Regulatory effects of arachidonate 5-lipoxygenase on hepatic microsomal TG transfer protein activity and VLDL-triglyceride and apoB secretion in obese mice

Marta López-Parra,* Esther Titos,* Raquel Horrillo,* Natàlia Ferré,* Ana González-Périz,* Marcos Martínez-Clemente,* Anna Planagumà,* Jaime Masferrer,§ Vicente Arroyo,† and Joan Clària[1,*]

Department of Biochemistry and Molecular Genetics,* Liver Unit, Hospital Clínic,† Centro de Investigación Biomédica Esther Koplowitz (CIBEK) and Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona 08036, Spain; and Pfizer, St. Louis, MO 63017§

Abstract **As 5-lipoxygenase (5-LO) is an emerging target in obesity and insulin resistance, we have investigated whether this arachidonate pathway is also implicated in the progression of obesity-related fatty liver disease. Our results show that 5-LO activity and 5-LO-derived product levels are significantly elevated in the liver of obese ob/ob mice with respect to wild-type controls. Treatment of ob/ob mice with a selective 5-LO inhibitor exerted a remarkable protection from hepatic steatosis as revealed by decreased oil red-O staining and reduced hepatic triglyceride (TG) concentrations. In addition, 5-LO inhibition in ob/ob mice downregulated genes involved in hepatic fatty acid uptake (i.e., L-FABP and FAT/CD36) and normalized peroxisome proliferator-activated receptor alpha (PPARα) and acyl-CoA oxidase expression, whereas the expression of lipogenic genes [i.e., fatty acid synthase (FASN) and SREBP-1c] remained unaltered. Furthermore, 5-LO inhibition restored hepatic microsomal TG transfer protein (MTP) activity in parallel with a stimulation of hepatic VLDL-TG and apoB secretion in ob/ob mice. Consistent with these findings, 5-LO products directly inhibited MTP activity and triggered cytosolic TG accumulation in CC-1 cells, a murine hepatocyte cell line. Taken together, these findings identify a novel steatogenic role for 5-LO in the liver through mechanisms involving the regulation of hepatic MTP activity and VLDL-TG and apoB secretion.—** López-Parra, M., E. Titos, R. Horrillo, N. Ferré, A. González-Périz, M. Martínez-Clemente, A. Planagumà, J. Masferrer, V. Arroyo, and J. Clària. **Regulatory effects of arachidonate 5-lipoxygenase on hepatic microsomal TG transfer protein activity and VLDL-triglyceride and apoB secretion in obese mice. *J. Lipid Res.* 2008.** 49: **2513–2523.**

Supplementary key words arachidonate 5-LO • VLDL-TG • hepatic steatosis

In the early stages of liver injury, triglycerides (TG) accumulate in the cytoplasm of hepatocytes leading to hepatic steatosis. Although generally asymptomatic, hepatic steatosis or fatty liver is no longer regarded as a neutral and innocent bystander but rather as a premorbid condition that increases the vulnerability of this organ to progress to advanced liver disease (1, 2). Indeed, hepatic steatosis is frequently complicated by the presence of a prominent inflammatory component, a condition known as steatohepatitis, which may evolve in time to tissue damage and scar accumulation, progressive fibrosis, and eventually to liver cirrhosis (3–5). The prevalence of fatty liver disease and steatohepatitis is increasing worldwide in parallel with the rise in obesity, dyslipidemia, and insulin resistance, and they are considered the hepatic manifestations of the metabolic syndrome (1, 2).

The arachidonate 5-lipoxygenase (5-LO) pathway has recently been identified as an emerging target in obesity, insulin resistance, and atherosclerosis (6–14). In fact, 5-LO activating protein is overexpressed in the adipose tissue of obese patients with insulin resistance, and linkage analysis and gene mapping have identified 5-LO as a gene with pleiotropic actions on adipose fat accumulation (7–9). In

Supported by a grant from the Ministerio de Educación y Ciencia (MEC) (SAF 06/03191). Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD) is funded by the Instituto de Salud Carlos III. M. López-Parra has a contract with Instituto de Salud Carlos III, E. Titos is a recipient of a CIBEREHD contract, and N. Ferré is under a MEC Juan de la Cierva contract. A. González-Périz (BES-2004-5054) and M. Martínez-Clemente (BES-2007-16147) are supported by MEC fellowships. R. Horrillo is supported by Generalitat de Catalunya-European Social Funds (2006FI-00091).

Manuscript received 26 February 2008 and in revised form 14 July 2008.

Published, JLR Papers in Press, July 21, 2008.
DOI 10.1194/jlr.M800101-JLR200

Abbreviations: 5-LO, 5-lipoxygenase; ACO, acyl-CoA oxidase; DPBS, Dulbecco's Phosphate Buffered Saline; EMEM, Eagle's minimal essential medium; FBS, fetal bovine serum; FASN, fatty acid synthase; FAT/CD36, fatty acid translocase; L-FABP, liver fatty acid-binding protein; LTB_4, leukotriene B_4; LTD_4, leukotriene D_4; MTP, microsomal TG transfer protein; NEAA, nonessential amino acids; OCT, optimal cutting temperature; PPARα, peroxisome proliferator-activated receptor alpha; SREBP-1c, sterol response element-binding protein-1c; TG, triglyceride.

[1] To whom correspondence should be addressed.
e-mail: jclaria@clinic.ub.es

addition, augmented formation of 5-LO-derived products has been detected in cells growing under hyperglycemic and hyperinsulinemic conditions (10). Likewise, the 5-LO pathway has been established as a risk factor in atherosclerosis, and an allelic variant of the 5-LO gene has been identified in patients with high-risk of atherosclerosis (11, 12). Importantly, 5-LO colocalizes with inflammatory cells in the atheroma plaque and appears to promote the pathogenesis of hyperlipidemia-dependent vascular complications (6, 13, 14). However, the potential involvement of the 5-LO pathway in the progression of fatty liver disease remains to be clarified.

In the present study, we examined the expression, activity, and functional role of 5-LO in hepatic steatosis in ob/ob mice, an experimental model of obesity-related fatty liver disease (3, 15). In these animals, we assessed key factors governing the progression of hepatic steatosis (i.e., fatty acid uptake, lipogenesis, and fatty acid oxidation), the activity of the microsomal TG transfer protein (MTP) and VLDL-TG and apoB secretion. In addition, we examined the effect of 5-LO products [leukotriene (LT) B_4 and LTD_4] on mechanisms regulating TG synthesis and export in CC-1 cells, a murine hepatocyte cell line.

MATERIALS AND METHODS

Experimental Studies

Adult male wild-type (control) and leptin-deficient mice (ob/ob mice) in the C57BL/6J background (The Jackson Laboratory, Bar Harbor, ME) received either a standard mouse chow (placebo group) or a chow containing a 5-LO inhibitor (5-LO inhibitor group) for 14 days. The 5-LO inhibitor used in this study was the compound CJ-13,610, a nonredox-type 5-LO inhibitor provided by Pfizer (St. Louis, MO), with proven efficacy in vivo and in vitro (16–18). CJ-13,610 was incorporated into the diet by PMI Nutrition International (Brentwood, MO) to yield approximately 10 mg/kg body weight. Feed was made available ad libitum in wire feeders above the floor of the cage. Feed was changed every day and food intake was monitored throughout the experiment. Mice consumed approximately 3–6 g food/day. This drug regimen exerts effective 5-LO inhibition in wild-type mice (16) and in ob/ob mice (see later discussion). At the end of the intervention period, mice were anesthetized with isoflurane and blood was collected by cardiac puncture. The liver was immediately excised, rinsed in Dulbecco's Phosphate Buffered Saline (DPBS), and fixed in 10% formalin for histological analyses. The remaining liver tissue was snap-frozen in liquid nitrogen for RNA extraction and other analysis or was cryopreserved in optimal cutting temperature (OCT) compound for oil red-O staining. All animal studies were conducted in accordance with the criteria of the Investigation and Ethics Committee of the Hospital Clinic and the European Community laws governing the use of experimental animals.

Histological analysis

Hepatic steatosis was assessed by oil red-O staining in OCT-embedded cryosections. Briefly, cryosections were fixed in 60% isopropanol for 10 min and stained with 0.3% oil red-O in 60% isopropanol for 30 min and subsequently washed with 60% isopropanol. Sections were counterstained with Gill's hematoxylin, washed with acetic acid solution (4%), and mounted with aqueous solution. Sections were visualized under a Nikon Eclipse E600 microscope (Kawasaki, Japan) at a magnification of ×100 and relative areas of steatosis (expressed as % oil red-O staining) were quantified by histomorphometry using a computerized image analysis system (AnalySIS®, Soft Imaging System, Munster, Germany). A minimum of 25 independent fields per sample were evaluated.

Hepatic VLDL-TG production rate and apoB secretion

The in vivo VLDL-TG production rate was assessed as described (19–21), with modifications. Wild-type and ob/ob mice received standard mouse chow or a chow containing the 5-LO inhibitor CJ-13,610 (10 mg/kg body weight) for 14 days (n = 3–4 per group). On the day of experimentation, mice received an iv injection of 500 mg/kg body weight of Triton WR-1339 (Tyloxapol, Sigma, St. Louis, MO) as a 15 mg/100 μl solution of saline in the tail vein. Blood samples were taken by tail bleeding before (t_0) and 30, 60, and 120 min after Triton injection. At the end of the experiment, a large blood sample was obtained by heart puncture for isolation of VLDL particles (see below). Plasma VLDL-TG levels were determined with the Triglycérides Enzymatique PAP 150 kit (BioMérieux, Marcy l'Etoile, France) and read at a wavelength of 492 nm in a FluoStar Optima plate reader (BMG LabTech, Offenburg, Germany). The VLDL-TG production rate was calculated from the slope of TG concentration versus the time curve and was expressed as μmol/kg/h. ApoB content in nascent VLDL particles was determined as described (19–21) with modifications. Briefly, 30 μl of isolated VLDL samples were resuspended in SDS-containing Laemmli sample buffer, heated for 5 min at 95°C, and separated through 5% SDS-PAGE. Proteins were electroblotted during 2 h into PVDF membranes. Blots were soaked for 1 h at room temperature in Tris-buffered saline containing 0.1% (v/v) Tween 20 and 5% (w/v) nonfat dry milk and were incubated overnight at 4°C with an anti-apoB polyclonal antibody (dilution 1:1000) (Chemicon, Millipore Co., Billerica, MA). After washing, blots were incubated for 1 h at room temperature with a horseradish peroxidase-linked donkey anti-goat antibody (dilution 1:4000) (Abcam, Cambridge, MA) and subsequently visualized using an enhanced chemiluminiscence detection system. Bands were quantified by densitometry using the Image Gauge 4.0 software (Fuji Photo Film Co., Japan).

Isolation of VLDL

Plasma VLDL-containing fraction (d = 1.006 g/ml) was isolated by density gradient ultracentrifugation with a NaCl solution (d = 1.006 g/ml) at 45,000 rpm for 18 h at 15°C in an Optima L-90K ultracentrifuge using a fixed-angle rotor (model 50 Ti; Beckman Coulter Inc., Fullerton, CA). The top fraction containing VLDL was collected and frozen at −80°C until analysis of apoB levels.

Biochemical analyses

Serum concentrations of cholesterol, TG, and free fatty acids, as well as serum alanine and aspartate aminotransferase (ALT and AST) activities were determined by standard laboratory procedures. Serum insulin levels were determined using an Ultrasensitive Mouse Insulin ELISA kit (Mercodia, Uppsala, Sweden). The total hepatic content of cholesterol and TG was determined by standard laboratory procedures following lipid extraction with isopropyl alcohol-hexane. Briefly, tissue samples (100 mg) were homogenized in 10 ml hexane/isopropanol mixture (3:2) and placed on an orbital shaker overnight at the maximum speed (250–300 rpm). Subsequently, 3 ml of anhydrous sodium sulfate (0.47 M) were added, and the samples were shaken again for 15 min. After centrifugation (200 g for 5 minutes), the upper

phase was placed into a clean borosilicate tube, evaporated under N_2, and resuspended in 1 ml of sodium cholate (0.5%, w/v). To determine TG concentrations in samples from cell culture experiments, supernatants were collected and evaporated under a stream of N_2, whereas cells were trypsinized, placed into borosilicate glass tubes, and centrifuged for 5 min at 800 *g*. Cell extracts and supernatants were resuspended in 1 ml hexane/isopropanol (3:2) and placed on an orbital shaker for 3 h. Subsequently, 300 μl of natrium sulfate anhydride (0.47 M) were added and the samples shaken again for 15 min. After centrifugation (200 *g* for 5 minutes), TG concentrations were analyzed with a commercially available kit (BioMérieux).

5-LO activity assay

5-LO activity was measured in liver explants obtained from wild-type and ob/ob mice. Liver sections of about 40 mg were incubated with 0.5 ml of Ringer buffer containing arachidonic acid (50 μM) (Cayman Chemical, Ann Arbor, MI) and ionophore A23187 (5μM) (Sigma) for 15 min at 37°C in a 24-well plate. At the end of the incubation period, supernatants were collected, centrifuged for 5 min at 3000 rpm at 4°C, and frozen at −80°C until the determination of 5-LO products by enzyme immunoassay (22, 23). 5-LO activity was calculated as the sum of LTB_4 and cysteinyl-LTs produced per time unit and expressed as ng/min. The accumulation of LTB_4 and cysteinyl-LTs in the liver of wild-type mice and ob/ob mice was determined in samples of about 0.2–0.4 g that were individually homogenized with a Ultra-Turrax T25 (Ika, Werke Staufen, Germany) in 2 ml of cold $DPBS^{2+}$ and extracted with Sep Pak C_{18} columns, prior to enzyme immunoassay analysis (22, 23).

Cell incubations

CC-1 cells, an adult rat hepatocyte cell line (24), were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). CC-1 cells were grown at 37°C in a 5% CO_2 atmosphere in EMEM media supplemented with 10% FBS (Biowest, Nuaillé, France), L-glutamine (4 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), sodium pyruvate (1 mM), and NEAA (0.1 mM). For experiments assessing TG levels and gene expression studies, the cells were seeded on 12-well plates at a density of 1×10^5 and 2.5×10^5 cells/well, respectively. Twenty-four hours later, the cells were incubated overnight with serum-free medium supplemented with 25 mM glucose. Thereafter, the cells were exposed to vehicle (<0.5% ethanol) or increasing concentrations (0, 0.1, 1, and 10 μM) of synthetic LTB_4 and LTD_4 (Cayman Chemical). Twenty-four hours later, supernatants and cell extracts were obtained as described above in the Biochemical analyses section, and stored at –20°C until further analysis. For gene expression studies, RNA was isolated from cell extracts as described below. In some experiments, insulin (100 nM) was added to the medium or the cells were pretreated with 10 μM of CP-105,696 (a B-LT_1 receptor antagonist) (kindly provided by Pfizer) or 1 μM of MK-571 (a cys-LT_1 receptor antagonist) (Cayman Chemical) for 15 min, followed by the addition of either vehicle, LTB_4 or LTD_4.

MTP activity assay

MTP activity was determined by a rapid and sensitive fluorescent assay in which samples were incubated with donor vesicles containing quenched fluorescent lipids and acceptor vesicle (Chylos Inc., Woodbury, NY). Briefly, 50–100 mg of tissue samples were homogenized in 1 ml of hypotonic Buffer K (1 mM TrisHCl, 1 mM EGTA, 1 mM $MgCl_2$, pH 7.6, 4°C) containing 1/50 protease-inhibitor cocktail (Roche, Manheim, Germany). Liver homogenates were stored on ice, and protein concentrations were determined using the MicroBCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL). For each sample, the MTP assay was performed in duplicate in black fluorescence microtiter plates using 5 μl of substrate vesicles (containing both donor and acceptor vesicles) and 10 μg of homogenate liver protein. Plates were incubated at 37°C for 30 min and read in a FluoStar Optima fluorescence plate reader at 450 nm excitation and 520 nm emission wavelengths. To determine blank values, 5 μl of vesicles were added to 95 μl of homogenization buffer. The total fluorescence of donor vesicles was determined by adding 95 μl isopropanol to 5 μl vesicles. MTP activity (percentage transfer) was then calculated with equation 1:

$$\%\,\text{Transfer} = (\text{Sample}_{\textit{fluorescence units}} - \text{Blank}_{\textit{fluorescence units}}) / (\text{Total}_{\textit{fluorescence units}} - \text{Blank}_{\textit{fluorescence units}}) \times 100 \quad (\textit{Eq. 1})$$

Specific MTP activity was expressed as the mean of % transfer/mg total protein/hour of duplicate quantifications.

To determine MTP activity in samples from cell culture experiments, CC-1 cells were seeded on 6-well plates at a density of 5×10^5 cells/well; grown overnight with serum-free medium supplemented with 25 mM glucose; and then exposed to vehicle (<0.5% ethanol), LTB_4 (0.1 and 1 μM), or LTD_4 (0.1 and 1 μM). Twenty-four hours later, cells were washed twice with ice-cold DPBS followed by one wash with ice-cold Buffer K. Subsequently, cells were incubated for 2 min with Buffer K prewarmed at 37°C, scraped with 300 μl ice-cold Buffer K containing 1/50 protease-inhibitor cocktail, and homogenized using a 21G needle syringe. In these experiments, 24 μg of protein extracts were assayed with an incubation time period of 6 h. To assess whether LTB_4 and LTD_4 directly affect MTP activity, homogenates from CC-1 cells containing a total of 24 μg of protein were incubated with different concentrations of LTB_4 (0.1 and 1 μM) and LTD_4 (0.1 and 1 μM) and immediately processed in triplicate for MTP activity for an incubation time period of 6 h.

RNA isolation

Isolation of total RNA from liver tissue and cultured cells was performed using Trizol (Invitrogen, Carlsbad, CA) and the RNAqueous Kit (Ambion Inc., Austin, TX), respectively. RNA concentration was assessed in a ultraviolet spectrophotometer and its integrity tested on a 6,000 LabChip in a 2,100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

RT Real-Time PCR

cDNA synthesis from 1 μg of total RNA was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Validated and predesigned TaqMan primers and probes from Assays-on-Demand products were used to quantify 5-LO (Mm01182743_m1), MTP (Mm00435015_m1 and Rn01522970_m1), sterol response element-binding protein-1c (Srebp-1c, ID: Mm00550338_m1 and Rn01495754_m1), fatty acid synthase (FASN, ID: Mm00662319_m1 and Rn00569117_m1), liver fatty acid-binding protein (L-FABP, ID: Mm00444340_m1 and Rn00561171_m1), fatty acid translocase (FAT/CD36, ID: Mm00432403_m1 and Rn00580728_m1), peroxisome proliferator-activated receptor α (PPARα, ID: Mm00440939_m1 and Rn00566193_m1), and acyl-CoA oxidase (Aco, ID: Mm00443579_m1 and Rn00569216_m1) using β-actin (Actb, ID: Mm00607939_s1 and Rn00667869_m1) as an endogenous control. Real-time PCR amplifications were carried out in an ABI Prism 7900HT Sequence Detection System. PCR results were analyzed with the Sequence Detector Software version 2.1 (Applied Biosystems). Relative quantification of gene expression was performed using the comparative C_t method. The amount of target gene, normalized to β-actin and relative to a calibrator, was

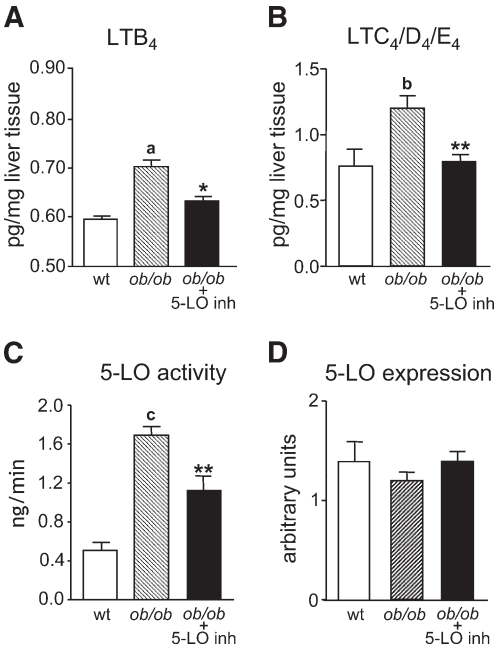

Fig. 1. Increased 5-lipoxygenase (5-LO) product formation in ob/ob mouse liver and inhibition by a selective 5-LO inhibitor. Hepatic levels of leukotriene B_4 (LTB_4) (A) and $LTC_4/LTD_4/LTE_4$ (B) in wild-type (wt) mice (n = 5), ob/ob mice (n = 8), and ob/ob mice treated with a 5-LO inhibitor [ob/ob + 5-LO inhibitor (inh)] (n = 8). C: 5-LO activity was determined in liver sections from wt and ob/ob mice incubated with arachidonic acid. D: 5-LO expression in liver tissue samples from wild-type and ob/ob mice was assessed by real-time PCR and normalized to β-actin levels. Results are expressed as mean ± SEM. a, $P < 0.05$, b, $P < 0.01$, and c, $P < 0.001$ vs. wt mice; * $P < 0.05$ and ** $P < 0.01$ vs. untreated ob/ob mice.

determined by the arithmetic formula $2^{-\Delta\Delta Ct}$ described in the comparative C_t Method (User Bulletin #2; http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf).

Statistical analysis

Statistical analysis of the results was performed using the ANOVA and unpaired Student's *t*-test. Results were expressed as mean ± SEM, and differences were considered significant at $P < 0.05$.

RESULTS

The amounts of LTB_4 and $LTC_4/LTD_4/LTE_4$, the most potent and representative products of the arachidonate 5-LO pathway, were significantly higher in homogenates of liver samples from ob/ob mice compared with those from wild-type mice (**Fig. 1A, B**). Moreover, liver tissue explants obtained from ob/ob mice and incubated with arachidonic acid produced significantly higher amounts of 5-LO products than liver tissue samples from wild-type animals (Fig. 1C). 5-LO mRNA levels were essentially unaltered in the liver of ob/ob mice (Fig. 1D). In the ob/ob mouse liver, 5-LO activity and LTB_4 and $LTC_4/LTD_4/LTE_4$ levels were effectively reduced by CJ-13,610, a selective nonredox type 5-LO inhibitor (Fig. 1A–C).

We next examined the contribution of increased hepatic 5-LO activity to the development of hepatic steatosis in ob/ob mice. **Table 1** shows that body and liver weights and serum cholesterol and insulin concentrations were significantly increased in ob/ob mice. In addition to severe obesity, hypercholesterolemia, and hyperinsulinemia, ob/ob mice showed increased serum ALT and AST activities (Table 1) and remarkable hepatic steatosis as revealed by histomorphometrical analysis of oil red-O-stained liver sections (**Fig. 2A**) and by the presence of increased hepatic TG content (Fig. 2B). The administration of the selective 5-LO inhibitor, CJ-13,610, to these mice significantly alleviated hepatic steatosis as the percentage of the area stained with oil red-O was drastically reduced in these obese mice (Fig. 2A). This antisteatotic effect was associated with a normalization of hepatic TG content (Fig. 2B). Body weight decreased and serum TG and free fatty acid levels increased in ob/ob mice following 5-LO inhibition (Table 1). Hepatic cholesterol levels (Fig. 2B) and serum cholesterol and insulin concentrations and serum ALT and AST activities (Table 1) were unaltered by 5-LO inhi-

TABLE 1. Body and liver weights and serum biochemistry values in wild-type mice (wt) (n = 5), leptin-deficient mice (ob/ob) (n = 8), and ob/ob mice receiving the 5-LO inhibitor (ob/ob + 5-LO inhibitor) (n = 8)

Parameter	Wt	ob/ob	ob/ob + 5-LO inhibitor
Body weight (g)	23.0 ± 1.2	33.0 ± 0.6[d]	31.0 ± 1.0[d,e]
Liver weight (g)	1.10 ± 0.01	1.70 ± 0.07[d]	1.60 ± 0.12[c]
Liver to body weight ratio (%)	5.02 ± 0.24	4.99 ± 0.14	5.01 ± 0.25
Serum TG (mg/dl)	90 ± 12.8	72.13 ± 8.09	147.1 ± 12.9[c,f]
Serum cholesterol (mg/dl)	78.2 ± 3.8	98.8 ± 8.9[a]	97.0 ± 6.8[b]
Serum free fatty acids (μmol/L)	ND	1023.0 ± 508.9	2447.0 ± 257.9[e]
Serum insulin (ng/ml)	0.69 ± 0.05	2.99 ± 0.38[d]	3.22 ± 1.28[a]
Serum ALT (U/L)	26.6 ± 2.8	80.8 ± 12.9[d]	105.3 ± 7.7[d]
Serum AST (U/L)	163.0 ± 43.0	312.3 ± 70.2[a]	415.3 ± 48.2[d]

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ND, not detected; TG, triglycerides. Data are expressed as mean ± SEM.

[a] $P < 0.05$ vs. wt.
[b] $P < 0.01$ vs. wt.
[c] $P < 0.005$ vs. wt.
[d] $P < 0.001$ vs. wt.
[e] $P < 0.05$ vs. ob/ob.
[f] $P < 0.001$ vs. ob/ob.

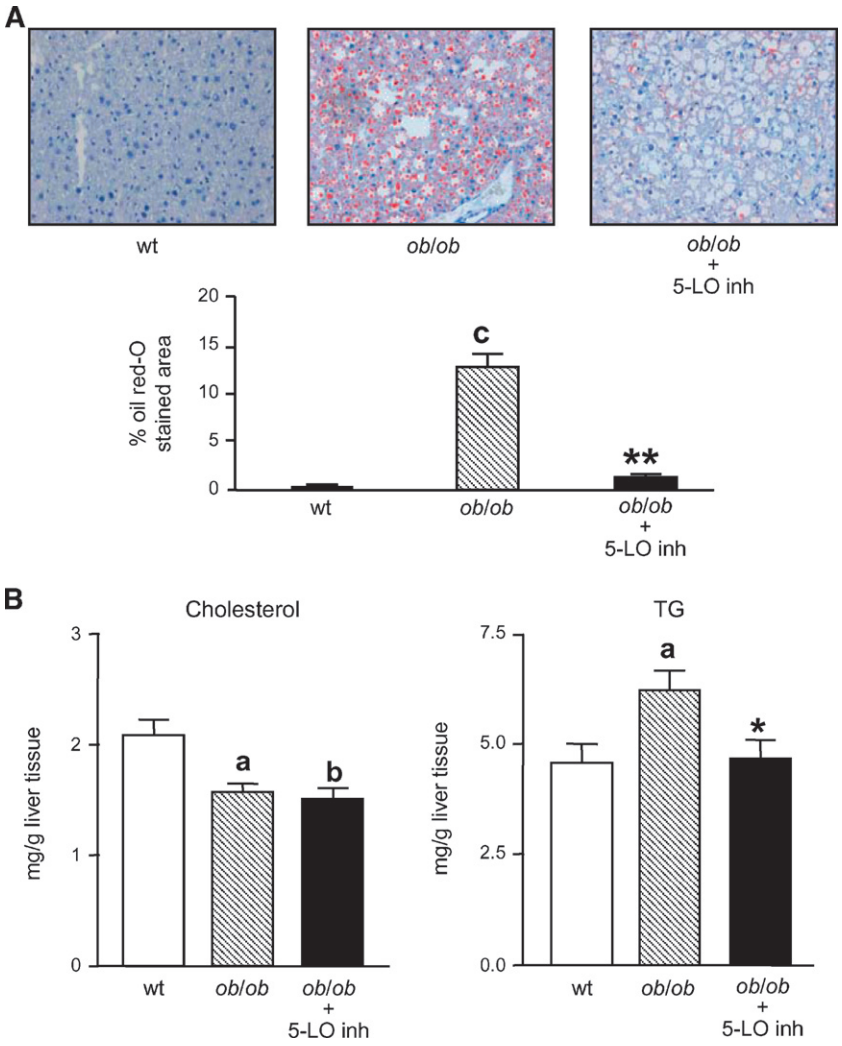

Fig. 2. Antisteatotic actions of 5-LO inhibition. A: Representative photomicrographs (original magnification, ×200) of liver sections stained with oil red-O from wild-type mice (wt, left panel) (n = 5), leptin-deficient mice (ob/ob, middle panel) (n = 8), and ob/ob mice receiving the 5-LO inhibitor (ob/ob + 5-LO inh, right panel) (n = 8). The histomorphometrical analysis of oil red-O-stained liver sections is shown below. B: Liver samples from wt mice, ob/ob mice, and ob/ob mice receiving the 5-LO inhibitor were extracted with isopropyl alcohol-hexane to determine the hepatic content of cholesterol and triglycerides (TG). Results are expressed as mean ± SEM. a, $P < 0.01$, b, $P < 0.005$, and c, $P < 0.001$ vs. wt mice; * $P < 0.01$ and ** $P < 0.001$ vs. untreated ob/ob mice.

bition. No changes in serum biochemistry parameters were observed in wild-type mice treated with the 5-LO inhibitor (data not shown).

To understand the molecular mechanisms underlying the antisteatotic effects associated with 5-LO inhibition, we explored key genes governing hepatic lipogenesis, fatty acid uptake, and fatty acid oxidation in ob/ob mice. Expression levels of key genes involved in de novo hepatic lipogenesis [i.e., fatty acid synthase (FASN)] (**Fig. 3A**), fatty acid uptake [i.e., fatty acid translocase (FAT/CD36)] (Fig. 3D) and peroxisomal oxidation of fatty acids [i.e., PPARα and the PPARα-responsive gene, acyl-CoA oxidase (ACO)] (Fig. 3E, F) were clearly upregulated in livers of ob/ob mice. In these obese mice, 5-LO inhibition significantly downregulated the expression of genes associated with fatty acid uptake [i.e., liver fatty acid-binding protein (L-FABP) and FAT/CD36] (Fig. 3C, D) and normalized the expression of PPARα and ACO (Fig. 3E, F). By contrast, the expression of genes involved in lipogenesis, such as FASN and sterol response element-binding protein-1c (SREBP-1c), remained unchanged (Fig. 3A, B). No changes were observed in wild-type mice treated with the 5-LO inhibitor (Fig. 3).

Because of its central role in the production of apoB-containing VLDL and, therefore, in the export of TG from hepatocytes, we examined the effects of 5-LO inhibition on MTP activity. As shown in **Fig. 4A**, compared with wild-type animals, the activity of this microsomal transfer protein was significantly decreased in livers from ob/ob mice and was partially restored following the inhibition

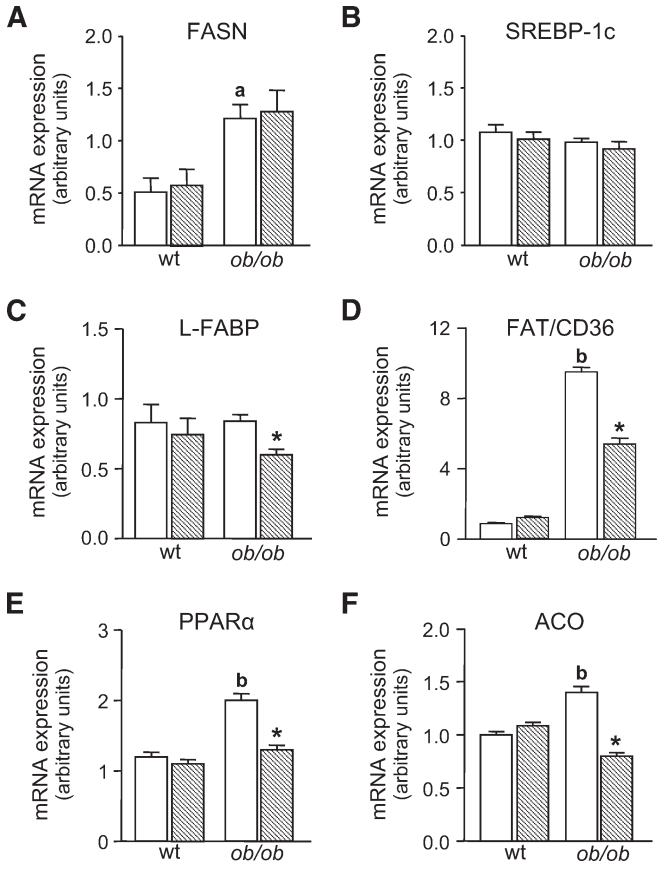

Fig. 3. Inhibition of arachidonate 5-LO downregulates liver fatty acid-binding protein (L-FABP) and fatty acid translocase (FAT/CD36), and normalizes peroxisome proliferator-activated receptor alpha (PPARα) and acyl-CoA oxidase (ACO) expression in the liver of ob/ob mice. mRNA levels of fatty acid synthase (FASN) (A), SREBP-1c (B), L-FABP (C), FAT/CD36 (D), PPARα (E), and ACO (F) in liver samples from wild-type (wt) and ob/ob mice receiving placebo (open bars) or the 5-LO inhibitor (hatched bars) were assessed by real-time PCR and normalized to the levels of β-actin. Results are expressed as mean ± SEM. a, $P < 0.005$ and b, $P < 0.001$ vs. placebo-treated wt mice; * $P < 0.001$ vs. placebo-treated ob/ob mice.

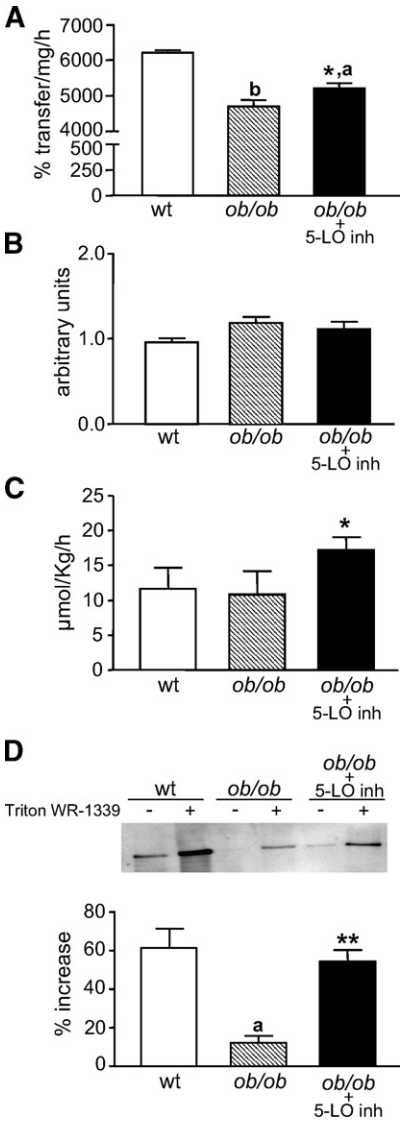

Fig. 4. The arachidonate 5-LO pathway modulates in vivo MTP activity and hepatic VLDL-TG and apoB secretion in ob/ob mice. A: MTP activity was determined in triplicate using 5 µl of substrate vesicles and 10 µg of homogenate liver protein. Plates were incubated at 37°C for 30 min and read in a fluorescence plate reader at 450 nm excitation and 520 nm emission wavelengths. B: Hepatic MTP mRNA expression in wild-type (wt) mice, ob/ob mice, and ob/ob mice treated with the 5-LO inhibitor was assessed by real-time PCR. C: VLDL-TG production rate in µmol/kg/h, calculated from the plasma TG versus time curve after injection of Triton WR-1339 in wt, ob/ob, and ob/ob mice treated with the 5-LO inhibitor. D: Representative Western blot of apoB content in VLDL particles before (−) and after (+) Triton WR-1339 injection. The VLDL fraction was isolated by density gradient ultracentrifugation and apoB levels were determined as described in Materials and Methods. The % increase in apoB secretion was calculated by comparing band intensities before and after Triton WR-1339 injection. Results are expressed as mean ± SEM from 3–6 different experiments. a, $P < 0.005$ and b, $P < 0.001$ vs. wt mice; * $P < 0.05$ and ** $P < 0.001$ vs. untreated ob/ob mice.

of the 5-LO pathway. MTP mRNA expression was not different in ob/ob mice and was not affected by 5-LO inhibition (Fig. 4B). We next determined in vivo the effect of 5-LO inhibition on VLDL-TG production rate by injecting ob/ob and control mice with Triton WR-1339. Consistent with previous studies (20), hepatic VLDL-TG production remained unaffected in ob/ob mice (Fig. 4C). Interestingly, 5-LO inhibition induced a 60% increase in hepatic VLDL-TG production in ob/ob mice (Fig. 4C). In parallel with findings in MTP activity, the apoB content of the VLDL fraction was significantly reduced in ob/ob mice and was completely restored by inhibition of the 5-LO pathway (Fig. 4 D).

Given that compounds that reduce MTP activity are known to be steatogenic, we next tested the effects of 5-LO products (i.e., LTB_4 and LTD_4) on MTP activity in cultured CC-1 cells, an adult hepatocyte cell line. Consistent with findings observed after 5-LO inhibition in vivo, LTB_4 reduced MTP activity in a concentration-dependent manner in CC-1 cells (**Fig. 5A**). LTD_4 produced similar effects on hepatocyte MTP activity, although the changes were more modest (Fig. 5A). To determine whether 5-LO products can directly affect MTP activity, homogenates

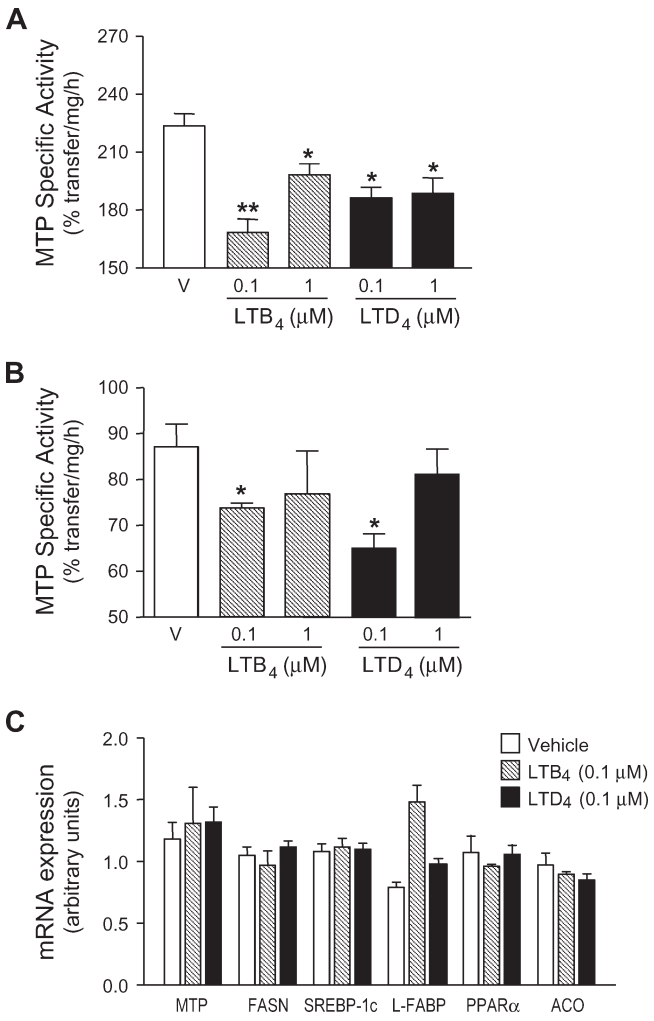

Fig. 5. 5-LO products modulate in vitro MTP activity in a murine hepatocyte cell line. A: MTP activity was determined in CC-1 cells exposed to vehicle (V) (<0.5% ethanol), LTB_4 (0.1 and 1 μM), or LTD_4 (0.1 and 1 μM). MTP activity was assayed in duplicate using 5 μl of substrate vesicles and 24 μg of protein extracts with an incubation period of 6 h. B: Direct effects of 5-LO products on MTP activity. Homogenates from CC-1 cells were incubated with different concentrations of LTB_4 and LTD_4 (0.1 and 1 μM) during the assessment of MTP activity in the fluorescent assay. C: Quantitative real-time PCR analysis of MTP, FASN, SREBP-1c, L-FABP, PPARα, and ACO mRNA expression in CC-1 cells incubated with vehicle (open bars), LTB_4 (hatched bars), and LTD_4 (solid bars). Results are expressed as mean ± SEM from 4–8 different experiments. * $P < 0.05$ and ** $P < 0.001$ vs. vehicle (V).

from CC-1 cells were incubated with different concentrations of LTB_4 and LTD_4, and MTP activity was monitored in the fluorescent assay. As shown in Fig. 5B, MTP activity was decreased in the presence of 0.1 μM concentrations of LTB_4 and LTD_4. These findings are consistent with the observation that neither CP-105,696, a selective B-LT_1 receptor antagonist, nor MK571, a selective Cys-LT_1 receptor antagonist, was able to block the effects of LTB_4 and LTD_4 on hepatocytes (data not shown), suggesting that their actions are apparently not mediated by binding to their cell surface receptors. The effects of incubating CC-1 cells with LTB_4 and LTD_4 on the expression of genes encoding MTP, FASN, SREBP-1c, L-FABP, PPARα, and ACO are shown in Fig. 5C. Consistent with in vivo findings, expression of MTP in CC-1 cells was unaffected by 5-LO products, and neither did LTB_4 and LTD_4 show any effect on FASN and SREBP-1c mRNA levels, while L-FABP expression was upregulated by LTB_4. However, incubation of CC-1 cells with LTB_4 and LTD_4 did not result in any significant change in PPARα and ACO expression, suggesting that the changes observed in ob/ob mice following 5-LO inhibition in vivo were likely to reflect the improvement in lipid homeostasis, since PPARα is a fine sensor of intrahepatic lipid levels (25).

The steatogenic effects of 5-LO products on hepatocytes were further substantiated by analyzing intracellular and extracellular TG levels in CC-1 cells incubated with increasing concentrations of LTB_4 and LTD_4 and growing under hyperglycemic conditions (25 mM glucose). As shown in **Fig. 6A**, LTB_4 induced a concentration-dependent increase in intracellular TG content in parallel with a decrease in extracellular TG concentrations. At the highest concentration (10 μM), however, LTB_4 produced an insignificant accumulation of TG (Fig. 6A). This effect was accompanied by a significant downregulation of FASN and SREBP-1c expression (Fig. 6B), indicating that, in the presence of an excessive concentration of 10 μM LTB_4, lipogenesis might be decreased. Similar findings, albeit more modest, were obtained with LTD_4 (Fig. 6A, B). The steatogenic effects induced by 5-LO products were partially attenuated by the addition of insulin to the cell incubations (data not shown).

DISCUSSION

Recent studies have demonstrated that, in addition to its well-documented participation in inflammatory response, the 5-LO pathway is also associated with metabolic disease encompassing atherosclerosis, obesity, and insulin resistance (6–14). In the present study, we provide evidence, for the first time to our knowledge, that the 5-LO pathway is also implicated in the pathogenesis of fatty liver disease. In fact, our findings clearly demonstrate increased hepatic 5-LO activity and 5-LO product formation (LTB_4 and $LTC_4/D_4/E_4$) in ob/ob mice, a well-established model of obesity-related hepatic steatosis (3, 15). Our findings also support a role for 5-LO products in hepatic steatosis by mechanisms including a decrease in hepatic MTP activity and VLDL-TG and apoB secretion and an upregulation of key genes involved in the uptake of free fatty acids (i.e., L-FABP).

MTP, a microsomal protein present in the endoplasmic reticulum lumen, is the rate limiting factor for the production of apoB-containing VLDL particles in hepatic cells (26–29). The principal role of MTP is to facilitate the transfer of lipids to the nascent apoB-containing lipoprotein particle, which is then converted to VLDL by the addition of lipid droplets (26–29). Although MTP is capable of transferring the four major lipid classes (free cholesterol, phospholipids, TG, and cholesteryl esters), this

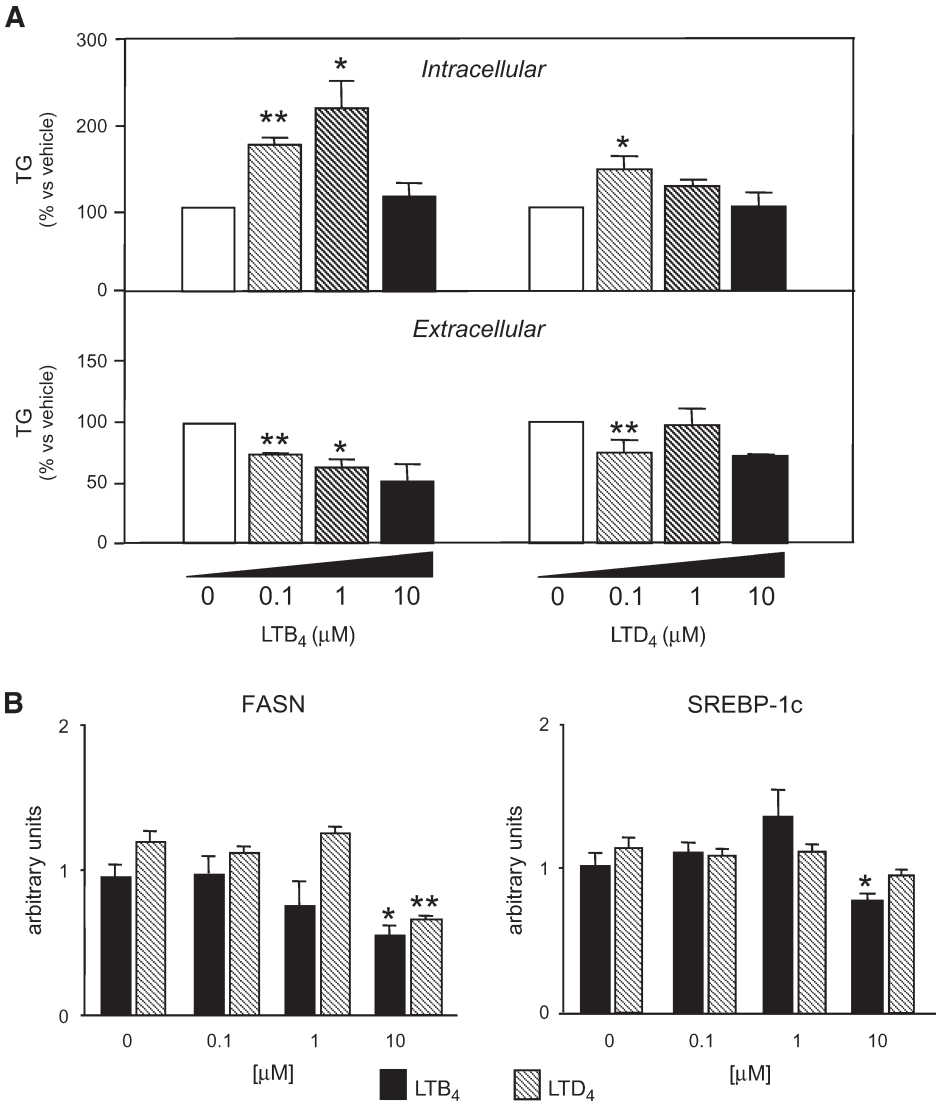

Fig. 6. 5-LO products modulate in vitro TG levels in a murine hepatocyte cell line. A: CC-1 cells were incubated with serum-free medium supplemented with 25 mM of glucose and exposed for 24 h to increasing concentrations (0, 0.1, 1, and 10 μM) of LTB_4 or LTD_4. TG concentrations were determined in both intracellular (upper panel) and extracellular (lower panel) compartments. (B) Quantitative real-time PCR analysis of FASN and SREBP-1c in CC-1 cells incubated with increasing concentrations (0, 0.1, 1, and 10 μM) of LTB_4 (solid bars) or LTD_4 (hatched bars). Data are expressed as mean ± SEM of three different experiments. * $P < 0.05$ and ** $P < 0.01$ vs. vehicle.

transfer protein strongly prefers TG and cholesteryl esters (30). Previous studies have shown that a defective mutation in MTP causes a β lipoproteinemia characterized by the absence of apoB lipoproteins accompanied by fatty liver disease (31). In addition, compounds that inhibit MTP activity are known to be steatogenic (28). In the present study, the two major 5-LO products (LTB_4 and LTD_4) (32, 33) significantly inhibited MTP activity in vitro (Fig. 5), whereas 5-LO inhibition re-established MTP activity, stimulated VLDL-TG production, and restored apoB secretion in vivo in steatotic ob/ob mice livers (Fig. 4). Interestingly, this effect was reproduced in vitro when LTB_4 and LTD_4 were directly added to homogenates of control cells, indicating a direct inhibitory effect of 5-LO products on MTP activity (Fig. 5). Although it is evident that more studies are required to fully elucidate the mechanisms implicated, LTB_4 and LTD_4 may bind to the surface of lipids to prevent MTP action. In fact, compounds with a carboxylic group, as is the case of LTB_4 and LTD_4, might conceivably be associated with TG to hamper MTP activity (28).

The uptake of free fatty acids is another of the most important contributing factors to hepatic steatosis since these lipid components are taken up by hepatocytes and are readily sterified to produce TG (4). The uptake of free fatty acids is facilitated by two major proteins: L-FABP, a fatty acid-binding protein of approximately 15 kDa, which is highly abundant in the cytosol of liver parenchymal

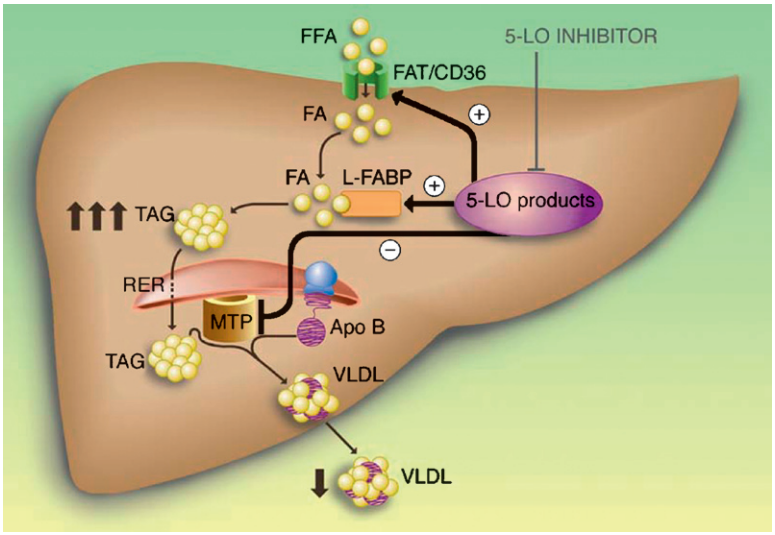

Fig. 7. Summary of the proposed mechanism for the steatogenic actions of 5-LO products. The uptake of free fatty acids (FFA) is one of the most important contributing factors to hepatic steatosis since these lipid components are taken up by hepatocytes and readily sterified to triglycerides (TAG). TAG are then stored within the intracellular compartment or incorporated in the rough endoplasmic reticulum (RER) into very low density lipoproteins (VLDL) particles for export to the interstitial space. The uptake of FFA is facilitated by two major proteins: a membrane fatty acid translocase (FAT/CD36) and a liver fatty acid-binding protein (L-FABP), whereas the transfer of TAG to the nascent apoB-containing VLDL is facilitated by a microsomal TAG transfer protein (MTP) present in the RER lumen. 5-LO products upregulate the expression of key genes involved in FFA uptake (i.e., L-FABP) and induce a reduction of MTP-mediated TAG export, thus contributing to the intracellular accumulation of TAG in the liver.

cells; and FAT/CD36, a fatty acid translocase of approximately 88 kDa (34, 35). Previous studies have shown that L-FABP-deficient mice manifest decreased fatty acid binding capacity and are protected against obesity-induced hepatic steatosis (34). On the other hand, FAT/CD36 is markedly overexpressed in the liver of ob/ob mice and its mRNA levels increase in parallel with liver TG content in experimental fatty liver (36, 37). Therefore, our results showing a reduction in L-FABP and FAT/CD36 after 5-LO inhibition contribute to explain the overall antisteatotic action of this experimental maneuver in ob/ob mice. It is important to note that no changes in the expression of these genes were observed following the administration of the 5-LO inhibitor to lean wild-type mice (Fig. 3), suggesting a lack of effect of these compounds in tissues carrying low 5-LO activity. This finding is consistent with previous studies showing no effects of inhibitors of the 5-LO pathway in lean control animals (16, 22, 23).

In our study, 5-LO inhibition dramatically improved fatty liver, but aggravated the severity of serum hypertriglyceremia and circulating free fatty acids in ob/ob mice. Similar undesired effects on the metabolic phenotype have been described in previous studies with other antisteatotic strategies (38–41). The mechanisms by which TG and free fatty acids levels are triggered in these studies remain to be defined, but intriguing possibilities are that excess lipids from other major storage depots, such as adipose tissue, are concomitantly removed and delivered to the circulation or that 5-LO products regulate TG homeostasis, contributing to hepatic steatosis but protecting other tissues from TG accumulation (39–41). The worsening of hyperlipidemia following inhibition of 5-LO in obese ob/ob mice was not observed after 5-LO inhibition in mice fed a high-fat diet, another model of diet-induced obesity-related steatosis (R.H., personal communication), suggesting that this undesired response to 5-LO inhibition could be restricted to the leptin-deficient ob/ob mouse model.

The presence of a metabolically active 5-LO pathway in the liver has been ascribed to Kupffer cells, and these macrophages are the only hepatic cell type endowed with a complete enzymatic machinery for the biosynthesis of 5-LO products (22, 23, 42). On the other hand, many of the known biological effects of 5-LO products are mediated through interaction with specific G-protein coupled receptors (33). To date, two receptors for LTB_4 (B-LT_1 and B-LT_2) and two receptors for LTD_4 (Cys-LT_1 and Cys-LT_2) have been cloned and their presence in hepatocytes has been characterized (43, 44). In our study, the induction of intracellular TG accumulation and the inhibition of MTP activity by synthetic 5-LO products was not mediated by binding of LTB_4 and LTD_4 to cell surface receptors since neither a selective B-LT_1 receptor antagonist nor a selective Cys-LT_1 receptor antagonist was able to block their effects. Although more studies are needed to clarify this issue, the existence of distinct uptake systems for LTB_4 and LTD_4 at the sinusoidal membrane of hepatocytes has been previously demonstrated (45). Moreover, in addition to cell surface receptors, LTB_4 is known to bind to nuclear receptors (46).

In summary, our results indicate that 5-LO products directly modulate two key steps in lipid transport within the hepatic cells: the expression of key genes involved in the uptake of free fatty acids (i.e., L-FABP) and the secretion of VLDL-TG and apoB under the control of MTP activity (**Fig. 7**). The finding that 5-LO inhibition exerts antisteatotic effects in a murine model of obesity-related fatty liver disease adds more weight to the evidence obtained in previous studies demonstrating a protective effect of 5-LO inhibition against liver inflammation and fibrosis (16, 22, 23).

REFERENCES

1. Angulo, P. 2002. Nonalcoholic fatty liver disease. *N. Engl. J. Med.* **346:** 1221–1231.
2. Sanyal, A. J. 2005. Mechanisms of Disease: pathogenesis of nonalcoholic fatty liver disease. *Nat Clin Pract Gastroenterol Hepatol.* **2:** 46–53.

3. Koteish, A., and D. A. Mae. 2002. Animal models of steatohepatitis. *Best Pract. Res. Clin. Gastroenterol.* **16:** 679–690.
4. Browning, J. D., and J. D. Horton. 2004. Molecular mediators of hepatic steatosis and liver injury. *J. Clin. Invest.* **114:** 147–152.
5. den Boer, M., P. J. Voshol, F. Kuipers, L. M. Havekes, and J. A. Romijn. 2004. Hepatic steatosis: a mediator of the metabolic syndrome. Lessons from animal models. *Arterioscler. Thromb. Vasc. Biol.* **24:** 644–649.
6. Zhao, L., M. P. Moos, R. Grabner, F. Pedrono, J. Fan, B. Kaiser, N. John, S. Schmidt, R. Spanbroek, K. Lotzer, et al. 2004. The 5-lipoxygenase pathway promotes pathogenesis of hyperlipidemia-dependent aortic aneurysm. *Nat. Med.* **10:** 966–973.
7. Kaaman, M., M. Ryden, T. Axelsson, E. Nordstrom, A. Sicard, A. Bouloumie, D. Langin, P. Arner, and I. Dahlman. 2006. ALOX5AP expression, but not gene haplotypes, is associated with obesity and insulin resistance. *Int. J. Obes.* **30:** 447–452.
8. Back, M., A. Sultan, O. Ovchinnikova, and G. K. Hansson. 2007. 5-Lipoxygenase-activating protein: a potential link between innate and adaptive immunity in atherosclerosis and adipose tissue inflammation. *Circ. Res.* **100:** 946–949.
9. Mehrabian, M., H. Allayee, J. Stockton, P. Y. Lum, T. A. Drake, L. W. Castellani, M. Suh, C. Armour, S. Edwards, J. Lamb, et al. 2005. Integrating genotypic and expression data in a segregating mouse population to identify 5-lipoxygenase as a susceptibility gene for obesity and bone traits. *Nat. Genet.* **37:** 1224–1233.
10. Shashkin, P. N., N. Jain, Y. I. Miller, B. A. Rissing, Y. Huo, S. R. Keller, G. E. Vandenhoff, J. L. Nadler, and T. M. McIntyre. 2006. Insulin and glucose play a role in foam cell formation and function. *Cardiovasc. Diabetol.* **5:** 13.
11. Dwyer, J. H., H. Allayee, K. M. Dwyer, J. Fan, H. Wu, R. Mar, A. J. Lusis, and M. Mehrabian. 2004. Arachidonate 5-lipoxygenase promoter genotype, dietary arachidonic acid, and atherosclerosis. *N. Engl. J. Med.* **350:** 29–37.
12. Mehrabian, M., H. Allayee, J. Wong, W. Shi, X. P. Wang, Z. Shaposhnik, C. D. Funk, and A. J. Lusis. 2002. Identification of 5-lipoxygenase as a major gene contributing to atherosclerosis susceptibility in mice. *Circ. Res.* **91:** 120–126.
13. Spanbroek, R., R. Grabner, K. Lotzer, M. Hildner, A. Urbach, K. Ruhling, M. P. Moos, B. Kaiser, T. U. Cohnert, T. Wahlers, et al. 2003. Expanding expression of the 5-lipoxygenase pathway within the arterial wall during human atherogenesis. *Proc. Natl. Acad. Sci. USA.* **100:** 1238–1243.
14. Qiu, H., A. Gabrielsen, H. E. Agardh, M. Wan, A. Wetterholm, C. H. Wong, U. Hedin, J. Swedenborg, G. K. Hansson, B. Samuelsson, et al. 2006. Expression of 5-lipoxygenase and leukotriene A4 hydrolase in human atherosclerotic lesions correlates with symptoms of plaque instability. *Proc. Natl. Acad. Sci. USA.* **103:** 8161–8166.
15. Anstee, Q. M., and R. D. Goldin. 2006. Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. *Int. J. Exp. Pathol.* **87:** 1–16.
16. Horrillo, R., A. Planagumà, A. González-Périz, N. Ferré, E. Titos, R. Miquel, M. López-Parra, J. L. Masferrer, V. Arroyo, and J. Clària. 2007. Comparative protection against liver inflammation and fibrosis by a selective COX-2 inhibitor and a nonredox-type 5-LO inhibitor. *J. Pharmacol. Exp. Ther.* **323:** 778–786.
17. Fischer, L., D. Steinhilber, and O. Werz. 2004. Molecular pharmacological profile of the nonredox-type 5-lipoxygenase inhibitor CJ-13,610. *Br. J. Pharmacol.* **142:** 861–868.
18. Zweifel, B. S., M. M. Hardy, G. D. Anderson, D. R. Dufield, R. A. Pufahl, and J. L. Masferrer. 2008. A rat air pouch model for evaluating the efficacy and selectivity of 5-lipoxygenase inhibitors. *Eur. J. Pharmacol.* **584:** 166–174.
19. Li, X., F. Catalina, S. M. Grundy, and S. Patel. 1996. Method to measure apolipoprotein B-48 and B-100 secretion rates in an individual mouse: evidence for a very rapid turnover of VLDL and preferential removal of B-48- relative to B-100-containing lipoproteins. *J. Lipid Res.* **37:** 210–220.
20. Wiegman, C. H., R. H. Bandsma, M. Ouwens, F. H. van der Sluijs, R. Havinga, T. Boer, D. J. Reijngoud, J. A. Romijn, and F. Kuipers. 2003. Hepatic VLDL production in ob/ob mice is not stimulated by massive de novo lipogenesis but is less sensitive to the suppressive effects of insulin. *Diabetes.* **52:** 1081–1089.
21. van der Veen, J. N., R. Havinga, V. W. Bloks, A. K. Groen, and F. Kuipers. 2007. Cholesterol feeding strongly reduces hepatic VLDL-triglyceride production in mice lacking the liver X receptor alpha. *J. Lipid Res.* **48:** 337–347.
22. Titos, E., J. Clària, A. Planagumà, M. López-Parra, N. Villamor, M. Párrizas, A. Carrió, R. Miquel, W. Jiménez, V. Arroyo, et al. 2003. Inhibition of 5-lipoxygenase induces cell growth arrest and apoptosis in rat Kupffer cells: implications for liver fibrosis. *FASEB J.* **17:** 1745–1747.
23. Titos, E., J. Clària, A. Planagumà, M. López-Parra, A. González-Périz, R. Miquel, V. Arroyo, and J. Rodés. 2005. Inhibition of 5-lipoxygenase-activating protein abrogates experimental liver injury: role of Kupffer cells. *J. Leukoc. Biol.* **78:** 871–878.
24. Gerschenson, L. E., M. Andersson, J. Molson, and T. Okigaki. 1970. Tyrosine transaminase induction by dexamethasone in a new rat liver cell line. *Science.* **170:** 859–861.
25. Reddy, J. K., and M. S. Rao. 2006. Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. *Am. J. Physiol. Gastrointest. Liver Physiol.* **290:** G852–G858.
26. Hussain, M. M., J. Shi, and P. Dreizen. 2003. Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. *J. Lipid Res.* **44:** 22–32.
27. Jamil, H., C. H. Chu, J. K. Dickson, Jr., Y. Chen, M. Yan, S. A. Biller, R. E. Gregg, J. R. Wetterau, and D. A. Gordon. 1998. Evidence that microsomal triglyceride transfer protein is limiting in the production of apolipoprotein B-containing lipoproteins in hepatic cells. *J. Lipid Res.* **39:** 1448–1454.
28. Letteron, P., A. Sutton, A. Mansouri, B. Fromenty, and D. Pessayre. 2003. Inhibition of microsomal triglyceride transfer protein: another mechanism for drug-induced steatosis in mice. *Hepatology.* **38:** 133–140.
29. Levy, E., S. Stan, E. Delvin, D. Menard, C. Shoulders, C. Garofalo, I. Slight, E. Seidman, G. Mayer, and M. Bendayan. 2002. Localization of microsomal triglyceride transfer protein in the Golgi: possible role in the assembly of chylomicrons. *J. Biol. Chem.* **277:** 16470–16477.
30. Domitrovich, A. M., D. J. Felmlee, and A. Siddiqui. 2005. Hepatitis C virus nonstructural proteins inhibit apolipoprotein B100 secretion. *J. Biol. Chem.* **280:** 39802–39808.
31. Wetterau, J. R., L. P. Aggerbeck, M. E. Bouma, C. Eisenberg, A. Munck, M. Hermier, J. Schmitz, G. Gay, D. J. Rader, and R. E. Gregg. 1992. Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. *Science.* **258:** 999–1001.
32. Samuelsson, B., S. E. Dahlen, J. A. Lindgren, C. A. Rouzer, and C. N. Serhan. 1987. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science.* **237:** 1171–1176.
33. Funk, C. D. 2001. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science.* **294:** 1871–1875.
34. Newberry, E. P., Y. Xie, S. M. Kennedy, J. Luo, and N. O. Davidson. 2006. Protection against Western diet-induced obesity and hepatic steatosis in liver fatty acid-binding protein knockout mice. *Hepatology.* **44:** 1191–1205.
35. Abumrad, N. A., M. R. el Maghrabi, E. Z. Amri, E. Lopez, and P. A. Grimaldi. 1993. Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. Homology with human CD36. *J. Biol. Chem.* **268:** 17665–17668.
36. Memon, R. A., J. Fuller, A. H. Moser, P. J. Smith, C. Grunfeld, and K. R. Feingold. 1999. Regulation of putative fatty acid transporters and Acyl-CoA synthetase in liver and adipose tissue in ob/ob mice. *Diabetes.* **48:** 121–127.
37. Degrace, P., B. Moindrot, I. Mohamed, J. Gresti, Z. Y. Du, J. M. Chardigny, J. L. Sebedio, and P. Clouet. 2006. Upregulation of liver VLDL receptor and FAT/CD36 expression in LDLR−/− apoB100/100 mice fed trans-10,cis-12 conjugated linoleic acid. *J. Lipid Res.* **47:** 2647–2655.
38. Hong, F., S. Radaeva, H. N. Pan, Z. Tian, R. Veech, and B. Gao. 2004. Interleukin 6 alleviates hepatic steatosis and ischemia/reperfusion injury in mice with fatty liver disease. *Hepatology.* **40:** 933–941.
39. Matsusue, K., M. Haluzik, G. Lambert, S. H. Yim, O. Gavrilova, J. M. Ward, B. Brewer, Jr., M. L. Reitman, and F. J. Gonzalez. 2003. Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. *J. Clin. Invest.* **111:** 737–747.
40. Schroeder-Gloeckler, J. M., S. M. Rahman, R. C. Janssen, L. Qiao, J. Shao, M. Roper, S. J. Fischer, E. Lowe, D. J. Orlicky, J. L. McManaman, et al. 2007. CCAAT/enhancer-binding protein beta deletion reduces adiposity, hepatic steatosis, and diabetes in Lepr (db/db) mice. *J. Biol. Chem.* **282:** 15717–15729.

41. Gavrilova, O., M. Haluzik, K. Matsusue, J. J. Cutson, L. Johnson, K. R. Dietz, C. J. Nicol, C. Vinson, F. J. Gonzalez, and M. L. Reitman. 2003. Liver peroxisome proliferator-activated receptor gamma contributes to hepatic steatosis, triglyceride clearance, and regulation of body fat mass. *J. Biol. Chem.* **278:** 34268–34276.
42. Decker, K. 1985. Eicosanoids, signal molecules of liver cells. *Semin. Liver Dis.* **5:** 175–190.
43. Ogasawara, H., S. Ishii, T. Yokomizo, T. Kakinuma, M. Komine, K. Tamaki, T. Shimizu, and T. Izumi. 2002. Characterization of mouse cysteinyl leukotriene receptors mCysLT1 and mCysLT2: differential pharmacological properties and tissue distribution. *J. Biol. Chem.* **277:** 18763–18768.
44. Yokomizo, T., K. Kato, K. Terawaki, T. Izumi, and T. Shimizu. 2000. A second leukotriene B(4) receptor, BLT2. A new therapeutic target in inflammation and immunological disorders. *J. Exp. Med.* **192:** 421–432.
45. Leier, I., M. Müller, G. Jedlitschky, and D. Keppler. 1992. Leukotriene uptake by hepatocytes and hepatoma cells. *Eur. J. Biochem.* **209:** 281–289.
46. Devchand, P. R., A. K. Hihi, M. Perroud, W. D. Schleuning, B. M. Spiegelman, and W. Wahli. 1999. Chemical probes that differentially modulate peroxisome proliferator-activated receptor alpha and BLTR, nuclear and cell surface receptors for leukotriene B(4). *J. Biol. Chem.* **274:** 23341–23348.