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Lipid atherogenic risk markers can be more favourably influenced by the *cis*-9,*trans*-11-octadecadienoate isomer than a conjugated linoleic acid mixture or fish oil in hamsters

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The aim of our present study was to compare the efficiency of conjugated linoleic acids (CLA) and fish oil in modulating atherogenic risk markers. Adult male hamsters were given a cholesterol-rich diet (0·6 g/kg) for 8 weeks; the diet was supplemented with 5 g *cis*-9,*trans*-11-CLA isomer/kg, 12 g CLA mixture (CLA-mix)/kg, 12 g fish oil/kg or 12 g fish oil + 12 g CLA-mix/kg. The plasma cholesterol status was improved only with the *cis*-9,*trans*-11-CLA (HDL-cholesterol and HDL-cholesterol:LDL-cholesterol ratio, $P<0·05$), but was of borderline significance for CLA-mix (HDL-cholesterol:LDL-cholesterol ratio, $P=0·06$), with an increase (33–40 %) in the liver lipoprotein receptors (scavenger receptor-type I and LDL ApoB/E receptor) and HDL-binding protein 2 ($P<0·05$). A 100 % pigment gallstones incidence and a slight insulin resistance (homeostatic model assessment index) were observed in the CLA-mix-fed hamsters ($P=-0·031$). In comparison, fish-oil feeding alone improved merely the scavenger receptor-type I and HDL-binding protein 2 liver status and faeces sterol output. For most of our present observations, the concomitant intake of fish oil and CLA-mix gave dominant effects that were exclusive and specific to one or the other oil. In conclusion, part of the beneficial effects of CLA in the present study can be ascribed to the *cis*-9,*trans*-11-isomer, and these did not generally overlap with those of fish oil. In addition, the CLA-mix effects are clearly affected by the marine (*n*-3) fatty acids.

Conjugated linoleic acid: Rumenic acid: Fish oil: Lipid atherosclerosis risk markers: Hamsters

Conjugated linoleic acid (CLA) is a collective term describing positional and geometrical isomers of linoleic acid. Among them, the *cis*-9,*trans*-11-isomer, so-called rumenic acid, occurs naturally in foodstuffs from ruminant animal fat sources. CLA have received growing attention in the past 10 years because of their pleiotropic biological activities. For instance, these fatty acids are effective anti-carcinogens, anti-atherosclerotic agents and potent modulators of the immune function (Pariza *et al.* 2001; Martin & Valeille, 2002). Studies dealing with the anti-atherosclerotic properties of CLA are scarce but promising. For instance, a CLA mixture (CLA-mix; 94·0 % (range 1–10 g/kg *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers/kg of diet) decreased early aortic atherosclerosis when given to male hamsters receiving a pro-atherogenic diet (F_1B strain; Wilson *et al.* 2000), and even caused regression of pre-established atherosclerosis in the male rabbit (New Zealand White; Kritchevsky *et al.* 2000) (10 g/kg diet). On the other hand, a detrimental effect on early aortic lesions outcome of a similar CLA-mix (5 g/kg diet) has been observed in C57Bl/6 mice fed a pro-atherogenic diet (Munday *et al.* 1999). Nevertheless, the studies dealing with the effect of CLA on the plasma lipid profile are less clear. Animal studies and studies with human subjects have had discordant results, as reviewed in Roche *et al.* (2001). In addition, animal experiments (hamsters) indicated that the *cis*-9,*trans*-11-isomer was ineffective in this regard (de Deckere *et al.* 1999; Gavino *et al.* 2000), whereas a study on human subjects demonstrated a slight beneficial effect on plasma VLDL-cholesterol (Noone *et al.* 2002). These discrepant results on plasma lipid variables arise from many factors, including duration, isomeric CLA composition, dose, species, gender, genomic polymorphism, initial metabolic status and diet, which could all have influence on the

Abbreviations: CLA, conjugated linoleic acid; CLA-mix, conjugated linoleic acid isomer mixture; CYP, cytochrome P450; HB2, HDL-binding protein 2; HMG, hydroxymethylglutaryl; HOMA, homeostatic model assessment; LDL-r, LDL ApoB/E receptor; SR-BI, scavenger receptor class B type I.

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effect of CLA. Since the cholesterol metabolism of the hamster is similar to that of man (Spady & Dietschy, 1983) and is similarly sensitive to changes in dietary fats (Kris-Etherton & Dietschy, 1997), we have examined the effects of CLA-supplemented diets on various aspects of cholesterol and lipid metabolism in this species together with the atherosclerotic risk. We used inbred male hamsters to minimize genomic polymorphism and variation due to gender. In addition, the biological effect of the main isomer found in foodstuffs, i.e. *cis*-9,*trans*-11-CLA, was examined by comparison with a CLA-mix containing equal amounts of both *cis*-9,*trans*-11-isomer and the *trans*-10,*cis*-12-isomer. To get a better quantitative assessment of the potencies of CLA, an additional comparison was made among hamsters fed the same amount of either long-chain *n*-3 fatty acids as fish oil or the CLA-mix, since the *n*-3 PUFA of fish oil have a well-recognized effect on the atherosclerotic risk (Harris, 1997). It is also clear from other studies that other fatty acids in the diet can modify the effect of CLA (Li *et al.* 1999), and this might be one of the reasons for the discrepant results from studies described earlier. We also examined the possible interaction of the marine *n*-3 fatty acids with CLA in our present model.

Experimental methods

Chemicals and isotopes

Kits for cholesterol, triacylglycerol and phospholipid assays were purchased from Boehringer-Mannheim (Meylan, France). A polyclonal antibody raised against the bovine adrenal cortex LDL-receptor was kindly provided by Paul Roach (Adelaide, South Australia, Australia). A rabbit polyclonal antibody against a peptide containing residues 495–509 from murine scavenger receptor class B type I (SR-BI; kindly prepared by André Mazur, Theix, France) was used to detect SR-BI as described by Loison *et al.* (2002). Hydroxymethylglutaryl (HMG) CoA, [5-^{3}H]mevalonolactone, [4-^{14}C]cholesterol, 25-[26,27-$^{3}H_2$]hydroxycholesterol and [24-^{14}C]chenodeoxycholic acid were obtained from Dupont-NEN Products (Les Ulis, France).

Animal and diets

Official French regulations for the general experimental conditions (no. 87848) and for the specific care and use of laboratory animals were followed (no. 03056). Male hamsters bred in our own animal facility (LPN strain) were housed ten per cage in plastic cages (with a wire bottom positioned 15 mm above the floor to prevent coprophagia and sawdust ingestion as much as possible), in a controlled environment, with constant temperature (22°C ± 1) and humidity (70 %) and a 12 h light–dark cycle (lights off at 19.30 hours). They were fed a standard non-purified diet (105; UAR, Villemoisson-sur-Orge, Epinay-sur-Orge, France) until 7 weeks of age. They were then fed a semi-synthetic diet containing 48·9 % energy as carbohydrate, 17·9 % energy as casein and 32·9 % energy as lipid (lard–high-oleic-acid sunflower oil–rapeseed oil (3·0 : 0·5 : 0·5, by wt) + 0·5 g cholesterol/kg) for 2 weeks before the experimental dietary period (Table 1). The nutritional adequacy of the diet has been previously evaluated (Bouthegourd *et al.* 2002; Loison *et al.* 2002). A stock-based diet was prepared in advance for 1 week of food consumption and stored at 4°C in a hermetically closed container. CLA and fish oil were stored in portions under an atmosphere of N_2 at −20°C. Food provided to the hamsters was prepared from the stock diet every 2 d.

The hamsters were then housed individually and assigned to one of five diets: (1) the standard diet (control group); (2) the standard diet augmented with 6 g *cis*-9,*trans*-11-isomer/kg; (3) the standard diet augmented with 12 g CLA-mix/kg; (4) the standard diet augmented with 12 g CLA-mix + 12 g fish oil/kg; (5) the standard diet + 12 g fish oil/kg. The hamsters (ten per group) were fed these diets for 8 weeks. Body weight was measured twice per week and food intake was measured daily at the end of the dark period (see Bouthegourd *et al.* 2002). CLA and fish oil were added to the basal diet with no substitution for carbohydrate, fat or protein. The *cis*-9,*trans*-11-isomer (81·5 g/100 g total fatty acids) was synthesized from dehydrated castor oil (see Bouthegourd *et al.* 2002), and the final product was found to contain (g/100 g total fatty acids): *cis*-9,*trans*12-18 : 2 5·8, *cis*-9,*cis*-12-18 : 2 2·0, *cis*-9,*trans*-11-18 : 2 81·5, *cis*-9,*cis*11-18 : 2 7·5, *trans*-9,*trans*-11-18 : 2 1·8, unidentified 1·4. The CLA mixture (78·2 g/100 g total fatty acids) was kindly donated by Seah International (Boulogne sur Mer, France). Its fatty acids composition was (g/100 g total fatty acids): 16 : 0, 4·0, 18 : 0 1·2, *cis*-9-18 : 1 12·0, *cis*-11-18 : 1 0·6, *cis*-9,*trans*-12-18 : 2 0·2, *trans*-9,*cis*-12-18 : 2*n*-6 0·1, *cis*-9,*cis*-12-18 : 2 2·0, all 8,10-18 : 2 isomers 0·8, all 7,9-18 : 2 isomers 0·7, all 11,13-18 : 2 isomers 0·4, all 12,14-18 : 2 isomers 0·1, *cis*-10,*cis*-12-18: 2 0·6, *cis*-9,*cis*-11-18 : 2 0·8, *cis*-9,*trans*-11-18 : 2 35·6, *trans*-10,*cis*-12-18 : 2 36·4, unidentified 4·2 (i.e. CLA isomeric repartition was (g/100 g total fatty acids): *cis*-9,*trans*-11 47·2, *trans*-10,*cis*-12 48·2, other CLA isomers 4·6). Determination of fatty acid composition is detailed elsewhere (Bouthegourd *et al.* 2002). The total CLA in the fat of the basal diet

Table 1. Composition of the basal diets

Components	g/kg	% Energy
Casein	200	17·9
Sucrose	200	17·9
Maize starch	350	31·3
Cellulose	50	–
Mineral mixture*	50	–
Vitamin mixture†	25	–
Fat‡	125	33

* Contained (mg/kg diet): NaCl 5·00, KCl 5·00, $CaHPO_4$ 21·50, $MgCl_2$ 5·00, $MgSO_4$ 2·50, Fe_2O_3 0·15, $FeSO_4.7H_2O$ 0·25, $MnSO_4.H_2O$ 0·1225, $CuSO_4.5H_2O$ 0·025, $ZnSO_4.7H_2O$ 0·1004, $CoSO_4.7H_2O$ 0·0002, KI 0·0004.

† Contained (mg/kg diet): retinol 27·00, ergocalciferol 2·00, thiamin 50·00, riboflavin 37·50, calcium panthotenate 175·00 pyridoxin 25·00, meso-inositol 375·00, cyanocobalamin 0·125, ascorbic acid 2000·00, DL-α tocopherol 425·00, menadiol 100·00, nicotinic acid 25·00, choline 34 000·00, folic acid 12·50, *p*-aminobenzoic acid 125·00, biotin 0·75.

‡ Lard–high-oleic-acid sunflower oil–rapeseed oil (1·0:0·5:0·5, by wt) + 0.5 g cholesterol/kg; fatty acid composition is detailed on p. 192.

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amounted to 0·45 g/100 g total fatty acids, and the isomeric distribution can be tentatively given as (g/100 g total fatty acids): *cis*-9,*trans*-11 + *trans*-8,*cis*-10 0·280, *trans*-9,*cis*-11 0·015, *trans*-10,*cis*-12 0·009, all *cis*,*cis*-isomers 0·067, *trans*-11,*trans*-13 0·019, other *trans,trans*-isomers 0·064. Lard contained 0·1 g cholesterol/kg, which made the overall cholesterol in the basal diet 0·6 g/kg. The fatty acid composition of the basal diet was (g/100 g total fatty acids): 12 : 0 0·8, 14 : 0 1·2, 16 : 0 21·0, 16 : 1 1·9, 18 : 0 11·8, 18 : 1 (*cis* and *trans*) 47·9, 18 : 2*n*-6 12·7, 18 : 3*n*-3 1·60, CLA 0·45, 20 : 0 0·2, 20 : 1*n*-9 0·7. CLA was added in the non-esterified form in addition to the fat: 6 (*cis*-9,*trans*-11-isomer diet) or 12 (CLA-mix diet) g/kg diet. When added, fish oil amounted to 12 g/kg diet and contained (g/100 g total fatty acids): 14 : 0 9·2, 16 : 0 18·4, 16 : 1 9·9, 18 : 0 6·0, *cis*-18 : 1 15·2, 18 : 2*n*-6 1·8, 20 : 1*n*-9 2·7, 20 : 5*n*-3 22·1, 22 : 5*n*-3 2·3, 22 : 6*n*-3 12·3.

Tissue removal and blood sampling

The hamsters were deeply anaesthetized with Zoletil® 50 (250 mg/kg; Tiletamine chlorhydrate–Zolazepam chlorhydrate, 50:50, w/w; Reading Laboratory, Nice, France) after 18 h food deprivation. Blood samples were taken by cardiac puncture between 09.00 and 10.00 hours. Blood (250 μl) was taken in a first puncture approximately 2 min after anaesthesia. Blood (10 μl) was used immediately for measurement of glucose. The plasma of the remaining blood was then separated by centrifugation (4°C, 20 min, 3000 ***g***) and stored at −80°C for further determination of insulin. Blood (2 ml) was collected from a second puncture. The plasma was immediately separated by centrifugation (4°C, 20 min, 3000 ***g***) and stored in portions at −80°C for further determination of lipoproteins. The liver was removed, rinsed in cold saline (9 g NaCl/l), blotted and finely chopped. A portion (1–2 g) was dipped in liquid N_2 and stored at −80°C for further analysis. The adrenal glands were excised, weighed and immediately frozen in liquid N_2 and kept at −80°C until processed for receptor analysis.

Plasma, bile, faeces and tissue analyses

Plasma lipids were measured by enzymatic procedures, using commercial kits, by means of an automatic analyser (Abbott VP, Rungis, France). Insulin was assayed by RIA (rat insulin RIA kit; Linco Research, St Louis, MO, USA). Glucose was measured in whole blood using a glucometer (Glucometer encore R; Bayer Corp., Elkhart, IN, USA). The adequacy of the glucometer for hamster blood was tested before the experimental assays. The homeostatic model assessment (HOMA) for insulin resistance was calculated from the insulin and glucose values using the equation of Matthews *et al.* (1985):

$$\mathrm{HOMA} = \mathrm{insulin(mU/l)}/(9 \times 22{\cdot}5^{-\ln \mathrm{glucose(mmol/l)}}).$$

Bile samples were diluted (1:20) with physiological saline (9 g NaCl/l) and analysed for cholesterol, phospholipid and total bile acid concentrations as previously described (Boehler *et al.* 1999). Lipids were extracted from homogenates of liver samples (0·5 g) and assayed enzymatically as described previously (Boehler *et al.* 1999). Lipids (neutral and acidic sterols) in faecal samples were extracted by boiling with ethanol in a Soxhlet apparatus for 48 h; they were then analysed and quantified by GLC (Boehler *et al.* 1999).

Lipoproteins were fractionated by ultracentrifugation of plasma samples (0·4 ml) in a KBr density gradient, using an SW41 rotor in an L8-70 apparatus (Beckman Instruments, Gagny, France) (Loison *et al.* 2002). Twenty-two fractions (0·5 ml) were collected and analysed for total cholesterol, triacylglycerol and phospholipid as described earlier.

Hepatic enzyme activities

Tissue processing and enzyme assays are detailed in Loison *et al.* (2002) and Souidi *et al.* (2001). The microsomal and mitochondrial fractions were isolated using conventional ultracentrifugation techniques. HMG-CoA reductase activity was determined in the microsomal fractions in the presence of alkaline phosphatase. Cytochrome P450 (CYP) 7A1, CYP27A1 and CYP7B1 were assayed in the microsomal fractions with a radioisotopic method using either [4-^{14}C]cholesterol (CYP7A1, CYP27A1) or 25-[26,27-$^{3}H_2$]hydroxycholesterol (CYP7B1), solubilized and carried by hydroxypropyl-β-cyclodextrin.

Scavenger receptor class B type I, LDL-receptor and HDL binding protein 2

Total membranes from frozen liver samples stored at −20°C (1 g) were prepared and membrane proteins were solubilized in a buffer containing Triton-X 100 (20 ml/l) (Milliat *et al.* 2000). Specific dot-blot immunodetection was then carried out for SR-BI, LDL ApoB/E receptor (LDL-r) and HDL-binding protein 2 (HB2), essentially as previously detailed by Milliat *et al.* (2000).

Statistical analyses

All assays were performed on one occasion each. Results are expressed as mean values with their standard errors. Comparisons were made using one-way ANOVA and tested by a *post hoc* protected least significant difference Fisher test. Differences in gallstones incidence were tested by the χ^2 test. All differences (including *post hoc* analysis) were considered significant at $P < 0{\cdot}05$.

Results

Plasma lipids

Plasma total cholesterol content was the highest in the *cis*-9,*trans*-11-group (Fig. 1), but the difference was significant only with control ($P = -0{\cdot}0239$) and fish-oil-fed animals ($P = -0{\cdot}0282$). This was not the case in the CLA-mix group (having as much of the *cis*-9,*trans*-11-isomer as in the *cis*-9,*trans*-11 group), although this group had a similar trend to the *cis*-9,*trans*-11 group (Fig. 1). The addition of fish oil to the CLA-mix gave rise to a HDL-cholesterol:LDL-cholesterol ratio similar to that of the fish-oil

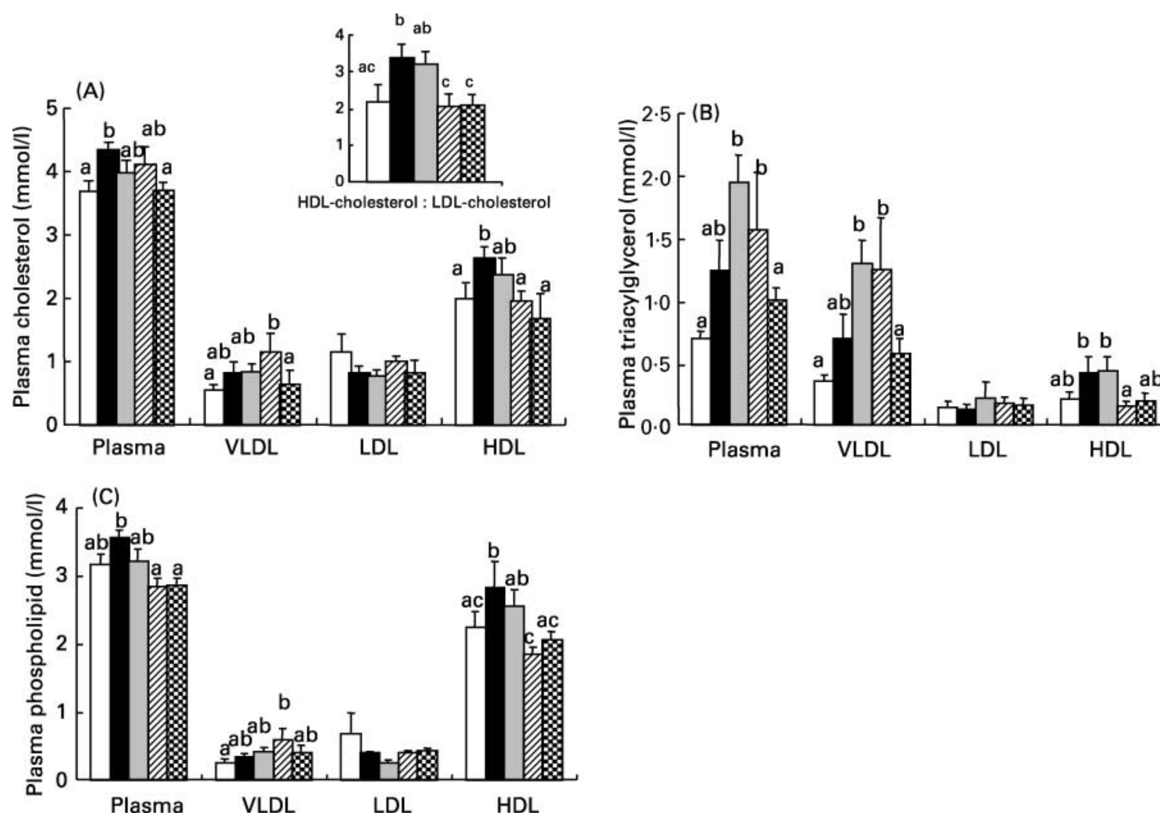

Fig. 1. Distribution of plasma total cholesterol (A), triacylglycerol (B) and phospholipid (C) in lipoprotein fractions obtained by density-gradient ultracentrifugation of plasma from hamsters fed the control diet (□), the *cis*-9,*trans*-11-isomer-supplemented diet (■), the conjugated linoleic acid isomer mixture (CLA-mix)-supplemented diet (▒), CLA-mix + fish oil-supplemented diet (▨) or the fish oil-supplemented diet (▦). For details of diets and procedures, see Table 1 and p. 192. Values are means with their standard errors shown by vertical bars (six hamsters per group). VLDL d < 1·006, LDL 1·019 < d < 1·063, HDL 1·063 < d < 1·210. [a,b]Mean values with unlike superscript letters were significantly different among each lipid lipoprotein fraction (one-way ANOVA; $P<0.05$).

group. Triacylglycerolaemia (total plasma and VLDL) significantly increased over control values only in groups consuming the CLA mixture ($P<0.025$) (Fig. 1). The addition of fish oil to the CLA-mix lowered the HDL-triacylglycerol values to those of fish oil alone or control (Fig. 1). The pattern of phospholipid distribution in plasma lipoproteins was close to that of cholesterol (Fig. 1). In particular, the HDL-phospholipid was higher in the *cis*-9,*trans*-11-CLA group ($P=-0.0317$ compared with control group). As for plasma cholesterol, the addition of fish oil to the CLA-mix brought about a similar effect as with fish oil alone for plasma phospholipid distribution. When expressed as % lipid per lipoprotein particles, the VLDL-triacylglycerol was significantly increased in the group given the CLA-mix alone compared with other groups ($P<0.05$, results not shown).

Lipoprotein receptors and binding proteins

The amount of LDL-r expressed per whole organ was greater in hamsters fed the *cis*-9,*trans*-11-CLA and the CLA-mix diets ($P<0.05$) compared with the other groups, with both having a similar content of receptors (Fig. 2). The amount of the liver HDL receptor SR-BI was also increased in groups fed on all supplemented diets compared with control, although the difference *v*. control was weaker in the CLA-mix group ($P=-0.0715$). The results were similar to those for HB2, another HDL-binding proteins, although the differences when compared with hamsters fed the control diet were greater than for SR-BI. In particular, fish-oil feeding alone increased the expression of this protein to a greater extent compared with the other groups (Fig. 2).

Interestingly, the addition of fish oil to the CLA-mix resulted either in a dominating CLA effect (HB2) or in a dominating fish-oil effect (LDL-r). No statistical differences were shown in the SR-BI and LDL-r content in a steroidogenic tissue expressing these receptors (adrenal glands, results not shown).

Liver lipid composition and enzyme activities

The cholesterol concentration in the liver, whether as total, esterified or non-esterified, was greater in the hamsters of the *cis*-9,*trans*-11-CLA group ($P=-0.0274$, $P=-0.0470$, $P=-0.0025$ compared with control respectively) (Table 2). On the other hand, the phospholipid concentration was decreased in the hamsters of the CLA-mix group ($P=-0.0021$ compared with control). There were no differences in the liver triacylglycerol concentrations of the hamsters fed the various diets ($P\geq0.09$).

The activities of the enzymes for the biosynthesis of cholesterol or its processing into bile acids, namely HMG-CoA reductase (rate limiting for cholesterol biosynthesis),

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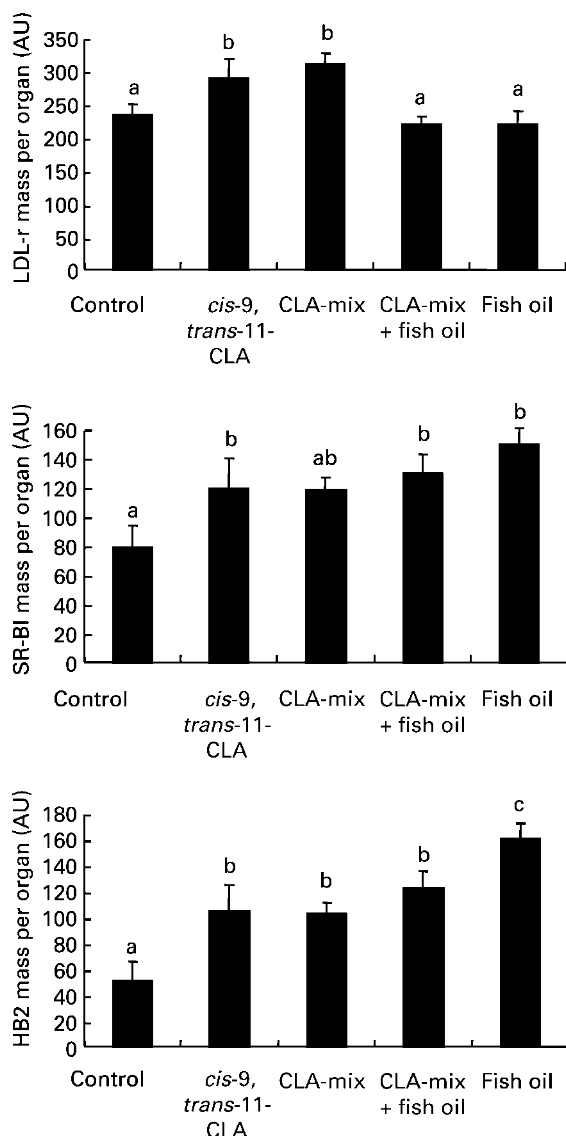

Fig. 2. Hepatic lipoprotein binding protein level determined by dot-blot analysis and expressed as protein mass per liver in arbitrary units (AU) per liver of hamsters fed the control diet, the *cis*-9,*trans*-11-isomer-supplemented diet, the conjugated linoleic acid isomer mixture (CLA-mix)-supplemented diet, the CLA-mix + fish-oil-supplemented diet or the fish-oil-supplemented diet. For details of diets and procedures, see Table 1 and p. 192. Values for the LDL-receptor (LDL-r), scavenger receptor class BI (SR-BI) and HDL-binding protein 2 (HB2) are means with their standard errors shown by vertical bars (seven to eight hamsters per group). [a,b,c]Mean values with unlike superscript letters were significantly different among each lipid lipoprotein fraction (one-way ANOVA, $P<0{\cdot}05$).

CYP7A1, CYP27A1 (both rate limiting for bile acid biosynthesis) and CYP7B1 were not different among treatment groups, whether expressed in specific activities (nmol/min per mg protein) or in liver capacities (nmol/min per whole liver) (results not shown).

Biliary compartment and faecal output

The overall cholesterol concentration in bile, whether as non-esterified cholesterol or as bile acids, was increased only in the hamsters of the CLA-mix group compared with control animals ($P=-0{\cdot}0004$) (Fig. 3). The addition of fish oil to the CLA mixture gave results similar to fish oil alone.

Both CLA-mix and the fish-oil feeding increased similarly the phospholipid output into bile ($P=-0{\cdot}0071$ and $P=-0{\cdot}0033$ *v.* control group respectively). Nonetheless, the effect was not additive when both were present in the diet (e.g. CLA-mix + fish-oil group, $P=-0{\cdot}0135$) (Fig. 3).

CLA-mix feeding, alone or along with fish oil, brought about a dramatic 100 % incidence of pigment gallstones (Fig. 4). The *cis*-9,*trans*-11-CLA and fish-oil-supplemented diet had no effect. No pure cholesterol gallstones were observed in the gallbladder of any hamsters.

In contrast to bile, only fish-oil feeding increased the sterol output in the faeces compared with the other treatment groups ($P<0{\cdot}05$), but this was due to acidic sterols (e.g. lithocholic acid and deoxycholic acid; 2·5-fold more in the fish-oil group than in the control group) (Fig. 3). Co-administration of a CLA mixture with fish oil in the diet completely antagonized this fish-oil effect.

Glucose and insulin concentrations in the food-deprived hamster

There were slight but non-significant differences in glucose and insulin concentrations among hamsters fed the various diets (except for the CLA-mix + fish-oil group for insulin, $P=-0{\cdot}0379$) (Fig. 5). Nonetheless, the HOMA calculated from these values was indicative of slight insulin resistance only for the CLA-mix group when compared with control ($P=-0{\cdot}031$).

Discussion

Two recent studies have addressed the potency of CLA-mix and individual CLA isomers to modulate plasma lipid levels in the hamster model (de Deckere *et al.* 1999; Gavino *et al.* 2000). If we compare the matched groups for CLA isomers among these studies including our present study, it is obvious that there is a lack of consistency in plasma lipids among the three studies. A close examination of the dietary setting and experimental conditions indicates that differences in the strain of hamsters used, the fat-based diet and its cholesterol content, and the caging conditions could explain these apparent discrepancies (Kris-Etherton & Dietschy, 1997). Therefore, we applied an experimental dietary setting that had already been shown to cause the same changes in plasma lipids in both our strain of hamster (LPN strain) and human subjects in response to an identical dietary challenge (Delplanque *et al.* 2002).

There is evidence to show that high plasma HDL-cholesterol as well as an elevated HDL-cholesterol:LDL-cholesterol ratio are protective against atherosclerosis (Rudel *et al.* 1998). In our present study, an increase in these two factors was achieved especially with the *cis*-9,*trans*-11-CLA diet, although the CLA-mix diet was borderline significant for the HDL-cholesterol:LDL-cholesterol ratio ($P=-0{\cdot}06$). Since the *cis*-9,*trans*-11-CLA intake is similar in the *cis*-9,*trans*-11-group and in the CLA-mix group, this

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Table 2. Lipid content (μmol/g liver) in the liver of hamsters fed for 8 weeks on either the control diet or the control diet supplemented with *cis*-9,*trans*-11-CLA, CLA mixture (CLA-mix), fish oil, or CLA-mix + fish oil (50:50 w/w)*†

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

	Total cholesterol		Cholesteryl esters		Non-esterified cholesterol		Triacylglycerol		Phospholipid	
Dietary groups	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Control	31·6[a]	2·6	48·2[a]	4·1	2·8[a]	0·3	8·9	0·6	26·6[b]	1·5
cis-9,*trans*-11	39·6[b]	2·3	59·8[b]	10·6	3·9[b]	0·3	9·5	1·1	26·7[b]	0·9
CLA-mix	34·2[ab]	2·3	52·1[ab]	3·9	3·1[a]	0·3	10·1	1·0	20·4[a]	0·5
CLA-mix + fish oil	37·6[ab]	2·3	58·3[ab]	3·9	2·8[a]	0·3	9·8	0·5	27·0[b]	0·6
Fish oil	33·2[ab]	3·1	51·0[ab]	5·2	2·6[a]	0·3	8·3	0·5	23·4[ab]	1·8

[a,b]Values with unlike superscript letters in the same column were significantly different (one-way ANOVA, $P<0·05$).
* For details of diets and procedures, see Table 1 and p. 192.
† Liver weight did not differ among groups.

suggests that the effect is mainly caused by the *cis*-9,*trans*-11-CLA when present as the main CLA isomer in food. Interestingly, a study has addressed the potency of the sole *trans*-10,*cis*-12-isomer to improve the plasma lipid status in obese men with the metabolic syndrome, and demonstrated that compared with placebo, the *trans*-10,*cis*-12-CLA decreased the HDL-cholesterol concentrations (Riserus *et al.* 2002). This is of concern, but cannot constitute a definitive conclusion because of the unusual metabolic status of the test group (with the metabolic syndrome). Other studies with human subjects, however, failed to show differences in HDL-cholesterol while given CLA-mix (Blankson *et al.* 2000; Benito *et al.* 2001; Noone *et al.* 2002) or a *cis*-9,*trans*-11-CLA-fortified blend (Noone *et al.* 2002) when compared with the respective control groups.

All the lipoprotein-binding proteins are upregulated in the liver of hamsters fed the *cis*-9,*trans*-11-CLA and the CLA-mix diets. Nonetheless, greater levels were measured with fish-oil feeding for HB2 and SR-BI but not LDL-r. This indicates an increased potential of the liver to clear plasma cholesterol. In particular, the fact that HDL-cholesterol is much elevated when hamsters fed the diet containing the *cis*-9,*trans*-11-CLA together with an increased expression of the liver SR-BI indicates that the flux of cholesterol through the reverse pathway could be even greater in this group. This assumption can be substantiated by a higher cholesteryl ester (and non-esterified cholesterol) content in the liver of the *cis*-9,*trans*-11-CLA-fed group. This increase was not due to an increase in endogenous cholesterol synthesis, since the activity of the rate-limiting HMG-CoA reductase was not modified (results not shown).

In contrast to the potential improvement in the plasma cholesterol clearance, the overall sterol output into bile (in terms of concentration) and in the faeces (mg/d) is not enhanced by *cis*-9,*trans*-11-CLA. This agrees well with the lack of effect on the rate-limiting enzymes of bile acid synthesis (CYP7A1 and CYP27A1). Only the hamsters fed the CLA-mix diet had higher bile sterol concentration, whereas those fed the fish-oil diet had a greater faecal acidic sterols output. Nonetheless, in a recent study Yeung *et al.* (2000) observed a greater faecal neutral sterol (cholesterol and coprostanol) output in CLA-mix-fed hamsters. Differences in the experimental setting might provide an explanation between that study and our present study. Strikingly, there was 100 % incidence of pigment gallstones in the gallbladder of hamsters fed the CLA-mix. The incidence in the control group was also unusually high (40 %). We did not distinguish whether the gallstones were mixed or solely pigmentary. One may hypothesize that this effect is attributable to the *trans*-10,*cis*-12-isomer contained in the mixture and not to *cis*-9,*trans*-11-CLA, since the hamsters fed only *cis*-9,*trans*-11-isomer did not differ from the control group with regard to gallstones incidence. The pathogenesis of black pigment stones has been associated with changes in haem metabolism or bilirubin absorption (Donovan, 1999) and to the pathophysiology of liver cirrhosis in a hamster model (Sakata *et al.* 1997). Therefore, further studies are necessary to clarify the relationship between the formation of such gallstones, hepatotoxicity and the dietary intake of the *trans*-10,*cis*-12-CLA.

Similarly to the results of the study by de Deckere *et al.* (1999), but not those of Gavino *et al.* (2000), in our present experiment only hamsters fed CLA-mix had greater fasting total plasma triacylglycerol and VLDL-triacylglycerol at 8 weeks, both in mmol/l and as % lipid per lipoprotein particle (results not shown). This cannot arise from higher total liver weight and therefore higher *en masse* VLDL-triacylglycerol secretion in the CLA-mix group, since the liver weight of the hamsters did not differ among the groups (results not shown). In our present hamsters, we previously found that the CLA-mix prevented whole-body triacylglycerol accumulation (Bouthegourd *et al.* 2002); this effect could be related to the inhibitory effect of the *trans*-10,*cis*-12-isomer on adipose tissue lipoprotein lipase (Pariza *et al.* 2001). This hypothesis is also substantiated by the slight insulin resistance (HOMA index) detected in the CLA-mix group in our present study, which could account for a lower activity of the lipase. Therefore, a lower clearance through the lipoprotein lipase pathway could explain at least in part some of the increase of the VLDL-triacylglycerol in the CLA-mix group. A greater flux of NEFA from adipose tissue to the liver in the CLA-mix group (de Deckere *et al.* 1999) can be used for *de novo* VLDL-triacylglycerol synthesis and could also offer a complementary explanation. Nevertheless, we did not measure the plasma concentrations of these metabolites.

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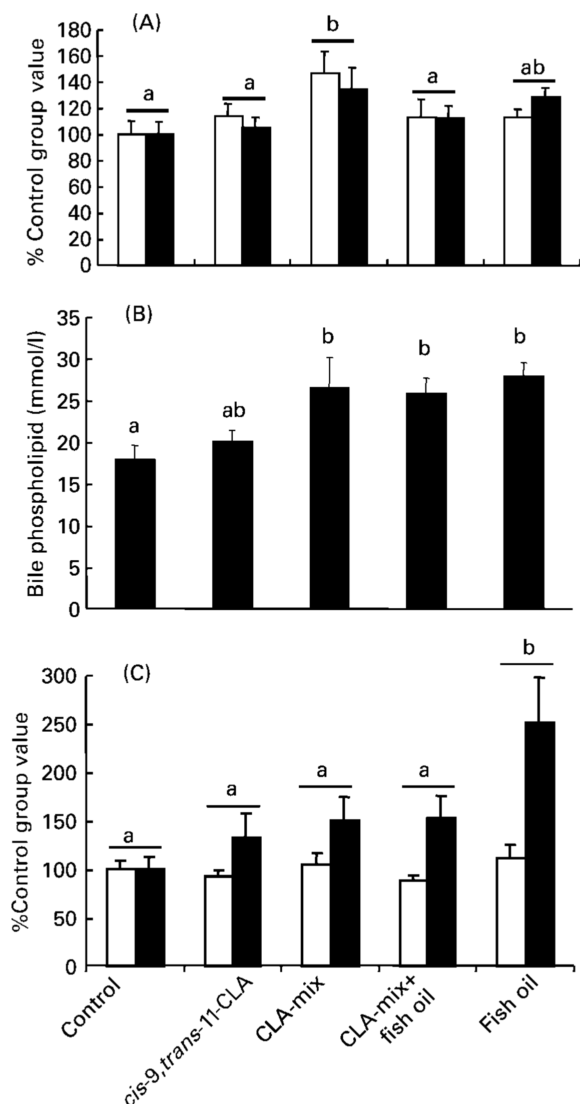

Fig. 3. Selected lipid composition in bile and faeces of hamsters fed the control diet, the *cis*-9,*trans*-11-isomer-supplemented diet, the conjugated linoleic acid isomer mixture (CLA-mix)-supplemented diet, the CLA-mix + fish-oil-supplemented diet or the fish-oil-supplemented diet. For details of diets and procedures, see Table 1 and p. 192. (A), bile sterols (non-esterified cholesterol (□) and bile acids (■)); non-esterified cholesterol 2·7 (SEM 0·3) mmol/l, bile acids 172 (SEM 17) mmol/l. (B), bile phospholipids. (C), neutral (total cholesterol; □) and acidic sterols (lithocholic and chenodeoxicholic acids; ■) in faeces; neutral sterols (control) 5·3 (SEM 0·5) mmol/d, acidic sterols 434 (SEM 29) mmol/d. Values are means with their standard errors shown by vertical bars (seven to eight hamsters per group). [a,b]Mean values with unlike superscript letters were significantly different (one-way ANOVA, $P<0·05$).

As mentioned earlier, our present study also established that only the hamsters in the CLA-mix group developed a relative insulin resistance as determined by the HOMA values. This result is consistent with results published from studies in mice (Tsuboyama-Kasaoka *et al.* 2000), swine (Stangl *et al.* 1999) and even in human subjects (Riserus *et al.* 2002; Medina *et al.* 2000), and therefore seems to be a common feature for CLA-mix containing the *trans*-10,*cis*-12-isomer. It remains to be determined

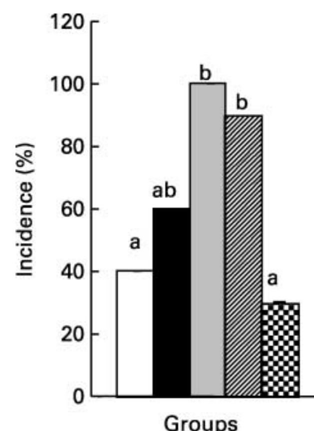

Fig. 4. Incidence of pigment gallstones outcome in the gallbladder of hamsters fed the control diet (□), the *cis*-9,*trans*-11-isomer-supplemented diet (■), the conjugated linoleic acid isomer mixture (CLA-mix)-supplemented diet (▒), the CLA-mix + fish-oil-supplemented diet (▨) or the fish-oil-supplemented diet (▦). For details of diets and procedures, see Table 1 and p. 192. Values are means for seven to eight hamsters per group. [a,b]Mean values with unlike superscript letters were significantly different (χ^2 test, $P<0·05$).

whether this phenomenon is transient (Hamura *et al.* 2001) or prolonged.

One of the most striking results of our present study is the demonstration that the addition of fish-oil fatty acids at the same level as the CLA-mix (i.e. 10 g/kg diet) in the basal diet can completely abolish the CLA effect. In particular, this is the case for the HDL-phospholipid, HDL-triacylglycerol and hepatic LDL-r status. The reverse holds true in other instances, since co-administration of CLA-mix with fish oil can lead to a dominating CLA-mix effect (pigment gallstones, VLDL-triacylglycerol, faecal sterols, hepatic HB2 status). Unless otherwise stated, for most of our present observations there are dominating

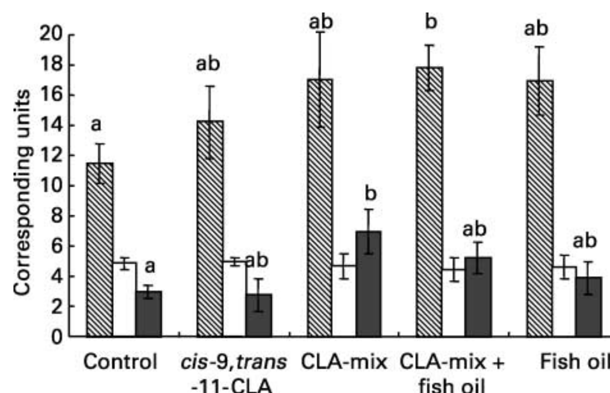

Fig. 5. Glucose and insulin values in 18 h food-deprived hamsters and calculated index of insulin resistance (homeostatic model assessment, HOMA) in hamsters fed the control diet, the *cis*-9,*trans*-11-isomer-supplemented diet, the conjugated linoleic acid isomer mixture (CLA-mix)-supplemented diet, the CLA-mix + fish-oil-supplemented diet or the fish-oil-supplemented diet. For details of diets and procedures, see Table 1 and p. 192. ▧, Insulin glucose (pmol/l × 10; □, glucose (mmol/l); ■, HOMA. Values are means with their standard errors shown by vertical bars (seven to eight hamsters per group). [a,b]Mean values with unlike superscript letters were significantly different (one-way ANOVA, $P<0·05$).

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effects arising from the consumption of either the CLA-mix or the fish oil. The reason for this could be a competition between CLA and *n*-3 long-chain fatty acids for common biochemical pathways at the nuclear transcription factors level, such as PPAR, sterol regulatory element binding protein and liver X receptors (Tsuboyama-Kasaoka *et al.* 2000; Jump, 2002; Roche *et al.* 2002). Such an issue would certainly be interesting to investigate in further detail. A similar effect of *n*-3 long-chain PUFA (Li *et al.* 1999) or even 18 : 2*n*-6 (Brown *et al.* 2001) in the modulation of CLA has already been observed in other studies. This underlines further the care that should be taken in the dietary environment when evaluating the health effect of CLA.

In conclusion, we have established that part of the effects of CLA evaluated in our present study can be ascribed to the *cis*-9,*trans*-11-isomer, and these are generally distinct from those of fish oil. Our present results also emphasize that the physiological activity of CLA can be clearly affected by the PUFA content of the diet, especially the long-chain *n*-3 fatty acids. It is now necessary to study the influence of this dietary factor more carefully in order to understand the variations in the results among studies and to determine the conditions that provide the best potential for CLA effects in human subjects. Finally, the effects of CLA on plasma lipids appeared modest, and one should now evaluate the real impact of the CLA isomers on the development of atherosclerosis.

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