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A *cis*-9,*trans*-11-conjugated linoleic acid-rich oil reduces the outcome of atherogenic process in hyperlipidemic hamster

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Valeille, Karine, Jacqueline Férézou, Ghislaine Amsler, Annie Quignard-Boulangé, Michel Parquet, Daniel Grippo, Victoria Dorovska-Taran, and Jean-Charles Martin. A *cis*-9,*trans*-11-conjugated linoleic acid-rich oil reduces the outcome of atherogenic process in hyperlipidemic hamster. *Am J Physiol Heart Circ Physiol* 289: H652–H659, 2005. First published March 18, 2005; doi:10.1152/ajpheart.00130.2005.—Conjugated linoleic acid (CLA) mixtures demonstrated antiatherogenic properties in several animal models, including hamsters, but the mechanism of action of the main food-derived CLA isomer is unknown in this species. This study thus focused on *cis*-9,*trans*-11-CLA (rumenic acid), and its effect was compared with that of fish oil, which is known to influence several aspects of atherogenesis. Syrian hamsters were fed (for 12 wk) diets containing 20% (wt/wt) butter fat (B diet) or the same diet augmented with either 1% (wt/wt) of a *cis*-9,*trans*-11-CLA-rich oil (BR diet) or 1% (wt/wt) fish oil (BF diet). The BR diet induced the lowest aortic lipid deposition (from –30% to –45%) among the butter oil-fed hamsters. In this group, plasma also displayed a reduced non-HDL-to-HDL-cholesterol ratio (21% less than in the butter oil group) and inflammatory serum amyloid A levels (70–80%) and an improvement of anti-oxidized LDL paraoxonase activity (all $P < 0.05$). Compared with the B group, the beneficial effects of the BR diet could be further explained in part by preventing the high VCAM-1 expression rate, increasing (30%) ATP-binding cassette subfamily A1 expression in the aorta, and downregulating expression of inflammatory-related genes (TNF- α , IL-1 β , and cyclooxygenase 2, 2- to 2.8-fold, $P < 0.05$). This effect was partly associated with an activation of peroxisome proliferator-activating receptor (PPAR)/liver X receptor (LXR)- α signaling cascade. Interestingly, activation of PPAR/LXR- α signaling was not observed in hamsters fed the BF diet, in which the early signs of atherogenesis were increased. In conclusion, this study demonstrated that milk fat-rich *cis*-9,*trans*-11-CLA reduces the atherogenic process in hyperlipidemic hamsters.

fish oil; rumenic acid; atherosclerosis; risk factors

THE TERM CONJUGATED LINOLEIC ACID (CLA) defines all the isomers of linoleic acid with conjugated double bonds. Although present in minor amounts in diets, CLA has been receiving growing interest for its multiple health-related effects reported in various animal models (35). Especially, the anti-atherogenic properties of CLA are now supported by many animal studies in rabbits (16), mice (32), and hamsters (37). Studies in cell cultures have demonstrated protective properties (8, 11, 15, 38). In animals, these properties can be observed

while using either the CLA isomeric mixture (*cis*-9,*trans*-11- and *trans*-10,*cis*-12-CLA, 50:50) (17, 37) or selected CLA isomers (*cis*-9,*trans*-11- or *trans*-10,*cis*-12-CLA) (16, 32). In most studies, these fatty acids consistently attenuate the formation of primary vascular lesions (fatty streaks) or induce regression of preestablished lesions (16, 17). There is, paradoxically, no clear relationship between this beneficial effect and an improvement of the lipid status in blood [i.e., blood levels of triglycerides (TG), total cholesterol, low-density lipoprotein (LDL)-cholesterol, and high-density lipoprotein (HDL)-cholesterol], which are usually considered as etiological factors in atherosclerosis processes (for a review, see Ref. 21). On the other hand, both in vitro and in vivo data recently indicated that part of the beneficial effects of CLA occurs through the modulation of vascular inflammatory and oxidant stress factors [cytokines and cyclooxygenase (COX)] (8, 15, 38) as well as through the expression of adhesion molecules (VCAM and ICAM) (7, 11). This regulation involves the interaction of CLA with transcription factors such as peroxisome proliferator-activating receptors (PPARs), liver X receptors (LXRs), and NF- κ B targeting the gene expression clusters downstream (2, 8, 15, 38). Interestingly, both *trans*-10,*cis*-12- and *cis*-9,*trans*-11-CLA appeared equally potent in this area.

Clinical studies, even those using pure CLA isomers, are no more conclusive than the animal experiments with regard to the contribution of CLA to reliably modulate blood lipid parameters (21). Nevertheless, in contrast to animal models, some but not all studies highlight the proatherogenic role of CLA, as demonstrated by the increase in markers of oxidative stress (isoprostanes) [induced by the *trans*-10,*cis*-12-CLA isomer (26) and *cis*-9,*trans*-11-CLA isomer (27)] and inflammation [C-reactive protein and the *trans*-10,*cis*-12-CLA isomer (26)]. However, this tendency was observed only in obese subjects (body mass index ≥ 30).

In the present study, we addressed whether a rumenic acid-rich oil can prevent the onset of atherosclerosis in the Syrian hamster. For this, we chose a strain prone to develop aortic fatty streaks when fed a high-fat diet [Janvier strain (1)]. Indeed, this condition is necessary to trigger early signs of atherogenesis and to test the protective effects of drugs or dietary compounds (10, 37). To get a better assessment of the specificity and efficacy of the rumenic acid-rich oil and to validate our experimental settings, we compared its action with

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fish oil, because long-chain n-3 polyunsaturated fatty acids have been well documented in their modulation of several markers of atherosclerosis including blood TG and cholesterol (13, 14, 36). The severity of the atherogenic process was compared among the dietary groups by measuring lipid deposition in the aortic wall and by analyzing the expression of genes involved in early arteriosclerosis events. These observations were related to the changes observed in liver lipids, in plasma lipoproteins, and in the expression of plasma markers for inflammation [serum amyloid A (SAA)] and oxidative stress (paraoxonase activity).

MATERIALS AND METHODS

Chemicals. Fish oil and the synthetic CLA mixture, specially enriched in rumenic acid, were kindly provided by Loders Croklaan (Wormerveer, The Netherlands). Zoletil 50 was purchased from Virbac (Carros, France), heparin was purchased from Sanofi (Gentilly, France), and aprotinin was purchased from Trasylol (Bayer Pharma; Puteaux, France). Most chemicals were of the highest purity and were purchased from Sigma (l'Isle d'Abeau-Chesnes, France).

Animals. All the experiments were conducted according to French Regulations for Animal Experimentation (Article 19.Oct 1987, Ministry of Agriculture) after approval of our institutions' referee for animal care and conformed with the principles of the APS' "Guiding Principles in the Care and Use of Animals." The forty male golden Syrian hamsters used were of the Janvier strain, 9 wk old and weighing 110 ± 10 g when purchased from the breeding center of Janvier (Le Genest-St Isle, France). They were housed in colony cages (6 hamsters/cage) and fed the commercial chow diet (C diet; 25/18 standard diet from Mucedola, Settimo, Milanese, Italy) for a 2-wk adaptation period. The animals were then caged by pairs and fed the experimental diets [butter fat diet (B diet), *cis*-9,*trans*-11-CLA-rich diet (BR diet), or fish oil-rich diet (BF diet); 10 animals/group] for the next 12 wk in a controlled environment (22°C, 14:10-h light-dark cycle). Diet and water, available ad libitum, were provided 4 times/wk. Body weight and food intake were measured weekly.

Composition and preparation of experimental diets. Butter was purchased from a local supermarket, and the butter oil was extracted by gravimetry. The same batch of C diet was used throughout the experiment. The C diet was composed of 12% moisture, 56% cereals, 18% proteins, 5% fiber, 6% vitamin and mineral mix, and 3% lipids (C16:0, 14%; C18:0, 3.5%; C18:1, 21.5%; C18:2n-6, 49%; C18:3n-3, 4.9%; C20:5n-3, 2.9%; C22:6n-3, 1.2%; and others, 3%). The diet contained ~140 mg sterols (21% cholesterol and 79% phytosterols) per 100 g, as assayed by gas liquid chromatography (GLC) analysis of the unsaponifiable material (12). This basal diet was grounded to powder and used as such in the control group or mixed with 20% butter oil in the other groups. The fatty acid composition and CLA content of the butter oil have been published elsewhere (34). The main fatty acids were as follows: C12:0, 4.3%; C14:0, 12.6%; C16:0, 35.6%; C18:0, 8.8%, all *trans*-C18:1, 4.2% (including *trans*-vaccenic acid, 1.0%), C18:2n-6, 1.4%, C18:3n-3, 0.3%; and all CLA isomers, 0.4% (including the *cis*-9,*trans*-11-CLA isomer, 0.37%).

CLA and fish oil were analyzed by the same gas-chromatographic method as butter fat. The synthetic CLA mixture contained 72% CLA, with the following isomeric distribution: *cis*-9,*trans*-11-CLA, 84.31%, *trans*-10,*cis*-12-CLA, 13.38%, and other CLA, 2.31%. The CLA preparation was provided as triacylglycerol, 81%; diacylglycerol, 17.6%; monoacylglycerol, 0.5%; and free fatty acids (FFA), 0.9%; peroxide value: 0.4 meq O₂/kg. In fish oil, the main long-chain n-3 fatty acids were as follows: C16:4n-3, 2.4%; C18:4n-3 (STA), 4.2%; C20:5n-3 (EPA), 20.6%; C22:5n-3 (DPA), 2.8%; and C22:6n-3 (DHA), 17.3%, peroxide value: 0.6 meq O₂/kg. CLA or fish oil were added to ~1% (wt/wt) in the butter oil diet.

The cholesterol content was adjusted to 0.12% by weight in all the lipid-enriched diets. All diets were prepared once, divided in portions for 2–3 days-feeding, and stored at –20°C under an atmosphere of N₂ until use.

Blood and tissue sampling. After 12 wk of dietary treatment and 18 h of food deprivation, the hamsters were anesthetized between 9:00 and 11:00 AM by an intramuscular injection of Zoletil 50 at a dose of 250 mg/kg. Two minutes later, the first cardiac puncture (250 µl blood) was performed with heparin (10 IU/ml) and aprotinin (500 IU/ml). Plasma was quickly isolated by centrifugation (4°C, 20 min, 3,000 g) and stored at –80°C for further insulin and glucose assays. A second cardiac puncture allowed us to collect 2 ml blood for lipid analysis. Plasma aliquots were stored at –80°C. The liver was removed, rinsed in cold saline, and weighed. Two portions (0.5 g) were stored at –20°C for lipid analysis. The epididymal adipose tissue was carefully dissected and weighed. Aortas were flash frozen in liquid nitrogen and stored at –80°C for further RNA isolation and lipid analysis.

Chemical and biochemical assays. Lipids were extracted from liver samples (0.3 g) by the method of Bligh and Dyer (4). Part of the extract was taken to dryness and dissolved in isopropyl alcohol for cholesterol, TG, and phospholipid assays, as described above for plasma lipids. Fatty acids from total lipids were derivatized by transmethylation following the two-steps method (6), which allows preservation of CLA. The resulting fatty acid methyl esters were then analyzed by GLC (model 3800, Varian; Les Ulis, France) using a polar column (BPX70, 60 m length, 0.32 mm inner diameter, 0.25 µm film thickness, SGE; Les Ulis, France) fitted with a split/splitless injector (temperature program: 60°C hold 1 min and then 170°C at 20°C/min, hold 35 min).

The cholesterol content in the aorta was measured from the initial mixture prepared for RNA isolation (see below), which consisted of a colorless upper aqueous phase (containing RNA) and a lower green phenol-chloroform phase containing lipids. The organic phase was transferred to a glass tube with a Teflon-lined cap and extracted using 5 ml petroleum ether containing 30 µl tri-tridecanoylglycerol (1 mg/ml) as an internal standard. The colored phase was aspirated and eliminated. Five milliliters of distilled water were added to the ether phase, and the mixture was mixed by vigorous shaking. The organic phase was collected and taken to dryness under vacuum. The residue was solubilized with 1.2 ml diethyl ether, and 1 ml Tris·HCl buffer (17.5 mM, pH 7.3) was then added, followed by 2 ml of a solution (1 U/ml Tris buffer, pH 7.3) of phospholipase C (type III, Sigma). The mixture was vortexed and incubated at 32°C for 2 h with shaking. The lower organic phase was collected and dried under vacuum (40°C). The dry residue was redissolved in 300 µl hexane, and 1 µl was analyzed by GLC on a short nonpolar capillary column (8 m length, 0.32 mm inner diameter, 0.25 µm film thickness, HP5, Hewlett-Packard; Les Ulis, France) (22).

Plasma lipids were measured by enzymatic procedures using commercial kits (Biomerieux; Lyon, France) by means of an automatic analyzer (Abbott VP; Rungis, France): total cholesterol (Ready-to-use kit method) and TG and phospholipids (pheno-amino-antipyrine 150 ml kit). Free cholesterol was measured by a manual enzymatic procedure adapted from that used for total cholesterol but in the absence of cholesterol esterase. Plasma glucose was assayed enzymatically (Biochem Immunosystems; Aix-en-Provence, France). Insulin was assayed by radioimmunoassay using the rat insulin radioimmunoassay kit from Linco Research (St. Louis, MO) after preliminary assays for testing the adequacy of the method to the hamster species (5). The homeostatic model assessment (HOMA) for insulin resistance was calculated from insulin and glucose values using the following formula: $HOMA = \text{insulin (mU/l)} / [9 \times 22.5^{-\ln \text{glucose (mmol/l)}}]$ (23).

The paraoxonase activity was determined using the method of paraoxon [*O,O*-diethyl-*O*-(4-nitrophenyl)phosphate, Sigma] hydrolysis, by measuring generated *p*-nitrophenol at 405 nm at 37°C on a Titertek Multiskan plus, as previously described (33). The enzyme

activity was expressed in arbitrary units (AU) per minute and per milliliter of plasma.

Lipoproteins were fractionated by density gradient ultracentrifugation of plasma samples (0.4 ml) using a SW41 rotor in a L8-70 ultracentrifuge (Beckman Coulter; Villepinte, France) (18). Twenty-two fractions (0.5 ml) were collected and analyzed for total cholesterol, as described before, to obtain individual profiles for the cholesterol distribution among the lipoproteins. Fractions were pooled by main lipoprotein classes, very LDL (fractions 1–2: density, <1.010 g/l), intermediate-density lipoprotein (IDL; fractions 3–6: 1.010 < density < 1.030), LDL (fractions 7–10: 1.030 < density < 1.063), and HDL (fractions 11–20: 1.063 < density < 1.190), to analyze their overall composition. Total and free cholesterol, TG, and phospholipids were assayed as described above, and proteins were assayed by the Lowry method (19) using BSA as a standard.

To analyze plasma apolipoproteins, total lipoproteins were isolated by flotation from pooled plasma samples (0.8 ml from 6 animals/group on EDTA at 1 µg/ml) previously adjusted to a density of 1.21 g/l with solid KBr and centrifuged in a Ti70 rotor (Beckman) at 100,000 g for 40 h at 15°C. After extensive dialysis (against 0.15 M NaCl and 1 mM EDTA, pH 7.4 at 4°C), apolipoproteins were separated without prior delipidation by SDS-PAGE in the presence of glycerol (9) on a 5.5%–10% polyacrylamide discontinuous gradient (Mini-Protein 3, electrophoresis cell, Bio-Rad; Marnes la Coquette, France). Sample volumes were adjusted to contain the same protein amount (30 µg) for each group after dilution [2:1 (vol/vol)] with a mixture (in vol/vol) of glycerol and isopropyl alcohol, 20% SDS, 50 mM DTT, 0.05% bromophenol blue and heated for 5 min at 95°C. The migration was performed at 20 mA per gel for about 3 h. After fixation and staining with Coomassie blue R-250, the apolipoprotein bands were analyzed by computer analysis (Scion Image). Final results were expressed in AUs per milliliter of plasma.

Gene expression analysis by semiquantitative and quantitative RT-PCR. Isolation of total RNA in aortas was performed using RNAsplu2 (Q.Biogene; Illkirch, France) according to the manufacturer's protocol. The RNA amount in the sample was quantified by spectrophotometry at 260 nm.

Levels of transcripts encoding for ATP-binding cassette sub-family A type 1 (ABCA1), VCAM-1, and fatty acid transporter (FAT)/CD36 scavenger receptor mRNA were determined by semiquantitative RT-PCR. Others, such as PPAR-α and PPAR-γ, LXR-α, IL-1β, TNF-α, COX-2, and inducible nitric oxide synthase (iNOS) mRNA, were determined by quantitative RT-PCR using the SYBR green method. The choice of the oligonucleotide sequences were based on available hamster data or on mice data (Table 1). Primers design was performed from the infobiogen website for RT-PCR and with Primer express (Applied Biosystems; Foster City, CA) for quantitative RT-PCR. Oligonucleotides were synthesized by PROLIGO (Primers and Probes; Boulder, CO). RNA (2 µg) underwent a RT reaction with Moloney murine leukemia virus (MMLV) reverse transcriptase (Ambion; Cliniscience, France) in the presence of 10 µmol/l of random hexanucleotides (Pharmacia Biotech; Orsay, France), 400 µmol/l of each dNTP, and 2 U/µl of placenta RNase inhibitor. The final volume was adjusted to 20 µl with buffer [50 mmol/l Tris·HCl (pH 8.3), 3 mmol/l MgCl₂, 75 mmol/l KCl, and 10 mmol/l DTT]. After a 30-min incubation at 37°C, MMLV reverse transcriptase was heat inactivated at 95°C. To check that no contamination with genomic DNA occurred, a control without MMLV reverse transcriptase was included for each RNA sample. For semiquantitative PCR, cDNA was amplified in the presence of 1 unit *Taq* DNA polymerase (Qiagen; Les Ulis, France), 0.3 µl of 10 mmol/l each dNTP, 2.5 µl of 10× PCR buffer [500 mmol/l KCl, 100 mmol/l Tris·HCl (pH 8.3), and 0.1 g/l gelatin], 2.5 µl of 25 mmol/l MgCl₂, and 0.5 µl of 20 µmol/l each sense and antisense oligonucleotides for all genes, and sterilized distilled water was added to give a final volume of 25 µl. After 40 µl mineral oil

Table 1. Oligonucleotide sequence for retrotranscription and real-time PCR

Genes	Species	Accession No.	Primer Sequences	Length, bp	Temperature, °C
PPAR-α	<i>Mesocricetus auratus</i>	AJ555631	Sense: 5'-GAGAAAGCAAACCTGAAAGCAGAGA-3' Antisense: 5'-GAAGGGCGGGTTATTGCTG-3'	179	59
LDL receptor	<i>Cricetulus griseus</i>	M94387	Sense: 5'-GATCCCAACCTGAAGAAGC-3' Antisense: 5'-AGTCTACGGCCAGCCCATCT-3'	179	58 60
18S	<i>Mus musculus</i>	X00686	Sense: 5'-GTGGGCTGCGGCTTAAT-3' Antisense: 5'-GCCAGAGTCTCGTTTCGTATC-3'	170	59
VEGF	<i>Mesocricetus auratus</i>	AF297627	Sense: 5'-CAGCGAGCTATTGCCATC-3' Antisense: 5'-GCTTGACCCGATGATCTG-3'	190	59
TNF-α	<i>Mesocricetus auratus</i>	AF046215	Sense: 5'-CCAGACACTCACACTCAGATCATCTT-3' Antisense: 5'-AGCCCGTCTGTGGTATCAC-3'	177	60 59
iNOS	<i>Mesocricetus auratus</i>	AY297461	Sense: 5'-CGGAGTGATGGGAAGCATG-3' Antisense: 5'-TCAGAGGCAGCACATCGAAG-3'	175	59
PPAR-γ	<i>Cricetulus griseus</i>	Z30972	Sense: 5'-CACTCCCATTCCTTTGACATCA-3' Antisense: 5'-AGCTGGGCCTTTTCAGAATAATAA-3'	200	58
COX-2	<i>Mesocricetus auratus</i>	AF345331	Sense: 5'-TGATCCCCAAGGCACGAA-3' Antisense: 5'-ACCTCTCCACCAATGACCTGA-3'	216	60 58
IL-1β	<i>Mesocricetus auratus</i>	AB028497	Sense: 5'-CGTGGACCTTCCAGGATGAG-3' Antisense: 5'-TGTTGGTTTATGTTCTGTCCGTTG-3'	220	59 60
LXR-α	<i>Mus musculus</i>	U22662	Sense: 5'-GCAACTCAATGATGCCGAGTT-3' Antisense: 5'-CGTGGGAACATCAGTCGGTC-3'	171	59 60
ABCA1*	<i>Mus musculus</i>	NM013454	Sense: 5'-TACAACATGGACATCCTGAAGC-3' Antisense: 5'-TCCAATTCTGTTCCTCCAAACT-3'	671	60
FAT/CD36*	<i>Mesocricetus auratus</i>	MAU42430	Sense: 5'-CTTTGAGCCTTCCCTGTCTG-3' Antisense: 5'-CAGCGTAGATGGATCTGAA-3'	470	60
VCAM-1*	<i>Mus musculus</i>	U12881	Sense: 5'-AAGAGAACCCAGGTGGAGGT-3' Antisense: 5'-TGAGCAGGTGAGGTTACAG-3'	588	60
18S*	<i>Mus musculus</i>	MMRNA18	Sense: 5'-CGGTACAGTGAACTGCGAAT-3' Antisense: 5'-TCTGATAAATGCAGCATCC-3'	156	60

PPAR, peroxisome proliferator-activating receptor; iNOS, inducible nitric oxide synthase; COX, cyclooxygenase; LXR, liver X receptor; ABCA1, ATP-binding cassette subfamily A1; FAT, fatty acid transporter. *Designed for semi-quantitative RT-PCR.

were added (Sigma Chemical; St. Louis, MO), cDNA was denatured for 5 min at 92°C and amplified (92°C, 30 s; 60°C, 30 s; and 72°C, 30 s, followed by a final extension of 10 min at 72°C) in a thermal cycler (TRIO-Thermoblock, Biometra; Gottingen, Germany); 100 ng of cDNA from the aorta were amplified as described above. The PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining, signals were quantified by scanning densitometry using NIH Image 1.56 software (Scion Image), and results were normalized to those of 18S rRNA.

Quantitative RT-PCR was performed on a MyiQ Cyclor Real-Time Detection system (Bio-Rad) using a qPCR Master Mix for SYBR green I No. ROX (Eurogentec; Angers, France). Because SYBR green I is a general double-stranded DNA intercalating dye, it may result in the detection of nonspecific products and primers/dimers as well as the amplicon of interest. To determine the number of products generated in the PCR and the specificity of the assay, a melting curve analysis was performed on each sample. Specifically, after the final extension step of PCR, the temperature was increased from 72°C up to 95°C at a rate of 0.5°C. Standard curves were then plotted and analyzed (PCR efficiency close to 100%, slope close to -3, r^2 close to 1). This allowed for discarding primers that did not meet the selection criteria, such as the matrix metalloproteinase, IL-6, and lectin-like oxidized LDL receptor primers tested in the present study (not shown).

Statistics. Results are expressed as means $\pm$ SE. Comparisons were made using one-way ANOVA, followed by a protected least-significant difference Fisher test. Differences were considered significant at $P < 0.05$.

RESULTS

As shown in Table 2, all hamsters fed the lipid-rich diets reduced their daily food consumption to adjust their energy intake to that of chow-fed controls ($P < 0.05$). The BF diet showed by far the lowest food efficiency, especially when compared with the BR diet. Part of this effect between these two high-fat diets can be ascribed to the relative higher fat deposition in the BR-fed hamsters compared with the BF-fed hamsters, as shown by the amount of epididymal adipose tissue (Table 2).

Liver lipids. All hyperlipidic diets similarly increased cholesterol and phospholipid concentrations in the liver (Table 2). TG concentrations remained almost unaffected, although *cis*-9,*trans*-11-CLA tended to lower them. Hamsters consuming the BR diet had a commensurate elevation of the *cis*-9,*trans*-11-CLA isomer in their liver lipids (2.2% vs. 0.3% in the BR and in the B or BF groups, respectively).

Plasma parameters. SDS-PAGE analysis of total apolipoproteins (Fig. 1) showed a clear elevation of plasma apolipoprotein B levels in all hyperlipidic diet-fed hamsters compared with controls; this effect was more pronounced in the BF group but was attenuated in the BR group. All of the lipid-rich diets increased the concentration of apolipoprotein E and decreased that of apolipoprotein AI. As a result, the apolipoprotein B-to-apolipoprotein AI ratio among the butter oil-fed hamsters was the most favorable in the BR group and the least in the BF group. Interestingly, the intensity of a minor band, corresponding to apolipoprotein-SAA (mol mass = 14 kDa) (3), a marker of inflammatory status ferried by HDL, was reduced in the group supplemented with *cis*-9,*trans*-11-CLA (BR diet) compared with the others.

All plasma lipid concentrations were increased by the hyperlipidic diets compared with the low-fat C diet (Table 2), with a maximal effect on cholesterol observed with the BF diet.

Table 2. *Physiological status of hamsters fed for 12 wk a low-fat diet or high-fat atherogenic diets (20% by weight, butter oil based) augmented with either 1% of the cis-9, trans-11-CLA or 1 % of fish oil*

	Dietary Groups			
	C	B	BR	BF
Food intake, g/day	8.8 $\pm$ 0.2*	6.8 $\pm$ 0.1	6.6 $\pm$ 0.1	6.6 $\pm$ 0.1
Energy intake, MJ/day	12.8 $\pm$ 0.2	13.4 $\pm$ 0.3	13.0 $\pm$ 0.3	13.1 $\pm$ 0.2
BW/12 wk, g	128 $\pm$ 2*†	130 $\pm$ 3*†	141 $\pm$ 3†	122 $\pm$ 4*
BW gain, g	22 $\pm$ 2*	20 $\pm$ 3*	28 $\pm$ 3*	9 $\pm$ 3†
Food efficiency	3.0 $\pm$ 0.2*	3.5 $\pm$ 0.4*†	5.0 $\pm$ 0.8†	1.6 $\pm$ 0.2‡
Epididymal adipose tissue, g/100 g	2.0 $\pm$ 0.08*†	1.9 $\pm$ 0.09*	2.3 $\pm$ 0.1†	1.8 $\pm$ 0.1*
Liver				
Weight, %BW	2.0 $\pm$ 0.1*†	1.9 $\pm$ 0.1*	2.3 $\pm$ 0.1†	1.8 $\pm$ 0.1*
FC, μ mol/g	4.1 $\pm$ 0.3*	9.8 $\pm$ 0.8	10.1 $\pm$ 1.3	11.4 $\pm$ 1.3
CE, μ mol/g	4.1 $\pm$ 0.5*	36.0 $\pm$ 2.8	37.6 $\pm$ 2.3	33.7 $\pm$ 2.6
TG, μ mol/g	15.3 $\pm$ 2.4*	12.5 $\pm$ 0.6*†	11.4 $\pm$ 0.7†	15.0 $\pm$ 0.9*
PL, μ mol/g	17.0 $\pm$ 2.2*	21.2 $\pm$ 1.4†‡	21.3 $\pm$ 1.9†‡	25.0 $\pm$ 1.0‡
CLA, %	ND	0.30 $\pm$ 0.05	2.20 $\pm$ 0.07*	0.30 $\pm$ 0.01
Plasma				
Glucose, mmol/l	6.6 $\pm$ 0.3*†	7.2 $\pm$ 0.4*	6.6 $\pm$ 0.2*†	6.1 $\pm$ 0.1†
Insulin, pmol/l	405 $\pm$ 28	389 $\pm$ 27	503 $\pm$ 66	390 $\pm$ 62
HOMA	17.0 $\pm$ 1.4	17.6 $\pm$ 1.4	21.1 $\pm$ 3.0	15.0 $\pm$ 2.4
TC, mmol/l	2.9 $\pm$ 0.1*	9.9 $\pm$ 0.60†	9.2 $\pm$ 0.6†	13.9 $\pm$ 1.2‡
TG, mmol/l	0.9 $\pm$ 0.10*	2.1 $\pm$ 0.26†	4.6 $\pm$ 0.4‡	2.4 $\pm$ 0.40†
PL, mmol/l	2.8 $\pm$ 0.1*	4.8 $\pm$ 0.17	4.9 $\pm$ 0.2	4.8 $\pm$ 0.2
Aorta				
Total CE, nmol/aorta	10.0 $\pm$ 0.7*	44.3 $\pm$ 3.4†	32.5 $\pm$ 3.2‡	57.4 $\pm$ 6.4§
FC, nmol/aorta	183 $\pm$ 11*	242 $\pm$ 10†	214 $\pm$ 18*†	263 $\pm$ 21‡
TC, nmol/aorta	193 $\pm$ 11*	286 $\pm$ 11†	245 $\pm$ 21*†	320 $\pm$ 27‡
FC/TC	0.95 $\pm$ 0.00*	0.85 $\pm$ 0.01†	0.89 $\pm$ 0.01‡	0.82 $\pm$ 0.01†

Values are means $\pm$ SE; $n = 8$ –10 animals/group. Food efficiency was calculated as (Weight gain $\times$ 100)/(Total food intake over 12 wk). C, low-fat diet; B, butter oil-based diet; BR, *cis*-9,*trans*-11-conjugated linoleic acid-augmented diet; BF, fish oil-augmented diet; CE, cholesteryl ester; BW, body weight; FC, free cholesterol; HOMA, homeostatic model assessment; PL, phospholipids; TC, total cholesterol (free and esters); TG, triglycerides. *,†,‡,§ Values in the same row not sharing the same symbol are statistically different ($P < 0.05$ by one-way ANOVA followed by a Fisher test).

Adding a high-rumenic acid blend up to 1% to the butter oil-based diet increased plasma TG. Relatively smaller differences were observed in plasma glucose or insulin concentrations as well as in HOMA values.

The plasma paraoxonase activity (Fig. 2) was lowered by all hyperlipidic diets used. However, the fish oil diet further amplified the decrease, whereas adding rumenic acid-rich oil improved the activity, without restoring the level observed in control normolipidemic hamsters.

The distribution of plasma cholesterol among the main lipoproteins (Fig. 3) showed that, compared with control hamsters in which HDL were the major carriers of plasma cholesterol, hamsters fed lipid-rich diets displayed an important shift of cholesterol toward a density inferior to 1.063, with a new population of IDL particles and no change or only a slight increase in the HDL peak. The maximal increase in the non-HDL-cholesterol concentration was observed in hamsters supplemented with fish oil ($P = 0.001$ for IDL and $P = 0.002$ for LDL) with the greatest accumulation of IDL and LDL particles. By comparison, BR-fed hamsters had lower IDL- and LDL-cholesterol concentrations. These differences were even more robust for the non-HDL-to-HDL-cholesterol ratio calcu-

	C	B	BR	BF
AU / mL plasma				
Apo B	18	129	78	160
Apo AIV	13.4	19.3	21.1	5.7
Apo E	5.4	31.7	20.5	38.5
Apo AI	211	184	177	138
Apo SAA	7.1	17.4	1.9	8.8
Apo SAA/AI	0.03	0.09	0.01	0.06
Apo B/ AI	0.09	0.70	0.44	1.16

Fig. 1. *Top*: polyacrylamide gel electrophoresis of apolipoproteins (Apos) from total plasma lipoproteins extracted from the different groups of hamsters fed various diets. C diet, control chow diet; B diet, C diet supplemented with 20% by weight of butter oil; BR diet, B diet supplemented with 1% by weight of rumenic acid-rich oil; BF diet, B diet supplemented with 1% by weight of fish oil. A same amount (30 μ g) of proteins from total lipoproteins extracted from pooled plasma (6 hamsters/group) was layered on the gel. In the table (*bottom*), the concentrations of apolipoproteins are expressed in arbitrary units (AU) per milliliter of plasma, taking into account the plasma concentration of total apolipoproteins in each group (C diet: 0.9 mg/ml plasma, B diet: 1.9 mg/ml plasma, BR diet: 1.6 mg/ml plasma, BF diet: 1.9 mg/ml plasma). SAA, serum amyloid A.

lated from these profiles (Fig. 4A). This index of the atherogenic risk was very low in controls but was significantly increased in hamsters fed the butter oil-based diets (5.2-fold, $P < 0.05$), with the highest values found again in those supplemented with fish oil (BF diet; 9.7-fold, $P < 0.05$) and the least in those supplemented with rumenic acid-rich oil (BR diet; 4.1-fold, $P < 0.05$).

Aortic cholesterol content and gene expression. Among hamsters fed the atherogenic diets, only those supplemented with rumenic acid-rich oil displayed total and free cholesterol loading in aortic tissues close to that of normolipidic hamsters (Table 2). Interestingly, the non-HDL-to-HDL-cholesterol ratio was highly predictive of the cholesteryl-ester deposition in the aorta ($r^2 = 0.78$, $P < 0.001$; Fig. 4B), which leads to fatty streak formation. A close observation could be done while using the apolipoprotein B-to-apolipoprotein AI ratio (Fig. 1).

Fig. 2. Paraoxonase activity from plasma samples collected from hamsters fed the various diets. Results are expressed as means $\pm$ SE for 8 animals/group. *†‡§ Different symbols indicate statistical differences (at $P < 0.05$ by one-way ANOVA followed by a Fisher test).

Also, the free cholesterol-to-total cholesterol ratio, taken as a severity end point of atherogenesis, was closer to that of control hamsters in the rumenic acid-supplemented animals (Table 2).

Fig. 3. Distribution of plasma cholesterol in lipoprotein fractions separated by density gradient ultracentrifugation from plasma samples collected from hamsters after 12 wk being fed the C, B, BR, or BF diet. Each point represents the mean $\pm$ SE for 8 individual determinations. HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; d, density.

Fig. 4. Plasma non-HDL-to-HDL-cholesterol ratio (A) and its relationship with cholesteryl ester (CE) deposition in the aorta (B). Results are expressed as means $\pm$ SE for 8 (A) or 5–7 (B) animals/group. In A, *†‡§ different symbols indicate statistical differences (at $P < 0.05$ by one-way ANOVA followed by a Fisher test). In B, the CE deposition in the aorta appeared significantly correlated to the plasma non-HDLto-HDL-cholesterol ratio ($r = 0.88$, $P < 0.0001$).

Concerning the gene expression in the aorta, the atherogenic butter-based diet (compared to the C diet) significantly induced the transcription of inflammatory-related genes (TNF- α , 2-fold; COX-2, 3.9-fold; and IL-1 β , 11-fold; all $P < 0.05$), unrelated to PPAR and LXR- α nuclear factor transcription (Fig. 5). Conversely, adding rumenic acid-rich oil to the atherogenic diet clearly induced an upregulation of these nuclear transcription factors (2.8- to 4.5-fold for PPARs and LXR- α , respectively). This was accompanied by a relative downregulation (IL-1 β , COX-2, TNF- α , and VCAM-1) or upregulation (ABCA1) of some target genes compared with the B group. Interestingly, adding fish oil to the atherogenic diet produced a response somewhat similar to that of rumenic acid oil for the inflammation mediator genes (iNOS, COX-2, IL-1 β , and TNF- α). Nevertheless, fish oil was less efficient in preventing cellular cholesterol uptake (FAT/CD36) and monocyte recruitment (VCAM-1), which were even further potentially increased. All these effects were unrelated to the induction of PPAR and LXR- α transcription (Fig. 5).

DISCUSSION

The present study points out that adding a rumenic acid-rich oil (*cis*-9,*trans*-11-CLA isomer) in a high-fat diet attenuates the early signs of atherosclerosis in the hamster.

More specifically, the analysis of the aortic cholesterol loading (Table 2) indicated a lower atherogenic outcome in hamsters supplemented with the rumenic acid oil (BR diet) than in nonsupplemented animals (B). Such a comparable finding has been reported in hamsters with another CLA mixture (50% each *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA isomer) and never with such a high *cis*-9,*trans*-11-CLA preparation (25, 37). Recently, both rumenic acid and the *trans*-10,*cis*-12-isomer have been found equally potent to inhibit the progression of atherosclerosis and to induce the regression of preestablished atherosclerosis in a rabbit model (16). Hence, the present results highly suggest that rumenic acid, and not only a CLA mixture, is efficient in reducing atherogenesis in hamsters. In contrast, adding fish oil to the

Fig. 5. Gene transcription levels measured in the total aorta after the extraction of RNA. PPAR, peroxisome proliferator-activated receptor (α and γ subtypes); LXR- α , liver X receptor- α ; ABCA1, ATP-binding cassette type 1; COX-2, cyclooxygenase type 2; FAT/CD36, fatty acid transporter/cluster of differentiation; IL-1 β , interleukin-1 β ; TNF- α , tumor necrosis factor- α ; VCAM-1, vascular cell adhesion molecule type 1. The expression rates are expressed in fold induction to normalized control values (C diet-fed hamsters, dotted line set at 1). Results are expressed as means $\pm$ SE for 5–7 animals/group. *†‡ Different symbols indicate statistical differences among high-fat groups; ‡ statistical difference with C diet-fed controls only (at $P < 0.05$ by one-way ANOVA followed by a Fisher test).

butter-based diet (BF diet) did not reduce the aortic cholesterol loading but even increased it further compared with the B diet. In the present study, part of the beneficial effect of rumenic acid and of the deleterious outcome of fish oil can be ascribed to the different plasma lipid profiles induced by these two diets. Hence, although all hyperlipidic diets induced an atherogenic lipoprotein pattern dominated by high LDL- and IDL-cholesterol levels, the shift was less evident in hamsters supplemented with rumenic acid-rich oil than with fish oil. As a result, the atherogenic index in plasma, calculated as the non-HDL-to-HDL-cholesterol ratio (and apolipoprotein B-to-apolipoprotein AI ratio) was highly predictive of the deposition of cholesteryl esters in the aorta (Figs. 1 and 4B and Table 2). The somewhat deleterious effects of fish oil in our study could seem to be in conflict with the beneficial effect of long-chain n-3 polyunsaturated fatty acids (PUFA) usually reported in cardiovascular diseases (36). In fact, these PUFA are considered more efficient in preventing cardiac dysfunction (antiarrhythmic agents), improving vascular endothelial function, and helping to lower blood pressure, platelet sensitivity, and serum TG levels (for a review, see Ref. 36) than improving the blood cholesterol levels (36). Indeed, in many species, fish oil addition increased non-HDL-cholesterol and lowered HDL-cholesterol (13). This holds true especially in hamsters fed cholesterol-rich diets (29, 31) as well as sometimes in rabbits reared on atherogenic diets (28). In this situation, such deleterious cholesterol profile can be associated with an unfavorable aortic lesion formation (28), as in the present study. Thus, in our hamster model, rumenic acid-rich diet but not fish oil-rich diet improved the atherogenic outcome, and this effect was at least partly sustained by an improvement of blood cholesterol transport.

Plasma TG levels were higher in hamsters consuming the rumenic acid preparation, but this appeared insufficient to produce deleterious consequences. In fact, whereas CLA (including rumenic acid) is able to attenuate quite consistently the outcome of primary vascular lesions (fatty streaks) in animal models [except in one mice study (24)], a clear relationship between this protective action and an improvement of circulating lipids is not always observed (for a review, see Ref. 21). We thus investigated other potential mechanisms whereby a rumenic acid-rich diet could elicit its beneficial effect on atherogenesis. Among them, plasma paraoxonase activity, involved in the protection of LDL against oxidation, was used as an index of the antiatherogenic, anti-inflammatory, and anti-oxidative defenses (20). Indeed, among hamsters fed the atherogenic diets, an increase of this activity was observed in hamsters consuming the *cis*-9,*trans*-11-CLA diet, whereas an inverse effect was observed in those consuming fish oil (Fig. 2), which suggests a protective role of this CLA isomer toward the formation of proatherogenic LDL particles. This effect can be ascribed to a possible direct effect of CLA in protecting the enzyme from oxidative inactivation, as demonstrated *in vitro* (30).

To give further insights into the molecular mechanisms whereby our CLA preparation could act as an antiatherogenic agent, we then studied the expression of several genes implicated in initial events of atherosclerosis in the vascular wall. Schematically, the onset of atherosclerosis is thought to proceed from endothelial microinjuries, which will then trigger a local inflammation involving cytokines and also COX-2 and iNOS

production. This response promotes the recruitment of monocytes (mediated by adhesion molecules such as VCAM-1), their differentiation into macrophages, and the uptake of oxidized LDL, which produces an imbalance between cholesterol uptake and efflux (through FAT/CD36 and ABCA1, for instance, respectively) leading to foam cell formation and ultimately to plaque formation. The induction/repression of the genes encoding much of these factors is under the control of nuclear transcription factors such as PPARs and LXR- α and is critical in the evolution of the disease. Indeed, the results indicate that part of the CLA action is compatible with the activation of the anti-inflammatory PPAR/LXR signaling cascade. Hence, high-rumenic acid blend intake brought about upregulation of these transcription factors, an effect that in turn was accompanied by a downregulation of the inflammatory gene cluster expression (IL-1 β , COX-2, and TNF- α , but not iNOS), maintained VCAM-1 to low expression, and slightly but significantly upregulated ABCA1 (Fig. 5). Although modest, this latter effect in the CLA-fed hamsters might at least partially account for the improvement of the free cholesterol-to-total cholesterol ratio in the aorta (Table 2). In addition, the anti-inflammatory action of the high-rumenic acid preparation is in agreement with the lower plasma concentration of apolipoprotein-SAA (Fig. 1), an acute-phase heat shock protein synthesized in response to inflammatory stress (3). In the BR group compared with the B group, this profile of genes induction can be interpreted as a result of an improvement of the local inflammatory status and of cholesterol trafficking (lower uptake and higher efflux).

Conversely, fish oil supplementation only lowered the transcription rate of inflammatory factors and less distinctly increased that of ABCA1, whereas it specifically enhanced that of FAT/CD36 involved in lipid uptake and fatty streak formation. As a whole, the beneficial effects do not appear sufficient to counteract the unfavorable atherogenic outcome.

In conclusion, the enrichment of an atherogenic diet with a CLA blend high in rumenic acid produced a beneficial effect in hamsters by reducing diet-induced atherosclerosis, whereas fish oil supplementation did not afford a similar advantage. In the present study, such an effect was mediated through an improvement of several aspects of the disease, including blood cholesterol transport (non-HDL-to-HDL-cholesterol ratio), LDL protection against oxidation (plasma paraoxonase activity), and inflammatory status (plasma apolipoprotein-SAA, vascular inflammatory mediators). Similar to *in vitro* results obtained with activated RAW macrophages (8), our *in vivo* results suggest an effect of the *cis*-9,*trans*-11-CLA intake on the PPAR/LXR signaling cascade in vascular cells. The study is in agreement with the antiatherogenic role of *cis*-9,*trans*-11-CLA in animals and brings some new mechanistic explanations. Nevertheless, the effects are likely much more complex than those described in the present study, and other key determinants of the disease require to be examined, such as the expression of matrix metalloproteinases and anti-inflammatory cytokines (IL-10, for instance) or NF- κ B activation in the vessel wall. Within the limitation of the hamster model in atherosclerosis, which requires the use of unrealistic diets, our findings suggest that long-term consumption of rumenic acid rich diets could be beneficial to human health.

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