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ORIGINAL ARTICLE

Vaccenic and Elaidic Acid Equally Esterify into Triacylglycerols, but Differently into Phospholipids of Fed Rat Liver Cells

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Abstract Elaidic acid (*trans*-9-$C_{18:1}$ or *trans*-9) is assumed to exert atherogenic effects due to its double bond configuration. The possibility that *trans*-9 and vaccenic acid (*trans*-11-$C_{18:1}$ or *trans*-11), its positional isomer, were biochemically equivalent and interchangeable compounds, was investigated by reference to their *cis*-isomers through esterification-related activities using rat liver cells and subcellular fractions. In hepatocytes, both *trans*-$C_{18:1}$ were incorporated to the same extent in triacylglycerols, but *trans*-9 was more esterified than *trans*-11 into phospholipids ($P < 0.05$). Glycerol-3-phosphate acyltransferase activity in microsomes was lower with *trans*-11 than with *trans*-9, while this activity in mitochondria was ~40% greater with *trans*-11 than with *trans*-9 ($P < 0.05$). Activity of 2-lysophosphatidic acid acyltransferase in microsomes was of comparable extent with both *trans* isomers, but activity of 2-lysophosphatidylcholine acyltransferase was significantly greater with *trans*-9 than with *trans*-11 at $P < 0.01$. Lipoproteins secreted by hepatocytes reached equivalent levels in the presence of any isomers, but triacylglycerol production was more elevated with *trans*-11 than with *trans*-9 at $P < 0.05$. Cholesterol efflux from previously labelled hepatocytes was lower with *trans*-11 than with *trans*-9. When these cells were exposed to either *trans*-$C_{18:1}$, the gene expression of proteins involved in fatty acid esterification and lipoprotein synthesis was unaffected, which indicates that the biochemical differences essentially depended on enzyme/substrate affinities. On the whole, vaccenic and elaidic acid were shown to incorporate cell phospholipids unequally, at least in vitro, which suggests they can differently affect lipid metabolic pathways in normal cells.

Keywords Vaccenic and elaidic acid · Esterification · Phospholipid · Cholesterol efflux · Gene expression

Abbreviations

ACC	Acetyl-CoA carboxylase
cis-9	*cis*-9-$C_{18:1}$, oleic acid
cis-11	*cis*-11-$C_{18:1}$
CLA	Conjugated linoleic acid
DGAT	Diacylglycerol acyltransferase
FA	Fatty acid
GPAT	Glycerol-3-phosphate acyltransferase
HMG-Red	Hydroxymethylglutaryl-CoA reductase
LDL-R	Low-density lipoprotein-receptor
mtGPAT	Mitochondrial GPAT
trans-9	*trans*-9-$C_{18:1}$, elaidic acid
trans-11	*trans*-11-$C_{18:1}$, vaccenic acid

Introduction

The consumption of fats containing *trans*-fatty acids (FA) has been correlated with an increased risk of coronary

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heart disease, principally through elevated levels of plasma total cholesterol, LDL cholesterol, apolipoprotein B and increased LDL/HDL ratios [1, 2]. The partial hydrogenation of vegetable oils for margarine production [3, 4] gave rise to by-products as *trans*-FA whose levels may reach 60% of total FA [5]. Among these, elaidic acid (*trans*-9) could represent 30–40% and vaccenic acid (*trans*-11) 10% [6, 7]. *trans*-9 was assumed to exert atherogenic effects because its concentration in margarines was associated with abnormal plasma lipid concentrations [2, 8]. Some *trans*-$C_{18:1}$ are also produced in the digestive system of ruminants through the microbial hydrogenation of vegetable oils and are consequently recovered in related products as milk and meat. In dairy fats, *trans*-11 was found to represent more than 80% of all the identified *trans*-$C_{18:1}$ isomers [9, 10]. Epidemiological surveys suggested that *trans*-9 increases more the cardiovascular risk than *trans*-11 [11–13]. However with hamsters, plasma LDL/HDL-cholesterol ratios were more elevated with diets enriched in *trans*-11 than in *trans*-9 [14]. Yet with the same animal model, the hypercholesterolemic effects of partially hydrogenated vegetable oils was demonstrated not to directly depend on the presence of *trans*-9 or *trans*-11 [15]. In the whole, the use of versatile experimental models, atherosclerosis markers and dietary fat blends provide inconsistent results, which suggests that *trans*-9 and *trans*-11 exert similar effects, but whose amplitude would depend in humans on particular parameters as specific dietary lipid levels [16] or hormonal status [6].

Geometrical isomers exhibit comparable fates as regards their hydrolysis from triacylglycerols and their absorption in the intestinal tract [17], as well as their distribution among lymph lipids [18]. Thus the specific effects of *trans* isomers would occur beyond the intestinal step and then affect most organs with possible interrelations. This prompted us to limit the whole issue to the comparison of actions of pure *trans*-$C_{18:1}$ isomers by using only one tissue model. Liver was chosen because of its pivotal role in the re-distribution of ingested lipids to other organs via lipoprotein secretion. *trans*-9 and *trans*-11 were all the more recovered in lipoproteins because they were shown to be weakly oxidised in liver mitochondria [19, 20] and were then available for the esterification reactions. Besides, the incorporation of FA into membrane lipids is associated with cholesterol efflux, which should increase plasma cholesterol levels [1, 2]. The present study was designed to compare first the esterification capacities of *trans*-9 and *trans*-11, relative to their respective *cis*-isomers, in rat liver extracts, second the effects of the four $C_{18:1}$ isomers on membrane cholesterol efflux and esterification-related gene expression in isolated hepatocytes. Rats were fed a standard diet, instead of diets enriched in the tested isomers, to prevent alterations of enzyme activities induced by these isomers within membranes [21].

Experimental Procedure

Chemicals

Oleic (*cis*-9-$C_{18:1}$), elaidic (*trans*-9-$C_{18:1}$), *cis*-11-octadecenoic (*cis*-11-$C_{18:1}$) and vaccenic (*trans*-11-$C_{18:1}$) acids, abbreviated *cis*-9, *trans*-9, *cis*-11 and *trans*-11, respectively, were purchased from Sigma Chemical Co. (St. Louis, MO). [1-^{14}C] oleic acid and [4-^{14}C] cholesterol were obtained from Amersham Biosciences Europe (Saclay, France). The other [1-^{14}C] isomers were specifically prepared by CEA (Gif sur Yvette, France) from commercial unlabelled FA and $^{14}CO_2$ using procedures described in [22]. Radiochemical purity (>98%) of [1-^{14}C] labelled compounds was determined by RP-HPLC (Zorbax SB C18; acetone/(methanol/water/acetic acid, 50:50:1, by vol), 60:40, by vol) and TLC (Silicagel Merck 60F254, pentane/diethylether/acetic acid, 80:20:0.1, by vol). They were all stored in chloroform/ethyl alcohol (50:50, by vol) at −30 °C. Evaporation of solvents under nitrogen was followed by the saponification of FA with NaOH or KOH for assays with whole cells or intracellular organelles, respectively, and their binding to essentially FA-free bovine serum albumin (BSA). These preparations were added to the other components of media immediately prior to incubations. [U-^{14}C]Glycerol-3-phosphate was obtained from NEN Life Science Products (NEN, Paris, France). BSA, cholesterol, collagenase (type I) and other biochemicals were from Sigma. Methylene chloride, 2,4,6-collidine, ethyl chloroformate and tetrahydrofuran, supplied by Aldrich Chemical Co. (Milwaukee, WI), were used for acyl-CoA synthesis [23]. Concentrations of synthesised acyl-CoAs were determined through their respective molar extinction coefficients at 260 nm and corrections based on their 232/260 nm ratios, relative to those with pure products [24–26].

Animals

Official French regulations (No. 87848) for the use and care of laboratory animals were followed throughout and the experimental protocol was approved by the local ethics committee for animal experimentation (No. BL0612). Male Wistar rats were purchased from Janvier (Le Genest St Isle, France). They were kept at 23 °C in a light-controlled room (light period fixed between 08:00 a.m. and 08:00 p.m.). They had free access to tap water and were fed a standard laboratory chow (ref AO4 from UAR, Epinay-sur-Orge, France) containing 58.7% carbohydrate, 17% protein, and 3% fat (palmitic, stearic, oleic and linoleic acids

representing 8.7, 1.7, 26.7 and 48.3%, respectively, of the total FA mass). When rats were 7–8 weeks old (250–300 g), they were anaesthetised with isoflurane and killed by exsanguination at 08:00 a.m.

Activity Rate Measurements

All the biochemical activity measurements were performed with increasing substrate concentrations, but the concentration retained for each experimental presentation was selected among those giving rise to the increasing part of activity curves.

FA Esterification by Isolated Hepatocytes and Liver Slices (Intact Cells)

Hepatocytes were isolated through a two-step collagenase perfusion as in [27]. Cell viability was assessed by trypan blue exclusion and suspensions with viability lower than 85% were discarded. Immediately after isolation, cells were preincubated in William's Medium E (WME) from PAN-Biotech GmbH (Aidenbach, Germany) containing 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic antimycotic solution (Sigma Chemical, Co.) in water-saturated atmosphere (5% CO_2, 95% air) for ~3 h at 37 °C, in dishes of 60-mm diameter (~3×10^6 cells per dish). The attached cell monolayer was gently washed with 3 mL of fresh WME that was replaced by 2.5 mL of Krebs-Henseleit buffer (KHB) containing 118 mM NaCl, 4.7 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.5 mM $CaCl_2$, and 25 mM $NaHCO_3$, pH 7.4. The reaction was started by addition of 1 mL of [1-^{14}C] FA (2.25 Ci/mol) with BSA (0.4 and 0.1 mM of final concentration, respectively) under 95% O_2 and 5% CO_2 atmosphere. Liver slices were also prepared using the procedure detailed in [19] and were incubated (0.2 g per assay) in 2.5 mL of KHB. The reaction was initiated by addition of 1 mL of labelled FA with BSA, as above for the cell monolayers. After 2 h of incubation at 37 °C, the supernatants were decanted, and the cell monolayers or tissue stripes were washed two times with KHB containing 2% FA-free BSA at 37 °C. Total lipids of both liver extracts were obtained using the Folch procedure [28] and were separated by TLC in hexane/ethyl ether/acetic acid/methanol (90:20:2:3, by vol). The radioactivity of triacylglycerol (TAG) and phospholipid (PL) spots was measured by AR-2000 TLC Imaging Scanner (Bioscan, Inc., Washington DC).

FA Esterification by Liver Homogenates (Disrupted or Disorganised Cells)

Liver pieces were homogenised by manual rotation of a Teflon pestle in an ice-cooled Potter–Elvehjem apparatus and diluted (1:40, w/v) in a chilled mixture of 0.25 M sucrose, 2 mM EGTA and 10 mM Tris/HCl, pH 7.4 [29]. The reaction was initiated by the injection of 0.1 mL homogenate into 1.5 mL of a mixture containing 2 mM ATP, 50 μM CoA, 4 mM $MgCl_2$, 1% glucose and 120 μM [1-^{14}C] FA (3.7 Ci/mol). Incubations were performed at 37 °C for 30 min and total lipids were extracted and separated as above for whole cells.

Microsomal and Mitochondrial Esterification onto the *sn*-1 Position of Glycerol-3-phosphate

The isolation of mitochondrial fractions was performed as in [29], using a medium containing 1% FA-free BSA to bind free FA released during and after the cell disruption. Microsomal fractions were isolated after mitochondria pelleting as in [30]. The protein content of these fractions cleared from BSA was measured using the bicinchoninic acid procedure [31] and their purity was checked through activity of enzymes specific to mitochondria, peroxisomes and microsomes, as detailed in [30]. Glycerol-3-phosphate acyltransferase (GPAT) activity was measured in 1 mL of medium buffered at pH 7.4 and containing 75 mM KCl, 0.2 mM EGTA, 4 mM $MgCl_2$, 80 mM mannitol, 25 mM HEPES, 2 mM KCN, 2 mM ATP, 50 μM CoA, 0.5 mM L-[H^3] glycerol-3-phosphate (0.72 mCi/mol), 0.1 mM of each $C_{18:1}$ isomer and 50 μM BSA, at 37 °C. The reaction was initiated by addition of 0.3 mg of microsomal or mitochondrial protein, or of 0.1 mL of liver homogenate (2.5 mg liver). After 7 min, 3 mL of 0.1 M HCl containing 10 mM DL-glycerol-3-phosphate (Prolabo, Paris, France) was added and the radioactivity of 1-monoacyl-[H^3] glycerol phosphate extracted with butan-1-ol [32] was determined after mixing with Ultima Gold XR (Packard, Meriden, CT).

Microsomal Esterification onto the *sn*-2 Position of Monoacylglycerolipids

2-lysophosphatidic acid acyltransferase and 2-lysophosphatidylcholine acyltransferase activities were measured according to the method described in [33]. Briefly, a preincubation time of 2 min was performed at 25 °C by addition of 0.2 mg of microsomal protein to 2 mL of a medium containing 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 5 mM Tris–boric acid and 5 μM of $C_{18:1}$-CoA, at pH 7.4. The specific reactions of esterification were recorded over 4 min after addition of 50 mM 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate or 150 mM 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL). The change in absorption at 413 nm results from the binding to DTNB of thiol groups released by acyl-CoA hydrolysis during the esterification reactions.

Results were corrected from control values obtained in the absence of $C_{18:1}$-CoA.

Components of Lipoprotein Secreted by Hepatocytes Exposed to $C_{18:1}$ Isomers

Hepatocytes prepared as already described were incubated in 4 mL of WME containing 100 μM of each $C_{18:1}$ isomer bound to 50 μM BSA, 100 nM insulin and 1% antibiotic antimycotic solution, under a water-saturated atmosphere (5% CO_2, 95% air) at 37 °C. Control assays were performed in the same way, but in the absence of exogenous $C_{18:1}$ isomer. After 24 h, the media were centrifuged at 2,000×*g* for 5 min and the supernatants containing the cell lipid secretions were assayed with commercial kits from Orion Diagnostica (Espoo, Finland) for apolipoproteins A1 and B100, and from BioMérieux (Marcy l'Etoile, France) for triacylglycerols (TG PAP 150) and cholesterol (Cholesterol RTU). The efflux of cholesterol originating from all the cell structures was measured as follows: Hepatocytes prepared as above were cultured until the attachment step (∼4 h), then were added with 4 mL of WME containing 10% BSA, 4 μCi [4-^{14}C] cholesterol and 75 μL of fresh rat plasma. In this medium, the final amounts of cholesterol and LDL, expressed per mL, were 65 nmol and 50 μg, respectively. After 24 h of incubation at 37 °C in water-saturated atmosphere (5% CO_2, 95% air), radioactive and unlabelled forms of cholesterol in cells were assumed to balance each other. The supernatants were decanted and cells were washed once with KHB containing 1% BSA. Hepatocytes were then incubated in 4 mL of WME in the presence of 0.2 mM $C_{18:1}$ isomer and 70 μM BSA, with control assays performed in the absence of isomer. After 5 h, the supernatants were centrifuged to discard cells detached from the ground monolayer and the radioactivity of clarified samples, mixed with Ultima Gold XR, was measured in a scintillation spectrometer.

Gene Expression in Hepatocytes Exposed to $C_{18:1}$ Isomers

Hepatocytes prepared as above were incubated in 4 mL of WME containing 100 μM of $C_{18:1}$ isomer, 50 μM BSA, 100 nM insulin and 1% antibiotic antimycotic solution, in water-saturated atmosphere (5% CO_2, 95% air) at 37 °C. After 24 h, total mRNA of hepatocyte monolayers was extracted using Tri-Reagent (Euromedex, Souffelweyersheim, France) as described in [34]. Total mRNA was reverse-transcripted using Iscript cDNA kit (Bio-Rad, Marnes-La-Coquette, France). Real-time quantitative PCR was performed in 96-well plate using an iCycler iQ (Bio-Rad). Each well contains: 12.5 μL of iQ Sybr Green Supermix (Bio-Rad), 2.5 μL of forward primer (0.3 μM), 2.5 μL of reverse primer (0.3 μM), 5 μL of cDNA (25, 2.5, 0.25 and 0.025 ng for standards and 2.5 ng for samples) and 2.5 μL of water. Primer pairs were designed using 'Primers!' software and were synthesised by MWG-Biotech AG (Ebersberg, Germany). The sequences of the forward and reverse primers used were, respectively, as follows: 5′-caccggctgaaggacatac-3′ and 5′-gctcaaaggggtct caaaaga-3′ for acetyl-CoA carboxylase 2 (ACC 2); 5′-tt caaccccagtatcccatct-3′ and 5′-ggtgccttgtgtgagtttca-3′ for mitochondrial glycerol-3-phosphate acyltransferase (mtG-PAT); 5′-tcctaccgggatgtcaatct-3′ and 5′-ctcggtaggtcagg ttgtcc-3′ for diacylglycerol acyltransferase (DGAT); 5′-tgagcttagcgttgcttttg-3′ and 5′-agctttctttggggtcacg-3′ for HMG-CoA reductase; 5′-tgggttccatagggtttctg-3′ and 5′-ttg gggatcaggctggtat-3′ for LDL-receptor (LDL-R); and 5′-aat cgtgcgtgacatcaaag-3′ and 5′-gaaaagagcctcagggcat-3′ for *β*-actin. The relative expression of each gene was calculated with cDNA standard curves and normalised with actin cDNA.

Statistics

Differences between groups were determined performing repeated measures of ANOVA followed by the Tukey–Kramer multiple comparison post-hoc test using GraphPad Instat 3.1 software. Difference were considered significant at $P < 0.05$.

Results

Esterification of $C_{18:1}$ Isomers in Intact Cells

In hepatocytes (Fig. 1a) and liver slices (data not presented), the results were qualitatively comparable and showed that all the isomers were ∼2 times as much esterified into TAG as into PL. In TAG, *trans*-$C_{18:1}$ were ∼2-fold less incorporated than the *cis*-ones, while in PL, the amount of *trans*-9 was 25% greater than that of *trans*-11, and 3 times as elevated as that of *cis*-9 at $P < 0.05$. In liver homogenates, i.e. in disrupted cells, all the isomers were ∼2 times more incorporated into PL than into TAG (Fig. 1b). In TAG, the *trans*-$C_{18:1}$ were 40% less abundant than the *cis*-forms, while in PL, both *trans*-$C_{18:1}$ and *cis*-11 amounted to ∼50% more than *cis*-9 at $P < 0.05$.

Esterification onto the *sn*-1 Position of Glycerophosphate in Microsomes and Mitochondria

GPAT, through the esterification of acyl groups in *sn*-1 position of glycero-3-phosphate, initiates the synthesis of glycerolipids. In liver homogenates (Fig. 2a), GPAT activity was maximum with *cis*-9 and minimum with *trans*-11,

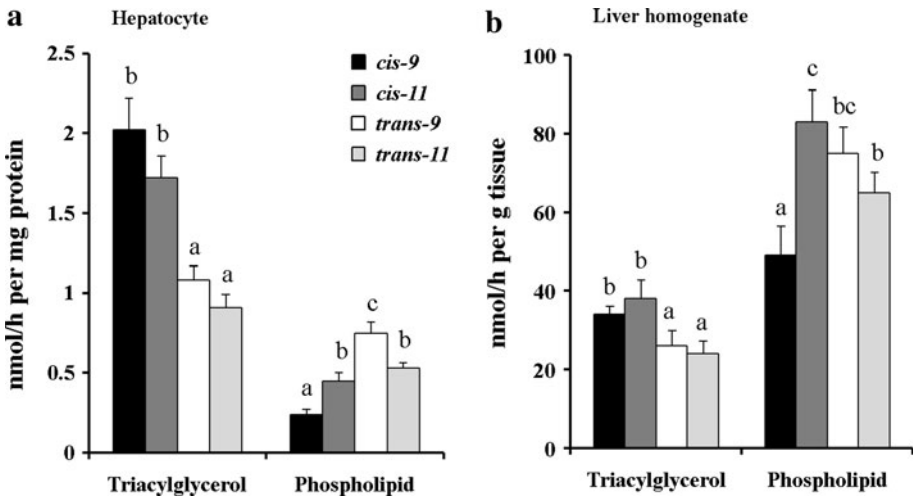

Fig. 1 Incorporation of $C_{18:1}$ isomers into TAG and PL of intact (**a**) and disrupted (**b**) liver cells. Concentrations of [1-^{14}C] isomer substrates in the final media were 0.4 and 0.12 mM with hepatocytes and liver homogenates, respectively. All results are expressed as nmol of $C_{18:1}$ isomers esterified per hour per mg hepatocyte protein or g wet liver. *T-bars* show SEM ($n = 3$). For each series and lipid class, values with different *letters* on columns statistically differ at $P < 0.05$

this latter exhibiting activity values lower than those of *trans*-9 at $P < 0.05$. The same pattern was observed in microsomes (Fig. 2b) except that the enzyme activity was comparable with *cis*-9 and *cis*-11. In mitochondria (Fig. 2c), GPAT (mtGPAT) was shown to exert activities of different extents as the considered $C_{18:1}$ isomer, in the following order: *cis*-11 > *cis*-9 > *trans*-11 > *trans*-9 ($P < 0.05$).

Esterification onto the *sn*-2 Position of Monoacylglycerolipids in Microsomes

The acylation in *sn*-2 position of 1-palmitoyl-2-hydroxyl-*sn*-glycero-3-phosphate is catalysed by 2-lysophosphatidic acid acyltransferase and regards as a step of the synthesis of glycerolipids as TAG and PL. Fig. 3a indicates that both *trans*-isomers were 40% less esterified in the *sn*-2 position than their *cis*-isomers ($P < 0.05$). The acylation at the same position of 1-palmitoyl-2-hydroxy-*sn*-3-phosphocholine is carried out by 2-lysophosphatidylcholine acyltransferase and reflects the renewing of cellular PL. This reaction was shown to be highly sensitive to the position of the double bond in the acyl chain, both Δ-11 isomers being ~4.5 times less esterified than both Δ-9-$C_{18:1}$ (Fig. 3b).

Lipid Secretion by Hepatocytes Exposed to $C_{18:1}$ Isomers

Total apolipoprotein A1 secretion was, compared to the control assay performed in the absence of FA, unaffected after 24 h-exposure of hepatocytes to any $C_{18:1}$ isomers, except that this production was decreased with *cis*-11 by reference to the control and *trans*-9 assays (Fig. 4a). Secretion of apolipoprotein B100 and total cholesterol was also unchanged after the same time of exposure to $C_{18:1}$ isomers (Fig. 4b, d). The TAG secretion was greater with *cis*-9 and *trans*-11 than with the control and *trans*-9 assays (Fig. 4c). When the efflux of cholesterol was studied with hepatocytes in which labelled cholesterol was previously balanced with the unlabelled cholesterol of cell structures (Fig. 5), values obtained after 5 h of incubation with the

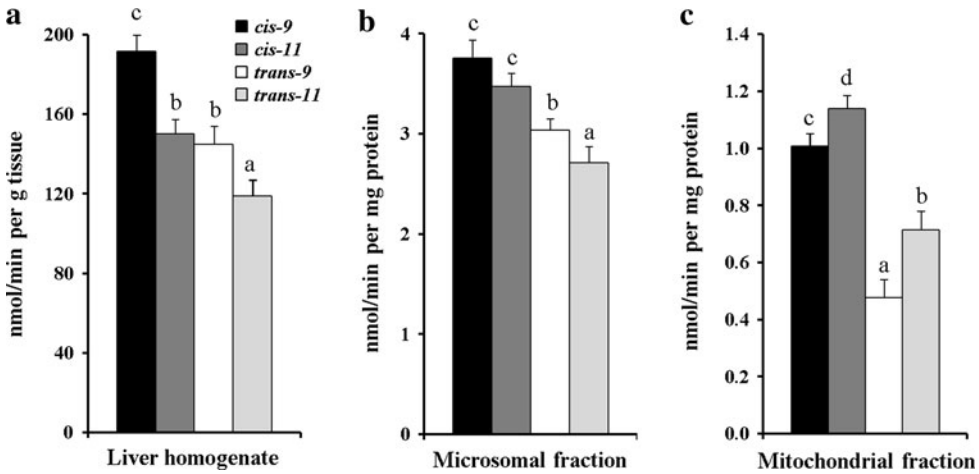

Fig. 2 Activity of glycerol-3-phosphate acyltransferase in the presence of 0.1 mM $C_{18:1}$ isomers with liver homogenates (2.5 mg wet liver) and 0.3 mg of microsomal or mitochondrial protein. All results are expressed as nmol of $C_{18:1}$ bound to glycerol-3-phosphate per min per g wet liver or mg microsomal protein. *T-bars* show SEM ($n = 3$). For each series, values with different *letters* on columns statistically differ at $P < 0.05$

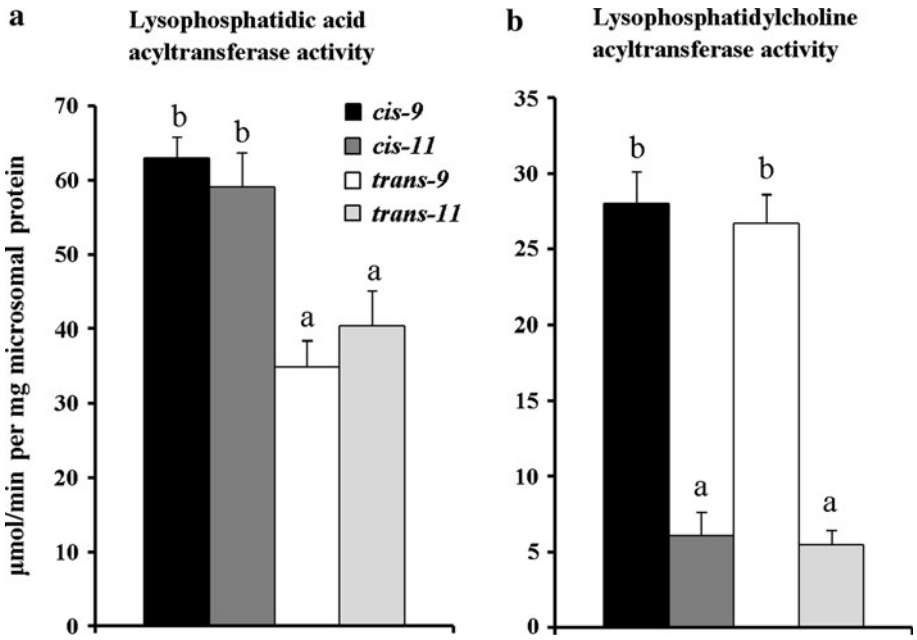

Fig. 3 Activity of 2-lysophosphatidic acid or 2-lysophosphatidylcholine acyltransferase in the presence of 5 µM $C_{18:1}$ isomers as CoA-esters with 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate (**a**) or 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (**b**), respectively, in liver microsomes. Enzyme activities were measured by following the absorbance at 413 nm of the binding of CoA (released from CoA-esters) to DTNB. Results are expressed as µmol of $C_{18:1}$ esterified per min per mg microsomal protein. *T-bars* show SEM ($n = 3$). For each activity, values with different *letters* on columns statistically differ at $P < 0.05$

isomers were all greater than in their absence, but were ~30% lower with *cis*-11 and *trans*-11 than with *cis*-9 and *trans*-9 at $P < 0.05$.

Esterification-Related Gene Expression

In hepatocytes cultured over 24 h with each $C_{18:1}$ isomer, the levels of gene expression for mtGPAT, DGAT and LDL-R were comparable to those obtained in the absence of FA (Fig. 6). A decrease in expression levels was observed only for ACC-2 with the *cis*-11 isomer and for HMG-CoA reductase with *trans*-11 at $P < 0.05$.

Discussion

The aim of the study was to determine whether the small difference in the double bond position of *trans*-11 versus *trans*-9 gave rise to comparable effects on FA esterification-related parameters. In this goal, rats were used in the fed state because this nutritional condition was shown to depress FA oxidation in liver and most other organs [35], which made all unbound FA available for TAG and PL synthesis. This ex vivo study shows that *trans*-11 and *trans*-9 were not interchangeable substrates for the esterification reactions in intact or disrupted liver cells, as well

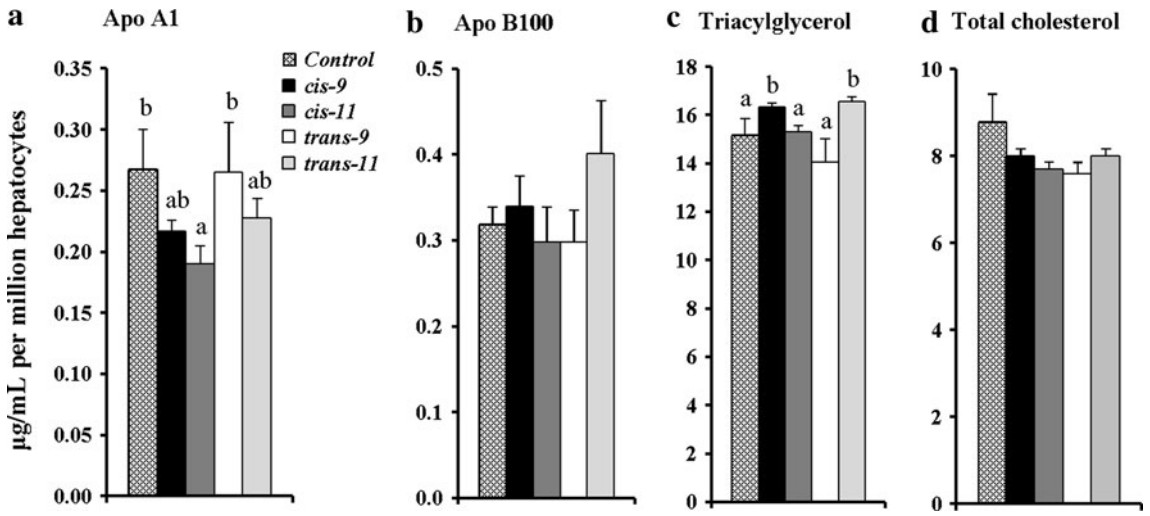

Fig. 4 Components of lipoproteins secreted by hepatocytes cultured for 24 h in the absence (*control assay*) or presence of 0.1 mM $C_{18:1}$ isomers. Results are expressed as µg of each component per mL of incubation medium per million hepatocytes. *T-bars* show SEM ($n = 3$). For each measurement, values with different *letters* on columns statistically differ at $P < 0.05$

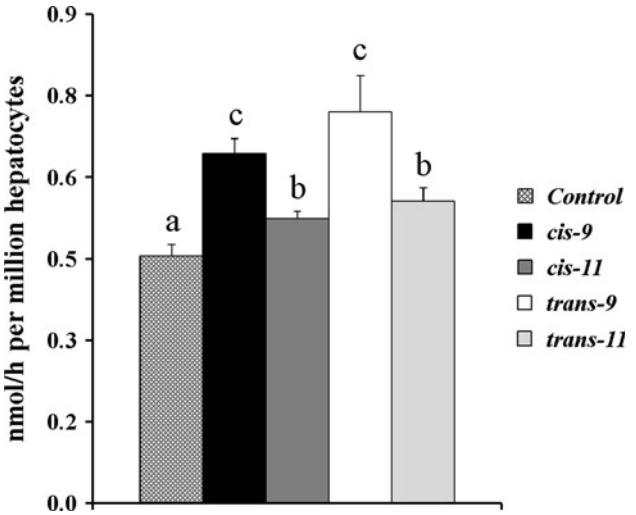

Fig. 5 Efflux of cholesterol from cholesterol-prelabelled hepatocytes in the presence of 0.2 mM $C_{18:1}$ isomers. After 5 h of incubation, the media were centrifuged and the radioactivity of their supernatants was determined as indicated in the "Experimental procedure" section. Results are expressed as nmol labelled cholesterol released per hour per million hepatocytes. *T-bars* show SEM ($n = 3$). Efflux values with different *letters* on columns statistically differ at $P < 0.05$

as in isolated microsomes and mitochondria. Yet the *trans* configuration of the single double bond gives both *trans*-11 and *trans*-9 a common linear aspect as for saturated FA [36–38]. Enzyme activities mainly depend on specific protein synthesis regulated by genes and on affinity of enzymes for their substrates. The genic expression of enzymes related to lipogenesis (FA and cholesterol synthesis) and FA esterification was about of the same range in the assays performed in the presence or not of isomers. These data may indicate that the expression levels of genes were increased with insulin, but without additional effects of isomers. Indeed, after various treatments, weakly higher or lower expression levels of the involved genes were found in fed animals [39–42]. Consequently, under this nutritional condition, the study of biochemical reactions related to TAG and PL synthesis appeared to be convenient to test the possible similarity of both *trans*-isomers through activity levels only depending on enzyme/substrate affinities.

Esterification of Isomers into TAG and PL of Liver Cells

In liver slices and isolated hepatocytes, i.e. in intact cells, the esterification of isomers into TAG and PL implied that some of their OH groups were available from pre-existent TAG and PL after specific hydrolysis [43–45]. In each of our assays, the labelled isomers, once within cells, were expected to compete for the esterification reactions with the endogenous FA present in the surroundings of partially hydrolysed TAG and PL. This competition also depended on the position of available OH groups in these lipids and the amounts of compatible FA. For instance, as regards a convenient position of esterification, the labelled isomers were recovered on TAG and PL to a greater extent when these lipids were previously weakly hydrolysed.

In perfused liver, *trans*-9 and *trans*-11, as well as stearic acid ($C_{18:0}$), were principally recovered on the 1- and 3-positions of TAG, while *cis*-9 and -11 isomers were mainly present on the 2-position [36]. With this specific information, our results show that, in intact and disrupted cells, the affinities of either *trans*-$C_{18:1}$ for the external 2 positions of TAG were equivalent and always at a

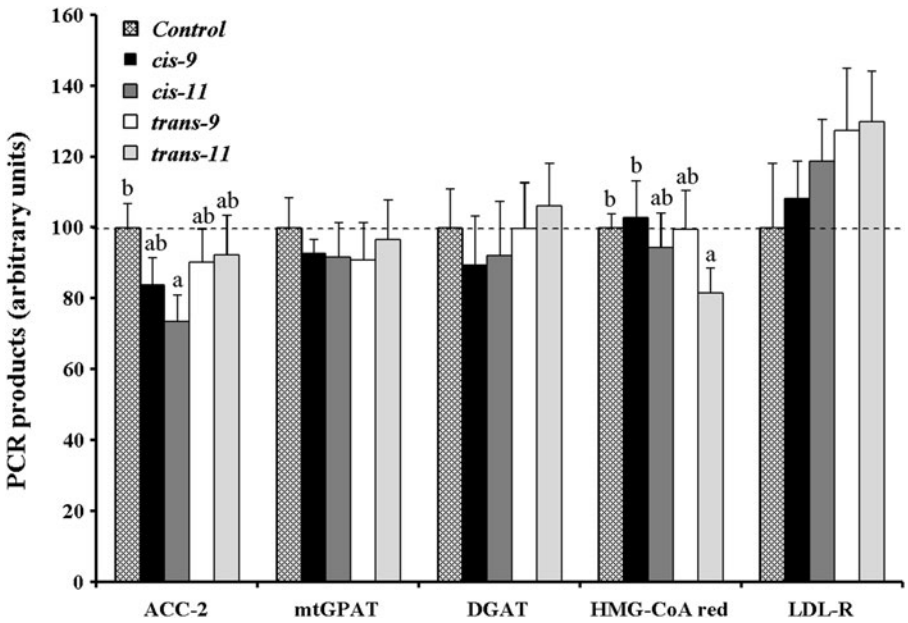

Fig. 6 mRNA levels of FA synthesis- and esterification-related proteins after incubation of fed rat hepatocytes with 0.1 mM of each $C_{18:1}$ isomer for 24 h. Arbitrary units ± SEM with $n = 3$ (different hepatocyte culture dishes) are percentages expressed by reference to control assays (*dotted line*) performed in the absence of any $C_{18:1}$ isomer (=100 for each gene). Different *letters* on columns denote statistically different results at $P < 0.05$

significantly less extent than those of either *cis*-$C_{18:1}$ for the internal position alone. The inversion of the rough distribution of the labelling between TAG and PL in intact and disrupted cells (Fig. 1a vs. b) could originate, as mentioned above, from a weak rate of TAG hydrolysis in intact cells and, after cell disruption, from increased hydrolysis of both TAG [44, 46] and PL through phospholipase activation [46, 47].

In PL, as the *sn*-2 position was preferentially occupied by *cis*-unsaturated FA [36–38], the low level of esterification of labelled *cis*-9 (see Fig. 1a) could result from the dilution of this exogenous isomer with endogenous *cis*-9 and other unsaturated FA, as linoleic acid representing ~50% of total FA of dietary lipids (see "Experimental procedure" section). The incorporation of *cis*-11 also occurred onto the *sn*-2 position, but was significantly greater than that of *cis*-9 both in intact and disrupted cells. This may be due to the fact that, contrary to oleic acid, *cis*-11 is not present in the natural lipids and its labelled form, once within cells, had not to compete with any endogenous equivalent, allowing it to be better incorporated than *cis*-9 into PL. In spite of the presence of endogenous *cis*-unsaturated FA, the notable incorporation of both *trans*-$C_{18:1}$ into PL suggests that these isomers, like saturated FA, were more convenient substrates for the *sn*-1 position, as has already been shown [36–38]. In this context, *trans*-9 was esterified significantly more than *trans*-11 in hepatocytes and marginally more in disrupted cells, which could result from greater or lower cell concentrations of unesterified *trans*-9 or *trans*-11, respectively. In this line, *trans*-9 that is less oxidised than *trans*-11 in liver mitochondria from fasted rats [19], should be present in concentration greater than that of *trans*-11 within cells. However in fed rat livers, the FA-oxidation pathway is strongly inhibited [48], which should normally maintain labelled *trans*-9 and *trans*-11 concentrations to equivalent levels in cells. A possibility was that either *trans*-isomer was transformed into another compound exhibiting distinct affinities for the available OH groups of PL. Indeed, *trans*-11 was found to give rise to *cis*-9, *trans*-11-$C_{18:2}$ (as a conjugated linoleic acid, CLA) through Δ-9 desaturation in liver microsomes, while under the same experimental conditions *trans*-9 stayed unchanged [49]. In whole rats, dietary *trans*-11 was also transformed into *cis*-9, *trans*-11-$C_{18:2}$ in most organs [50]. This CLA added to the diet of pigs was abundantly recovered in liver PL [51]. The gain of a *cis*-9 double bond could be thought to confer to the resulting CLA a high affinity for the *sn*-2 position of PL, but being then in competition, as labelled *cis*-9, with endogenous FA for this position, which would decrease its incorporation into PL via the initial labelling of its precursor. This hypothesis however was not relevant under our experimental conditions because the Δ-9 desaturation of *trans*-11 into CLA has been shown to be very weak in liver and to take place exclusively in peripheral tissues [52]. Further, contrary to what could be expected, *cis*-9, *trans*-11-CLA preferentially esterified as *trans*-11 onto the *sn*-1 position of PL [53].

Esterification-Related Enzyme Activities

As previously mentioned, the esterification of isomers in intact and disrupted cells could be distorted because of the competition with endogenous FA and the fact that this reaction was connected or not to lipoprotein formation and membrane remodeling. By contrast, the measurement of biochemical activities with isolated subcellular particles in appropriate medium strongly limited the mentioned interferences and allowed exogenous *trans*-11 and *trans*-9 to be strictly compared as substrates in the chosen activities. GPAT activity represents an early step common to TAG and PL synthesis with the esterification of acyl groups onto the *sn*-1 position of glycerol-3-phosphate. The about same decreasing order of activities with all the isomers in liver homogenates and microsomal fractions suggests that the whole cell GPAT activity was mainly supported by endoplasmic reticulum. The differences of activities found between both *trans*-$C_{18:1}$ could result from a preferential channeling of *trans*-9 towards the PL synthesis pathway in hepatocytes (Fig. 1a). Unexpectedly, these differences did not concomitantly give rise to comparable effects on the incorporation of both *trans*-$C_{18:1}$ into TAG. GPAT activities in mitochondria (mtGPAT) were qualitatively inversed with these isomers, compared to those in microsomes, but were likely of too low amplitude to affect the distribution of these labelled isomers in TAG and PL of intact or disrupted cells (Fig. 1a, b). This effect could however influence the FA esterification/oxidation balance in mitochondria, since mtGPAT and carnitine palmitoyltransferase I activities have been shown to interfere negatively each other in fasted rats [54].

With glycerol-derived compounds whose *sn*-1 and *sn*-3 positions are already occupied, the esterification activity in microsomes was comparable with both *trans*-isomers when the *sn*-3 position was bound to phosphate, but totally different when this position was bound to phosphocholine. The latter activity was much more greater with *cis*-9 and *trans*-9 isomers than with their *cis*-11 and *trans*-11 counterparts, which indicates that the position of the double bond in the acyl chain was then more important than its geometric configuration. This reaction, catalysed by 2-lysophosphatidylcholine acyltransferase, was shown to possess lysophospholipase and transacylase activities [55], and was believed to take part in the permanent remodeling of membrane PL [56]. Further the purified enzyme exhibited much affinity for arachidonoyl-CoA and to a lesser extent for linoleoyl- and oleoyl-CoA [57, 58]. The comparable

enzyme activity in our assays with oleoyl- and elaidoyl-CoA suggests that *trans*-9 could participate much more actively than *trans*-11 in the PL remodeling. The higher GPAT and 2-lysophosphatidylcholine acyltransferase activities exhibited by isolated microsomes could explain, at least partly, that *trans*-9 was incorporated to a greater extent than *trans*-11 into PL of hepatocytes (Fig. 1a). This point was supported by other studies carried out in hamsters fed diets containing both *trans*-isomers and showing that *trans*-9 was recovered more abundantly than *trans*-11 in *sn*-2 position of platelet PL [14].

Lipoprotein Secretion by Hepatocytes

Measurements of apolipoproteins, TAG and total cholesterol did not reveal any manifest effect of the exposure of hepatocytes to isomers, relative to control assays. Indeed similar experiments performed in the presence of insulin demonstrated that TAG synthesis and secretion were stimulated [59, 60], but that apolipoprotein secretions were equivalent [59] or decreased [60, 61], depending on the time of incubation. When oleic acid was added to incubation medium [59], TAG synthesis and secretion were increased, which meets our results (Fig. 4c). The same effect was observed with *trans*-11, but not with *trans*-9 that could be used preferentially for PL synthesis/remodeling as already suggested. The same amounts of cholesterol secreted by hepatocytes exposed to *trans*-11 versus *trans*-9 (Fig. 4d) appeared to disagree with the different levels of labelled cholesterol released from cells previously incubated with [^{14}C] cholesterol after exposure to both *trans*-$C_{18:1}$ (Fig. 5). In fact during the preincubation time of this latter experiment, exogenous cholesterol was expected to settle down between membrane PL. The collected data suggest that the greater incorporation of *trans*-9 versus *trans*-11 into whole PL of hepatocytes (Fig. 1a) allowed more cholesterol molecules to be shifted from their unsteady positions into the surrounding medium for lipoprotein formation. This would not be the case for *trans*-11 that, contrary to *trans*-9, was associated with lower blood total and LDL-cholesterol in rats [62].

This study carried out with intact cells and cell subfractions demonstrates that vaccenic and elaidic acids, in spite of their chemical resemblance, differ as substrates in esterification-related activities, particularly for PL synthesis and remodeling. It could be assumed that the differences observed would be modified by the fasting period, known to be associated with increased FA oxidation [48], reduced mitochondrial GPAT activity [54] and greater oxidability of vaccenic versus elaidic acid [19]. All these differences found in ex vivo and in vitro assays with liver extracts could be modulated in whole animals, for instance because *cis*-9, *trans*-11-CLA mainly synthesised from *trans*-11 in extrahepatic organs might interfere in the liver lipid metabolism. In the same way, hormonal specificities could differently affect FA oxidation and esterification pathways in humans ingesting both *trans*-$C_{18:1}$ [6]. These data support the fact that vaccenic and elaidic acids are dissimilar substrates for crucial steps of the PL synthesis pathway in liver.

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