CLA-Enriched Diet Containing t10,c12-CLA Alters Bile Acid Homeostasis and Increases the Risk of Cholelithiasis in Mice1–3

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4Physiologie de la Nutrition, UMR INSERM U 866/ Université de Bourgogne, AgroSup Dijon, 21000 Dijon, France; 5Department of Nutrition and Food Science, Faculty of Pharmacy, University of Pais Vasco, 01006 Victoria, Spain; and 6Plateau Technique Lipidomique, IFR100 / Université de Bourgogne, 21000 Dijon France

Abstract

Mice fed a mixture of CLA containing t10,c12-CLA lose fat mass and develop hyperinsulinemia and hepatic steatosis due to an accumulation of TG and cholesterol. Because cholesterol is the precursor in bile acid (BA) synthesis, we investigated whether t10,c12-CLA alters BA metabolism. In Expt. 1, female C57Bl/6J mice were fed a standard diet for 28 d supplemented with a CLA mixture (1 g/100 g) or not (controls). In Expt. 2, the feeding period was reduced to 4, 6, and 10 d. In Expt. 3, mice were fed a diet supplemented with linoleic acid, c9,t11-CLA, or t10,c12-CLA (0.4 g/100 g) for 28 d. In Expt. 1, the BA pool size was greater in CLA-fed mice than in controls and the entero-hepatic circulation of BA was altered due to greater BA synthesis and ileal reclamation. This resulted from higher hepatic cholesterol 7α-hydroxylase (CYP7A1) and ileal apical sodium BA transporter expressions in CLA-fed mice. Furthermore, hepatic Na+/taurocholate co-transporting polypeptide (NTCP) (~52%) and bile salt export pump (BSEP) (~77%) protein levels were lower in CLA-fed mice than in controls, leading to a greater accumulation of BA in the plasma (+500%); also, the cholesterol saturation index and the concentration of hydrophobic BA in the bile were greater in CLA-fed mice, changes associated with the presence of cholesterol crystals. Expt. 2 suggests that CLA-mediated changes were caused by hyperinsulinemia, which occurred after 6 d of the CLA diet before NTCP and BSEP mRNA downregulation (10 d). Expt. 3 demonstrated that only t10,c12-CLA altered NTCP and BSEP mRNA levels. In conclusion, t10,c12-CLA alters BA homeostasis and increases the risk of cholelithiasis in mice. J. Nutr. 141: 1437–1444, 2011.

Introduction

CLA refers to a group of geometric and positional conjugated dienoic isomers produced during biological or industrial hydrogenation of linoleic acid [18:2(n-6), Δc9, c12] (1). The major dietary sources of CLA are ruminant meat, dairy products, and partially hydrogenated vegetable oils. In the rumens of sheep and cattle, microbial bioconversion mainly produces the c9,t11-CLA isomer, whereas dietary supplements and functional foods contain c9,t11-CLA and t10,c12-CLA isomers in approximately equal amounts (2).

The possible beneficial effects of CLA on health as reported in various experimental models have resulted in increasing interest in these molecules. Besides the anticarcinogenic and antiatherogenic effects of CLA, CLA-enriched diets lead to a decrease in fat stores in several species, including pigs, rats, hamsters, chickens, mice, and humans (3), suggesting that CLA might be useful as weight-loss agents. However, several side effects have been reported in mice fed diets supplemented with a CLA mixture. CLA-mediated fat loss is associated with insulin resistance, severe hyperinsulinemia, massive liver steatosis (4), and inflammation of white adipose tissue (5). This lipoatrophic syndrome is due to only the t10,c12-CLA isomer (6). These side effects, found in mice, which are particularly sensitive to t10,c12-CLA, might also occur in humans. A meta-analysis of 18 clinical studies reported a slight but significant reduction in fat stores in overweight or obese participants receiving CLA supplementation (7). This reduction was associated with impaired insulin sensitivity (8–12) or an increase in lipid peroxidation or biomarkers of inflammatory diseases (13,14). Despite these data, dietary supplements and functional foods containing high levels of t10,c12-CLA are currently sold in several countries.

1 Supported by Groupe Lipides et Nutrition and by a fellowship from the Council of Burgundy. Amaia Zabala was supported by grants from the Ministerio de Ciencia y Tecnología (BFI2002-00273) and the Ministerio de Educación y Ciencia (AGL2005-02494).
3 Supplemental Table 1 is available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.
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Liver steatosis triggered by the t10,c12-CLA isomer is due to the concomitant activation of the lipogenic pathway and repression of fatty acid β-oxidation leading to a huge accumulation of TG (15,16). CLA-enriched diets also lead to cholesterol accumulation in the liver through an unknown mechanism (17,18). Because hyperinsulinemia, hyperadiponectinemia, and the rapid loss of body mass observed in mice fed with t10,c12-CLA are risk factors for cholelithiasis (19,20), the accumulation of cholesterol in the liver could be associated with an alteration of bile acid (BA) homeostasis.

The complete synthesis of BA occurs only in the liver and includes several enzymatic steps involving hepatic P-450 cytochromes, such as cholesterol 7α-hydroxylase (CYP7A1), cholesterol 27α-hydroxylase (CYP27A1), 25-hydroxycholesterol 7α-hydroxylase (CYP7B1), and sterol 12α-hydroxylase (CYP8B1). Among these, CYP7A1 and CYP27A1 are considered rate limiting for the respective neutral and acidic pathways of BA synthesis. After conjugation with glycine or taurine in the hepatocyte, BA are secreted into bile by the bile salt export pump (BSEP) then stored in the gallbladder. During a meal, BA are released into the duodenum where they are required for the efficient absorption of dietary fat and lipid-soluble molecules. More than 95% of BA is reabsorbed, mainly in the ileum, by the apical sodium BA transporter (ASBT). Then they return via the portal blood to the liver where they are efficiently transported across the sinusoidal membrane of hepatocytes by the Na+-taurocholate co-transporting polypeptide (NTCP) and, to a lesser extent, by theorganic anion transporting polypeptide-1 (OATP1). This entero-hepatic circulation, which is essential for the maintenance of BA and cholesterol homeostasis (21), is controlled by a specific hormonal pathway. Indeed, CYP7A1 is regulated by fibroblast growth factor 15 (FGF15), which is secreted in the portal blood by ileocytes when BA are reclaims in the ileum. In the liver, FGF15 binds to fibroblast growth factor receptor 4 (FGFR-4), which works in concert with β-Klotho, a membrane-bound glycosidase, leading to the inhibition of CYP7A1 (22). Quantitatively, the most important way for the body to eliminate cholesterol is by converting it into BA and then subsequently eliminating it in the feces. BA are known to have other important functions in the body. They contribute to the solubilization of cholesterol in the gallbladder and thus provide protection against the risk of cholelithiasis. Moreover, BA are important regulators in the digestive tract, because they activate several nuclear receptors such as the farnesoid-X-receptor (FXR), pregnane-X-receptor, and vitamin-D receptor (23). Finally, it recently has been shown that BA are involved in the regulation of energy expenditure through the binding and activation of the G proteincoupled bile acid receptor (TGR5) (24).

This study was designed to determine how CLA intake affects hepatic cholesterol metabolism and whether or not it disturbs BA homeostasis. To test this hypothesis, we used female C57Bl/6j mice, which are prone to gallstone formation (25) and are highly responsive to CA (26). We report herein that a CLA diet containing t10,c12-CLA is responsible for profound quantitative and qualitative alterations in the hepatic metabolism and entero-hepatic circulation of BA. These changes increase the risk of cholelithiasis.

### Materials and Methods

#### Animals and experimental procedures

French guidelines for the use and care of laboratory mice were followed and protocols were approved by the ethics committee of the University of Burgundy. Female C57Bl/6j mice (10 wk old) purchased from Centre d’Elevage Janvier were maintained in a temperature-controlled room with 12-h-light/-dark cycle (lights on 0700–1900 h).

**Expt. 1.** To explore the effects of a CLA mixture on cholesterol and BA metabolism, 6 female mice/diet were housed in physiological cages and consumed ad libitum a purified diet (SAFE) containing either 2.4% Iso 4 oil (Lesieur; 10% SFA, 45% MUFA, and 45% PUFA, with 43.1% of linoleic acid and 1.9% of linolenic acid) (control diet), or 1.4% oil plus a 1% CLA mixture obtained from Natural Lipids. The composition of the mixture was 92% CLA isomers, 5.7% oleic acid, 0.5% palmitic acid, 0.4% linoleic acid, and 0.1% stearic acid. The main CLA isomers were c9,t11-CLA (42.8%) and t10,c12-CLA (44.6%) (g/100 g) (Table 1). The mice were freshly prepared every day. Body mass gain and food intake were monitored and stools and urine were removed at regular intervals and kept at −20°C before analysis. After 28 d of diet and 4 h of food deprivation, the isoflurane-anesthetized mice were bled to death by sectioning the axillary vessels. Blood and bile were rapidly removed and the liver, perirenal adipose tissue, and total small intestine were collected, weighed, and frozen in liquid nitrogen before being stored at −80°C. To measure BA transporter expression, the ileal mucosa was removed by scraping the last 3rd of the small intestine in the other mice. To estimate the risk of cholelithiasis, an aliquot of fresh gallbladder bile from each mouse was placed on a glass slide at room temperature (22°C) and viewed using polarized light microscopy (Nikon Eclipse TE2000-U).

**Expt. 2.** To determine the effects of a CLA-enriched diet over time, female C57Bl/6j mice were fed with the same CLA-enriched diet as in Expt. 1 for 0, 4, 6, and 10 d. At each time point, 5 mice were killed and

<table>
<thead>
<tr>
<th>TABLE 1 Composition of the experimental diets</th>
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<tr>
<td><strong>Ingredients</strong></td>
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<tr>
<td>Iso 4 oil a</td>
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<td>CLA mixture b</td>
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<tr>
<td>Linolic acid c</td>
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<tr>
<td>c9,t11-CLA d</td>
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<tr>
<td>t10,c12-CLA d</td>
</tr>
<tr>
<td>Cholesterol</td>
</tr>
<tr>
<td>Casein</td>
</tr>
<tr>
<td>Glucose + starch</td>
</tr>
<tr>
<td>Cellulose</td>
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<tr>
<td>Mineral mixture d</td>
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<tr>
<td>Vitamin mixture e</td>
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<td><strong>g/kg</strong></td>
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a Abbreviations used: ASBT, apical sodium bile acid transporter; BA, bile acid; BSEP, bile salt export pump; CSI, cholesterol saturation index; CYP7A1, cholesterol 7α-hydroxylase; CYP27A1, cholesterol 27α-hydroxylase; CYP7B1, 25-hydroxycholesterol 7α-hydroxylase; CYP8B1, sterol 12α-hydroxylase; FGF15, fibroblast growth factor 15; FGFR4, fibroblast growth factor receptor 4; FXR, farnesoid-X-receptor; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; HNF, hepatocyte Nf; LDLr, LDL receptor; NTCP, Na+taurocholate co-transporting polypeptide; SREBP1a and 2, sterol regulatory element-binding protein 1a and 2; TGR5, G-protein-coupled bile acid receptor.

b CLA mixture: 92% of CLA including 42.8% of c9,t11-CLA and 44.6% of t10,c12-CLA; linearic acid, 99%; c9,t11-CLA, 91.6%; t10,c12-CLA, 96.2%.

c Linoleic acid 487. 487, 487, 487, 487.

d CLA mixture: 92% of CLA including 42.8% of c9,t11-CLA and 44.6% of t10,c12-CLA; linearic acid, 99%; c9,t11-CLA, 91.6%; t10,c12-CLA, 96.2%.

1 Iso 4 oil (Lesieur): 10% SFA, 45% MUFA, and 45% PUFA, with 43.1% of linoleic acid and 1.9% of linolenic acid.

2 Purify: CLA mixture, 92% of CLA including 42.8% of c9,t11-CLA and 44.6% of t10,c12-CLA; linearic acid, 99%; c9,t11-CLA, 91.6%; t10,c12-CLA, 96.2%.

3 CaCO3, 12 g; K2HPO4, 10.75 g; CaHPO4, 10.75 g; MgSO4, 7H2O, 5 g; NaCl, 3 g; MgO, 2 g; FeSO4, 7H2O, 400 mg; ZnSO4, 7H2O, 350 mg; MnSO4, 7H2O, 100 mg; CuSO4, 5H2O, 50 mg; Na2SO4, 3H2O, 25 mg; AlK2SO4, 12H2O, 10 mg; K2CrO4, 7.5 mg; NaF, 5 mg; NiSO4.6H2O, 5 mg; H2BO3, 5 mg; CoSO4, 7H2O, 2.5 mg; KI, 2 mg; (NH4)2MnO4, 4H2O, 1 mg; LiCl, 0.75 mg; Na2SeO3, 0.75 mg; Na2VO4, 0.5 mg; sucrose, 5.5 g.

4 Retinol acetate, 2.75 mg; cholecalciferol, 31.25 μg; α-tocopherol acetate, 100 μg; phytolchinone, 1 mg; thymine chloride hydrate, 10 mg; riboflavin, 10 mg; nicotinic acid, 50 mg; Ca-pantothenate, 25 mg; pyridoxin chloride hydrate, 10 mg; D-biotin, 0.2 mg; folic acid, 2 mg; cyanoacobalamin, 25 μg; choline chloride hydrate, 1 g; β-methionine, 2 g; p-aminobenzoic acid, 50 mg; inositol, 100 mg; sucrose, 5.5 g.

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their livers were weighed then rapidly frozen in liquid nitrogen and stored at -80°C.

Expt. 3. To explore the effects of the 2 main CLA isomers found in the CLA mixture on hepatic BA transporters, female mice were fed ad libitum for 4 wk on a purified diet (SAFE) containing either 2.4% oil (control diet) or 2% oil plus 0.4% linoleic acid (LA diet; Sigma; 99% purity; n = 5), highly purified CLA isomers (i.e. c9,t11-CLA; 91.6% purity; n = 5), or t10,c12-CLA (96.2% purity; n = 5) diets (Natural Lipids) (Table 1). The mice were killed and their livers were collected, weighed, and rapidly frozen in liquid nitrogen and stored at -80°C.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CLA</th>
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<tbody>
<tr>
<td>Body mass, g</td>
<td>21.1 ± 0.4</td>
<td>18.6 ± 0.5**</td>
</tr>
<tr>
<td>Energy intake, kcal/d</td>
<td>65.4 ± 1.2</td>
<td>66.8 ± 0.9</td>
</tr>
<tr>
<td>Perirenal adipose tissue mass, % body mass</td>
<td>2.56 ± 0.10</td>
<td>0.17 ± 0.04***</td>
</tr>
<tr>
<td>Liver mass, % body mass</td>
<td>5.21 ± 0.21</td>
<td>14.1 ± 1.42**</td>
</tr>
<tr>
<td>Hepatic TG, μmol/g</td>
<td>27.3 ± 1.7</td>
<td>54.9 ± 2.7***</td>
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### Plasma analytes

- Glucose, mmol/L: Control: 7.77 ± 0.06, CLA: 6.66 ± 0.06
- TG, mmol/L: Control: 0.60 ± 0.07, CLA: 0.47 ± 0.06
- Cholesterol, mmol/L: Control: 1.66 ± 0.10, CLA: 2.27 ± 0.19*
- Insulin, pmol/L: Control: 78 ± 12, CLA: 202 ± 15***
- Albumin, g/L: Control: 13.5 ± 0.8, CLA: 15.5 ± 1.2
- Bilirubin, μmol/L: Control: <2, CLA: <2
- Aspartate aminotransferase, μkat/L: Control: 2.60 ± 0.62, CLA: 5.65 ± 0.68***
- Alanine aminotransferase, μkat/L: Control: 0.47 ± 0.10, CLA: 2.53 ± 0.20***
- Alkaline phosphatase, μkat/L: Control: 1.48 ± 0.11, CLA: 3.52 ± 0.22***

1 Values are means ± SEM, n = 6. Asterisks indicate different from control: *P < 0.05; **P < 0.01; ***P < 0.001.

### Serum analysis

Serum glucose (Gluose RTU kit, Biotrak), TG (GPO-PAP method, Boehringer Mannheim), cholesterol (CHOD-PAP method, Boehringer Mannheim), albumin (Randox Albumin kit, Randox), bilirubin (Randox Bilirubin kit, Randox), aspartate aminotransferase (Randox AST kit, Randox), alanine aminotransferase (Randox ALT kit, Randox), and alkaline phosphatase (Randox Alkaline Phosphatase kit, Randox) were determined by enzymatic methods. Serum insulin was assayed by RIA (no. 621NSPBE, CIS Bio).

### Hepatic lipid analysis

Total hepatic lipid content was assayed by the Delsal method (27). Briefly, liver (0.5 g) was homogenized and extracted twice with dimethoxymethane/methanol (4:1, v:v) then allowed to settle and was filtered. The filtrate was evaporated under vacuum to remove the solvent. Dried extracts were weighed to estimate total hepatic lipid content then dissolved in 1% Triton X-100 in chloroform, dried under vacuum, and redissolved in water to measure TG, phospholipids, and free and total cholesterol using commercial kits (Triglycerides FS, Phospholipids FS, Free cholesterol FS, Cholesterol FS, DiaSys). Finally, the concentration of esterified cholesterol was calculated from the difference between the concentrations of total and free cholesterol.

### Quantification of BA

BA were extracted from livers and bile from gallbladders, plasma, intestines, stools, and urine, as described by Keller et al. (28), using norcholanic acid as the internal standard and derivatized as pentfluorobenzyl esters and trimethylsilyl ethers (Steraloids). The BA derivatives were analyzed on an Agilent Technologies quadrupole GC-MS instrument (MSD 5975, GC 7890A, Column HP5-MS, 30 m × 0.25 mm). The ion source was operated in the negative ion chemical ionization mode at 150°C, with methane as the reagent gas. Analysis was performed in the selected ion monitoring mode on [M-PF2]⁻ ions.

### Determination of the cholesterol saturation index

Biliary lipids were extracted as described by Folch et al. (29) and biliary cholesterol and phospholipid concentrations were analyzed using commercial kits (Cholesterol kit, Phospholipid kit, BioSystems). The cholesterol saturation index (CSI) was determined according to (30).

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**FIGURE 1** Hepatic cholesterol metabolism in mice fed a control diet (C) or CLA mixture-supplemented diet (1 g/100 g) for 28 d (Expt. 1). (A) Liver total cholesterol (TC), nonesterified cholesterol (NEC), and esterified cholesterol (CE). (B) Relative mRNA level of genes involved in cholesterol uptake, synthesis, and excretion. Values were normalized to 36B4 mRNA level and compared with controls. (C) HMG-CoA reductase immunoblotting performed on liver homogenates. These data were normalized to β-Actin. (D) mRNA level of SREBP1a and SREBP2. Values were normalized to the 36b4 mRNA level. Values are means ± SEM, n = 6 or 4 (C). Asterisks indicate different from control: *P < 0.05; **P < 0.01; ***P < 0.001.
Real-time qPCR
cDNA was reverse transcribed from 1 μg of total RNA pretreated with
DNase I Amplification grade (Invitrogen Life Technologies) and Omni-
script reverse transcriptase (Qiagen). The cDNA was diluted to 12.5 ng
in 1 μL using sterilized water, and real time PCR was done in duplicate
with 2 μL of cDNA, 12.5 μL of qPCR MasterMix from Eurogentec,
10.5 μL of distilled water, and 1 μL of forward and reverse primers (200
nmol/L) for a final reaction volume of 25 μL. The primers and fluorogenic
probes used in this study are described in Supplemental Table 1. PCR
was run on the iCycler iQ system (Bio-Rad Laboratories). The fluorescence
used to calculate the threshold cycle was measured at 60°C. Data were
quantified using the comparative ΔΔCt method (31) with 36B4 and 18S
as housekeeping genes.

Western blotting
Total proteins extracted from the liver (50 μg) and ileum mucosa (15 μg)
were prepared in ice-cold buffer (0.154 mol/L KCl, 0.01 mol/L phosphate
buffer, pH 7.4), separated on 7.5% or 12% SDS-PAGE, and then blotted
onto a Polyscreen membrane (PerkinElmer Life Sciences) according to the
manufacturer’s protocol (Bio-Rad). Anti-3-hydroxy-3-methylglutaryl
CoA (HMG-CoA) reductase (H300) and anti-CYP7A1 (1/400) anti-
bodies were purchased from Santa-Cruz (1/200); anti-NTCP (1/5000)
and anti-BSEP (1/1000) antibodies were generously donated by Dr. B.
Steiger, University Hospital Zurich, Division of Clinical Pharmacology
and Toxicology, Zurich, Switzerland and anti-ASBT (1/3000) was generated
by our laboratory (32), anti-actin (1/10000) (Sigma), anti-Hsc70 (1/10000)
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(Santa-Cruz), or anti-intestinal fatty acid-binding protein (I-FABP) (1/1000)
(33) antibodies were used as controls. Peroxidase-conjugated secondary
antibodies were purchased from Sigma. Detection was performed using
the ECL blotting kit (PerkinElmer Life Sciences), Blot size was quantified
using a GS-800 calibrated densitometer (Bio-Rad).

Statistical analysis
The results are expressed as means ± SEM. Means in Expt. 1 were
compared by Student’s t test to determine significance. Data in Expts. 2
and 3 were statistically analyzed with a 1-way ANOVA and significant
differences between the CLA-treated groups and the control group were
identified by Dunnett’s test. When the variances were not equal, the data
differences between the CLA-treated groups and the control group were
statistically analyzed with a 1-way ANOVA and significant
0.05 was considered significant. The SigmaStat statistical software
package was used for all the statistical analyses.

Western blotting
Total proteins extracted from the liver (50 μg) and ileum mucosa (15 μg)
were prepared in ice-cold buffer (0.154 mol/L KCl, 0.01 mol/L phosphate
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differences between the CLA-treated groups and the control group were
identified by Dunnett’s test. When the variances were not equal, the data
were transformed logarithmically, and the transformed data were then
analyzed by Student’s t tests or ANOVA followed by Dunnett’s test. P <
0.05 was considered significant. The SigmaStat statistical software
package was used for all the statistical analyses.

Results
CLA supplementation induces cholesterol accumulation in the liver (Expt. 1). In Expt. 1, a 4-wk supplementation with a
CLA mixture triggered a lipoatrophic syndrome in mice charac-
terized by a lower body weight (~12%) and lower perirenal adipose tissue mass (~93%) associated with hyperinsulinemia
and liver enlargement (~270%) with steatosis compared with controls (Table 2). Food intake did not differ between the 2
groups after 4 wk of diet (Table 2). Steatosis in the livers of CLA-
Fed mice was due to an accumulation of TG and cholesterol,
maintly in the form of cholesteryl esters (Table 2; Fig. 1A). More-
over, hepatic injury was characterized by greater plasma trans-
aminase and phosphatase activity in the CLA-fed mice than that
in controls (Table 2). Cholesterol accumulation in the liver of CLA-fed mice was probably due to higher levels of LDL receptor
(LDLr; mRNA) and HMG-CoA reductase (mRNA and protein)
in CLA-fed mice than in controls (Fig. 1B,C). The gene expression analysis of
transcriptional factors involved in LDLr and HMG-CoA reduc-
tase regulation demonstrated that only the level of sterol regu-
larity element-binding protein (SREBP1a) mRNA was greater in
CLA-fed mice than in controls (Fig. 1D).

CLA supplementation increases BA pool size (Expt. 1). The
BA content in the liver (~72%) and gallbladder + intestine

Table 2

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Liver, mmol/kg body weight</td>
<td>4.8 ± 0.6</td>
<td>8.2 ± 1.6*</td>
</tr>
<tr>
<td>Gallbladder + intestine, mmol/kg body weight</td>
<td>120 ± 12</td>
<td>284 ± 43***</td>
</tr>
<tr>
<td>Plasma, μmol/L</td>
<td>2.1 ± 0.3</td>
<td>12.6 ± 3.9**</td>
</tr>
<tr>
<td>Urine, mmol/d</td>
<td>2.8 ± 0.7</td>
<td>7.8 ± 1.3**</td>
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<tr>
<td>Feces, μmol/d</td>
<td>6.5 ± 1.3</td>
<td>2.0 ± 0.3***</td>
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</table>

1 Values are means ± SEM, n = 6. Asterisks indicate different from control: *P < 0.05; **P < 0.01; ***P < 0.001.

FIGURE 2 Entero-hepatic circulation of BA in mice fed a control diet (C) or CLA mixture-supplemented diet (1 g/100 g) for 28 d (Expt. 1) or
0, 4, 6, or 10 d (Expt. 2). (A) NTCP and BSEP mRNA levels. Values were normalized to 36B4 and compared with controls. (B) NTCP and BSEP protein
levels. β-Actin served as an internal control for protein loading. (C) ASBT expression from ileum mucosa. Homodimer (49 kDa) and heterodimer
(98 kDa) forms of ASBT were detected. These data were normalized to I-FABP. (D) Kinetics of CLA feeding effect on NTCP and BSEP mRNA levels.
Values were normalized to 36B4 and compared with values at 0 d. Values are means ± SEM, n = 6 (A,D) or 4 (B,C). Asterisks indicate different from control:
*P < 0.05; **P < 0.01; ***P < 0.001.

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1 Values are means ± SEM, n = 6. Asterisks indicate different from control: *P < 0.05; **P < 0.01; ***P < 0.001.
CLA supplementation alters enterohepatic circulation of BA (Expts. 1 and 2). In Expt. 1, NTCP and BSEP expression (mRNA and protein), which are responsible for BA circulation through the liver, were lower in CLA-fed mice than in controls (Fig. 2A,B). The OATP-1 mRNA level was also lower in CLA-fed mice (0.15 ± 0.02) than in controls (1.00 ± 0.27) (P < 0.01) (data not shown). In contrast, homo- and heterodimer forms of ASBT protein were greater in the ileum of CLA-treated mice than in controls (Fig. 2C). These effects are consistent with the higher plasma BA levels in CLA-fed mice than in control mice (Table 3). Expt. 2 demonstrated that it was necessary to feed the mice with the CLA-supplemented diet for at least 10 d to observe the decrease in hepatic NTCP and BSEP mRNA levels compared with controls (Fig. 2D). Altogether, these data demonstrate that chronic supplementation with a CLA mixture profoundly affects the entero-hepatic circulation of BA in mice.

t10,c12-CLA supplementation specifically alters NTCP and BSEP gene expression (Expt. 3). NTCP and BSEP mRNA levels were lower only in mice fed the t10,c12-CLA isomer-supplemented diet (Fig. 3). These data suggest that t10,c12-CLA supplementation specifically affects the entero-hepatic circulation of BA in mice.

CLA supplementation affects hepatic BA synthesis (Expt. 1). Liver CYP27A1, CYP7B1, and CYP8B1 mRNA levels were lower in mice fed a CLA-enriched diet for 28 d than in controls (Fig. 4A). Although the mRNA level of CYP7A1 was not significantly modified by CLA supplementation (Fig. 4A), its protein level was greater in CLA-fed mice than in control mice (Fig. 4B).

To gain insight into the molecular mechanisms responsible for CLA-mediated effects in hepatic BA synthesis, the expression of nuclear receptors known to regulate these pathways in the liver was explored. Mice fed the CLA-supplemented diet for 28 d had lower expression of nuclear receptors known to inhibit BA influx and synthesis and to stimulate BA excretion (FXR, liver receptor homolog 1 (LRH-1), and small heterodimer partner) (Fig. 4C) than did controls. Similar results were found for hepatocyte nuclear factor (HNF)-1α and HNF4α, which are involved in the activation of neutral and acidic BA synthesis (Fig. 4C).

Interestingly, the expression of liver-X-receptor-α, which is a major regulator of the CYP7A1, was similar in the different groups (Fig. 4C). Moreover, intestinal levels of FGF15 mRNA and hepatic levels of FGFR-4 and β-Klotho mRNA were lower in CLA-fed mice than in controls, suggesting that the FGF15 pathway was downregulated (Fig. 4D). Therefore, dietary supplementation with a CLA mixture promotes the BA biosynthetic pathway via the upregulation of CYP7A1.

CLA supplementation increases the risk of cholelithiasis (Expt. 1). To explore whether the CLA-supplemented diet can also alter the quality of BA as suggested by the downregulation of the enzyme involved in the acidic pathway, the BA profile in bile was determined in mice fed a diet supplemented with a CLA mixture for 28 d. CLA led to a more hydrophobic BA profile (+136%) in the mice fed the CLA-enriched diet for 28 d was greater than in controls (Table 3). Moreover, the plasma BA concentration was higher in CLA-fed mice than in controls and was associated with a greater excretion of BA in urine. In contrast, BA levels in the feces were lower in the CLA-fed mice than in controls (−70%) (Table 3). Therefore, the BA pool size in mice fed the CLA-enriched diet for 28 d was greater than that in control mice.

Discussion

Mice fed a diet supplemented with a CLA mixture containing t10,12-CLA develop a lipoatrophic syndrome mainly character-
cholesterol and BA. A concomitant rise in the expression of genes encoding for LDLr and HMG-CoA reductase involved in cholesterol uptake and synthesis by hepatocytes, respectively, was found in CLA-fed mice. Consistent with these changes, these mice also had higher levels of cholesterol in the liver than did controls. CLA-fed mice also had an increased BA pool size, probably due to the rise in the CYP7A1 protein level. They also displayed profound alterations in the entero-hepatic circulation of BA. Increased intestinal ASBT protein level was found, suggesting a more efficient reclamation of BA. This change, as well as a drop in hepatic BA transporters NTCP and BSEP that was exclusively due to the t10,c12-CLA isomer, led to an accumulation of BA in the blood and urine and a decrease in BA in feces. Finally, the CLA-enriched diet also led to a more hydrophobic BA profile, which is in accordance with the 2-fold decrease in mRNA encoding for the main enzymes involved in the acidic pathway: CYP27A1, CYP7B1, and CYP8B1. In the liver, the coordinated downregulation of both key enzymes of the acidic BA pathway and BA transporters NTCP and OATP1 might be attributed to a decrease in HNF4, which is one of their common regulators (35,36). This hypothesis is reinforced by a recent study that demonstrated that in polygenic obese mice fed a diet supplemented with t10,c12-CLA, several HNF4 target genes were downregulated (37). Moreover, we report herein that CLA supplementation also decreases the expression of FXR, the major regulator of BSEP (38). This downregulation might explain the decrease in BSEP expression despite the accumulation of BA in the liver.

These adverse CLA-induced alterations in cholesterol and BA metabolic pathways promote the formation of cholesterol crystals, which is considered as a preliminary step in gallstone synthesis. This phenomenon was due to a 2.1-fold rise in the biliary CSI induced by a concomitant increase in the cholesterol level and a decrease in the content of both phospholipid and BA in the bile. Importantly, it is noteworthy that in our study, this increased risk of cholelithiasis occurred in mice fed a low-fat diet (3% lipids, 0.02% cholesterol, wt:wt). This finding strongly suggests that CLA supplementation to a lithogenic diet or a high-cholesterol diet might dramatically increase the risk of cholelithiasis. In keeping with this hypothesis, hamsters, in which the metabolism of cholesterol and BA is similar to that in humans, fed a hypercholesterolemic diet supplemented with 1% CLA rapidly developed gallstones (39,40).

The origin of the CLA-mediated effects on cholesterol and BA pathways remains elusive. Both direct (genic) and indirect (endocrine) effects are expected. However, several lines of evidence strongly suggest that hyperinsulinemia triggered by t10,c12-CLA plays a fundamental role. First, insulin is known to regulate the transcriptional level and activity of HMGCoA reductase. Therefore, cholesterol accumulation in the liver observed in CLA-fed mice might be due to the upregulation of HMGCoA reductase by SREBP1a, which is induced by insulin (41) or a decrease in cAMP triggered by insulin, which, in turn, is known to increase HMG-CoA reductase activity (42). Second, hyperinsulinemia occurred at 6 d, thus before the alteration of NTCP and BSEP mRNA levels that decreased only after 10 d of the CLA-enriched diet (Fig. 2). Third, it has been reported that hyperinsulinemia increases the CSI in bile and thus the risk of cholelithiasis (19).

The accumulation of BA in plasma triggered by nutritional supplementation with a CLA mixture might lead to the activation of TGR5 (44). The signaling pathway of this receptor triggers an increase in energy expenditure by controlling triiodothyronine synthesis (24). Moreover, it induces insulinemia by stimulating the secretion of the incretin glucagon-like peptide 1 (45). Finally,
TGR5 seems to be involved in the development of cholesterol gallstones (46). Interestingly, these TGR5 effects are similar to those observed in CLA-treated mice. Indeed, CLA leads not only to increased energy expenditure (47,48) and plasma triiodothyronine levels (49) but also to hyperinsulinemia and an increased risk of cholelithiasis (Fig. 5) (39,40). More investigations are required to check whether the effects of t10,c12-CLA are mediated by TGR5.

Taken together, these data raise the question of the effects of t10,c12-CLA on BA homeostasis in humans even though the dose of t10,c12-CLA used here, alone, or in a mixture (containing other isomers like rumenic acid) was higher than that used in human studies when evaluated per kilogram of body mass. This disturbance of BA homeostasis might be verified in humans by measuring levels of BA in the plasma. Such measurements could then constitute a biomarker of the t10,c12-CLA effect.

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FIGURE 5 BA profile of bile (A), cholesterol saturation index (CSI) (B), and presence of cholesterol crystals in freshly taken bile observed by polarizing light microscopy (C) in mice fed with a control diet (C) and a CLA mixture-supplemented diet (1 g/100 g) for 28d (Expt. 1). Values were means ± SEM, n = 6. Asterisks indicate different from control: *P < 0.05; **P < 0.01; ***P < 0.001.


