Down-regulation in muscle and liver lipogenic genes: EPA ethyl ester treatment in lean and overweight (high-fat-fed) rats

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Abstract

The precise mechanisms by which ω-3 fatty acids improve fat metabolism are not completely understood. This study was designed to determine the effects of eicosapentaenoic acid (EPA) ethyl ester administration on the expression levels of several muscle, liver and adipose tissue genes involved in lipogenesis and fatty acid oxidation pathways. Male Wistar rats fed a standard diet (control animals) or a high-fat diet were treated daily by oral gavage with EPA ethyl ester (1g/kg) for 5 weeks. The high-fat diet caused a very significant increase in plasma cholesterol (P < .01) levels, which was reverted by EPA (P < .001). A significant decrease in circulating triglyceride levels (P < .05) was also observed in EPA-treated groups. EPA administration induced a significant down-regulation in some lipogenic genes such as muscle acetyl CoA carboxylase β (ACCβ) (P < .05) and liver fatty acid synthase (FAS) (P < .05). Furthermore, a decrease in glucokinase (GK) gene expression was observed in EPA-treated animals fed a control diet (P < .01), whereas a significant increase in GK mRNA levels was found in groups fed a high-fat diet. On the other hand, no alterations in genes involved in β-oxidation, such acetyl CoA synthase 4 (ACS4), acetyl CoA synthase 5 (ACS5) or acetyl CoA oxidase (ACO), were found in EPA-treated groups. Surprisingly and opposite to the expectations, a very significant decrease in the expression levels of liver PPARGα (P < .01) was observed after EPA treatment. These findings show the ability of EPA ethyl ester treatment to down-regulate some genes involved in fatty acid synthesis without affecting the transcriptional activation of β-oxidation-related genes.

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Keywords: High-fat diet; Eicosapentaenoic acid (EPA); Lipid metabolism; Muscle; Liver; Adipose tissue

1. Introduction

Over two decades, numerous research studies and clinical investigations have focused on the healthy beneficial effects of consuming marine polyunsaturated fatty acids (PUFAs) [1,2]. There is much evidence suggesting that the intake of these ω-3 fatty acids, especially eicosapentaenoic acid (EPA), produces some benefits on cardiovascular disease markers [3], insulin resistance [1], obesity features [4] and regulation of serum lipid level [5]. In fact, several studies have demonstrated that fish oil administration caused hypolipidemic effects by coordinately suppressing new fatty acid synthesis and by inducing fatty acid oxidation in different tissues such as liver, skeletal muscle and white adipose tissues [6–8]. However, the intimate mechanisms by which specific PUFAs improve lipid metabolism, particularly by reduction of blood lipids such as triglycerides (TGs), are not completely understood.

Some studies have suggested that ω-3 fatty acids are important mediators of gene expression acting through the peroxisome proliferator-activated receptor (PPAR) and sterol regulatory element binding protein (SREBP) pathways [9,10], which are two critical transcriptional factors involved in β-oxidation and lipogenesis, respectively. In this sense, Neschen et al. [11] have recently confirmed that ω-3 fatty acids act as PPARα ligands and thereby induce hepatic β-oxidation. It has been also reported that mRNA levels of acetyl CoA oxidase (ACO), the first and rate-limiting enzyme of peroxisomal fatty acid β-oxidation [12], are increased by fish oil rich in EPA and docosahexaenoic acid (DHA) [13,14]. However, it has been suggested that EPA in the form...
of ethyl ester could not mimic the physiological activity of fish oil, particularly regarding hepatic fatty acid oxidation in rats [15].

On the other hand, it was shown that lipogenic gene expression of several enzymes was decreased by ω-3 fatty acids such as EPA and DHA in triacylglycerol form [16]. Indeed, experimental studies showed that fish oils regulate SREBP-1c mRNA levels, a transcriptional factor that controls some lipogenic genes [10,17]. It was also observed that liver fatty acid synthase (FAS) is down-regulated by fish oils [13,18]. The same effects on this enzyme elicited by EPA ethyl ester were found in adipose tissue [19]. Moreover, the effect of EPA ethyl ester on acetyl CoA carboxylase β (ACCβ) — another important enzyme involved in muscle lipogenesis and, in an indirect manner, fatty acid oxidation [20] — apparently has not been determined, although controversial effects on this enzyme in the liver of EPA-fed mice have been described [21,22]. Furthermore, several authors indicated that dietary ω-3 fatty acids could inhibit glucokinase (GK), the enzyme responsible for glucose phosphorylation in the liver [1,23].

Furthermore, the importance of these circulating TGs and cholesterol levels in the development of fatty liver and in the accumulation of lipids in skeletal muscle, which are both involved in obesity and insulin resistance, is well known. Thus, fish oil induces improvement in nonalcoholic steatohepatitis in rats [24,25]. However, the effects of EPA ethyl ester on fatty liver development in overweight rats have not been determined, although it has been reported that this pure ω-3 fatty acid induces TG and cholesterol accumulation in skeletal muscle despite its healthy effects [26].

In order to clarify the mechanisms by which EPA ethyl ester improves lipid metabolism, the aim of the present work was to determine the effects of EPA ethyl ester administration on the expression levels of several liver, muscle and adipose tissue target genes involved in lipogenesis and β-oxidation pathways in rats fed a standard diet (control animals) or a high-fat diet (overweight rats).

2. Materials and methods

2.1. Animals and treatment

Twenty-nine male Wistar rats (6 weeks old) supplied by the Center of Applied Pharmacology (CIFA, Pamplona, Spain) were housed in a temperature-controlled room (22±2°C) with a 12-h light–dark cycle. All experimental procedures were performed in accordance with the National and Institutional Guidelines for Animal Care and Use at the University of Navarra, Spain. Animals were distributed into four experimental groups: control, control+EPA (CEPA), overweight and overweight+EPA (OEPA). All animals were maintained for an adaptation period of 4 days, fed chow diet (Rodent Toxicology Diet; B&K Universal) and given deionized water ad libitum. After this period of time, the control and CEPA groups were fed a standard pelleted diet (Rodent Toxicology Diet; B&K Universal) containing 76% carbohydrates, 6% lipids and 18% proteins (362 kcal/100 g). On the other hand, the overweight and OEPA groups were fed a cafeteria diet composed of the following items: paté, chips, bacon, chocolate, biscuits and pelleted diet (relative ratio, 2:1:1:1:1) [27]. The composition of this diet was as follows: 9% energy as protein, 29% energy as carbohydrate and 62% energy as lipid, by dry weight. All animals had ad libitum access to water and food for 5 weeks.

The fatty acid composition of both control and high-fat diets was analyzed as previously reported [4], with the finding that both control and cafeteria diets have no EPA. Thus, the rats’ only source of this fatty acid is oral gavage. Thus, the CEPA and OEPA groups were treated, simultaneously with the maintenance of diets for 35 days, with 1 g/kg animal weight of highly purified EPA ethyl ester (Brudy S.L., Spain). This dose of EPA ethyl ester has been previously reported by Nobukata et al. [28] to have a beneficial effect on diabetes prevention. The same volume of water was orally administrated to the control and overweight groups, as previously described in other studies [26,29], for 35 days. These control and overweight groups without treatment with any other type of fatty acid (such as saturated fatty acids with equal chain length as EPA or oleic acid) are more likely to be considered as control groups in our study design [4]. This is because it has been demonstrated that supplementation with some other fatty acids is able to modify adiposity and circulating levels of biochemical and hormonal markers planned to be determined in the present study [29].

Body weight and food intake were measured daily. After 35 days, animals were submitted to overnight fasting and euthanized by decapitation. Gastrocnemius muscle, liver and epididymal adipose tissues were collected and frozen in liquid nitrogen before being stored at −80°C [4].

2.2. Gene expression analysis

Gene RNA levels were analyzed by Northern blot analysis or RT-PCR. Thus, liver SREBP-1c was determined by Northern blot analysis. Total RNA was extracted from the liver in accordance with Gibco Life Technologies procedure using Trizol (Life Technologies, Inc., Grand Island, NY). For each tissue sample, 15 μg of total RNA was fractionated by electrophoresis on a denaturing 1% agarose gel containing 2.2 M formaldehyde and 1× MOPS running buffer. One microliter of 50 μg/ml ethidium bromide (Gibco BRL, Gaithersburg, MD) stock solution was added in order to check RNA integrity and even loading. After electrophoresis, RNA was transferred to a nylon membrane (Duralon-UV; Stratagene, La Jolla, CA) by overnight capillary transfer and UV cross-linked (Stratalinker 1800; Stratagene). Blots were then hybridized for 1 h at 68°C in the presence of the previously labeled cDNA probe (2×106 cpm/ml Express Hyb solution; Clontech, Palo Alto, CA). After the blots had been washed at high stringency, they were exposed to X-ray film with an intensifying screen for enough time at −80°C. To allow loading of an equal mass of RNA in each well, after the
analysis of gene mRNA using a single-stranded cDNA probe followed by quantification of bands from film, the blots were reanalyzed using a probe complementary to mouse 18S ribosomal RNA. cDNA was synthesized from total liver RNA by PCR for 40 cycles using the following primers for SREBP-1c — sense: 5′-CACCACCTTCATCAAGGCAGACTCGC-3′ and antisense: 5′-CGTTGGCGTTCACCTGTTATCC-3′, resulting in a 640-pb fragment (GenBank accession no. L16995). The 18S ribosomal probe, used as a housekeeping gene, was obtained from Ambion (Austin, TX). cDNA probes were labeled by random priming (Rediprime kit; Amersham, Buckinghamshire, UK) in the presence of [32P]dCTP (3000 Ci/mmol; Amersham). Unincorporated nucleotides were removed using NucTrap probe purification columns (Stratagene) [30].

Acetyl CoA synthase 4 (ACS4), acetyl CoA synthase 5 (ACS5), PPARα, FAS, ACCβ, lipoprotein lipase (LPL), GK and ACO were analyzed by real-time PCR. Five micrograms of total RNA from muscle and liver was treated with DNA free (Ambion, Inc., Canada) in a total reaction volume of 24 μl for 30 min at 37°C. Thirteen microliters of this product was retrotranscribed to cDNA. Reaction conditions of retrotranscription were as follows: 60 min at 37°C and 5 min at 95°C. For real-time PCR amplification, 9 μl of cDNA was used per reaction. In this case, reaction conditions were as follows: 10 min at 25°C and 120 min at 37°C. TaqMan probes for ACS4 (Rn 00586179), ACS5 (Rn 00586013_m1), PPARα (Rn00566193_m1), FAS (Rn00569117_m1), ACCβ (Rn 00588290_m1), LPL (Rn00561482_m1), GK (Rn00561265_m1), ACO (Rn00569216-m1) and 18S (Hs99999901_s1) were Assay-on-Demand gene expression products. Reagents for real-time PCR analysis (Assays-on-Demand, TaqMan Reverse Transcriptase reagents and TaqMan Universal PCR Master mix) were purchased from Applied Biosystems (Foster City, CA), and the conditions were used in accordance with the manufacturer’s protocol. Amplification and detection of specific products were performed with the ABI PRISM 7000HT Sequence Detection System (Applied Biosystems). Universal 18S was used as reference to normalize the expression levels between samples, allowing data to be expressed relative to 18S RNA and therefore compensating for any differences in reverse transcriptase efficacy. All standards and samples were analyzed in duplicate. Data were obtained as Ct values (the cycle where the fluorescence signal emitted is significantly above background levels and is inversely proportional to the initial template copy number) in accordance with the manufacturer’s guidelines and used to determine ΔCt values (ΔCt = Ct of the target gene – Ct of the housekeeping gene) of each sample. Fold changes in gene expression were calculated by the 2−ΔΔCt method [31].

2.3. Serum analysis

Circulating levels of TGs, cholesterol, ketone bodies and serum free fatty acids were measured with a COBAS-Mira analyzer (Roche Diagnostic, Basel, Switzerland), as described by the commercial kit.

2.4. Tissue TGs and cholesterol content

To determine the TGs and cholesterol content in liver and muscle, 150 mg of tissue was homogenated with an ultraturrax (Ika-Werk, Janke y Kunkel) for 1 min in 1.5 ml of buffer (previously warmed at 50°C) composed of 150 mM NaCl, 0.1% Triton, and 10 mM Tris (pH 8). After centrifugation at 12,000g for 10 min, the obtained supernatant was used to determine the TG and cholesterol levels with a COBAS-Mira analyzer (Roche Diagnostic), as described elsewhere [32].

2.5. Statistical analysis

Results are given as mean±S.E. (standard error). Statistical differences and interactions were evaluated through a two-way [Diet (D)×EPA Treatment (E)] factorial analysis of variance (ANOVA). When statistically significant differences resulted at the interaction level, Student’s t test was carried out to compare the effects of each treatment (GraphPad Prism; GraphPad Software, Inc., San Diego, CA). Differences were considered statistically significant at P<.05.

3. Results

3.1. Effects of EPA ethyl ester on body weight gain, lipid profile, and TG and cholesterol content in muscle and liver

A previous study of our group revealed that the administration of EPA ethyl ester (1 g/kg) was able to partially prevent the body weight gain induced by the high-fat diet in rats, together with a significant reduction (P<.05) in retroperitoneal adipose tissue weight. In addition, EPA treatment induced a significant reduction (P<.05) in both insulin plasma levels and homeostasis model assessment index (HOMA), which is an index of insulin resistance [4].

Here we report that, in these animals, a marked improvement in lipid profile was also observed by oral administration of EPA ethyl ester (1 g/kg) (Table 1). Thus, EPA treatment was able to reverse (P<.001) the significant increase in cholesterol levels (P<.01) induced by high-fat diet. Also, EPA-ethyl-ester-treated rats exhibited a significant decrease in TG (P<.05) levels. However, no specific effect on free fatty acid serum levels was found. On the other hand, a significant increase in ketone body levels was observed with the effects of both high-fat diet and EPA treatment (P<.01).

High-fat diet produced a very significant increase in TG (P<.01) and cholesterol (P<.001) content in the liver (Table 2). However, EPA treatment did not modify these determinations either in control animals or in high-fat-diet-fed rats. Regarding muscle, no significant differences in TGs or cholesterol levels were caused either by the high-fat diet or by EPA ethyl ester treatment. However, it is
important to note that EPA administration produced a slightly marginally significant increase (P = .08) in cholesterol content in skeletal muscles.

3.2. Effects of EPA on expression levels of liver lipogenic genes

Gene expression levels of FAS were up-regulated (P < .001) in overweight rats, which was reversed (P < .05) by EPA ethyl ester administration (Fig. 1A). However, EPA treatment was not able to reverse the increase (P < .05) induced by high-fat diet in the gene expression level of the lipogenic transcriptional factor SREBP-1c (Fig. 1B). Because glucose disposal has been involved in the regulation of genes of fatty acid synthesis in the liver [33], we analyzed the effects of EPA on GK gene expression (Fig. 1C). A statistically significant interaction (P < .05) between diet and EPA treatment was observed in the expression levels of GK mRNA. A significant increase (P < .05) in GK was observed in high-fat-fed groups compared to control animals. However, the effect of EPA treatment on GK levels was diet-dependent. In fact, a significant decrease (P < .01) in the mRNA expression of this enzyme was detected in the CEPA group as compared with control animals, while EPA treatment did not induce any significant effect on GK mRNA in overweight rats.

3.3. Effects of EPA ethyl ester on expression levels of muscle lipogenic genes

ACCβ mRNA levels were significantly down-regulated by EPA ethyl ester treatment (P < .05) in gastrocnemius skeletal muscle (Fig. 2A). Regarding LPL, high-fat-diet animals exhibited a significant decrease in the mRNA expression levels of this enzyme (P < .05); however, although a tendency to decrease could be observed, no statistically significant changes in LPL gene expression were induced by EPA ethyl ester administration (Fig. 2B).

3.4. Effects of EPA ethyl ester on gene expression levels implicated in β-oxidation in the liver

Regarding ACO, a rate-limiting enzyme implicated in fatty acid β-oxidation, our data showed a significant decrease (P < .05) in this gene in high-fat-diet-fed rats, while no significant changes were observed in EPA-treated rats (Fig. 3A). The expression levels of ACS4 and ACS5 were not modified either by the high-fat diet or by EPA ethyl ester administration (Fig. 3B and C). However, EPA treatment caused a significant decrease (P < .01) in PPARα mRNA expression levels (Fig. 3D).

4. Discussion

Numerous studies have shown that ω-3 fatty acids, especially EPA, reduced plasma lipid levels in human and experimental animal models [5,34], mainly through changes in fatty acid synthesis and β-oxidation pathways [8,35]. For this purpose, the aim of the present study was to determine the mechanisms through which EPA ethyl ester improves lipid metabolism in an experimental animal model of obesity induced by high-fat (cafeteria) diet. As previously reported

### Table 1
Effects of EPA ethyl ester on plasma lipid parameters in lean and overweight (high-fat-fed) rats

<table>
<thead>
<tr>
<th></th>
<th>Control (n=8)</th>
<th>CEPA (n=7)</th>
<th>Overweight (n=7)</th>
<th>OEPA (n=7)</th>
<th>ANOVA 2×2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGs (mg/dl)</td>
<td>90.25±13.06</td>
<td>59.57±7.78</td>
<td>91.29±12.89</td>
<td>67.71±6.33</td>
<td>n.s.</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>47.56±1.48</td>
<td>39.47±1.39</td>
<td>56.53±2.85</td>
<td>43.09±2.09</td>
<td>**</td>
</tr>
<tr>
<td>Ketone bodies (mmol/L)</td>
<td>0.93±0.07</td>
<td>1.32±0.14</td>
<td>1.37±0.10</td>
<td>1.81±0.18</td>
<td>**</td>
</tr>
<tr>
<td>Free fatty acids (mg/dl)</td>
<td>0.58±0.03</td>
<td>0.59±0.04</td>
<td>0.59±0.04</td>
<td>0.60±0.03</td>
<td>n.s.</td>
</tr>
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</table>

Data (mean±S.E.) were analyzed by two-way ANOVA. n.s., not significant. * P<.05. ** P<.01. *** P<.001.

### Table 2
Effects of EPA ethyl ester on TG and cholesterol content in muscle and liver in lean and overweight (high-fat-fed) rats

<table>
<thead>
<tr>
<th></th>
<th>Control (n=8)</th>
<th>CEPA (n=7)</th>
<th>Overweight (n=7)</th>
<th>OEPA (n=7)</th>
<th>ANOVA 2×2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver TGs (mg/g tissue)</td>
<td>21.55±1.72</td>
<td>21.76±1.79</td>
<td>30.48±3.74</td>
<td>33.47±5.92</td>
<td>**</td>
</tr>
<tr>
<td>Liver cholesterol (mg/g tissue)</td>
<td>3.48±0.33</td>
<td>4.11±0.26</td>
<td>5.38±0.61</td>
<td>6.08±0.74</td>
<td>***</td>
</tr>
<tr>
<td>Muscle TGs (mg/g tissue)</td>
<td>150.10±26.88</td>
<td>161.69±36.02</td>
<td>229±47.32</td>
<td>151.47±15.02</td>
<td>n.s.</td>
</tr>
<tr>
<td>Muscle cholesterol (mg/g tissue)</td>
<td>9.71±0.98</td>
<td>11.80±0.94</td>
<td>10.08±1.92</td>
<td>11.59±0.99</td>
<td>n.s. P=.08</td>
</tr>
</tbody>
</table>

Data (mean±S.E.) were analyzed by two-way ANOVA. n.s., not significant. ** P<.01. *** P<.001.
by our group and others [4,27,36,37], this is an accepted model of diet-induced obesity that shares common features with Western diet and drives towards metabolic syndrome conditions. The dose of EPA ethyl ester used in the present study has been previously reported to have beneficial metabolic actions in rodents. Thus, Nobukata et al. [28] described the ability of EPA ethyl ester to decrease in a dose-dependent manner the incidence of diabetes at 0.1, 0.3 and 1.0 g/kg (92%, 50% and 17%, respectively) in WBN/Kob rats (a model of rats with spontaneous diabetes mellitus), indicating that, at 1 g/kg, animals obtained the most beneficial effect, improving their incidence of diabetes. Furthermore, Mori et al. [29] concluded that this fatty acid at 1 g/kg prevents the onset of insulin resistance in Otsuka Long–Evans Tokushima Fatty (OLETF) rats, which is another model of spontaneous non-insulin-dependent diabetes mellitus rats. In addition, in a previous study, we also found that a dose of 1 g/kg EPA ethyl ester increased insulin sensitivity in both control and high-fat-fed Wistar rats [4].

In the present article, we also observed that, at this same dose of EPA ethyl ester, these animals obtain a beneficial effect on serum lipid levels. These results are in concordance with the study of Minami et al. [34], which demonstrated that supplementation of the diet with 1 g/kg EPA ethyl ester, but not with 1g/kg oleic-acid-rich safflower oil, resulted in reduced plasma lipids in OLETF rats, supporting the attribution of hypolipidemic action to the EPA component.

Our data also suggest that the decrease in TG and cholesterol serum levels observed in EPA-ethyl-ester-treated groups could be mainly mediated by a diminution in the expression levels of some hepatic and muscle lipogenic genes. In fact, we observed that the administration of EPA ethyl ester induced a decrease in the expression levels of the

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Our data also suggest that the decrease in TG and cholesterol serum levels observed in EPA-ethyl-ester-treated groups could be mainly mediated by a diminution in the expression levels of some hepatic and muscle lipogenic genes. In fact, we observed that the administration of EPA ethyl ester induced a decrease in the expression levels of the
crucial hepatic enzyme involved in the synthesis of fatty acids, $FAS$. This effect elicited by the ethyl ester form was observed by Jump et al. [23] and also after the administration of $\omega$-3-rich fish oil [13], suggesting the implication of this hepatic enzyme in the improvement induced by EPA in lipid serum levels.

However, we also found that $FAS$ was regulated in a tissue-dependent manner [38], since we did not find any change in the expression levels of $FAS$ caused either by EPA administration or by the high-fat diet in epididymal adipose tissue (data not shown). These results contrast with those of others, which observed that EPA ethyl ester could downregulate $FAS$ in adipose tissues, explaining the decrease in adipose tissue weight observed in these animals [19].

The main transcriptional factor controlling lipid synthesis in the liver is SREBP. The isoform SREBP-1c is most relevant in rodent and human livers and is generally recognized as a key modulator of enzymes of fatty acid and TG synthesis [39]. Concerning the expression levels of this gene, our results revealed no changes in SREBP-1c mRNA levels in the liver after EPA ethyl ester administration. This observation has similarities with the data of Yahagi et al. [40], which described that mRNA expression levels of SREBP-1 in mice remained unchanged with EPA ethyl ester treatment. However, EPA-treated mice showed a suppressive effect on the mature SREBP-1 protein, indicating that EPA ethyl ester could regulate the abundance of mature SREBP-1 protein mainly at a posttranscriptional level, presumably through cleavage and/or degradation processes. In this sense, it was also reported that physiological doses of fish oil feeding effectively decrease the expression of liver lipogenic enzymes by inhibiting SREBP-1 proteolytic cascade in mice, while a substantial decrease in this transcriptional factor expression is only observed in its pharmacological doses [17].

Hepatic GK catalyzes the phosphorylation of glucose to glucose-6-phosphate, a step that is essential for glucose metabolism in the liver, as well as for the induction of lipogenic genes [41]. Our experimental data showed a significant decrease in the liver expression levels of $GK$ in EPA-treated chow-fed (CEPA) rats, whereas EPA treatment was not able to reduce the increase in $GK$ mRNA induced by the high-fat diet. The down-regulation of $GK$ mRNA levels mediated by EPA ethyl ester administration in chow-fed rats could induce, in a long term manner, a decrease in the synthesis and storage of new fatty acids [1,23].

Mitochondrial ACC$\beta$ catalyzes the formation of malonyl CoA in skeletal muscle [42], which regulates fatty acid synthesis and, in an indirect manner, fatty acid oxidation inhibiting the action of carnitine palmitoyltransferase.

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**Fig. 3. Effects of EPA ethyl ester on the expression levels of liver genes involved in $\beta$-oxidation.** mRNA levels of (A) $ACO$, (B) ACS4, (C) ACS5 and (D) PPAR$\alpha$ in the liver of control and overweight rats. The expression levels of all genes were obtained by RT-PCR and calculated by the $2^{-\Delta\Delta C_t}$ method (the mean value for control subjects was set to 1). Rat 18S RNA was used as reference to normalize the expression levels of these genes. Data are presented as mean±S.E. for at least seven independent experiments per group. Statistical analysis was performed by two-way ANOVA (D: diet; E: EPA treatment; D×E: interaction between diet and EPA treatment). *$P<.05$; **$P<.01$. 

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1 enzyme [20]. In fact, ACCβ-null mice had a reduced lipid content in the liver [43]. Moreover, the possible significance of ACCβ in the development of obesity and hyperlipidemia has led to the development of inhibitors of this enzyme [44]. Our results showed a significant fall in the expression levels of ACCβ in skeletal muscle, supporting the potential role of EPA ethyl ester in the inhibition of lipogenesis, as well as in the indirect stimulation of fatty acid oxidation and, consequently, improvement of hyperlipidemia at the skeletal muscle level.

Furthermore, it is well known that the level of circulating TGs is determined by the balance between delivery into the plasma and removal of TG-rich lipoproteins by tissues (i.e., muscle and adipose tissue). LPL catalyzes the hydrolysis of TG contained in very-low-density lipoproteins and chylomicrons, leading to the release of free fatty acids and their subsequent uptake by myocytes for energy production or by adipocytes for reesterification and storage [45]. Also, LPL activators have been shown to have antiobesity properties [46]. In our study, a significant decrease in the expression levels of muscle LPL was observed in high-fat-diet-fed rats. This is in agreement with previous studies describing that high-fat and/or high-sucrose diets provoke a diminution in the activity and/or abundance of this enzyme. Indeed, this change in LPL may decrease TG-rich lipoprotein clearance, contributing to increased plasma lipids and obesity [45,47]. However, we did not find any significant change in the gene expression levels of this enzyme produced by EPA ethyl ester. This result suggests that the improvement in lipid serum levels observed in EPA-ethyl-ester-treated rats is not mediated by any increase in muscle fatty acid utilization for energy production. In addition, no significant changes were observed in LPL mRNA expression levels in visceral adipose tissue by EPA ethyl ester treatment (data not shown), as previously described by Raclot et al. [19].

Previous studies described that EPA consumption increases the physiological activity of liver and adipose tissue fatty acid oxidation [35,48]. This effect was assumed in our study because of the significant rise found in serum ketone bodies under both high-fat-diet intake [49] and EPA ethyl ester treatment. However, this event was not reflected in the hepatic gene expression profile for genes involved in fatty acid oxidation. In fact, the effect of EPA ethyl ester on this metabolic pathway is controversial. Some studies have demonstrated that highly purified EPA in the form of ethyl ester increases fatty acid degradation [50], mainly affecting the activity and gene expression of hepatic fatty acid oxidation enzymes [51]. However, it was also observed that EPA ethyl ester, compared to fish oil, which contains this fatty acid as triacylglycerol, has different physiological activities in affecting hepatic fatty acid metabolism. Specifically, the EPA ethyl ester form is much less effective in increasing the activity and gene expression enzymes involved in hepatic β-oxidation [15]. Thus, ACO, the rate-limiting enzyme in liver peroxisomal β-oxidation, has not obtained the same regulation obtained by EPA ethyl ester and fish oil form. In this sense, it is well known that ACO increases their activity and/or mRNA levels in the presence of fish oil [52]. However, Hong et al. [15] showed that fish oil, compared to the ethyl ester form of EPA fatty acid, much greatly increases the mitochondrial and peroxisomal fatty acid oxidation in the liver of rats, as a result of a greater increase in both activity and mRNA levels of ACO enzyme in fish-oil-treated animals. These findings could explain the absence of significant differences in ACO gene expression in overweight EPA-ethyl-ester-treated animals shown in our trial. However, other studies showed an increase in ACO activity mediated by EPA ethyl ester administration, along with a chow background diet [53], which is also reflected in the tendency to increase the mRNA levels especially in our chow-fed EPA-ethyl-ester-treated (CEPA) animals. This event suggests a different regulation in the expression levels of ACO by the presence of EPA ethyl ester, depending on dietary composition. In fact, it has been proposed in humans that the beneficial effects of ω-3 fatty acids are more potent when they are accompanied by a decrease in saturated fat intake, suggesting that fish oil intake, accompanied by a low-saturated-fat hypocaloric diet, is the best combination to improve serum lipids levels [54].

The transcription factor PPARα is highly expressed in the liver, where it up-regulates genes involved in fatty acid oxidation. It has been described that PPARα mediates the effects of high-fat diet on hepatic gene expression. In fact, Patsouris et al. [55] described that PPARα mRNA and PPARα signaling are activated in the liver by chronic high-fat feeding (up to 26 weeks). This up-regulation of PPARα mRNA by high-fat diet is expected to serve a physiological role similar to the events occurring during fasting. Thus, as suggested by Patsouris et al. [55], intake of high-fat diet increased the amounts of fatty acids arriving at the liver and, concomitantly, there is an increased requirement for fatty acid oxidation. In our experiment, no significant changes in PPARα mRNA were observed in high-fat-fed rats, although a moderate nonsignificant increase could be observed in the overweight group (high-fat-fed non-EPA-treated rats). However, these animals were fed a high-fat diet only for 35 days (5 weeks) and, probably, a more chronic high-fat feeding is necessary in order to observe a significant increase in PPARα gene expression. A previous study of our group showed that these high-fat-fed rats treated with EPA ate less and exhibited decreased adiposity [4], and probably lower amounts of fatty acids reached the liver. Therefore, they would have a decreased requirement for fatty acid oxidation, which could explain, at least in part, the decrease in PPARα mRNA expression observed in EPA-treated animals. On the other hand, one study developed by Pawar and Jump [56] in rat hepatocytes indicated that the presence of EPA in an intracellular nonesterified fatty acid hepatic pool is the major determinant controlling PPARα activity, despite the fact that the fatty acid content in this pool represents <0.5% of the total fatty acids of the liver. Moreover, they suggested that dynamic changes in the composition of the nonesterified
fatty acid pool induce PPARα-regulated gene transcription. Probably because of that, EPA ethyl ester could not regulate the expression levels of PPARα as EPA triacylglycerol (fish oil form), and it could also contribute to explain the significant decrease in PPARα gene expression observed by the administration of EPA ethyl ester in our study. This interpretation could also explain the lack of effect of EPA ethyl ester administration on mRNA levels of some enzymes implicated in β-oxidation such as ACS4 and ACS5 [57] — two isoforms of one important enzyme that catalyze the first step in intracellular lipid metabolism: the conversion of fatty acids into acyl CoA thioesters. The resulting activated form of fatty acids can then be oriented toward the synthesis of esterified lipids or toward the β-oxidation pathway [58,59].

Some studies have suggested that muscle and liver TGs or cholesterol content in rats fed a high-fat diet is decreased by ω-3 and ω-6 fatty acids [60–62]. However, in our trial, only a slight but nonsignificant increase in cholesterol levels was found in skeletal muscle, which has also been observed by other authors administering EPA in ethyl ester form [26]. Therefore, the remarkable reducing effect of EPA ethyl ester on lipid serum levels was not reflected in TG and cholesterol accumulation in some critical tissues such as liver and skeletal muscles.

In summary, our data show that EPA ethyl ester treatment down-regulates some genes involved in fatty acid synthesis without affecting the transcriptional activation of β-oxidation-related genes. However, with the present data, we cannot rule out the possibility that EPA ethyl ester increases lipid β-oxidation by acting at the posttranscriptional level.

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References

in increased mitochondrial fatty acid oxidation followed by diminished lipogenesis. Lipids 1993;28:683–90.


