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Trans-10,*cis*-12-conjugated linoleic acid does not increase body fat loss induced by energy restriction

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Very little evidence exists concerning the effects of conjugated linoleic acid (CLA) on body fat reduction induced by energy restriction. Moreover, although an effect of *trans*-10,*cis*-12-CLA on lipolysis has been suggested, it has not been consistently shown. The aims of the present study were to determine whether *trans*-10,*cis*-12-CLA increases the reduction of body fat induced by energy restriction, and to analyse its effect on lipolysis and adipose tissue lipase expression (hormone-sensitive lipase (HSL) and adipose tissue TAG lipase (ATGL)). Male Syrian Golden hamsters were fed a high-fat diet during 7 weeks in order to make them fatter. Then they were submitted to a mild energy restriction (25 %) without or with supplementation of 0·5 % *trans*-10,*cis*-12-CLA for 3 weeks. Basal glycerol release and lipolysis stimulated by several drugs acting at different levels of the lipolytic cascade were measured in epididymal adipose tissue. The expression of HSL and ATGL was assessed by real-time RT-PCR. No differences were found in adipose tissues size between the experimental groups. Medium adipocyte size and total number of adipocytes were similar in both experimental groups. Animals fed the CLA-enriched diet showed similar lipolytic rates as well as HSL and ATGL expressions to the controls. In conclusion, *trans*-10,*cis*-12-CLA does not promote adipose tissue lipid mobilisation nor does it heighten body fat reduction induced by energy restriction. Consequently, this CLA isomer does not seem to be a useful tool to be included in body weight-loss strategies followed in obesity treatment.

Conjugated linoleic acid: Lipolysis: Energy restriction: Hamsters

Numerous studies performed on rodents and pigs have clearly demonstrated that the *trans*-10,*cis*-12 isomer of conjugated linoleic acid (CLA) reduces adiposity by decreasing fat deposition. This body fat-lowering effect has been observed when CLA is added to either normal-fat or high-fat diets[1–4].

Although several mechanisms have been proposed to explain this CLA effect, it seems that it acts primarily by reducing the activities of lipoprotein lipase and stearoyl-coenzyme A desaturase, two key enzymes of lipid storage. Indeed, the general consensus achieved concerning the effects of CLA on these enzymes supports the effect on fat accretion[2,5]. Lipoprotein lipase hydrolyses fatty acids from circulating TAG, thus enhancing fatty acid uptake, and re-esterification by the adipocytes, and stearoyl-coenzyme A desaturase is the rate-limiting enzyme for converting SFA to MUFA, the main substrate for fat deposit in adipose tissue. Consequently, the reduction in lipoprotein lipase and stearoyl-coenzyme A desaturase activities suggests an impairment in adipocyte replenishment.

In contrast, very little evidence exists concerning the effects of CLA on body fat reduction induced by energy restriction[6,7]. Moreover, although an effect of *trans*-10,*cis*-12-CLA on lipolysis has been suggested, it has not been consistently shown, and at present there is little support for this hypothesis[8–11].

As a result, it can be stated that *trans*-10,*cis*-12-CLA blocks body fat gain but does not necessarily reduce body fat level accumulated before CLA administration, so it is too early to predict the extent to which CLA supplements could be useful in enhancing body fat loss promoted by dietary treatment of obesity based on energy restriction.

The first aim of the present study was to determine whether *trans*-10,*cis*-12-CLA increases the reduction of body fat induced by an energy restriction in hamsters. A second objective, focused on lipolytic pathways, consisted of the analysis of the effects of this CLA isomer on lipolysis stimulated at different levels of the lipolytic cascade, as well as on the expression of two lipases present in adipose tissue, hormone-sensitive lipase (HSL) and adipose tissue TAG lipase (ATGL).

Experimental methods

Animals, diets and experimental design

The experiment was conducted with twenty-four male Syrian Golden hamsters (age 9 weeks; initial body weight

Abbreviations: ATGL, adipose tissue TAG lipase; CLA, conjugated linoleic acid; HSL, hormone-sensitive lipase; RC, restricted group fed a control diet; RCLA, restricted group fed a CLA-supplemented diet.

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82·6 ± 1·4 g) purchased from Harlan Ibérica (Barcelona, Spain) and took place in accordance with the Institution's guide for the care and use of laboratory animals. Animals were individually housed in polycarbonate metabolism cages (Techniplast Gazzada, Guguggiate, Italy) and placed in an air-conditioned room (22 ± 2°C) with a 12 h light–dark cycle. In order to increase body fat content in animals, after a 6 d adaptation period, they were fed *ad libitum* a semi-purified high-fat diet for 7 weeks. This diet consisted of (g/kg): casein, 200 (Sigma, St Louis, MO, USA); L-methionine, 4 (Sigma); wheat starch, 220 (Vencasser, Bilbao, Spain); sucrose, 320 (local markets); palm oil, 150 (Agra-Unilever, Leioa, Spain); sunflower-seed oil, 5 (local markets); cellulose, 46 (Vencasser); choline–HCl, 4 (Sigma). Vitamin (11 g/kg) and mineral (40 g/kg) mixes were formulated according to AIN-93 guidelines[12] and supplied by ICN Pharmaceuticals (Costa Mesa, CA, USA). Palm oil was used because it has been shown that saturated fats lead to greater body fat accumulation than unsaturated ones[13–15].

After this experimental period, the hamsters (128·7 ± 2·3 g) were divided into three weight-matched groups of eight animals each. Two groups were submitted to energy restriction and fed a semi-purified standard diet: a restricted group fed a control diet (RC) and a restricted group fed a CLA-supplemented diet (RCLA). To calculate the appropriate quantity of diet to be offered to these restricted groups, the third weight-matched group (reference group), fed the same semi-purified standard diet, was used only in order to know the spontaneous food intake. A 25 % reduction was applied to the mean food intake of this group. The semi-purified standard diet consisted of (g/kg): casein, 200; L-methionine, 4; wheat starch, 390; sucrose, 235; sunflower-seed oil, 70; cellulose, 46; choline–HCl, 4; vitamin mix, 11; mineral mix, 40. The diet provided to the RC group was supplemented with linoleic acid at 5 g/kg (supplied by sunflower-seed oil) and that provided to the RCLA group with *trans*-10,*cis*-12-CLA at 5 g/kg (Natural Lipids Ltd, Hovdebygda, Norway). Sunflower-seed oil was used as the fat source instead of palm oil in order to obtain a balanced fatty acid profile.

All the experimental diets were freshly prepared once per week, gassed with N_2, and stored at 0–4°C to avoid rancidity. Body weight was measured daily.

At the end of the experimental period the hamsters from the RC and RCLA groups were killed under anaesthesia (chloral hydrate) by exsanguination. Adipose tissue from abdominal subcutaneous, perirenal and epididymal locations were dissected and weighed, then immediately frozen. Serum was obtained from blood samples after centrifugation (1000 ***g*** for 10 min at 4°C). All samples were stored at − 80°C until analysis.

Serum measurements

Serum NEFA concentration was determined by using a commercial kit (Roche Diagnostics, Mannheim, Germany).

Adipocyte isolation and lipolysis

Isolated fat cells were obtained from epididymal adipose tissue by collagenase digestion (1·5 mg/ml; 37°C) in Krebs–Ringer bicarbonate buffer, containing bovine serum albumin (3·5 g/100 ml) and 5 mM-glucose at pH 7·4 (KRBA), under continuous vigorous shaking (90 cycles/min) according to the Rodbell method[16]. Adipocytes were filtered through nylon mesh and washed three times with the same incubation buffer (KRBA) to eliminate the stroma-vascular fraction and collagenase. Measurements of lipolytic activity were performed by incubating isolated adipocytes in 200 µl KRBA buffer with continuous gentle shaking (30 cycles/min), at 37°C with several drugs acting at different levels of the lipolytic cascade: (1) at the β-adrenoceptors (isoproterenol (ISO; 10^{-5} M)); (2) at the adenylate cyclase (forskolin (10^{-5} M)); (3) at the phosphodiesterase (isobutylmethylxanthine (10^{-4} M)); (4) at the protein kinase A (dibutyryl-cAMP (10^{-3} M)); (5) at adenosine release (adenosine deaminase (0·2 units/ml)); or without drugs (basal lipolysis), for 90 min. In all cases, the reaction was stopped with an ice bath and a sample (200 µl) was taken for enzymic determination[17] of glycerol released in the incubation buffer, which was used as the index of fat cell lipolysis. Total lipids were determined gravimetrically after their extraction, according to the method of Dole & Meinertz[18]. Results were expressed as µmol glycerol released by 100 mg lipids in 90 min.

Lipolytic drugs were supplied by Sigma (St Louis, MO, USA), and NAD^+, ATP, glycerol kinase, glycerol-3-phosphate-dehydrogenase and collagenase A by Roche Diagnostics (Mannheim, Germany).

Determination of adipocyte size and number

Adipocyte diameters were determined by direct microscopy (Olympus BX50F; Olympus Optical Co. Ltd, Tokyo, Japan) using a 50 µl sample of each isolated fat cell suspension. Cells (200 in each sample) with diameters from 20 to 240 µm were counted[19].

The total number of fat cells in adipose tissue was obtained by dividing the total lipid content of the tissue by the average lipid content of the fat cells. This value is derived by the mean cell volume (calculated from the diameter) × density of lipid[19].

Extraction and analysis of RNA and quantification by reverse transcription-polymerase chain reaction

Total RNA was isolated from 100 mg of epididymal adipose tissue using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA samples were then treated with a DNA-free kit (Applied Biosystems, Foster City, CA, USA) to remove any contamination with genomic DNA. The yield and quality of the RNA were assessed by measuring absorbance at 260, 270, 280 and 310 nm and by electrophoresis on 1·3 % agarose gels. Of the total RNA of each sample, 1·5 µg was reverse-transcribed to first-strand complementary DNA (cDNA) using an iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA, USA).

Relative HSL and ATGL mRNA levels were quantified using real-time PCR with an iCycler™–MyiQ™ Real-time PCR Detection System (BioRad). β-Actin mRNA levels were similarly measured and served as the reference gene. Of each cDNA, 0·1 µl were added to PCR reagent mixture, SYBR® Green Master Mix (Applied Biosystems), with the upstream and downstream primers (900 nM each for HSL and 300 nM for ATGL). Specific primers were synthesised commercially (Tib Molbiol, Berlin, Germany), and the sequences were:

HSL: 5′-GGT GAC ACT CGC AGA AGA CAA TA-3′ (forward), 5′-GCC GCC GTG CTG TCT CT-3′ (reverse); ATGL: 5′-CAC TTT AGC TCC AAG GAT GA-3′ (forward), 5′-TGG TTC AGT AGG CCA TTC CT-3′ (reverse); β-Actin: 5′-ACG AGG CCC AGA GCA AGA G-3′ (forward), 5′-GGT GTG GTG CCA GAT CTT CTC-3′ (reverse).

The PCR parameters were as follows: initial 2 min at 50°C, denaturation at 95°C for 10 min followed by forty cycles of denaturation at 95°C for 30 s, annealing at 60°C for HSL and 63·5°C for ATGL for 15 s and extension at 60°C for 30 s. All sample mRNA levels were normalised to the values of β-actin and the results expressed as -fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method[20].

Statistical analysis

Results are presented as mean values with their standard errors. Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Repeated measures of ANOVA statistical analysis was applied to analyse body weight loss time courses and Student's *t* test to the rest of measurements. Statistical significance was set-up at the $P<0{\cdot}05$ level.

Results

Body weight and adipose tissue size

No differences in body weight loss were found between both experimental groups throughout the experimental period (Fig. 1). Adipose depot sizes (epididymal, perirenal and abdominal subcutaneous) showed a tendency to reduced values in the RCLA group but this difference did not reach statistical significance when compared with the RC group (Table 1).

Serum measurements

Hamsters in both experimental groups showed similar values of serum NEFA (0·32 (SEM 0·01) nmol/l in the RC group and 0·28 (SEM 0·02) nmol/l in the RCLA group).

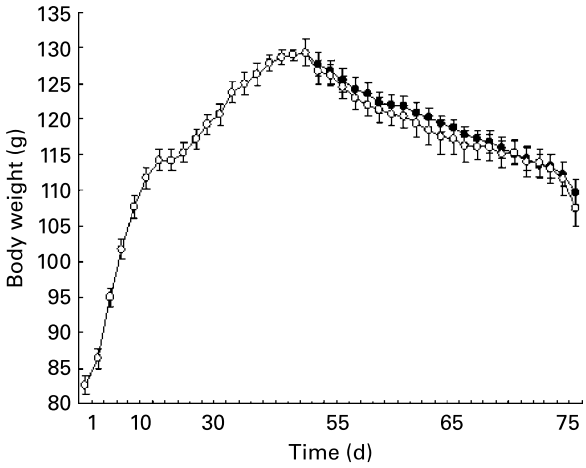

Fig. 1. Body-weight evolution in hamsters fed a high-fat diet for 7 weeks (–○–) and then 25 % restricted diets supplemented (–○–) or not (–●–; control diet) with *trans*-10,*cis*-12-conjugated linoleic acid for 3 weeks. Values are means, with their standard errors represented by vertical bars.

Adipocyte size and number

Microscopy revealed no significant differences in medium mature adipocyte diameter. A tendency to a reduced number of adipocytes was observed in hamsters fed the *trans*-10,*cis*-12-CLA (− 26·6 %), but this difference did not reach statistical significance (Table 1).

Lipolytic activity and lipase expression

There were no differences in epididymal adipocyte basal lipolysis between the experimental groups or in lipolysis induced by lipolytic drugs acting at different levels of the lipolytic cascade (Table 2). Due to the fact that no significant differences were found in adipocyte size, lipolysis results are expressed as μmol glycerol/100 mg lipids per 90 min. The results are similar when expressed as μmol glycerol/10^6 cells per 90 min (data not shown).

When comparing the expression of HSL between the experimental groups, a tendency towards increased values was found in the RCLA group, but the difference did not reach statistical significance (Fig. 2 (a)). With regard to ATGL, similar mRNA levels were observed in both experimental groups (Fig. 2 (b)).

Discussion

As described in the introduction section, numerous studies performed on experimental animals have clearly demonstrated that the *trans*-10,*cis*-12 isomer of CLA reduces adiposity by decreasing fat deposition[1–4]. In contrast, very little evidence exists concerning the effects of CLA on body fat reduction induced by energy restriction.

Stangl[6] carried out a study in young male rats fed a 30 % energy-restricted diet, supplemented or not with a CLA mixture (3 %) for 18 d. As a result of CLA feeding body fat loss was significantly increased. With regard to human subjects, a study has been performed with premenopausal women during a 12-week period of moderate energy restriction[7]. No differences in body weight or body fat losses were observed between the placebo group and the CLA-treated group.

In the present study, after a period of fattening feeding, hamsters were subjected to a mild energy restriction (25 %) for 3 weeks that led to a loss of 18·6 g in the RC group and 20·8 g in the RCLA group. No significant differences were found in body-weight loss between both experimental groups, and this situation persisted throughout the whole experimental period. This indicates that, although a CLA effect in a longer experimental period could not be discarded, in a 3-week or shorter restriction period *trans*-10,*cis*-12-CLA does not seem to be useful in increasing body-weight reduction. In good accordance with these results concerning body-weight loss, adipose tissue sizes in different anatomical locations were not different in CLA-fed hamsters from those in the controls, showing that CLA did not modify body fat reduction.

The discrepancy between these results and those published by Stangl[6] could be explained by the difference in the CLA dose used. Thus, whereas Stangl chose a 3 % CLA mixture (approximately 1·5 % *trans*-10,*cis*-12-CLA), in the present

British Journal of Nutrition

Table 1. Final body weight, adipose tissue weights and epididymal adipocyte size and number of hamsters fed the restricted diets for 3 weeks

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

	RC		RCLA		
	Mean	SEM	Mean	SEM	*P*
Final body weight (g)	109·7	1·9	107·5	2·01	NS
Adipose tissue weights (g)					
Epididymal	1·28	0·17	1·23	0·24	NS
Perirenal	1·67	0·19	1·43	0·13	NS
Subcutaneous	2·59	0·23	2·25	0·26	NS
Epididymal + perirenal + subcutaneous	5·54	0·43	4·91	0·46	NS
Epididymal adipocyte size and number					
Diameter (μm)	42	2	45	2	NS
Number (10^6 cells)	56	7	42	7	NS

RC, restricted group fed a control diet; RCLA, restricted group fed a conjugated linoleic acid-supplemented diet.

study 0·5 % *trans*-10,*cis*-12-CLA was selected; that is, a three-fold lower dose.

Further studies are warranted to gain a better understanding of the effects of CLA under energy-restriction conditions in other species. Nevertheless, taking into account that the young hamster has shown itself to be an experimental model having a good sensitivity to CLA under *ad libitum* feeding conditions[21–23] and considering that the dose of 0·5 % is higher than that used in human studies[4], it does not seem likely that a significant effect of CLA on lipid reduction induced by energy restriction in humans will be found, as they show a very moderate sensitivity to CLA.

White adipose tissue TAG is the major energy reserve in the body. During times of energy deprivation, white adipose tissue undergoes a shift to greater net rates of lipolysis, and TAG are rapidly mobilised, releasing NEFA that are oxidised to meet the energy requirement of the organism[24].

Thus, because lipolysis is one of the metabolic pathways activated during energy restriction we were interested in the analysis of potential changes induced by *trans*-10,*cis*-12-CLA in this metabolic pathway. Data in the literature concerning the effects of CLA on lipolysis are scarce and controversial, and refer to studies that have not been performed under energy-restriction conditions[8–11].

The results obtained in the present study show that the β-adrenergic lipolytic cascade was not a target for *trans*-10,*cis*-12-CLA. It did not modify lipolysis induced by forskolin, which directly stimulates adenylate cyclase without the participation of β-adrenergic receptor nor the Gs protein. Furthermore, the lack of effect on dibutyryl-cAMP-mediated lipolysis suggests that CLA does not interfere at the protein kinase A level and the lack of changes in lipolysis induced by isobutylmethylxantine indicates no effects of CLA on phosphodiesterase, the enzyme that catabolyses cAMP. Finally, taking into account that the lipolytic cascade was not modified downstream of the membrane receptors, the absence of an

Table 2. Epididymal adipose tissue lipolysis of hamsters fed the restricted diets for 3 weeks*

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

	RC		RCLA		
	Mean	SEM	Mean	SEM	*P*
Basal	0·43	0·04	0·42	0·04	NS
Isoproterenol (10^{-5} M)	1·54	0·11	1·56	0·17	NS
Forskolin (10^{-5} M)	1·60	0·14	1·79	0·30	NS
IBMX (10^{-4} M)	1·86	0·13	1·69	0·16	NS
dAMPc (10^{-3} M)	0·61	0·07	0·73	0·06	NS
ADA (0·2 units/ml)	2·49	0·23	3·59	0·20	NS

RC, restricted group fed a control diet; RCLA, restricted group fed a conjugated linoleic acid-supplemented diet; IBMX, isobutylmethylxanthine; dAMPc, dibutyryl-cAMP; ADA, adenosine deaminase.

* Results are expressed as μmol glycerol/100 mg lipids per 90 min.

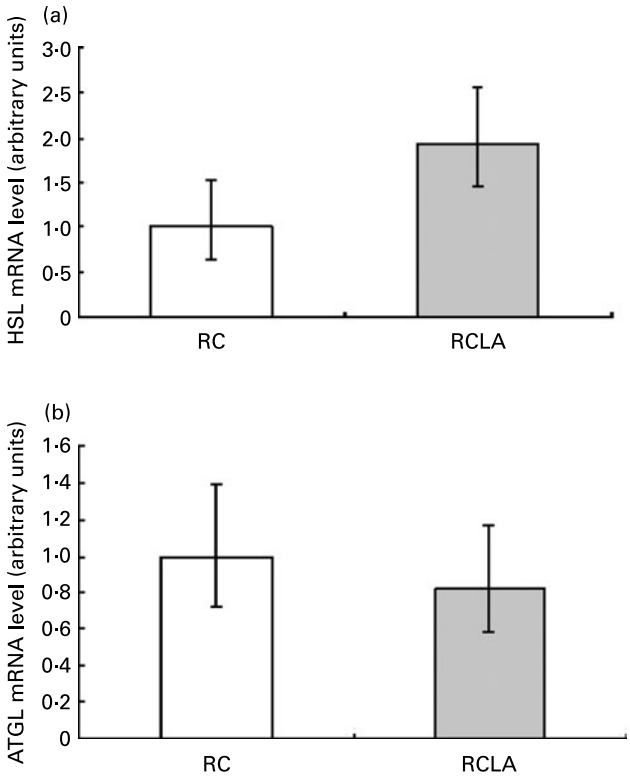

Fig. 2. Relative mRNA levels of hormone-sensitive lipase (HSL) (a) and adipose tissue TAG lipase (ATGL) (b) in epididymal adipose tissue from hamsters fed on the restricted diets for 3 weeks. Values are means, with their standard errors represented by vertical bars. RC, restricted group fed a control diet; RCLA, restricted group fed a conjugated linoleic acid-supplemented diet.

British Journal of Nutrition

effect on isoproterenol-induced lipolysis suggests that changes in β-adrenergic receptors are also discarded.

Adenosine deaminase is a drug that increases glycerol release by avoiding the antilipolytic action produced by the adenosine physiologically released by adipocytes. Thus, the lack of effect of *trans*-10,*cis*-12-CLA on adenosine deaminase-stimulated lipolysis shows that it does not modify adipocyte adenosine/A1 receptor-protein Gi signalling pathway.

The results concerning glycerol release stimulation are in good accordance with the lack of change in HSL expression after *trans*-10,*cis*-12-CLA feeding, and as a whole these data suggest that this CLA isomer does not modify adipose tissue lipid mobilisation.

Until recently, the hydrolysis of TAG in white adipose tissue was thought to be catalysed exclusively by the enzyme HSL. However, recent studies showing that adipocytes from HSL knockout mice retain a marked basal and adrenergically stimulated lipolysis demonstrated that HSL is not the sole lipase involved in TAG catabolism[25–27]. Three independent groups have discovered a new lipase termed ATGL, desnutrin or Ca-independent phospholipase A2/lipase[28–30]. ATGL acts as an additional rate-limiting lipase, catalysing the initial step of the breakdown of TAG[31]. This enzyme, unlike HSL, is not regulated by protein kinase A-dependent phosphorylation.

In order to gain a better understanding of the action of CLA on adipose tissue lipolysis, we analysed its effect on ATGL expression in epididymal adipose tissue. No significant differences were found in ATGL expression when comparing both experimental groups. These results show that *trans*-10,*cis*-12-CLA seems not to affect this new lipolytic pathway.

In conclusion, taking these results as a whole, it was shown that *trans*-10,*cis*-12-CLA does not promote adipose tissue lipid mobilisation nor heighten body fat reduction induced by energy restriction. Consequently, this CLA isomer seems not to be a useful tool for inclusion in body weight loss strategies followed during obesity treatment.

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British Journal of Nutrition

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