Interaction of Fish Oil and Conjugated Linoleic Acid in Affecting Hepatic Activity of Lipogenic Enzymes and Gene Expression in Liver and Adipose Tissue

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The interaction of dietary fish oil and conjugated linoleic acid (CLA) in affecting the activity of hepatic lipogenic enzymes and gene expression in liver and adipose tissue was examined in mice. A diet containing 1.0% CLA, mainly composed of 9*cis*,11*trans*- and 10*trans*,12*cis*-octadecadienoic acids at equivalent amounts, greatly decreased adipose tissue weight and serum concentrations of leptin and adiponectin and was accompanied by a downregulation of the expression of various adipocyte-abundant genes in epididymal adipose tissue. However, CLA increased the serum insulin concentration fourfold, and it caused hepatomegaly, with huge increases in the triacylglycerol level and the activity and mRNA levels of hepatic lipogenic enzymes. Different amounts (1.5, 3, and 6%) of fish oil added to CLA-containing diets dose-dependently downregulated parameters of lipogenesis and were accompanied by a parallel decrease in the triacylglycerol level in the liver. The supplementation of CLA-containing diets with fish oil was also associated with an increase in fat pad mass and mRNA levels of many adipocyte-abundant genes in epididymal adipose tissue along with a normalization of serum concentrations of leptin and adiponectin in a dose-dependent manner. However, in mice fed a diet containing 1.5% fish oil and CLA in whom fat pad mass was still low and comparable to that in the animals fed CLA alone, the serum insulin concentration greatly exceeded (twofold) the value observed in mice fed CLA alone, indicating an aggravation of insulin resistance. This hyperinsulinemia was ameliorated with increasing amounts of fish oil in the diets. Apparently, many of the physiological effects of CLA can be reversed by fish oil.
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ABCA1, ATP-binding cassette transporter A1; CLA, conjugated linoleic acid; IL, interleukin; LXR, liver X receptor; PPAR, peroxisome proliferator–activated receptor; SREBP, sterol regulatory element-binding protein; TNF-α, tumor necrosis factor-α; UCP, uncoupling protein.

Conjugated linoleic acid (CLA) is a generic name for a group of positional and geometrical isomers of conjugated dienoic derivatives of linoleic acid, and it occurs naturally in dairy products and beef fat (1). Studies have shown that CLA profoundly reduces body fat mass in mice (2–13). Regarding the mechanism behind the decrease in body fat, some studies indicated that dietary CLA increases energy expenditure (3). Alternatively, there is evidence to suggest that dietary CLA induces the apoptosis of adipocytes (2). But it has also been reported that CLA causes liver enlargement accompanied by an increase in tissue lipid content in mice (2,5,7–11,14).

With regard to the mechanism behind the increase in liver triacylglycerol content, we (9) previously showed that CLA greatly increased the activity and mRNA levels of various lipogenic enzymes in the liver. Moreover, CLA increased the mRNA expression of hepatic Δ^5- and Δ^6-desaturases and sterol regulatory element-binding protein-1 (SREBP-1), which regulates the expression of lipogenic enzymes (15) and these desaturases (16). Peters et al. (4) and Clement et al. (5) also showed that dietary CLA increased mRNA levels of lipogenic enzymes in mouse liver. These observations strongly indicated that an increase in lipogenesis is primarily responsible for the CLA-dependent accumulation of triacylglycerol in the liver. It is expected that the simultaneous ingestion of a dietary factor capable of reducing hepatic lipogenesis in CLA-treated animals would be effective at preventing hepatic lipid accumulation.

We hypothesized that a CLA-dependent increase in hepatic lipogenesis is the consequence of a large reduction in fat pad mass rather than a direct effect of CLA on the SREBP-1 signaling pathway (9). Because white adipose tissue plays a crucial role in metabolizing and converting glucose to fatty acid for storage purposes, the large decrease of fat pad mass caused by dietary CLA may result in a retardation of whole-body glucose metabolism. In fact, it has been reported that dietary CLA caused marked glucose intolerance and hyperinsulinemia (2,5,7,8,13). A large increase in lipogenesis and accumulation of triacylglycerol in the liver after CLA treatment may represent the physiological response of the animal to metabolize excess glucose to fatty acid and store it as triacylglycerol in the liver rather than in adipose tissue. Therefore, there is a possibility that the counteraction of CLA-mediated induc-

TABLE 1
Fatty acid contents of experimental diets

Fatty acid contents (g/100 g diet)	Dietary fatty acids and fish oil					
	Linoleic acid	Linoleic acid + 6% fish oil	CLA	CLA + 1.5% fish oil	CLA + 3% fish oil	CLA + 6% fish oil
16:0	5.94	4.09	5.94	5.48	5.02	4.09
n-7 16:1	0.01	0.40	0.01	0.11	0.21	0.40
18:0	0.60	0.52	0.60	0.58	0.56	0.52
n-9 18:1	5.28	4.28	5.30	5.05	4.80	4.30
n-6 18:2	2.35	1.91	1.27	1.16	1.05	0.83
n-3 20:5	—	0.57	—	0.14	0.28	0.57
n-3 22:6	—	1.85	—	0.46	0.93	1.85
CLA						
9*cis*, 11*trans*-18:2	0.00	0.00	0.49	0.49	0.49	0.49
10*trans*, 12*cis*-18:2	0.00	0.00	0.51	0.51	0.51	0.51
9*cis*, 11*cis*-/10*cis*, 12*cis*-18:2	0.00	0.00	0.03	0.03	0.03	0.03
9*trans*, 11*trans*-/10*trans*, 12*trans*-18:2	0.00	0.00	0.02	0.02	0.02	0.02
Total CLA	0.00	0.00	1.06	1.06	1.06	1.06

Diets contained 1.5% fatty acid preparation rich in linoleic acid (72.0%) or CLA (70.7%) and 0, 1.5, 3 or 6% fish oil. Total dietary lipid contents were adjusted to 15% by adding palm oil. The values for some minor fatty acids are not presented.

tion of hepatic lipogenesis aggravates glucose intolerance and hyperinsulinemia, despite being potentially effective in preventing fatty liver. To clarify the relationship between hepatic lipogenesis, glucose metabolism, and the function of adipose tissue in CLA-treated mice, we examined the interaction of dietary fish oil and CLA in affecting hepatic lipid content, the activity and mRNA levels of hepatic lipogenic enzymes, various serum metabolic parameters, and adipose tissue gene expression. It has been demonstrated that fish oil even at a low dietary levels strongly decreases the gene expression of hepatic lipogenic enzymes (17).

RESEARCH DESIGN AND METHODS

Male ICR mice obtained from Charles River Japan (Kanagawa, Japan) at 5 weeks of age were housed individually in animal cages in a room with controlled temperature (20–22°C), humidity (55–65%), and lighting (lights on from 0700 to 1900) and were fed a commercial nonpurified diet (type NMF; Oriental Yeast, Tokyo). After 7 days of acclimatization, mice were fed purified experimental diets for 22 days. Two groups of mice were fed CLA-free diets containing a 1.5% fatty acid preparation rich in linoleic acid (72.0%) and either 0 or 6% fish oil. Another four groups of animals were fed diets containing a 1.5% fatty acid preparation rich in CLA (70.7%) and either 0, 1.5, 3, or 6% fish oil. Dietary lipid levels in the experimental diets were adjusted to 15% by adding palm oil. The fatty acid preparations rich in linoleic acid and CLA were generously donated by Rinoru Oil Mills (Aichi, Japan). All of the experimental diets contained 12.8 g fatty acids as triacylglycerol and 1.5 g free fatty acids per 100 g of diet. The basal composition of the experimental diet was the same as that described previously (9). Fatty acid contents of experimental diets are listed in Table 1. The two CLA-free diets contained ocatadecadienoic acid exclusively as linoleic acid, whereas the four CLA-containing diets included this fatty acid as both linoleic acid and 1.06% CLA isomers, mainly as 9*cis*,11*trans*-18:2 and 10*trans*,12*cis*-18:2. Because both CLA and fish oil are susceptible to oxidation, diets were put into plastic bags in small portions (40 g), flushed with nitrogen, and stored at −30°C until use. New diets were served to animals in the morning (0900–1030) on each day of the experimental period, and old diets were discarded. Animals had free access to experimental diets and water until they were killed. Body weight at the start of the feeding period was 27–35 g. We followed our institute's guidelines for the care and use of laboratory animals.

Enzyme assays. At the end of the experimental period, the animals were killed by bleeding from the inferior vena cava under diethyl ether anesthesia. The livers were then quickly excised. Next, ~0.6 g of each liver was homogenized with 6 ml of 0.25 mol/l sucrose containing 1 mmol/l EDTA and 3 mmol/l Tris-HCl (pH 7.2). The activity of lipogenic enzymes was measured spectrophotometrically using the 200,000*g* supernatant fraction of the liver homogenate (9). The activity of various enzymes involved in the fatty acid oxidation pathway was measured spectrophotometrically, using the whole liver homogenate as an enzyme source (9).

RNA analysis. RNA in the liver and epididymal adipose tissue was extracted (18), and mRNA levels in these tissues were analyzed by quantitative real-time PCR, using a Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Total RNA was treated with RNase-free DNase I (Roche Diagnostics, Mannheim, Germany) and reverse-transcribed with random hexamers, using MultiScribe reverse transcriptase (Applied Biosystems) to generate cDNA. The PCR mixture, in a final volume of 20 μl, contained 20 ng cDNA, 900 nmol/l each of the forward and reverse primers, 250 nmol/l TaqMan probe, and 10 μl 2× PCR master mix (Applied Biosystems). The nucleotide sequences of primers and probes, designed using Primer Express Software (Applied Biosystems), are shown in Table 2. We also used primers and probes supplied by Applied Biosystems (Assay on Demand Products) to measure mRNA levels of leptin (an adipocytokine involved in the regulation of food intake and body fat mass), adiponectin (an adipocytokine presumed to be involved in the regulation of whole-body energy metabolism), adipocyte lipid-binding protein (a cytosolic fatty acid–binding protein highly expressed in adipocytes), fatty acid translocase/CD36 (a membrane-associated protein that is known to facilitate the uptake of fatty acids into adipocytes), lipoprotein lipase (an enzyme involved in the catabolism of the triacylglycerol core of circulating triacylglycerol-rich lipoproteins into nonesterified fatty acids and glycerol), GLUT4 (an insulin-responsive glucose transporter that plays an important role in whole-body glucose homeostasis), adiponutrin (an adipocyte-specific gene product of unknown function) (19), and tumor necrosis factor-α (TNF-α; a cytokine possessing a wide range of biological actions). The levels of 18S rRNA in cDNA samples were analyzed using TaqMan ribosomal RNA control reagents (Applied Biosystems). mRNA abundances were calculated as the ratio of mRNA to the 18S rRNA level in each cDNA sample, and they were expressed as percentages, assigning a value of 100 to the percentage in mice fed a CLA-free diet devoid of fish oil.

Analyses of serum and liver lipids and serum glucose, leptin, adiponectin, and insulin. Liver lipids were extracted and purified (20). Liver triacylglycerol, phospholipid, and cholesterol concentrations were determined as described before (21). Serum leptin (Morinaga, Tokyo), adiponectin (Otsuka Pharmaceutical, Tokushima, Japan), and insulin (Morinaga) concentrations were analyzed with commercial enzyme-linked immunosorbent assay kits.

Statistical analysis. StatView for Macintosh (SAS Institute, Cary, NC) was used for statistical analyses. The data were analyzed by one-way ANOVA, and a Tukey-Kramer post hoc analysis was used to detect significant differences between the means at a level of $P < 0.05$.

RESULTS

Animal growth, tissue weights, and liver and serum components. Although none of the differences were significant, food intake was ~10% lower in the three groups of mice fed diets containing CLA and various amounts of fish oil than in those fed CLA-free diets (Table 3). Also, growth was somewhat lower in mice fed CLA than in the other groups, and a significant difference was detected between mice fed a CLA-free diet containing 6% fish oil

and those fed a diet containing CLA and 1.5% fish oil in combination. Dietary fish oil did not affect adipose tissue weight in mice fed CLA-free diets. The weight of epididymal and perirenal depots in mice given a CLA diet free of fish oil became 22–25 and 11–14%, respectively, of that in the animals fed CLA-free diets. Fish oil dose-dependently increased adipose tissue weight among the groups of animals fed CLA-containing diets. Epididymal and perirenal adipose tissue weights were 2.5- and 2.6-fold higher, respectively, in mice fed CLA and 6% fish oil in combination, compared with those fed a CLA-containing diet free of fish oil.

Dietary fish oil did not affect liver weight in mice fed CLA-free diets. Although the difference was not significant, the hepatic triacylglycerol level in mice fed a CLA-free diet containing 6% fish oil was about one-half that in the animals fed a CLA-free diet devoid of fish oil. The hepatic cholesterol level was comparable between the two groups of animals fed CLA-free diets. The CLA diet free of fish oil, compared with the two types of CLA-free diets, caused liver enlargement accompanied by large increases in the triacylglycerol and cholesterol levels. The inclusion of fish oil in CLA-containing diets dose-dependently decreased liver weight and hepatic triacylglycerol and cholesterol levels.

Serum leptin and adiponectin concentrations were comparable between the animals fed CLA-free diets containing 0 and 6% fish oil. CLA feeding profoundly decreased these parameters. Fish oil dose-dependently increased these parameters among the animals fed CLA-containing diets, and the levels in mice fed a diet simultaneously containing CLA and 6% fish oil were comparable to those in the animals fed CLA-free diets. Fish oil at a 6% dietary level did not affect serum insulin levels in mice fed the CLA-free diet. A CLA diet devoid of fish oil, compared with CLA-free diets, greatly increased the serum insulin concentration (four- to fivefold). Supplementation of a CLA diet with 1.5% fish oil further increased this parameter, and the level became 9- to 11-fold higher than that in the animals fed CLA-free diets. The serum insulin level was also much higher in mice fed a diet simultaneously containing CLA and fish oil at the 3% level than in those fed CLA-free diets, but fish oil added to a CLA diet at the 6% level decreased the value to levels comparable to those in the animals fed CLA-free diets.

Activity and mRNA levels of hepatic enzymes involved in fatty acid synthesis and oxidation. In the animals fed CLA-free diets, fish oil reduced activity levels of lipogenic enzymes (Fig. 1). However, dietary CLA strongly increased these parameters. Fish oil added to CLA-containing diets dose-dependently decreased the activity levels, and dietary fish oil at the 3% level reduced the values to those observed in animals fed a CLA-free diet devoid of fish oil.

The mRNA levels of lipogenic enzymes were expressed as percentages, assigning a value of 100 to the percentage in mice fed a CLA-free diet devoid of fish oil (Fig. 2). Consistent with the results obtained for enzyme activity, between the two groups of animals fed CLA-free diets, mRNA levels of lipogenic enzymes were lower in mice fed fish oil than in those fed a fish oil–free diet. CLA greatly increased mRNA levels of lipogenic enzymes, and the values

TABLE 2
Primers and probes for real-time PCR of mRNAs

Genes	Sense primer	Antisense primer	Probe	Length of PCR products (bp)
Acetyl-CoA carboxylase	5′-GAGGTGGATCAGAGATTTCATAGAGA-3′	5′-AATGCGGTCCTCCTCAAACTT-3′	5′-TCCCCAAATTTTTCACATTCCGAGCA-3′	81
Acyl-CoA oxidase	5′-TTTGTTGTCCCTATCCGTGAGA-3′	5′-CCGATATCCCCAACAGTGATG-3′	5′-TGGGACCCACAAGCCTCTGCCA-3′	68
ABCA1	5′-GGTTTGGAGATGGTTATACAATAGTTGT-3′	5′-TTCCCGGAAACGCAAGTC-3′	5′-CGAATAGCAGGCTCCAACCCTGAC-3′	96
ATP-citrate lyase	5′-GTCAATGAACTGGCGAATTACG-3′	5′-GTGAGAGGATGGTCTTGGCATAG-3′	5′-CTCGGGTGCCCCCAGTGAACA-3′	85
Bifunctional enzyme	5′-TGGTGATTGGCACCCACTT-3′	5′-AGTATCGGCTAGGAATGACCTCTAGT-3′	5′-TTCTCCCCAGCCCACATCATGAGGT-3′	72
Carnitine palmitoyltransferase II	5′-ATCCCCTGGATATGTCCCAATA-3′	5′-CATCACGACTGGGTTTGGGTAT-3′	5′-TTCGGCTTTTCAATTCAACTCG-3′	69
Cholesterol 7α-hydroxylase	5′-TTTGATGACATGGAGAAGGCTAAG-3′	5′-CAGGAATGGTGTTTGCTTGAGA-3′	5′-CGCACCTCGTGATCCTCTGGGC-3′	70
Δ^5-Desaturase	5′-ACGGCATCAAGTATGAGTCCAA-3′	5′-CCTGACTCCTTCAGGGAGTAAACA-3′	5′-TGCTCACAGCCTTCGCGGA-3′	72
Δ^6-Desaturase	5′-CATAAAGAGCCTGCATGTGTTTG-3′	5′-TATTTCAGCTTCTTCTTGCCATACTC-3′	5′-CCTTGGCGAGTGGCAGCCCC-3′	72
Fatty acid synthase	5′-ATCCTGGAACGAGAACACGATCT-3′	5′-AGAGACGTGTCACTCCTGGACTT-3′	5′-CTGCGGAAACTTCAGGAAATGTCC-3′	140
IL-6	5′-CCAGAAACCGCTATGAAGTTCCT-3′	5′-CACCAGCATCAGTCCCAAGA-3′	5′-TCTGCAAGAGACTTCCATCCAGTTGCC-3′	72
LXRα	5′-CAGAGCCGACAGAGCTTCGT-3′	5′-AGCTCGTTCCCCAGCATTTT-3′	5′-CGGAAAAAGGGCCCAGCCCC-3′	70
Malic enzyme	5′-CATATCTCAGCAAGTGTCAGATAAACAC-3′	5′-AAACGCCTCGAATGGTATTCA-3′	5′-TGCAAGAAGGCCGGCTCTATCCTCC-3′	77
PPAR-γ1	5′-GGCTGAGGAGAAGTCACACTCTG-3′	5′-AAATCTTGTCTGTCACACAGTCCTG-3′	5′-CCAACAGCCTGACGGGTCTCGG-3′	119
PPAR-γ2	5′-AAACTCTGGGAGATTCTCCTGTTG-3′	5′-GTGCTCATAGGCAGTGCATCA-3′	5′-CCCAGAGCATGGTGCCTTCG-3′	67
Pyruvate kinase	5′-AAGGCAGGGATGAACATTGC-3′	5′-CGATGGACTCTGCATGGTACTC-3′	5′-CGACTCAACTTCTCCCATGGCTCCC-3′	70
SREBP-1a	5′-TTTCCTTAACGTGGGCCTAGTC-3′	5′-TGTCTTCGATGTCGTTCAAAACC-3′	5′-CTCTGGAACAGACACTGGCCGAGA-3′	140
SREBP-1c	5′-CGGCGCGGAAGCTGT-3′	5′-AGTCACTGTCTTGGTTGTTGATGAG-3′	5′-CCACGGAGCCATGGATTGCACA-3′	118
Trifunctional enzyme subunit α	5′-TGATCGGCATGCACTACTTCTC-3′	5′-TTGGAGGTTTTGTCAGTGGTGAT-3′	5′-CCCGTGGACAAGATGCAGCTCC-3′	76
Trifunctional enzyme subunit β	5′-CAGGAAAGGACACAGTTACCAAAGATA-3′	5′-GCAGGTTTTAGTTTGGCCATTT-3′	5′-TGGGATCCGTCCTTCCTCACTGGA-3′	76
UCP 2	5′-GCTTCTGCACCACCGTCAT-3′	5′-ACTGGCCCAAGGCAGAGTT-3′	5′-CCTCCCCTGTTGATGTGGTCAAGACG-3′	75

TABLE 3
Effect of dietary CLA and fish oil on growth parameters, tissue weights, and levels of liver lipids and serum components in mice

	Dietary fatty acids and fish oil					
	Linoleic acid	Linoleic acid + 6% fish oil	CLA	CLA + 1.5% fish oil	CLA + 3% fish oil	CLA + 6% fish oil
Body weight (g)	37.8 ± 0.9	40.1 ± 1.4	37.2 ± 0.7	36.2 ± 1.0	36.8 ± 1.0	36.9 ± 0.9
Body weight gain (g/22 days)	6.7 ± 0.9	9.0 ± 0.9	6.0 ± 0.5	5.3 ± 0.9	5.9 ± 0.9	5.8 ± 0.8
Food intake (g/day)	4.1 ± 0.1	4.1 ± 0.2	4.0 ± 0.2	3.6 ± 0.1	3.7 ± 0.1	3.7 ± 0.1
Epididymal white adipose tissue (g/100 g body wt)	3.3 ± 0.4*	3.7 ± 0.2*	0.83 ± 0.15†	1.0 ± 0.2†	1.3 ± 0.2†	2.1 ± 0.3†
Perirenal white adipose tissue (g/100 g body wt)	1.0 ± 0.1*	1.2 ± 0.1*	0.14 ± 0.03†	0.17 ± 0.04†	0.25 ± 0.04†	0.37 ± 0.09*‡
Liver (g/100 g body wt)	5.3 ± 0.2*	5.8 ± 0.2*	9.5 ± 0.7†	8.7 ± 0.4†	7.0 ± 0.4*†	6.2 ± 0.4*
Liver lipids (μmol/100 g body wt)						
Triacylglycerol	284 ± 16*	132 ± 11*	2,016 ± 400†	1,157 ± 194*†	459 ± 86*	185 ± 17*
Cholesterol	30.0 ± 1.2*	27.6 ± 1.7*	97.4 ± 14.9†	53.2 ± 2.7*§	36.9 ± 5.2*	32.0 ± 2.0*
Serum components						
Leptin (ng/dl)	1,059 ± 118*	1,199 ± 103*	173 ± 30†	471 ± 82†‡	647 ± 30*†	1011 ± 136*
Adiponectin (μg/dl)	1077 ± 75*	1363 ± 111*	190 ± 14†	375 ± 38†	523 ± 35†‡	907 ± 99*
Insulin (ng/dl)	738 ± 50‡	601 ± 54‡	3121 ± 276§	6,357 ± 1450*†	4,691 ± 744†	726 ± 166‡
Glucose (μmol/dl)	1258 ± 42	1,090 ± 52‡	1,496 ± 109	1927 ± 210†‡	1,457 ± 57	1333 ± 97

Data are means ± SE for 7–8 mice. Two groups of mice were fed CLA-free diets containing a 1.5% fatty acid preparation rich in linoleic acid (72.0%) and either 0 or 6% fish oil. Another four groups of animals were fed diets containing a 1.5% fatty acid preparation rich in CLA (70.7%) and either 0, 1.5, 3 or 6% fish oil. Total dietary lipid contents were adjusted to 15% by adding palm oil. The feeding period was 22 days. *$P <$ 0.01 vs. mice fed a CLA diet containing 0% fish oil; †$P < 0.01$ vs. mice fed a CLA-free diet containing 0% fish oil; ‡$P < 0.05$ vs. mice fed a CLA diet containing 0% fish oil; §$P < 0.05$ vs. mice fed a CLA-free diet containing 0% fish oil.

became 1.7- to 3.6-fold higher in mice fed a CLA diet free of fish oil relative to those fed a control diet without CLA and fish oil. We previously observed (9) that dietary CLA also caused a large increase in the mRNA levels of Δ^5- and Δ^6-desaturases, which catalyze the desaturation of polyunsaturated fatty acids. In contrast, available informa-

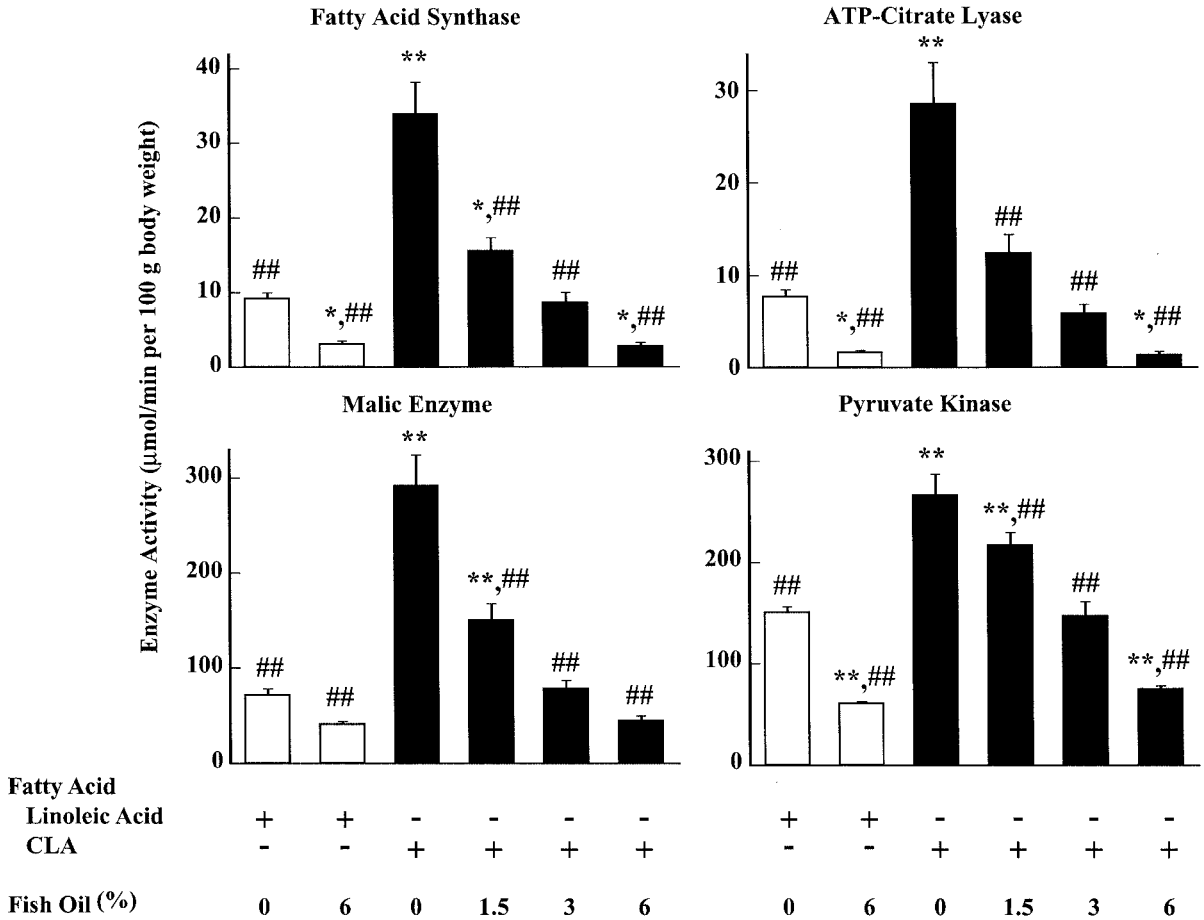

FIG. 1. Interaction of fish oil and CLA in affecting the activity of hepatic lipogenic enzymes. Values represent means ± SE for 7–8 mice. □, mice fed CLA-free diets; ■, mice fed CLA-containing diets. *$P < 0.05$, **$P < 0.01$ vs. mice fed a CLA-free diet containing 0% fish oil; #$P < 0.05$, ##$P <$ 0.01 vs. mice fed a CLA diet containing 0% fish oil.

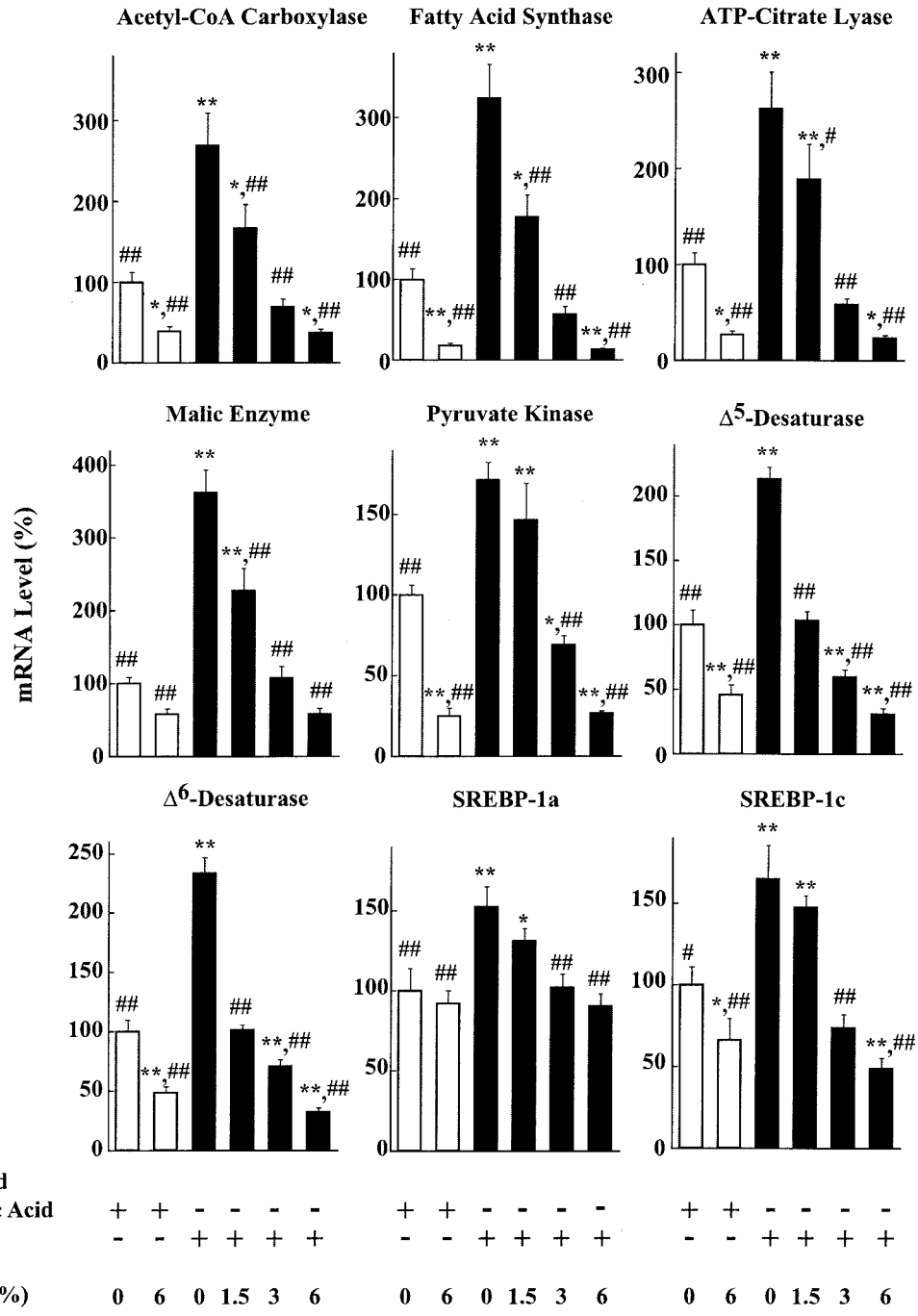

FIG. 2. Interaction of fish oil and CLA in affecting mRNA levels of hepatic lipogenic enzymes, Δ^5- and Δ^6-desaturases, and SREBP-1. mRNA levels were analyzed by quantitative real-time PCR using specific primers and probes. mRNA abundances were calculated as the ratio to the 18S rRNA level in each cDNA sample and expressed as percentages, assigning the value in mice fed a CLA-free diet devoid of fish oil as 100. Values represent the means ± SE for 7–8 mice. □, mice fed CLA-free dies; ■, mice fed CLA-containing dies. *$P < 0.05$, **$P < 0.01$ vs. mice fed a CLA-free diet containing 0% fish oil; #$P < 0.05$, ##$P < 0.01$ vs. mice fed a CLA diet containing 0% fish oil.

tion (17) indicated that dietary fish oil decreases the mRNA expression of desaturases. These observations were confirmed in the current study. Fish oil added to a CLA-free diet at the 6% level caused a >50% decrease in the mRNA levels of Δ^5- and Δ^6-desaturases. However, dietary CLA caused a 2.1- and 2.4-fold increase in the mRNA levels of Δ^5- and Δ^6-desaturases, respectively. The simultaneous addition of fish oil to CLA-containing diets dose-dependently decreased mRNA levels of lipogenic enzymes and Δ^5- and Δ^6-desaturases. The values in mice fed a diet simultaneously containing CLA and 3% fish oil became comparable to or even lower than those in the animals fed a CLA-free diet devoid of fish oil. We measured mRNA levels of SREBP-1a and -1c involved in the regulation of gene expression of lipogenic enzymes (15) and Δ^5- and Δ^6-desaturases (16). Fish oil added to a CLA-free diet at the 6% level significantly decreased the mRNA level of SREBP-1c (35% decrease) but not SREBP-1a. Dietary CLA caused a 1.5- and 1.7-fold increase in the mRNA level of SREBP-1a and -1c, respectively. Fish oil added to CLA-containing diets dose-dependently reduced mRNA levels of SREBP-1a and -1c, and the levels observed in diets

TABLE 4
Effect of dietary CLA and fish oil on the activity and mRNA levels of enzymes involved in fatty acid oxidation in the liver

	Dietary fatty acids and fish oil					
	Linoleic acid	Linoleic acid + 6% fish oil	CLA	CLA + 1.5% fish oil	CLA + 3% fish oil	CLA + 6% fish oil
Enzyme activity (μmol/min per 100 g body wt)						
Peroxisomal fatty oxidation	3.81 ± 0.35*	9.48 ± 0.98*†	7.18 ± 0.48†	10.8 ± 0.7*†	9.73 ± 0.85†‡	10.8 ± 0.71*†
Acyl-CoA oxidase	3.63 ± 0.40*	10.7 ± 1.5†	8.24 ± 0.83†	11.7 ± 1.1†‡	10.5 ± 1.0†	11.8 ± 1.4†‡
Carnitine palmitoyltransferase	6.90 ± 0.22*	16.5 ± 0.4*†	12.4 ± 1.3†	16.0 ± 0.5*†	17.5 ± 1.0*†	19.0 ± 1.2*†
Enoyl-CoA hydratase	3,175 ± 71*	5,723 ± 371†	5,368 ± 595†	6,106 ± 280†	5,860 ± 427†	6,466 ± 477†
3-Ketoacyl-CoA thiolase	668 ± 36*	1,259 ± 48†	1,194 ± 68†	1,425 ± 96†	1,333 ± 163†	1,384 ± 79†
mRNA level (%)						
Acyl-CoA oxidase	100 ± 8*	208 ± 29†‡	168 ± 9†	163 ± 6†	139 ± 6§	133 ± 8
Bifunctional enzyme	100 ± 4*	317 ± 24*†	202 ± 18†	218 ± 7†	202 ± 13†	226 ± 13†
Carnitine palmitoyltransferase II	100 ± 9*	160 ± 3†‡	141 ± 1†	133 ± 6†	118 ± 6*§	109 ± 4*
Trifunctional enzyme subunit α	100 ± 7*	174 ± 4†‡	149 ± 8†	120 ± 6*§	120 ± 8*§	129 ± 7†
Trifunctional enzyme subunit β	100 ± 8*	261 ± 10†‡	206 ± 8†	213 ± 14†	221 ± 29†	184 ± 10†

Data are means ± SE for 7–8 mice. mRNA levels of fatty acid oxidation enzymes were analyzed by quantitative real-time PCR using specific primers and probes. mRNA abundances were calculated as the ratio to the 18S rRNA level in each cDNA sample and expressed as percentages, assigning the value in mice fed a CLA-free diet devoid of fish. *$P < 0.01$ vs. mice fed a CLA diet containing 0% fish oil; †$P < 0.01$ vs. mice fed a CLA-free diet containing 0% fish oil; ‡$P < 0.05$ vs. mice fed a CLA diet containing 0% fish oil; §$P < 0.05$ vs. mice fed a CLA-free diet containing 0% fish oil.

containing CLA and 3 or 6% fish oil were comparable to the values in the animals fed CLA-free diets.

Liver X receptor-α (LXRα) is a transcription factor highly expressed in liver, and it plays a role in regulating the expression of the genes involved in cholesterol metabolism (22–23). Moreover, studies (24–27) have shown that ligand activation and increased expression of this transcription factor upregulate hepatic lipogenesis through direct and indirect mechanisms. To test the possible involvement of LXRα in the regulation of hepatic lipogenesis by CLA and fish oil, we analyzed mRNA levels of LXRα and its targets involved in cholesterol metabolism—cholesterol 7α-hydroxylase (22) and ATP-binding cassette transporter A1 (ABCA1) (23)—in the liver. No changes in mRNA levels of LXRα and ABCA1 were observed among the groups (data not shown). However, mRNA levels of cholesterol 7α-hydroxylase were significantly lower in mice fed a CLA-free 6% fish oil diet (53.4 ± 14.2%) and a fish oil–free CLA-containing diet (43.0 ± 17.4%) compared with levels in the animals fed a control diet free of CLA and fish oil (100 ± 17%). The values remained low in mice given diets containing CLA in combination with different amounts of fish oil (46.7 ± 13, 41.8 ± 4.4, and 37.0 ± 14.3% for mice given CLA diets containing 1.5, 3, and 6% fish oil, respectively).

We also analyzed the activity and mRNA levels of hepatic fatty acid oxidation enzymes (Table 4). Fish oil added at a 6% dietary level to a CLA-free diet significantly increased the activity (1.5- to 2.9-fold) and mRNA levels (1.6- to 3.2-fold) of hepatic fatty acid oxidation enzymes. A CLA diet free of fish oil compared with a control diet devoid of CLA and fish oil also increased these parameters (1.7- to 2.3-fold and 1.4- to 2.1-fold for the enzyme activity and mRNA levels, respectively). Apparent divergence was observed between enzyme activity and mRNA levels among mice fed CLA diets containing fish oil. Hepatic fatty acid oxidation enzyme activities of mice fed CLA diets containing 1.5–6% fish oil were indistinguishable and slightly higher than those observed with a CLA diet free of fish oil, and they were comparable to the values in animals fed a CLA-free diet containing 6% fish oil. However, mRNA levels of hepatic fatty acid oxidation enzymes in mice fed CLA diets rather decreased as dietary levels of fish oil increased in many cases, and they were lower than the values in mice fed a CLA-free diet containing 6% fish oil.

mRNA levels in epididymal adipose tissue. mRNA levels of proteins regulating lipid and carbohydrate metabolism in epididymal white adipose tissue were analyzed (Fig. 3). Fish oil added to a CLA-free diet did not affect mRNA levels of leptin, adiponectin, adipocyte lipid-binding protein, fatty acid translocase/CD36, lipoprotein lipase, GLUT4, adiponutrin, and interleukin-6 (IL-6; a multifunctional protein that plays important roles in host defense, acute-phase reactions, immune responses, and adipocyte function). CLA strongly decreased these parameters. The levels observed with a CLA diet free of fish oil were 9.2–40% of control values. The addition of fish oil to the CLA diet at the 1.5% level was totally ineffective in modulating the expression of these genes, except for IL-6. However, fish oil added to CLA diets at the 3 and 6% levels increased these parameters, and the values in mice fed a CLA diet containing 6% fish oil reached 50–112% of those observed in mice fed CLA-free diets. We also measured mRNA levels of two isoforms of peroxisome proliferator–activated receptor-γ (PPAR-γ; a transcription factor that controls adipocyte differentiation). Dietary CLA also decreased mRNA expression of PPAR-γ1 and -γ2, and the extent of the reduction was stronger with PPAR-γ2 (87%) than with PPAR-γ1 (60%). Fish oil added to CLA diets progressively increased mRNA levels of PPAR-γ1, and the value found in mice fed a CLA diet supplemented with 6% fish oil was indistinguishable from that observed with CLA-free diets. However, fish oil increased mRNA levels of PPAR-γ2 in CLA-fed mice to a limited extent. Fish oil added to a CLA-free diet did not affect mRNA levels of uncoupling protein 2 (UCP-2; a member of the UCP family highly expressed in adipocytes that allows dissipation of the proton electrochemical membrane) and TNF-α. Dietary CLA evoked a roughly twofold increase in these parameters, but the simultaneous addition of fish oil, even at the 6% level, did not affect these parameters.

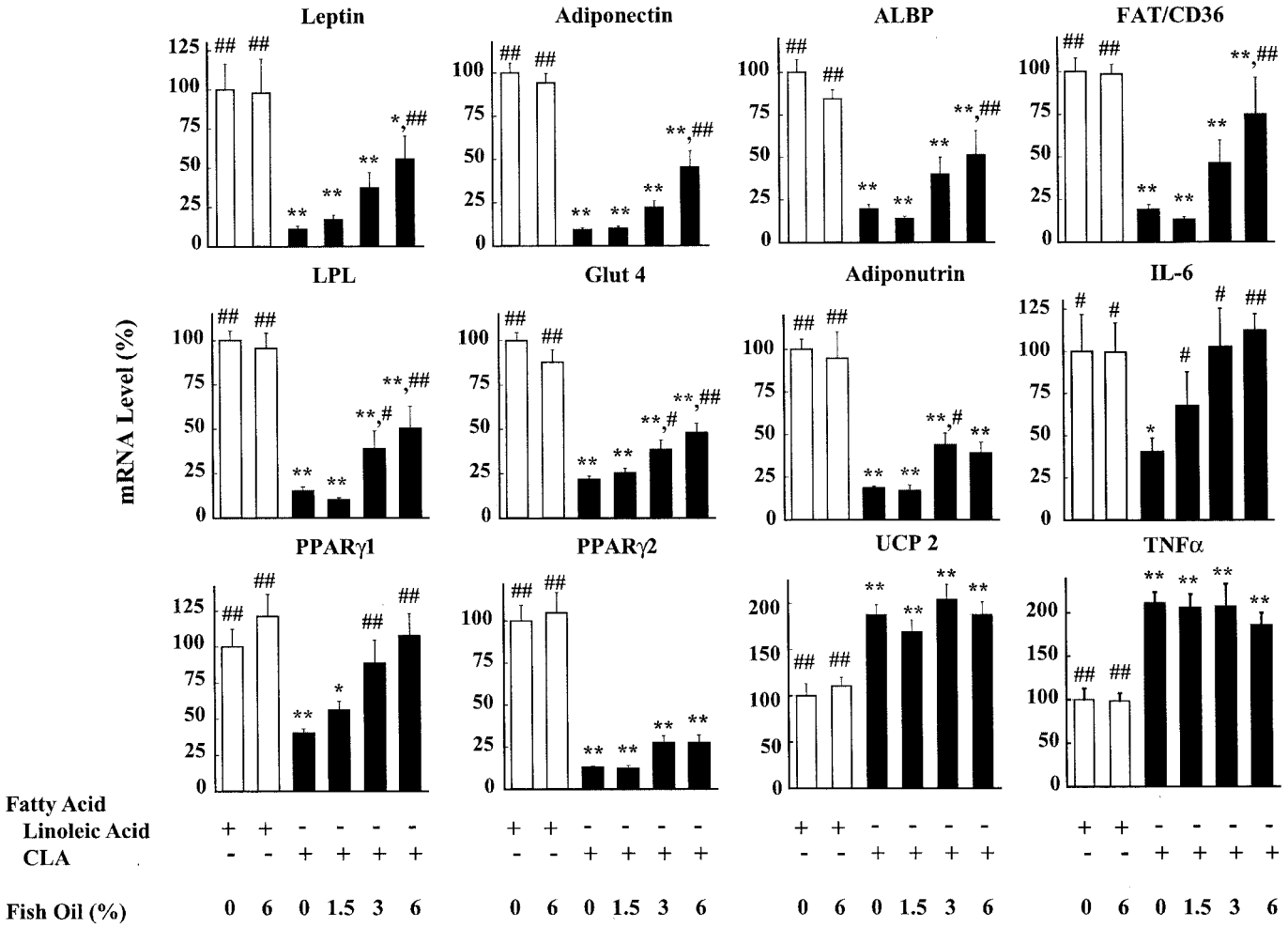

FIG. 3. Interaction of fish oil and CLA in affecting mRNA levels in epididymal adipose tissue. mRNA levels were analyzed by quantitative real-time PCR using specific primers and probes. mRNA abundances were calculated as the ratio of mRNA to the 18S rRNA level in each cDNA sample and expressed as percentages, assigning a value of 100 to the percentage in mice fed a CLA-free diet devoid of fish oil. Values represent means ± SE for 7–8 mice. □, mice fed CLA-free dies; ■, mice fed CLA-containing dies. *$P < 0.05$, **$P < 0.01$ vs. mice fed a CLA-free diet containing 0% fish oil; #$P < 0.05$, ##$P < 0.01$ vs. mice fed a CLA diet containing 0% fish oil. ALBP, adipocyte lipid-binding protein; FAT/CD36, fatty acid translocase/CD36; LPL, lipoprotein lipase.

We also analyzed mRNA levels of lipogenic enzymes in epididymal adipose tissue. In contrast to the situation in the liver, mRNA levels of lipogenic enzymes were rather unaffected by dietary treatments. No significant differences were observed in mRNA levels of acetyl-CoA carboxylase, fatty acid synthase, and malic enzyme among the groups (data not shown). The ATP-citrate lyase mRNA level was significantly lower in mice fed a CLA-free diet containing 0% fish oil (100 ± 8%) than in those fed a CLA diet containing 1.5% fish oil (146 ± 8%). However, no other significant differences in this parameter were detected among the groups.

Expression of adipocyte-abundant genes in the liver. One study (5) indicated that dietary CLA evoked increases in the expression of adipocyte-abundant genes in the liver, and this may represent one factor accounting for hepatic triacylglycerol accumulation in mice fed CLA. Therefore, we analyzed the mRNA expression of adipocyte-abundant genes not only in adipose tissue but also in the liver (Fig. 4). Preliminary experiments showed that in the liver, expression levels of mRNA for leptin, adiponectin, adipocyte lipid-binding protein, GLUT4, and IL-6 were very low and represented <0.05% of those in white adipose tissue, and it was difficult to obtain reliable values. Thus, we have not measured the expression of these genes in the liver. Hepatic mRNA levels of fatty acid translocase/CD36, lipoprotein lipase, adiponutrin, PPAR-γ1 and -γ2, UCP-2, and TNF-α in mice fed a control diet devoid of CLA and fish oil were 1.79 ± 0.20, 0.190 ± 0.051, 3.49 ± 0.62, 11.4 ± 2.3, 0.240 ± 0.089, 16.5 ± 1.8, and 18.8 ± 3.0%, respectively, of the levels observed in adipose tissue. Fish oil added to a CLA-free diet at the 6% level more than doubled the mRNA level of fatty acid translocase/CD36, but the difference was not significant. Relative to the value obtained in mice fed a CLA-free diet devoid of fish oil, mRNA levels were five- to sevenfold higher in mice fed CLA diets containing 0–3% fish oil, with a peak in the animals fed a diet containing CLA and 1.5% fish oil. However, a CLA diet containing 6% fish oil decreased the value to the level observed with a CLA-free diet containing 6% fish oil. Between the two groups of mice fed CLA-free diets, fish oil at the 6% dietary level more than doubled the lipoprotein lipase mRNA level. However, dietary CLA did not affect this parameter, and the mRNA levels remained unchanged on the inclusion of different amounts of fish oil in the diets. Although the effect was not significant, fish oil added to a

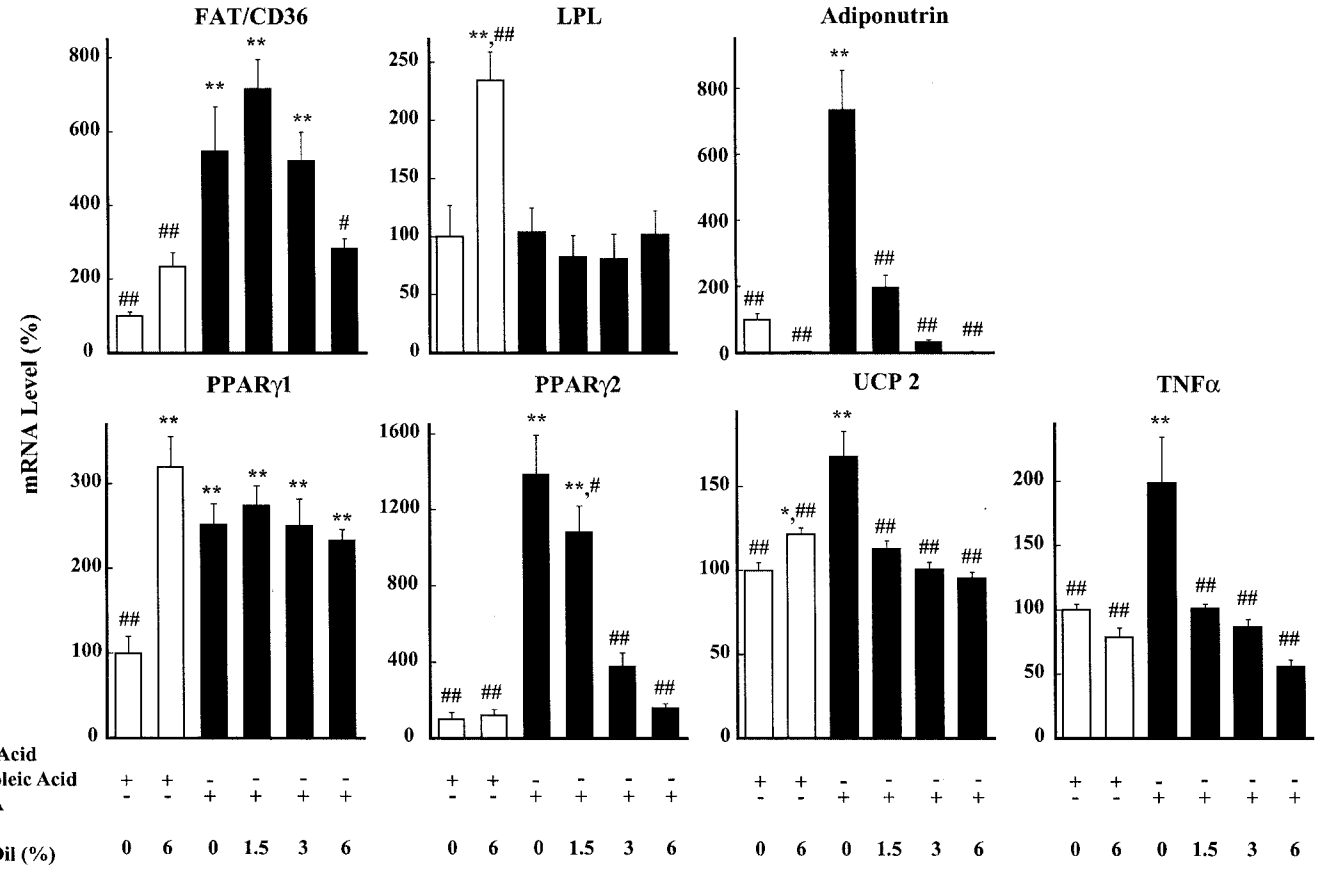

FIG. 4. Interaction of fish oil and CLA in affecting mRNA levels of adipocyte-abundant genes in the liver. mRNA levels were analyzed by quantitative real-time PCR using specific primers and probes. mRNA abundances were calculated as the ratio of mRNA to the 18S rRNA level in each cDNA sample and expressed as percentages, assigning a value of 100 to the percentage in mice fed a CLA-free diet devoid of fish oil. Values represent means ± SE for 7–8 mice. □, mice fed CLA-free diets; ■, mice fed CLA-containing diets. *$P < 0.05$, **$P < 0.01$ vs. mice fed a CLA-free diet containing 0% fish oil; #$P < 0.05$, ##$P < 0.01$ vs. mice fed a CLA diet containing 0% fish oil. FAT/CD36, fatty acid translocase/CD36; LPL, lipoprotein lipase.

CLA-free diet strongly reduced the mRNA expression of adiponutrin (a ~95% decrease). In contrast, CLA evoked a more than sevenfold increase in the mRNA level, and fish oil added to CLA diets dose-dependently reduced this parameter. Fish oil added to a CLA-free diet tripled the mRNA level of PPAR-γ1. Dietary CLA also more than doubled this parameter. Despite the fact that fish oil added to a CLA-free diet increased the PPAR-γ1 mRNA level, the simultaneous addition of different amounts of fish oil to CLA-containing diets did not affect this parameter. The expression level of hepatic PPAR-γ2 relative to that in adipose tissue was very low, but it was markedly affected by dietary CLA. Fish oil added to a CLA-free diet did not change this parameter. The mRNA level of this transcription factor was 11- to 14-fold higher in mice fed a fish oil–free CLA-containing diet than in animals fed CLA-free diets. However, the levels in mice fed CLA-containing diets progressively decreased as dietary levels of fish oil increased. Fish oil added to a CLA-free diet slightly but significantly increased the mRNA expression of UCP-2. Dietary CLA caused a 70% increase in this parameter, but the simultaneous addition of as little as 1.5% fish oil lowered this value to a level indistinguishable from that observed in mice fed CLA-free diets. Fish oil added to a CLA-free diet did not affect the mRNA expression of TNF-α. Dietary CLA caused a roughly twofold increase in the mRNA level of TNF-α. However, the values in mice fed CLA diets containing different amounts of fish oil were the same as those observed in the animals fed CLA-free diets.

DISCUSSION

Previous studies in mice showed that dietary CLA profoundly reduces body fat (2–13) mass but causes hepatomegaly accompanied by accumulation of lipids in the liver (2,5,7–11,14), as confirmed in the current study. In the current study, we used a CLA preparation consisting principally of 9*cis*,11*trans*- and 10*trans*,12*cis*-CLA amounts similar to those used by many investigators (2–4,6,8,9,12,13). The results of recent studies (5,7,10,11,14) indicate that 10*trans*,12*cis*- but not 9*cis*,11*trans*-CLA is primarily responsible for decreased body fat mass and liver steatosis in mice. The CLA mixture and 10*trans*,12*cis*-CLA appear equivalent in their physiological activities in affecting fat pad mass and liver lipid content (2–14). However, there is the possibility that they have antagonistic effects on lipid metabolism (7,14), and hence the outcome of the results obtained with the CLA mixture and 10*trans*,12*cis*-CLA differ.

We previously demonstrated (9) that CLA strongly increased the activity and mRNA levels of hepatic lipogenic enzymes. Therefore, we indicated that enhanced lipogenesis is a principle mechanism of fatty liver induced by CLA. This consideration was supported by the current finding that fish oil–mediated suppression of the activity and

mRNA levels of lipogenic enzymes was accompanied by a parallel decrease in hepatic triacylglycerol levels in mice fed CLA. We previously observed that dietary CLA increased the mRNA level of SREBP-1 involved in regulating the gene expression of lipogenic enzymes (9). Our previous assay did not distinguish the mRNAs of the two isoforms of this transcription factor (SREBP-1a and -1c). In the current study, using the real-time PCR method, we found that that the contours of the changes in mRNA levels of lipogenic enzymes and Δ^5- and Δ^6-desaturases that were caused by dietary treatments well paralleled those of SREBP-1c but not SREBP-1a. This supports the idea (15, 28,29) that SREBP-1c but not SREBP-1a is mainly involved in the regulation of hepatic lipogenesis.

LXRs belong to a family of nuclear hormone receptors that form obligate heterodimers with the retinoid X receptor. Two subtypes, LXRα and LXRβ, have been identified. LXRα is highly expressed in liver and plays a role in regulating cholesterol metabolism in this tissue. LXRs are activated by oxysterols and regulate intracellular cholesterol levels by upregulating the expression of several proteins involved in cholesterol absorption and metabolism (22,23). Moreover, LXR/retinoid X receptor was found to be a dominant activator of SREBP-1c promoter (24). Also, LXR/retinoid X receptor regulates fatty acid synthase expression through direct interaction with the fatty acid synthase promoter (25). Therefore, the ligand activation (24,25,27) and enhanced expression (26) of this transcription factor not only affect cholesterol metabolism but also upregulate lipogenesis through an indirect SREBP-1c–mediated mechanism and direct interaction with a lipogenic enzyme gene promoter. However, in the current study, both CLA and fish oil failed to affect hepatic mRNA levels of LXRα and ABCA1 (a gene targeted by LXR involved in cholesterol efflux from tissue) (23). However, the fish oil–dependent decrease in lipogenesis was associated with a downregulation of the mRNA expression of cholesterol 7α-hydroxylase, another LXR-dependent gene involved in the degradation of cholesterol (22). However, CLA, which induces hepatic lipogenesis, also decreased the mRNA level of this protein. Therefore, in this study we obtained no consistent evidence to indicate the involvement of the LXR-dependent mechanism in the alteration of hepatic lipogenesis by CLA and fish oil. Both CLA (30) and fish oil (31) are known to activate PPAR-α. It should be stated that the ligand activation of PPAR-α interferes with the LXR-dependent upregulation of SREBP-1c gene expression (32).

Alterations in hepatic fatty acid oxidation are expected to be an alternative factor affecting hepatic lipid levels. It is well demonstrated that both fish oil (31) and CLA (4,9,30) increase hepatic fatty acid oxidation through the activation of PPAR-α. As expected, in the current study, dietary fish oil and CLA caused increases in the activity and mRNA levels of various hepatic fatty acid oxidation enzymes. However, the impact of the combination of fish oil and CLA on the activity and mRNA levels of hepatic fatty acid oxidation enzymes was rather marginal and did not parallel the changes in hepatic lipid levels. Malonyl-CoA is the product of acetyl-CoA carboxylase and plays an important role in regulating hepatic fatty acid oxidation as a potent inhibitor of carnitine palmitoyltransferase I (33). Degrace et al. (11) reported that dietary CLA increased the hepatic level of malonyl-CoA and the sensitivity of carnitine palmitoyltransferase I to inhibition by malonyl-CoA. Therefore, it is plausible that the CLA-dependent increase in the activity and gene expression of hepatic fatty acid oxidation enzymes is not necessarily accompanied by a parallel change in the actual rate of hepatic fatty acid oxidation because of the strong inhibition by malonyl-CoA of carnitine palmitoyltransferase I. Fish oil included in CLA-containing diets is expected to lower the hepatic malonyl-CoA level through the downregulation of acetyl-CoA carboxylase. In addition, information (34) suggests that dietary fish oil decreases the sensitivity of carnitine palmitoyltransferase I to inhibition by malonyl-CoA. These consequences may result in an increase in the actual rate of hepatic fatty acid oxidation, even in a situation in which the activity and mRNA levels of hepatic fatty acid oxidation enzymes remained unchanged. Therefore, there is the possibility that alterations in the rate of fatty acid oxidation in addition to synthesis contribute to the fish oil–dependent decrease in hepatic triacylglycerol levels in mice fed CLA.

In the current study, we also found that CLA and fish oil interact to affect mRNA levels of various adipocyte-abundant genes in the liver, even though the expression levels in liver of these genes were considerably lower than those in adipose tissue. Reponses of mRNA levels to dietary fish oil and CLA are mutually different according to the respective genes, and changes in mRNA levels (especially of fatty acid translocase/CD36, adiponutrin, and PPAR-γ2) generally paralleled those in hepatic triacylglycerol levels among groups. Therefore, it is possible that alterations in the mRNA expression of these adipocyte-abundant genes in liver tissue at least partially contribute to the changes in hepatic triacylglycerol levels caused by dietary treatments.

Previous studies indicated that CLA reduces mRNA levels of leptin (6,10), GLUT4 (2,6,8), PPAR-γ (2,6), adipocyte lipid-binding protein (5), and adiponectin (10) in adipose tissue. In the current study, we confirmed these observations and further showed that fatty acid translocase/CD36, lipoprotein lipase, adiponutrin, and IL-6 are also targets to be downregulated by CLA. Moreover, we found that fish oil added to CLA diets dose-dependently increased expression levels of many adipocyte-abundant genes, accompanying an increase in fat pad mass. In the current study, we also showed that the serum concentrations of leptin and adiponectin were closely correlated with mRNA levels of these proteins in adipose tissue. It has been demonstrated that PPAR-γ1 and -γ2 are highly expressed in adipose tissue (35). PPAR-γ2 is found selectively in fat tissue, whereas PPAR-γ1 is also expressed at low levels in many tissues (35). It is generally considered that PPAR-γ2 but not PPAR-γ1 is mainly involved in regulating the expression of various adipocyte-abundant genes and adipocyte differentiation (36,37). In fact, dietary CLA strongly reduced mRNA levels of this transcription factor, with great reductions in the mRNA levels of many adipocyte-abundant genes. However, when fish oil was added to CLA-containing diets, the increase in the PPAR-γ2 mRNA level was minimal, and it did not parallel the responses of various adipocyte-abundant genes. Therefore, not only PPAR-γ2 but also other transcription factors, including

PPAR-γ1 (36,37), may be involved in the regulation of gene expression in adipose tissue.

We found that fish oil feeding counteracts the CLA-dependent decrease in fat pad mass. However, the mechanism by which fish oil increased fat pad mass in mice fed CLA-containing diets is not clear at present. It has become increasingly clear that adipose tissue produces and releases a number of cytokines and hormone-like proteins collectively known as adipocytokines, all of which may have crucial roles in adipocyte function and the development of obesity (38). Among the various adipocytokines, leptin, adiponectin, and resistin are almost exclusively expressed in adipose tissue, but cytokines such as IL-6, TNF-α, plasminogen activator inhibitor-1, and angiotensinogen are synthesized and released from many types of cell in addition to adipocytes (38). It is plausible that dietary CLA and fish oil affected the expression of these adipocytokines in adipose tissue and hence altered fat pad mass. In the current study, CLA strongly decreased the serum concentration and mRNA level in adipose tissue of leptin, consistent with other studies (6,10), and fish oil added to CLA-containing diets dose-dependently increased these parameters. However, fish oil added to a CLA-free diet did not affect the serum concentration or mRNA expression of leptin in adipose tissue. It was suggested that in the current study, alterations in the leptin mRNA level reflect those of fat pad mass, as has been demonstrated in a previous study (39), but do not represent the specific effect of dietary CLA and fish oil. In this study, we observed similar responses of the serum level and mRNA expression of adiponectin to dietary fish oil and CLA. Previous studies showed that plasma levels of adiponectin are lower in obese compared with lean humans (40), and overexpression of adiponectin offsets the development of diet-induced obesity in rats (41). Therefore, it is difficult to conclude that alterations in adiponectin mRNA expression are responsible for the CLA- and fish oil–dependent changes in fat pad mass observed in this study. It has been reported that CLA increases mRNA levels of TNF-α in adipose tissue (2,8). Tsuboyama-Kasaoka et al. (2) showed that dietary CLA caused apoptosis of adipocytes, and this may account for the reduction in fat pad mass. They hypothesized that upregulation of the mRNA expression of TNF-α in addition to UCP-2 can account for the apoptosis of adipocytes induced by CLA. Our current study confirmed previous findings that CLA increases mRNA levels of TNF-α (2,8) and UCP-2 (2,6,8) in adipose tissue. However, the mRNA levels remained unchanged among the groups of animals fed CLA diets containing different amounts of fish oil, even though the fish oil effectively increased fat pad mass. Therefore, it is difficult to conclude that TNF-α and UCP-2 are primarily involved in the dietary CLA- and fish oil–dependent changes in fat pad mass. Previous observations have indicated that increased production of IL-6 is associated with a reduced amount of adipose tissue together with increased hepatic lipid synthesis in mice (42). Also, Lagathu et al. (43) reported that IL-6 treatment interfered with the differentiation of 3T3-L1 adipocytes. Moreover, Brown et al. (44) recently reported that 10*trans*,12*cis*-CLA added to cultures of stromal vascular cells containing newly differentiated human adipocytes caused an increase in the expression of IL-6 and -8, which accompanied the decrease in cellular triacylglycerol content and insulin-stimulated glucose metabolism. These observations raised the interesting possibility that CLA also increases IL-6 expression in adipose tissue in vivo and hence decreases fat pad mass and causes hepatic lipid accumulation. Also, it is possible that the counteraction by fish of a CLA-dependent decrease in fat pad mass is mediated through changes in IL-6 production inasmuch as dietary fish oil decreases IL-6 production, at least in immune cells (45,46). However, in the current study, we found that CLA decreased mRNA expression of IL-6 in adipose tissue, but fish oil dose-dependently increased this parameter in mice fed CLA-containing diets. Given the above, it is difficult to conclude that in adipose tissue, changes in mRNA expression of adipocytokines, at least of leptin, adiponectin, TNF-α, and IL-6, can account for the CLA- and fish oil–dependent changes in fat pad mass.

Both dietary CLA and fish oil strongly affected hepatic activity and mRNA levels of lipogenic enzymes in the current study. However, these dietary lipids were ineffective in modulating mRNA levels of lipogenic enzymes in adipose tissue. Information on the effect of CLA on adipose tissue lipogenesis is controversial. Tsuboyama-Kasaoka et al. (2) and Clement et al. (5) showed that dietary CLA decreases mRNA levels of acetyl-CoA carboxylase (2) and fatty acid synthase (2,5) in mouse adipose tissue. In contrast, Kang et al. (12) reported that dietary CLA increased mRNA expression of fatty acid synthase in mouse adipose tissue. West et al. (13) observed that de novo fatty acid biosynthesis in mouse adipose tissue, measured by the incorporation of deuterium-labeled water, was not affected by CLA treatment, which is consistent with our current finding. Although the cause of these inconsistencies is not clear, our observation does not support the conclusion (2,5) that the alteration of adipocyte lipogenesis is a factor regulating fat pad mass. The observation that dietary fish oil did not alter the mRNA levels of lipogenic enzymes in adipose tissue is consistent with observations made by others in experiments using rats (47,48).

The results of the current study further support the conclusion made in our previous study (9) that enhanced hepatic lipogenesis is the mechanism for fatty liver in mice fed CLA. We hypothesized that the enhanced hepatic lipogenesis in mice fed CLA is a consequence of the depletion of adipose tissue because adipose tissue plays a crucial role in glucose metabolism. In mice fed CLA, lipogenesis in liver may function to dispose of excess glucose by converting it to fatty acid and storing it in the form of triacylglycerol in the liver rather than in adipose tissue. Thus, there is a concern that the inhibition of hepatic lipogenesis without large enough amounts of functionally active adipocytes may aggravate glucose intolerance and hyperinsulinemia, which have been observed in mice fed CLA (2,5,7,8,13). This was actually the case in the current study. Fish oil added at a level as low as 1.5% to a CLA-containing diet was effective in reducing the hepatic levels of activity and mRNA of both lipogenic enzymes and triacylglycerol to 50% of the levels in animals fed a fish oil–free CLA-containing diet; however, the effects were not accompanied by changes in adipose tissue mass and mRNA levels of genes involved in metabolism in adipose tissue. In this situation, the serum insulin concentration

was four- to fivefold higher in mice fed a fish oil–free CLA-containing diet than in those fed CLA-free diets, and it almost doubled with the simultaneous addition of 1.5% fish oil accompanied by a significant increase in the serum glucose level. Although an insulin tolerance test still would be required, the observation indicated that the aggravation of insulin resistance actually occurs under this nutritional condition. This super hyperinsulinemia was, however, ameliorated with increasing dietary levels of fish oil accompanying the increases in adipose tissue mass and expression of many genes in this tissue. Therefore, our results not only emphasize the significance of adipose tissue in controlling whole-body glucose metabolism, but they also suggest that hepatic lipogenesis serves as a tool to dispose of excess glucose, at least in particular situations where functionally active adipose tissue is lacking.

Studies have indicated that leptin and adiponectin can reverse insulin resistance and hepatic lipid accumulation associated with lipoatrophy (49,50). In fact, Tsuboyama-Kasaoka et al. (2) showed that leptin injection normalized the serum insulin level and hepatic lipid content in mice fed CLA. In the current study, dietary CLA depleted fat pad mass and induced hyperinsulinemia and fatty liver, but these were ameliorated by the simultaneous ingestion of fish accompanied by parallel increases in the mRNA levels in epididymal adipose tissue and the serum concentrations of leptin and adiponectin. Significant negative correlations ($P < 0.01$) were observed between serum insulin levels and both the serum concentrations and mRNA levels of leptin and adiponectin ($r = 0.465–0.594$). Also, liver triacylglycerol levels and both the mRNA levels and serum concentrations of leptin and adiponectin were significantly and negatively correlated ($P < 0.01$, $r = 0.524–0.633$). Our current observations, therefore, support the idea that these adipocytokines play crucial roles in the amelioration of insulin resistance and hepatic lipid accumulation associated with lipoatrophy induced by CLA.

In conclusion, we confirmed previous findings that dietary CLA decreased fat pad mass (2–13) but caused increases in the activity and mRNA levels of lipogenic enzymes (4,5,9) and triacylglycerol levels (2,5,7–11,14) in the liver of mice. Moreover, we demonstrated that simultaneous ingestion of fish oil reversed many of the physiological changes induced by dietary CLA.

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REFERENCES

1. Kelly GS: Conjugated linoleic acid: a review. *Altern Med Rev* 6:367–382, 2001
2. Tsuboyama-Kasaoka N, Takahashi M, Tanemura K, Kim HJ, Tange T, Okuyama H, Kasai M, Ikemoto S, Ezaki O: Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. *Diabetes* 49:1534–1542, 2000
3. Ohnuki K, Haramizu S, Ishihara K, Fushiki T: Increased energy metabolism and suppressed body fat accumulation in mice by a low concentration of conjugated linoleic acid. *Biosci Biotechnol Biochem* 65:2200–2204, 2001
4. Peters JM, Park Y, Gonzalez FJ, Pariza MW: Influence of conjugated linoleic acid on body composition and target gene expression in peroxisome proliferator-activated receptor α-null mice. *Biochim Biophys Acta* 1533:233–242, 2001
5. Clement L, Poirier H, Niot I, Bocher V, Guerre-Millo M, Krief S, Staels B, Besnard P: Dietary *trans*-10,*cis*-12 conjugated linoleic acid induces hyperinsulinemia and fatty liver in the mouse. *J Lipid Res* 43:1400–1409, 2002
6. Takahashi Y, Kushiro M, Shinohara K, Ide T: Dietary conjugated linoleic acid reduces body fat mass and affects gene expression of proteins regulating energy metabolism in mice. *Comp Biochem Physiol B Biochem Mol Biol* 133:395–404, 2002
7. Roche HM, Noone E, Sewter C, Mc Bennett S, Savage D, Gibney MJ, O’Rahilly S, Vidal-Puig AJ: Isomer-dependent metabolic effects of conjugated linoleic acid: insights from molecular markers sterol regulatory element-binding protein-1c and LXRα. *Diabetes* 51:2037–2044, 2002
8. Tsuboyama-Kasaoka N, Miyazaki H, Kasaoka S, Ezaki O: Increasing the amount of fat in a conjugated linoleic acid-supplemented diet reduces lipodystrophy in mice. *J Nutr* 133:1793–1799, 2003
9. Takahashi Y, Kushiro M, Shinohara K, Ide T: Activity and mRNA levels of enzymes involved in hepatic fatty acid synthesis and oxidation in mice fed conjugated linoleic acid. *Biochim Biophys Acta* 1631:265–273, 2003
10. Warren JM, Simon VA, Bartolini G, Erickson KL, Mackey BE, Kelley DS: *Trans*-10,*cis*-12 CLA increases liver and decreases adipose tissue lipids in mice: possible roles of specific lipid metabolism genes. *Lipids* 38:497–504, 2003
11. Degrace P, Demizieux L, Gresti J, Chardigny JM, Sebedio JL, Clouet P: Hepatic steatosis is not due to impaired fatty acid oxidation capacities in C57BL/6J mice fed the conjugated *trans*-10,*cis*-12-isomer of linoleic acid. *J Nutr* 134:861–867, 2004
12. Kang K, Miyazaki M, Ntambi JM, Pariza MW: Evidence that the anti-obesity effect of conjugated linoleic acid is independent of effects on stearoyl-CoA desaturase1 expression and enzyme activity. *Biochem Biophys Res Commun* 315:532–537, 2004
13. West DB, Blohm FY, Truett AA, DeLany JP: Conjugated linoleic acid persistently increases total energy expenditure in AKR/J mice without increasing uncoupling protein gene expression. *J Nutr* 130:2471–2477, 2000
14. Kelley DS, Bartolini GL, Warren JM, Simon VA, Mackey BE, Erickson KL: Contrasting effects of *t*10,*c*12- and *c*9,*t*11-conjugated linoleic acid isomers on the fatty acid profiles of mouse liver lipids. *Lipids* 39:135–141, 2004
15. Horton JD, Goldstein JL, Brown MS: SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 109:1125–1131, 2002
16. Matsuzaka T, Shimano H, Yahagi N, Amemiya-Kudo M, Yoshikawa T, Hasty AH, Tamura Y, Osuga J-I, Okazaki H, Iizuka Y, Takahashi A, Sone H, Gotoda T, Ishibashi S, Yamada N: Dual regulation of mouse Δ^5- and Δ^6-desaturase gene expression by SREBP-1 and PPARα. *J Lipid Res* 43:107–114, 2002
17. Nakatani T, Kim HJ, Kaburagi Y, Yasuda K, Ezaki O: A low fish oil inhibits SREBP-1 proteolytic cascade, while a high-fish-oil feeding decreases SREBP-1 mRNA in mice liver: relationship to anti-obesity. *J Lipid Res* 44:369–379, 2003
18. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
19. Baulande S, Lasnier F, Lucas M, Pairault J: Adiponutrin, a transmembrane protein corresponding to a novel dietary- and obesity-linked mRNA specifically expressed in the adipose lineage. *J Biol Chem* 276:33336–33344, 2001
20. Folch J, Lees M, Sloane-Stanley GH: A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 226:497–509, 1957
21. Ide T, Okamatsu H, Sugano M: Regulation by dietary fats of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in rat liver. *J Nutr* 108:601–612, 1978
22. Lehmann JM, Kliewer SA, Moore LB, Smith-Oliver TA, Oliver BB, Su JL, Sundseth SS, Winegar DA, Blanchard DE, Spencer TA, Willson TM: Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *J Biol Chem* 272:3137–3140, 1997
23. Costet P, Luo Y, Wang N, Tall AR: Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. *J Biol Chem* 275:28240–28245, 2000
24. Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, Shan B, Brown MS, Goldstein JL, Mangelsdorf DJ: Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRα and LXRβ. *Genes Dev* 14:2819–2830, 2000
25. Joseph SB, Laffitte BA, Patel PH, Watson MA, Matsukuma KE, Walczak R, Collins JL, Osborne TF, Tontonoz P: Direct and indirect mechanisms for

regulation of fatty acid synthase gene expression by liver X receptors. *J Biol Chem* 277:11019–11025, 2002
26. Tobin KA, Ulven SM, Schuster GU, Steineger HH, Andresen SM, Gustafsson JA, Nebb HI: Liver X receptors as insulin-mediating factors in fatty acid and cholesterol biosynthesis. *J Biol Chem* 277:10691–10697, 2002
27. Pawar A, Botolin D, Mangelsdorf DJ, Jump DB: The role of liver X receptor-α in the fatty acid regulation of hepatic gene expression. *J Biol Chem* 278:40736–40743, 2003
28. Kim HJ, Takahashi M, Ezaki O: Fish oil feeding decreases mature sterol regulatory element-binding protein 1 (SREBP-1) by down-regulation of SREBP-1c mRNA in mouse liver: a possible mechanism for down-regulation of lipogenic enzyme mRNAs. *J Biol Chem* 274:25892–25898, 1999
29. Shimomura I, Bashmakov Y, Ikemoto S, Horton JD, Brown MS, Goldstein JL: Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes. *Proc Natl Acad Sci U S A* 96:13656–13661, 1999
30. Moya-Camarena SY, Vanden Heuvel JP, Blanchard SG, Leesnitzer LA, Belury MA: Conjugated linoleic acid is a potent naturally occurring ligand and activator of PPARα. *J Lipid Res* 40:1426–1433, 1999
31. Ren B, Thelen AP, Peters JM, Gonzalez FJ, Jump DB: Polyunsaturated fatty acid suppression of hepatic fatty acid synthase and S14 gene expression does not require peroxisome proliferator-activated receptor α. *J Biol Chem* 272:26827–26832, 1997
32. Yoshikawa T, Ide T, Shimano H, Yahagi N, Amemiya-Kudo M, Matsuzaka T, Yatoh S, Kitamine T, Okazaki H, Tamura Y, Sekiya M, Takahashi A, Hasty AH, Sato R, Sone H, Osuga J, Ishibashi S, Yamada N: Cross-talk between peroxisome proliferator-activated receptor (PPAR) α and liver X receptor (LXR) in nutritional regulation of fatty acid metabolism. I. PPARs suppress sterol regulatory element binding protein-1c promoter through inhibition of LXR signaling. *Mol Endocrinol* 17:1240–1254, 2003
33. McGarry JD: Glucose-fatty acid interactions in health and disease. *Am J Clin Nutr* 67:500S–504S, 1998
34. Wong SH, Nestel PJ, Trimble RP, Storer GB, Illman RJ, Topping DL: The adaptive effects of dietary fish and safflower oil on lipid and lipoprotein metabolism in perfused rat liver. *Biochim Biophys Acta* 792:103–109, 1984
35. Vidal-Puig A, Jimenez-Liñan M, Lowell BB, Hamann A, Hu E, Spiegelman B, Flier JS, Moller DE: Regulation of PPAR γ gene expression by nutrition and obesity in rodents. *J Clin Invest* 97:2553–2561, 1996
36. Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM: Transcriptional regulation of adipogenesis. *Genes Dev* 14:1293–1307, 2000
37. Rosen ED, Spiegelman BM: PPARγ: a nuclear regulator of metabolism, differentiation, and cell growth. *J Biol Chem*: 276:37731–3734, 2001
38. Lyon CJ, Law RE, Hsueh WA: Minireview: adiposity, inflammation, and atherogenesis. *Endocrinology* 144:2195–2200, 2003
39. Qureshi A, Kopelman PG: Leptin - fat messenger or fat controller? *Clin Endocrinol* 47:169–171, 1997
40. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y: Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 257:79–83, 1999
41. Shklyaev S, Aslanidi G, Tennant M, Prima V, Kohlbrenner E, Kroutov V, Campbell-Thompson M, Crawford J, Shek EW, Scarpace PJ, Zolotukhin S: Sustained peripheral expression of transgene adiponectin offsets the development of diet-induced obesity in rats. *Proc Natl Acad Sci U S A* 100:14217–14222, 2003
42. Metzger S, Hassin T, Barash V, Pappo O, Chajek-Shaul T: Reduced body fat and increased hepatic lipid synthesis in mice bearing interleukin-6-secreting tumor. *Am J Physiol Endocrinol Metab* 281:E957–E965, 2001
43. Lagathu C, Bastard JP, Auclair M, Maachi M, Capeau J, Caron M: Chronic interleukin-6 (IL-6) treatment increased IL-6 secretion and induced insulin resistance in adipocyte: prevention by rosiglitazone. *Biochem Biophys Res Commun* 311:372–379, 2003
44. Brown JM, Boysen MS, Chung S, Fabiyi O, Morrison RF, Mandrup S, McIntosh MK: Conjugated linoleic acid induces human adipocyte delipidation: autocrine/paracrine regulation of MEK/ERK signaling by adipocytokines. *J Biol Chem* 279:26735–26747, 2004
45. Sadeghi S, Wallace FA, Calder PC: Dietary lipids modify the cytokine response to bacterial lipopolysaccharide in mice. *Immunology* 96:404–410, 1999
46. Venkatraman JT, Chu WC: Effects of dietary ω-3 and ω-6 lipids and vitamin E on serum cytokines, lipid mediators and anti-DNA antibodies in a mouse model for rheumatoid arthritis. *J Am Coll Nutr* 18:602–613, 1999
47. Sebokova E, Klimes I, Gasperikova D, Bohov P, Langer P, Lavau M, Clandinin MT: Regulation of gene expression for lipogenic enzymes in the liver and adipose tissue of hereditary hypertriglyceridemic, insulin-resistant rats: effect of dietary sucrose and marine fish oil. *Biochim Biophys Acta* 1303:56–62, 1996
48. Jang IS, Hwang DY, Chae KR, Lee JE, Kim YK, Kang TS, Hwang JH, Lim CH, Huh YB, Cho JS: Role of dietary fat type in the development of adiposity from dietary obesity-susceptible Sprague-Dawley rats. *Br J Nutr* 89:429–438, 2003
49. Shimomura I, Hammer RE, Ikemoto S, Brown MS, Goldstein JL: Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. *Nature* 401:73–76, 1999
50. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T: The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 7:941–946, 2001