining fatty acids, as well as that of other nutrients, was approximately the same in both diets, which therefore resulted in isocaloric balance. As shown in Table 2, the food intake and the liver weight were similar in the two groups of mice over the course of the study. The body weight was only slightly higher in the olive oil-fed mice than in the corn oil-fed animals (Table 2). Such a difference was statistically significant in the 2nd and 4th weeks of the dietary treatment.

Lipid composition of the plasma and liver
Table 3 reports the levels of triglycerides, cholesterol and phospholipids in the plasma of the corn oil- and olive oil-treated animals. A transient, yet significant, increase of about 1.2 fold in the plasma concentration of triglycerides was found in the mice in the 2nd and 4th weeks of the olive oil administration. On the contrary, the plasma cholesterol and phospholipids levels were slightly lower in the olive oil-fed animals than in the corn oil-fed mice at all times during the dietary

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Food intake (g/day)</th>
<th>Body weight (g)</th>
<th>Liver weight (g/100 g of body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Corn oil</td>
<td>Olive oil</td>
<td>Corn oil</td>
</tr>
<tr>
<td>0</td>
<td>8.9 ± 1.3</td>
<td>9.0 ± 1.3</td>
<td>26.7 ± 1.4</td>
</tr>
<tr>
<td>2</td>
<td>8.5 ± 1.7</td>
<td>9.0 ± 2.5</td>
<td>37.1 ± 1.0</td>
</tr>
<tr>
<td>4</td>
<td>8.6 ± 1.9</td>
<td>8.3 ± 1.7</td>
<td>38.6 ± 0.8</td>
</tr>
<tr>
<td>6</td>
<td>8.4 ± 1.7</td>
<td>8.2 ± 1.1</td>
<td>40.1 ± 0.5</td>
</tr>
<tr>
<td>8</td>
<td>8.2 ± 2.0</td>
<td>8.3 ± 1.3</td>
<td>42.0 ± 1.1</td>
</tr>
</tbody>
</table>

The food intake, body weight and liver weight of mice fed with the corn oil- or olive oil-supplemented diet are shown for the times indicated. Each point represents the mean ± SE for 5 animals. All three parameters (food intake, body weight and liver weight) were subjected to the t-test (*P < 0.05).

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Triglycerides (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>Phospholipids (mg/dl)</th>
</tr>
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<tr>
<td></td>
<td>Corn oil</td>
<td>Olive oil</td>
<td>Corn oil</td>
</tr>
<tr>
<td>0</td>
<td>80.0 ± 3.0</td>
<td>84.2 ± 3.4</td>
<td>162.1 ± 0.9</td>
</tr>
<tr>
<td>2</td>
<td>96.4 ± 0.2</td>
<td>115.2 ± 4.0*</td>
<td>168.6 ± 0.7</td>
</tr>
<tr>
<td>4</td>
<td>92.4 ± 1.7</td>
<td>107.2 ± 0.2*</td>
<td>154.3 ± 1.0</td>
</tr>
<tr>
<td>6</td>
<td>101.9 ± 1.3</td>
<td>102.6 ± 0.7</td>
<td>163.3 ± 0.3</td>
</tr>
<tr>
<td>8</td>
<td>97.2 ± 0.3</td>
<td>104.5 ± 0.6</td>
<td>170.0 ± 0.7</td>
</tr>
</tbody>
</table>

Each value reported in the table represents the mean ± SE (n = 5). All the values were subjected to the t-test (*P < 0.05).
the 8th treatment (lower by about 9% and 17%, respectively, in the 8th week).

As shown in Fig. 1, a more striking difference was detected in the level of hepatic lipids. In particular, an increase of about 2.6 fold in the level of hepatic triglycerides was detected in the mice fed for 8 weeks with the olive oil-enriched diet in comparison to the corn oil-fed mice (Fig. 1A). It is also interesting to note that about three times more triglycerides were found in the olive oil-fed animals in the 8th week with respect to the level at the beginning of this dietary treatment (10.68 versus 3.53 mg/g). The corn oil-fed mice, on the contrary, showed a level of triglycerides in the 8th week (4.17 mg/g) approximately comparable to that found at the beginning of this dietary treatment (3.53 mg/g). As shown in Fig. 1B, the level of hepatic cholesterol was roughly the same in the two groups of mice (the olive oil- and corn oil-fed mice) within the first month of the dietary treatment, whereas a significant increase was detected in the olive oil-fed animals over a longer period of time (an increase of 1.38 fold in the 8th week). Figure 1C shows that the level of liver phospholipids was significantly higher, starting from the 4th week, in the mice fed with olive oil than in the corn oil-treated animals. In the 8th week of dietary treatment, the level of liver phospholipids was 1.7 fold higher in the olive oil-fed mice.

Citrate transport into liver mitochondria and proteoliposomes

We then investigated the possibility of increased de novo fatty acid synthesis as the molecular mechanism responsible for the change in the hepatic lipid parameters found in the olive oil-fed mice. To this end, we measured the transport activity of the CIC in mouse liver mitochondria freshly isolated from the two groups of mice at different times (Fig. 2A). Whereas no effect of olive oil on the citrate uptake was found over time, a strong decrease in the CIC activity was detected in the corn oil-treated mice (62% in the 8th week).

The fatty acid composition of a diet is one of the factors capable of influencing the lipid composition of biological membranes and consequently their fluidity. Therefore, the assay of the CIC transport activity in intact mitochondria could be influenced, at least in principle, by the fluidity of the inner membrane in which this carrier protein is functionally inserted. In order to exclude this possibility, we used a different experimental approach involving the functional reconstitution of this carrier protein into liposomes. This method offers several advantages with respect to the assay carried out in intact mitochondria, including the well-established lipid composition of the liposomal membranes, the absence of possible protein effectors and a more defined internal and external substrate concentration. As shown in Fig. 2B, the results obtained with the reconstituted system were very similar to those previously found in the intact organelles. Again, in the 8th week of corn oil administration, a decrease of 57% in the CIC activity was found in comparison to that registered in the olive oil-fed mice, which remained substantially unaltered over time.

In order to obtain more insight into the molecular mechanism responsible for these findings, we carried out a western-blot experiment in which the amount of immuno-reactive CIC in the mitochondrial membranes from both the corn oil- and olive oil-fed mice was determined (Fig. 2C). As expected, whereas the amount of the CIC protein was virtually identical at any time of olive oil feeding, there was a time-dependent decrease in the CIC protein level in the corn oil fed animals (a 61% decrease in the 8th week). Such a decrease was consistent with the inhibition of the citrate transport activity previously shown in the corn oil-fed animals (Fig. 2A and B).

Hepatic enzymes involved in the synthesis and oxidation of fatty acids

De novo fatty acid synthesis starts when the carbon units transported outside the mitochondria by the CIC

![Fig. 1](Image)

**Fig. 1.** Lipid Analysis of the Liver from Corn Oil and Olive Oil Fed Mice.

The levels of liver triglycerides (A), cholesterol (B) and phospholipids (C) were determined at the times indicated. Triglycerides, total cholesterol and phospholipids were measured with commercial kits as described in the Materials and Methods section. Each point represents the mean ± SE of 3 liver samples. All the values were subjected to the t-test (*P < 0.05).
are made available for the cytosolic enzymes, ACC and FAS. Such enzymatic activities were therefore assayed in the liver cytosolic fractions from the corn oil- and olive oil-fed animals (Fig. 3). Similarly to the previous findings with the mitochondrial CIC (Fig. 2), no significant change in the activities of these enzymes was detected over time following the olive oil administration (Fig. 3A and B). On the contrary, a significantly lower level of both enzymatic activities was reproducibly found in the corn oil-treated animals than in the olive oil-fed ones (Fig. 3A and B). In the 8th week of corn oil feeding, 65% and 61% lower ACC and FAS activities were respectively found.

Besides de novo fatty acid synthesis, liver is also implicated in the oxidation of fatty acids. In this latter process, the key role is played by the mitochondrial CPT I which catalyzes the rate-limiting step for the /C12-oxidation of fatty acids. Figure 4 shows that the CPT I activity decreased over time in the olive oil-treated mice in comparison to the corn oil-fed animals, in which, on the contrary, it remained substantially unaltered over time in the first 6 weeks, with a slight increase in the 8th week. A lower level of about 30% in CPT I activity was found in the 8th week of olive oil treatment than that found in the corn oil-fed mice.

**Discussion**

We have investigated in this study the effect of olive oil and corn oil on the synthesis and oxidation of fatty acids in the mouse liver. The starting point of this study was the finding that an olive oil-enriched diet was able to increase the level of hepatic lipids when compared to a low-fat diet or to diets enriched with different dietary fats. However, the molecular mechanism for this finding is not clear.

The liver in an organism plays a fundamental role in lipid metabolism, because it is involved in such different processes as fatty acid uptake, storage, conversion, oxidation, synthesis and secretion. We focused in this study our attention on the specific aspects of fatty acid synthesis and oxidation, therefore investigating the activities of ACC, FAS and CPT I. Furthermore, we also studied the effect of olive oil and corn oil on the activity of the mitochondrial CIC which plays a key role in the metabolic cross-talk between mitochondria and the cytosol of hepatocytes. It is indeed the mitochondrial CIC which channels the carbon units derived from carbohydrate catabolism towards the anabolic process of fatty acid synthesis.

The results presented in this investigation indicate that the addition of 7.5% olive oil to a standard diet was not able to influence either the transport activity of the mitochondrial CIC, or the activities of the lipogenic enzymes, ACC and FAS (Figs. 2 and 3). This finding, on the one hand, confirms the close relationship existing between the CIC and the lipogenic enzymes and, on the other, excludes the possibility that stimulation of the hepatic lipogenic program was responsible for the strong accumulation of lipids, mainly triglycerides, in the liver of the olive oil-fed mice (Fig. 1A). On the contrary, in the mice fed on the diet enriched with 7.5% corn oil, there was a clear decrease in both the transport activity of the mitochondrial CIC and in the activities of
the cytosolic lipogenic enzymes (Figs. 2 and 3). Interestingly, the level of hepatic triglycerides, except for the initial increase seen in the first 2 weeks, progressively decreased during the corn oil treatment, reaching, in the 8th week, a value similar to that found at the beginning of the dietary administration (Fig. 1A). It is therefore tempting to speculate that the corn oil-induced inhibition of de novo fatty acid synthesis was one of the factors leading to the stabilization of liver triglycerides found in these animals.

An increase in the activities of the lipogenic enzymes in the liver of olive oil-fed animals had been found in previous studies, and such an increase had been positively correlated with the increase in the level of hepatic lipids.8,9 In those investigations, the activities of ACC and FAS in olive oil-fed animals were compared with those found in PUFA-treated animals. In principle, we should take into account the fact that different results among nutritional studies can depend on the experimental conditions used in each investigation, such as the animal spcief, dietary fat level, dietary fatty acid composition and duration of feeding. However, it is possible that the increase in the ACC and FAS activities found in previous studies8,9 was only apparent and due to the type of diet fed to the control animals. In fact, if a comparison between the hepatic lipogenic enzymes is done in the olive oil- and PUFA-treated animals only at a given time of feeding,8,9 a higher enzyme activity can be found in the olive oil-treated animals. This is also clearly evident in the results of the present study if we compare the lipogenic enzyme activities of the olive oil- and corn oil-fed animals only in the 8th week (Fig. 2 and Fig. 3). Yet, if the activities of these enzymes are followed over time, after the beginning of both dietary treatments, it is clear that olive oil did not influence them, while corn oil strongly decreased them. Therefore, further metabolic events, other than increased fatty acid synthesis, may have been responsible for the higher hepatic content of lipids found in the olive oil-fed mice.

We indeed found in this study significantly lower mitochondrial CPT I activity in the liver of the olive oil-fed mice than that in the corn oil-fed ones (Fig. 4). This decrease in fatty acid oxidation may therefore have played a role in the hepatic lipid accumulation detected in the olive oil-treated mice. In the corn oil-fed animals, on the other hand, the CPT I activity remained unaltered.
during the first 6 weeks of treatment and it then only slightly increased by the 8th week (Fig. 4). This relatively unaltered fatty acid oxidation may therefore have contributed, together with the decreased fatty acid synthesis, to the stabilization of the hepatic lipid levels found in the corn oil-fed animals.

A conclusive finding from this study is that the different effects of the two dietary oils on the hepatic lipid metabolism in mice can be attributed to their specific fatty acid composition. In fact, olive oil, as has been previously reported, contains more oleic acid in comparison to corn oil which, on the contrary, is rich in linoleic acid. The present data, therefore, reinforce the current view of fatty acids as being powerful and versatile modulators of lipid metabolism.1,2

References


