

## The *trans*-10,*cis*-12 isomer of conjugated linoleic acid reduces hepatic triacylglycerol content without affecting lipogenic enzymes in hamsters

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Conjugated linoleic acid (CLA) refers to the positional and geometric dienoic isomers of linoleic acid. The dietary intake of CLA has been associated with changes in lipid metabolism. The aim of the present work was to assess the effects of the two main isomers of CLA on sterol regulatory element binding protein (SREBP)-1a and SREBP-1c mRNA levels, as well as on mRNA levels and the activities of several lipogenic enzymes in liver. For this purpose hamsters were fed an atherogenic diet supplemented with 5 g linoleic acid, *cis*-9,*trans*-11 or *trans*-10,*cis*-12 CLA/kg diet for 6 weeks. The *trans*-10,*cis*-12 isomer intake produced significantly greater liver weight, but also significantly decreased liver fat accumulation. No changes in mRNA levels of SREBP-1a, SREBP-1c and lipogenic enzymes, or in the activities of these enzymes, were observed. There was no effect of feeding *cis*-9,*trans*-11 CLA. These results suggest that increased fat accumulation in liver does not occur on the basis of liver enlargement produced by feeding the *trans*-10,*cis*-12 isomer of CLA in hamsters. The reduction in hepatic triacylglycerol content induced by this isomer was not attributable to changes in lipogenesis.

**Conjugated linoleic acid: Lipogenesis: Sterol regulatory element binding protein: Liver: Hamster**

Conjugated linoleic acid (CLA) refers to different positional and geometric dienoic isomers of linoleic acid. These fatty acids are a natural food component occurring in the lipid fraction of meat, milk and other dairy products. The *cis*-9,*trans*-11 CLA isomer is the principal dietary form of CLA, but lower levels of *trans*-10,*cis*-12 and other isomers are also present in these food sources (Roche *et al.* 2001).

CLA intake is known to produce changes in lipid metabolism in adipose tissue and liver. A great deal of work has been performed on adipose tissue (Pariza *et al.* 2000; Roche *et al.* 2001). In contrast, data on the effects of CLA on liver are scarcer. In some studies it has been reported that CLA consumption may modify hepatic fatty acid oxidation and synthesis (Park *et al.* 1997; Martin *et al.* 2000; Takahashi *et al.* 2003; Tsuboyama-Kasaoka *et al.* 2003).

The transcription of genes encoding lipogenic enzymes, such as fatty acid synthase and acetyl-CoA carboxylase, is controlled by a family of membrane-bound transcriptional factors known as sterol regulatory element binding proteins (SREBP; Pai *et al.* 1998). Three types of SREBP have been identified. It has been shown that SREBP-1a and SREBP-1c, both derived from a single gene, participate in the regulation of the gene expression of enzymes involved in fatty acid synthesis (Shimano *et al.* 1997; Shimano, 2001; Amemiya-Kudo *et al.* 2002; Horton *et al.* 2002).

Numerous studies have found that SREBP respond to nutrients and to hormones to control lipid metabolism (Shimomura *et al.* 1999; Osborne, 2000). PUFA have been shown to decrease SREBP-1 mRNA levels, as well as the nuclear content of SREBP-1 in liver, thus leading to reduced lipogenic enzyme gene expression (Kim *et al.* 1999; Xu *et al.* 1999, 2002; Yahagi *et al.* 1999).

The aim of the present work was to assess the effects of the two main isomers of CLA, *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA, on SREBP-1a and SREBP-1c expression, as well as on the expression and activity of several lipogenic enzymes in livers from hamsters fed an atherogenic diet.

### Material and methods

#### *Animals, diets and experimental design*

The experiment was conducted with twenty-four 9-week-old male Syrian Golden hamsters (body weight 105 (SEM 1) g) purchased from Harlan Ibérica (Barcelona, Spain) and was in accordance with the Institution's guide for the care and use of laboratory animals. The hamsters were individually housed in polycarbonate metabolic cages (Techniplast Gazzada, Guguggiate, Italy) and placed in an air-conditioned room (22±2°C) with a 12 h light–dark cycle. After a 6 d adaptation period, hamsters were randomly divided into three groups of eight animals each and, over a period of 6 weeks, fed the semi-purified

atherogenic diets supplemented with 5 g linoleic acid, *cis*-9,*trans*-11 or *trans*-10,*cis*-12 CLA/kg diet (Table 1). The experimental diets were freshly prepared once per week, gassed with N<sub>2</sub> and stored at 0–4°C to avoid rancidity. DL-Methionine and cholesterol were supplied by Sigma (St Louis, MO, USA), wheat starch and cellulose by Ven-casser (Bilbao, Spain), conjugated linoleic isomers by Natural Lipids Ltd (Hovdebygda, Norway), sucrose and sunflower oil were obtained from local markets, and palm oil was a generous gift from Agra-Unilever (Leioa, Spain). Vitamin and mineral mixes were formulated according to AIN-93 guidelines (Reeves *et al.* 1993) and supplied by ICN Pharmaceuticals (Costa Mesa, CA, USA). All animals had free access to food and water. Food intake and body weight were measured daily.

At the end of the experimental period animals were killed under anaesthesia (diethyl ether) and blood was collected by cardiac puncture. Adipose tissue from different locations (perirenal, epididymal and subcutaneous) and liver were dissected, weighed and immediately frozen. Serum was obtained from blood samples after centrifugation (1000g for 10 min at 4°C). Samples were stored at –80°C until analysis.

#### Hepatic triacylglycerol content

Total lipids were extracted from liver following the method described by Folch *et al.* (1957). The lipid extract was dissolved in isopropanol. Triacylglycerol was measured using a commercial kit (Biosystems, Barcelona, Spain).

#### Extraction and analysis of RNA and semiquantification by RT-PCR

Total RNA was isolated from 100 mg liver using Ultraspec-II (Biotecx Laboratories, Houston, TX, USA) according to the manufacturer's instructions. RNA (1.5 µg) was used to synthesize first-strand complementary DNA (cDNA) after 60 min at 37°C treatment with RNase-free DNase I (Roche, Mannheim, Germany). The RT-PCR

was carried out as described by Corbalan *et al.* (1999). Specific sense and antisense primers used for SREBP-1a, SREBP-1c, fatty acid synthase and acetyl-CoA carboxylase mRNA quantification were those described by Field *et al.* (2003); primers used to amplify β-actin were designed using the Oligo 6.0 Primer Analysis Software for Windows (National Biosciences, Annapolis, MN, USA) (Table 2). cDNA was amplified using the variables shown in Table 2. A first step of denaturation (95°C for 30 s) and a final extension step (72°C for 7 min) were applied for all primers. Amplifications were linear under these conditions and carried out in a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). Our preliminary experiments demonstrated that the cycle numbers were well below the plateau phase for PCR products. Linearity of the PCR reactions were tested by amplification of 200 ng first strand cDNA per reaction from twenty to forty cycles. The amplified products were resolved in an agarose (12 g/kg) gel (MS-8, Pronadisa, Madrid, Spain) and stained with ethidium bromide. PCR band intensity was semiquantified by scanning densitometric analysis using the Gel Doc 1000 UV fluorescent gel documentation system and Molecular Analyst software for quantification of images (Bio-Rad Laboratories, Hercules, CA, USA). The values were normalized using β-actin as an endogenous internal standard.

#### Lipogenic activities

Liver samples (0.5 g) were homogenized in 5 ml buffer (pH 7.6) containing 150 mM-KCl, 1 mM-MgCl<sub>2</sub>, 10 mM-*N*-acetyl-cysteine and 0.5 mM-dithiothreitol.

After centrifugation at 100 000g for 40 min at 4°C, the supernatant fraction was used for quantification of enzyme activities. Glucose-6-phosphate dehydrogenase was determined from the rate of glucose-6-phosphate-dependent reduction of NADP<sup>+</sup> (Kuby & Noltmann, 1966), malic enzyme from the rate of malate-dependent NADP<sup>+</sup> reduction (Hsu & Lardy, 1969) and fatty acid synthase from the rate of malonyl-CoA dependent NADPH

**Table 1.** Composition of experimental diets (g/kg)\*

Ingredients	Linoleic acid	<i>cis</i> -9, <i>trans</i> -11 CLA	<i>trans</i> -10, <i>cis</i> -12 CLA
Casein†	200	200	200
DL-Methionine	4	4	4
Sucrose	404	404	404
Wheat starch	200	200	200
Palm oil	100	100	100
Sunflower oil‡	8.2	–	–
<i>cis</i> -9, <i>trans</i> -11 CLA§	–	5.6	–
<i>trans</i> -10, <i>cis</i> -12 CLA§	–	–	5.6
Cholesterol	1	1	1
Cellulose	30	30	30
Mineral mix	40	40	40
Vitamin mix	11	11	11
Choline chloride salt	4	4	4

CLA, conjugated linoleic acid.

\*For details of suppliers of ingredients, see p. 384.

†900 g casein/kg.

‡617 g linoleic acid/kg (Rodríguez *et al.* 2002).

§900 g CLA/kg.

|| Formulated according to the American Institute of Nutrition (AIN-93; Reeves *et al.* 1993).

**Table 2.** Primers for PCR amplification gene products and specific conditions used for the amplification of each gene studied

Primers...	Sense primer	Antisense primer	Length (bp)	GenBank accession no.
SREBP-1a	5'-GGCTGTGGAACAGGCACTG-3'	5'-AGCTGGAGCATGTCTTCGATG-3'	78	Field <i>et al.</i> (2003)
SREBP-1c	5'-GCGGACGCAGTCTGGG-3'	5'-ATGAGCTGGAGCATGTCTTCAA-3'	95	Field <i>et al.</i> (2003)
FAS	5'-AGCCCCTCAAGTGCACAGT-3'	5'-TGCCAATGTGTTTCCCTGA-3'	81	AF356086
ACC	5'-ACACTGGCTGGCTGGACAG-3'	5'-CACACAACCTCCCAACATGGT-3'	76	AF356089
β-Actin	5'-TCTACAATGAGCTGCGTGTG-3'	5'-GGTCAGGATCTTCATGAGGT-3'	300	J00691
Conditions...	Annealing	Elongation	No. of cycles	
SREBP-1a	60°C – 60 s	60°C – 60 s	29	
SREBP-1c	60°C – 60 s	60°C – 60 s	30	
FAS	60°C – 60 s	60°C – 60 s	30	
ACC	60°C – 60 s	60°C – 60 s	33	
β-Actin	59°C – 30 s	72°C – 30 s	32	

SREBP, sterol regulatory element binding protein; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase.

oxidation (Lynen, 1969). NADPH was measured by reading absorbance at 340 nm. Acetyl-CoA carboxylase was measured from the acetyl-CoA-dependent incorporation of <sup>14</sup>HCO<sub>3</sub><sup>-</sup> into acid-stable product (Chang *et al.* 1967; Chakrabarty & Leveille, 1969). All enzyme assays were conducted at 37°C. Soluble protein in the supernatant fraction was determined using bovine serum albumin as standard (Bradford, 1976). Glucose-6-phosphate dehydrogenase and malic enzyme activities were expressed as nmol NADPH produced/min per mg protein, fatty acid synthase activity as nmol NADPH consumed/min per mg protein and acetyl-CoA carboxylase activity as nmol bicarbonate incorporated/min per mg protein.

*Serum variables*

Serum glucose and insulin were measured by spectrophotometry and RIA respectively using commercial kits (BioSystems, Barcelona, Spain; Linco, St Charles, MO, USA).

*Statistical analysis*

Results are presented as mean values with their standard errors. Statistical analysis was performed using SPSS 8.0 (SPSS Inc., Chicago, IL, USA). Data were analysed by one-way ANOVA followed by Newman-Keuls *post hoc* test. Statistical significance was at *P*<0.05.

**Results**

*Body weight, food intake, adipose tissue and liver weights, and hepatic triacylglycerol content*

No differences in final body weight and total food intake were found among the experimental groups. There was a significant effect of *trans*-10,*cis*-12 CLA on liver weight and composition: the addition of this CLA isomer to the diet led to significantly greater liver weight, whether expressed as absolute value or as percentage of total body weight (*P*<0.05). This effect was not due to increased fat accumulation; on the contrary, hamsters from the *trans*-10,*cis*-12 CLA group had significantly lower triacylglycerol content (-34% *v.* linoleic group; *P*<0.01) (Table 3). No changes in these variables were induced by *cis*-9,*trans*-11 CLA intake.

The *trans*-10,*cis*-12 CLA isomer, but not the *cis*-9,*trans*-11 isomer, also reduced fat accumulation in adipose tissue (-36% *v.* linoleic group; *P*<0.001). The results in Table 3 show the weight of white adipose tissue (a pool of perirenal, epididymal and subcutaneous depots).

*Liver gene expression and activities of lipogenic enzymes*

mRNA levels of SREBP-1a and SREBP-1c were not affected by CLA feeding (Fig. 1). Likewise, mRNA

**Table 3.** Adipose tissue and liver weights, and hepatic triacylglycerol content in hamsters fed on the experimental diets for 6 weeks\*

(Mean values with their standard errors for eight hamsters per group)

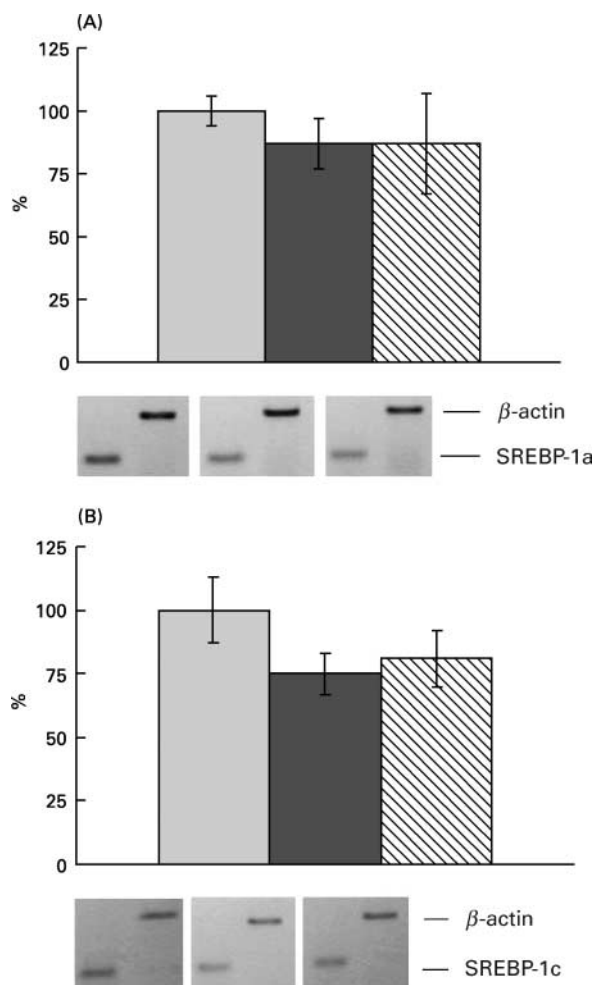
Experimental groups...	Linoleic acid		<i>cis</i> -9, <i>trans</i> -11 CLA		<i>trans</i> -10, <i>cis</i> -12 CLA		Statistical significance of effect (ANOVA)
	Mean	SEM	Mean	SEM	Mean	SEM	
Adipose tissue weight†							
g	6.80 <sup>a</sup>	0.43	6.07 <sup>a</sup>	0.26	4.36 <sup>b</sup>	0.25	<i>P</i> <0.001
% body weight	5.64 <sup>a</sup>	0.30	5.06 <sup>a</sup>	0.20	3.72 <sup>b</sup>	0.19	<i>P</i> <0.001
Liver weight							
g	4.62 <sup>b</sup>	0.16	4.48 <sup>b</sup>	0.19	5.10 <sup>a</sup>	0.11	<i>P</i> <0.05
% body weight	3.84 <sup>b</sup>	0.11	3.74 <sup>b</sup>	0.13	4.37 <sup>a</sup>	0.08	<i>P</i> <0.01
Hepatic triacylglycerol content							
mg/g	7.21 <sup>a</sup>	0.60	6.50 <sup>a</sup>	0.56	4.79 <sup>b</sup>	0.48	<i>P</i> <0.01
mg/liver	33.3 <sup>a</sup>	2.9	29.1 <sup>ab</sup>	2.7	24.2 <sup>b</sup>	2.1	<i>P</i> <0.05

CLA, conjugated linoleic acid.

<sup>a,b</sup> Mean values within the same row with unlike superscript letters were significantly different (*P*<0.05).

\* For details of diets and procedures, see Table 1 and pp. 383–384.

† A pool of perirenal, epididymal and subcutaneous adipose tissues.

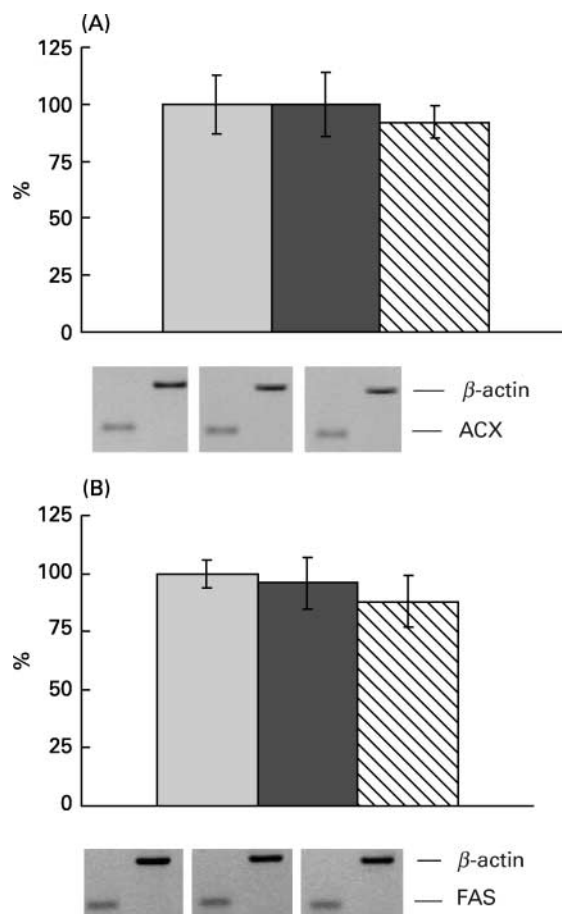


**Fig. 1.** RT-PCR analysis of sterol regulatory element binding protein (SREBP)-1a mRNA (A) and SREBP-1c mRNA (B) in the liver of hamsters fed the experimental diets. ■, Linoleic acid; ■, *cis*-9,*trans*-11 conjugated linoleic acid; ▨, *trans*-10,*cis*-12 conjugated linoleic acid. For details of diets and procedures, see Table 1 and pp. 383–384. The results are shown as values relative to mRNA levels in the linoleic acid group.

levels of acetyl-CoA carboxylase and fatty acid synthase were not modified by dietary CLA isomers (Fig. 2). We found no changes in the SREBP-regulated enzyme activities (acetyl-CoA carboxylase and fatty acid synthase) or in glucose-6-phosphate dehydrogenase and malic enzyme activities (enzymes that provide NADPH for reductive biosynthesis) (Table 4).

#### Serum variables

Neither serum glucose concentration (9.56 (SEM 0.91) mmol/l in linoleic acid group, 9.25 (SEM 1.27) mmol/l in *cis*-9,*trans*-11 CLA group and 9.35 (SEM 0.59) mmol/l in *trans*-10,*cis*-12 CLA group,  $P > 0.05$ ) nor serum insulin concentration (176 (SEM 25) pmol/l in linoleic acid group, 144 (SEM 12) pmol/l in *cis*-9,*trans*-11 CLA group and 184 (SEM 16) pmol/l in *trans*-10,*cis*-12 CLA group,  $P > 0.05$ ) were significantly modified by CLA isomer intake.



**Fig. 2.** RT-PCR analysis of acetyl-CoA carboxylase (ACC) mRNA (A) and fatty acid synthase (FAS) mRNA (B) in the liver of hamsters fed the experimental diets. ■, Linoleic acid; ■, *cis*-9,*trans*-11 conjugated linoleic acid; ▨, *trans*-10,*cis*-12 conjugated linoleic acid. For details of diets and procedures, see Table 1 and pp. 383–384. The results are shown as values relative to mRNA levels in the linoleic acid group.

#### Discussion

Several studies in mice have shown that feeding CLA leads to an increase in liver weight (DeLany *et al.* 1999; Martin *et al.* 2000; Hamura *et al.* 2001; Miner *et al.* 2001; Ealey *et al.* 2002; Terpstra *et al.* 2002; Warren *et al.* 2003). In some of these trials, hepatic triacylglycerol content was measured and it was concluded that hepatomegaly was due to lipid accumulation (Belury & Kempa-Steczko, 1997; West *et al.* 1998; Tsuboyama-Kasaoka *et al.* 2000; Takahashi *et al.* 2003). In contrast, the studies performed on rats showed no effects on liver weight induced by CLA (Chin *et al.* 1994; Sakono *et al.* 1999; Azain *et al.* 2000; Martin *et al.* 2000; Stangl, 2000; Clément *et al.* 2002; Wang *et al.* 2003).

These findings suggest the existence of a species-specific response of liver to CLA feeding (Moya-Camarena & Belury, 1999). Differences among species have also been described in terms of CLA-induced reductions in body-fat gain; thus, species responsiveness is of the following order: mice > hamster > rats (Kim *et al.* 2002). In order

**Table 4.** Hepatic lipogenic enzyme activities in hamsters fed on the experimental diets for 6 weeks\* (Mean values with their standard errors for eight hamsters per group)

Experimental groups...	Linoleic acid		<i>cis</i> -9, <i>trans</i> -11 CLA		<i>trans</i> -10, <i>cis</i> -12 CLA		Statistical significance of effect (ANOVA)
	Mean	SEM	Mean	SEM	Mean	SEM	
Glucose 6-phosphate dehydrogenase†	59.6	2.2	63.4	2.2	67.5	4.3	NS
Malic enzyme†	207	6	195	3	192	9	NS
Fatty acid synthase‡	150	8	174	16	165	11	NS
Acetyl-CoA carboxylase§	13.3	1.5	15.5	1.5	17.3	1.4	NS

CLA, conjugated linoleic acid.

\* For details of diets and procedures, see Table 1 and pp. 383–384.

† nmol NADPH formed/mg protein per min.

‡ nmol NADPH consumed/mg protein per min.

§ nmol HCO<sub>3</sub><sup>-</sup> incorporated/mg protein per min.

to gain insight into these differences among species, the present work was carried out using the hamster as experimental model.

The present results showed that feeding the *trans*-10,*cis*-12 CLA isomer led to greater liver weight. This effect was similar to that found in mice, but in hamsters the effect was not due to increased fat accumulation; on the contrary, the liver triacylglycerol content in animals fed this CLA isomer was significantly lower than that in the linoleic acid group. Moreover, a significant increase in the hepatocyte number was found (23%  $P < 0.01$ ; MT Macarulla, A Fernández-Quintela, A Zabala, V Navarro, E Echevarria, I Charruca, VM Rodríguez and MP Portillo, unpublished results). Tsuboyama-Kasaoka *et al.* (2003) proposed that liver fat accumulation in mice was probably a result of an important fat mobilization from adipose tissue. Thus, when they added 10 g CLA mixture/kg diet, which provided 4% total fat, animals showed lipodystrophy. This situation led to a great fatty acid delivery into liver, and probably not all fatty acids could be oxidized. In the present study, 5 g *trans*-10,*cis*-12 CLA/kg diet led to a significant, but not massive, reduction in adipose tissue size (-36% *v.* linoleic acid group) and thus, the net flux of NEFA to liver can be assumed to be lower. All these results demonstrate that the response of liver to CLA is different in the hamster compared with other rodent species, such as the mouse and the rat.

It is well known that lipogenesis and fatty acid oxidation are metabolic pathways controlling liver lipid content. We previously studied the effects of CLA feeding on mitochondrial-peroxisomal fatty acid oxidation and we found that *trans*-10,*cis*-12 CLA, but not *cis*-9,*trans*-11 CLA, induced a significant increase in the activity of hepatic carnitine palmitoyltransferase-I (43.0%,  $P < 0.01$ ) (Zabala *et al.* 2003) and acyl-CoA oxidase (157.3%,  $P < 0.01$ ), a rate-limiting enzyme in peroxisomal oxidation (MT Macarulla, A Fernández-Quintela, A Zabala, V Navarro, E Echevarria, I Charruca, VM Rodríguez and MP Portillo, unpublished results). Thus, increased fatty acid oxidation is probably responsible, at least in part, for the reduced hepatic triacylglycerol content.

Hepatic fatty acid oxidation and synthesis are regulated reciprocally under various nutritional and physiological conditions. Thus, in the present experimental design, where the activity of enzymes regulating fatty acid oxidation was significantly increased, a decreased lipogenesis

could be expected. In order to analyse the effects of CLA feeding on this metabolic pathway, relative mRNA levels and activity of lipogenic enzymes, as well as relative mRNA levels of the SREBP involved in its regulation, were assessed.

Although the predominant form of SREBP-1 in hamster, mouse and human liver is SREBP-1c (Kim *et al.* 1999; Shimano, 2001), in the present study both SREBP-1a and SREBP-1c were studied because it has been reported that some nutritional factors can affect these two transcripts differently. Thus, Horton *et al.* (1998) showed that 24 h fasting decreased SREBP-1c mRNA expression by 60%, but not SREBP-1a mRNA, and Kim *et al.* (1999) observed greater reduction in SREBP-1c than in SREBP-1a when mice were fed a high-fish-oil diet.

In the present study no changes in the expression of SREBP-1a, SREBP-1c and lipogenic enzymes (acetyl-CoA carboxylase and fatty acid synthase) induced by both CLA isomers were found. Despite this lack of effect, the possibility that mRNA levels of transcription factors and enzymes did not parallel enzyme activities should not be discarded. Thus, activities of acetyl-CoA carboxylase, fatty acid synthase and enzymes that provide NADPH for reductive biosynthesis (glucose-6-phosphate dehydrogenase and malic enzyme) were also assessed, and no effects of CLA isomers were found. Finally, serum concentrations of glucose and insulin, two important regulators of SREBP and lipogenic enzyme expression and activity, remained unchanged. These results suggest that changes in lipogenesis are not on the basis of the *trans*-10,*cis*-12 effects on liver composition. This is in accordance with the results published by Tsuboyama-Kasaoka *et al.* (2003). They found that lipogenesis was not affected when a CLA mixture (10 g/kg diet) accounted for 3% total dietary fat, but was significantly increased when the same amount of the CLA mixture accounted for 25% total dietary fat. In the present experimental design CLA isomers represented about 5% total dietary fat. Together these results suggest that the effect of CLA on hepatic lipogenesis depends on the CLA:total fat ratio.

In summary, when liver is studied, the responsiveness of hamsters to CLA feeding is different from that elicited by mice and rats. The *trans*-10,*cis*-12 CLA isomer, but not the *cis*-9,*trans*-11 CLA, produces an increase in liver weight, but a decrease in hepatic triacylglycerol content. The reduced fat deposition in liver is not apparently due

to changes in lipogenesis and should be ascribed to an increase in fatty acid oxidation.

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