Biochimica et Biophysica Acta 1862 (2016) 2043–2053

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Hepatic carboxylesterase 3 (Ces3/Tgh) is downregulated in the early stages of liver cancer development in the rat

Ariel D. Quiroga [a,b,*], María P. Ceballos [a], Juan P. Parody [a], Carla G. Comanzo [a], Florencia Lorenzetti [a], Gerardo B. Pisani [b], María T. Ronco [a], María de L. Alvarez [a,b], María C. Carrillo [a,b]

[a] Instituto de Fisiología Experimental (IFISE), Facultad de Ciencias Bioquímicas y Farmacéuticas, CONICET, UNR, Rosario, Argentina
[b] Área Morfología, Facultad de Ciencias Bioquímicas y Farmacéuticas, UNR, Rosario, Argentina

ARTICLE INFO

Article history:
Received 1 June 2016
Received in revised form 21 July 2016
Accepted 9 August 2016
Available online 12 August 2016

Keywords:
Carboxylesterases
Liver preneoplasia
Hypertriglyceridemia
Polyunsaturated fatty acids
Rat
Lipid metabolism

ABSTRACT

It is accepted that cancer development is associated with metabolic changes. Previously, we established a model of hepatic preneoplasia in which adult rats were subjected to a 2-phase model of hepatocarcinogenesis (initiated-promoted, IP) for 6 weeks until they develop altered hepatic foci (AHF). Here, we found that a whole metabolic shift occurs in order to favor cancer development. IP animals presented with increased plasma lipids due to increased VLDL secretion as well as increased liver lipid accretion due to stimulated transacetylase activity rather than lipogenesis, compared to control rats. We found that carboxylesterase 3/triacylglycerol hydrolase (Ces3/Tgh) presented with a perilobular distribution surrounding lipid droplets in normal livers. However, it is downregulated both at the protein and mRNA level in liver homogenates and is almost undetectable inside the AHF with no changes in the surrounding tissue. Ces3/Tgh expression is regulated by ω-3 fatty acids, thus, supplementation of diet with fish oil, allowed the restoration of Ces3/Tgh expression inside the foci and, more interestingly, led to the decrease in number and volume of the AHF. These studies show a preventive role of Ces3/Tgh in liver cancer development.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world [1,2]. Its incidence is increasing dramatically in the Western world due to increased underlying hepatic conditions such as non-alcoholic fatty liver disease (NAFLD). NAFLD is defined as a set of pathological conditions ranging from fatty liver to non-alcoholic steatohepatitis, which eventually progresses to cirrhosis and its associated complications, liver failure and HCC. Today it is accepted that an increase in lipid production is a general molecular phenomenon during the progression of hepatocarcinogenesis [3,4]. The mechanisms by which lipid accumulation occurs in the development of HCC are not exactly known; however, it was reported that insulin resistance associated with obesity, metabolic syndrome and diabetes lead to an increased secretion of adipose tissue fatty acid, together with secretion of several pro-inflammatory cytokines, as well as a decreased secretion of adiponectin [5,6]. Taken together or separately, these factors promote the development of hepatic steatosis and inflammation in the liver, and can eventually lead to development of cancer [1,6].

On the other hand, carboxylesterases are enzymes of the endoplasmic reticulum distributed in various tissues, with high expression in liver and intestine [7]. Carboxylesterases hydrolyze a variety of esters, amides, carbamates and similar structures of drugs and xenobiotics, although little is known about the physiology and the nature of their natural substrates. The nomenclature of carboxylesterases is confused [7], for example, carboxylesterase 3/triacylglycerol hydrolase (Ces3/Tgh) in the mouse and rat, corresponds to carboxylesterase 1 (CES1) in humans, which is also known as a triacylglycerol hydrolase (TGH). Interestingly, some carboxylesterases appear to play a role (directly or indirectly) in preventing the development of liver cancer. It

Abbreviations: Ces3/Tgh, carboxylesterase 3/triacylglycerol hydrolase; IP, initiated-promoted or initiation-promotion; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; TGH, triacylglycerol hydrolase; Ces, carboxylesterase; Es-x, esterase-x; DEN, diethylnitrosamine; 2-AAF, 2-acetylaminofluorene; TG, triacylglycerol; PUFA, polyunsaturated fatty acids; AHF, altered hepatic foci; C, control; PL, phospholipids; FC, free cholesterol; CE, cholesteryl ester; FA, fatty acid; NEFA, non-esterified fatty acid; VLDL, very low-density lipoprotein; apo, apolipoprotein; P-407, poloxamer-407; DMEM, Dulbecco's Modified Eagle Medium; TLC, thin layer chromatography; OA, oleic acid; BSA, bovine serum albumin; WAT, white adipose tissue; AST, aspartate aminotransferase; ALT, alanine aminotransferase; rGST P, rat glutathione S-transferase Pi class; MTP, microsomal TG transfer protein; UCP-1, Uncoupling protein-1; PCR, polymerase chain reaction; SREBP, sterol regulatory element binding protein; FAS, fatty acid synthase; SCD, stearoyl-CoA desaturase; DGAT, diacylglycerol acyl transferase; CYC, cyclophilin; CNX, calnexin; H&E, hematoxylin & eosin; A-FFA, adipose tissue derived-free fatty acids.

* Corresponding author at: Área Morfología, Facultad de Ciencias Bioquímicas y Farmacéuticas, UNR. Instituto de Fisiología Experimental (IFISE), CONICET, Rosario, Argentina.

E-mail addresses: quiroga@ifise-conicet.gov.ar (A.D. Quiroga), ceballos@ifise-conicet.gov.ar (M.P. Ceballos), parody@ifise-conicet.gov.ar (J.P. Parody), cgcomanzo@gmail.com (C.G. Comanzo), lorenzetti@ifise-conicet.gov.ar (F. Lorenzetti), gpisani@fbioyf.unr.edu.ar (G.B. Pisani), ronco@ifise-conicet.gov.ar (M.T. Ronco), alvarez@ifise-conicet.gov.ar (M.L. Alvarez), carrillo@ifise-conicet.gov.ar (M.C. Carrillo).

http://dx.doi.org/10.1016/j.bbadis.2016.08.006
0925-4439/

is known that CES1 expression decays in most cases of human HCC [8]. Similarly, preliminary data from our laboratory showed that protein expressions of Ces3/Tgh, and another structurally related carboxylesterase, carboxylesterase 1 or esterase-x (Ces1/Es-x) are reduced in liver of wild-type mice exposed to a model of initiation with diethylnitrosamine (DEN, complete carcinogen) and promotion with 2-acetylaminofluorene (2-AAF) and other proliferating models. A definitive and conclusive explanation for these observations is not available yet, but it is believed that triacylglycerols (TG) are possible substrates for these enzymes. In a recent study, we have demonstrated that Ces1/Es-x preferentially hydrolyzes polyunsaturated-fatty acid (PUFA)-containing TGs [9]. Thus, it is likely that decreasing TG by carboxylesterases would reduce chances for cancer cells to get energy from these molecules. Lipid metabolism becomes an attractive field of investigation for better understanding the complex relationship between carboxylesterases, their regulation by PUFA and the development of cancer in the liver.

In the present study, we focused on the evaluation of lipid metabolism in a model of hepatic preneoplasia in the rat. In normal livers Ces3/Tgh was expressed in hepatocytes around the central vein with no immunoreactivity in the periportal areas. Also, Ces3/Tgh was found around the cytosolic lipid droplets. In early stages of hepatic cancer development, we found that hepatocytes transform their metabolism by downregulating key enzymes involved in lipid metabolism. Indeed, Ces3/Tgh was downregulated both at the protein and mRNA level in liver homogenates and it was almost undetectable inside the altered hepatic foci (AHF). Regulation of carboxylesterases by the transformed hepatocytes, seem to be crucial for the promotion and progression of hepatocarcinogenesis.

2. Materials and methods

2.1. Animals

Adult male Wistar rats weighing 250–280 g were maintained in a room at constant temperature with a 12 h light–dark cycle, with food and water supplied ad libitum. Experimental protocols were performed according to the NIH "Guide for the Care and Use of Laboratory Animals" (Publication no. 25-28, revised 1996), and approved by the local animal care and use committee. Animals were divided into two groups 6 animals each. An overview of the experimental protocol is provided in Fig. 1A. Animals of the initiated–promoted (IP) group were subjected to a two-phase model of rat hepatocarcinogenesis, as previously described [10]. Briefly, animals received two intraperitoneal necrogenic doses of DEN (150 mg/kg body weight) 2 weeks apart. One week after the last injection of DEN, rats received 20 mg/kg body weight of 2-AAF by gavage for four consecutive days per week during 3 weeks. Control (C) rats received the vehicle df the drugs the length of the treatment [10]. At the end of the six-week treatment, animals were fasted overnight and some animals were used for VLDL secretion, others were used for hepatocyte isolation and others were anesthetized and sacrificed, and livers were removed and processed.

2.2. Plasma and tissue metabolic parameters

Plasma and tissue levels of TG, phospholipids (PL), free cholesterol (FC), and cholesteryl esters (CE) were determined by a chemical commercial kit (Weiner Lab, Rosario, Argentina). For tissue lipid levels, lipids were extracted previous to the determination by the Folch method [11]. Blood glucose was monitored using blood glucose strips and the Accu-Check glucometer (Roche Diagnostics, Vienna, Austria). Plasma insulin concentration was measured by ELISA (Millipore Corp., MA). Plasma fatty acids (FA) levels were determined using NEFA C commercial kit according to the manufacturer's protocol (Wako, Japan). Hepatic enzymes were measured using a commercial kit (Biotron Diagnostic, CA). Ketone bodies were measured according to manufacturer's instructions (Wako, Japan). Plasma β-hydroxybutyrate levels were measured via the cyclic enzymatic method using Autokit Total Ketone Bodies (Wako, Japan).

2.3. Analysis of very low density lipoprotein (VLDL) secretion in vivo

After a 12-h fasting period, rats received an i.p. injection of 1 g/kg body weight of poloxamer-407 (P-407, Sigma-Aldrich, St. Louis, MO). Blood was collected from tail veins before and at 90 and 180 min after the administration of the detergent. Plasma was prepared and TG levels were analyzed. For analysis of newly synthesized apoB, rats were fasted for 4 h and injected with 100 μL of PBS containing 250 μCi of [^{35}S]methionine via tail vein, together with an injection of P-407. Two hours following P-407 injection, blood samples were collected and plasma was isolated and processed for lipid analyses.

2.4. Preparation of primary rat hepatocytes

Primary rat hepatocytes were isolated by collagenase perfusion of livers from C and IP rats. Hepatocytes were either placed in 60 mm collagen-coated dishes or plated on collagen-coated coverslips in six-well dishes at 2×10^5 cells/dish. Hepatocytes were maintained in DMEM supplemented with 10% FBS at 37 °C in humidified air containing 5% CO_2.

2.5. RNA isolation and PCR analysis

Liver total RNA was isolated as previously described [12]. First strand cDNA synthesis from 2 μg of total RNA was performed using Super-Script II reverse transcriptase (Invitrogen) primed by oligo(dT)12–18 primers. The transcripts of Ces3/Tgh were evaluated by PCR analysis. Real-time qPCR was employed to detect other transcripts related to fatty acid oxidation, lipogenesis, and secretion. Detailed procedures for real-time qPCR are presented in the Supporting Information. Primers for the various genes are listed in Supporting Table 1.

2.6. Cytology

To visualize lipid droplets, freshly isolated rat hepatocytes were grown on collagen-coated coverslips for 4 h, washed three times with phosphate-buffered saline (PBS), followed by incubation with 2 g/mL Bodipy 493/503 in PBS. Slices were observed in a fluorescent microscope.

2.7. Labeling studies

Before performing these studies, primary rat hepatocytes were incubated overnight with serum-free DMEM. For incorporation of exogenous FA studies (general cellular acyltransferase activity), hepatocytes were incubated in 2 mL of DMEM containing 5 μCi [^3H]oleic acid (OA) dissolved in 0.4 mM OA/ 0.5% FA-free BSA. After 4-h incubation, cells and media were collected for analysis. Lipids were extracted in chloroform:methanol (2:1) containing TG, CE and PL as internal standards. Then lipids were separated by thin-layer chromatography (TLC) on silica gel H TLC plates with the solvent system hexane:isopropyl ether:acetic acid (15:10:1). Lipids were visualized by exposure to iodine, and lipids were recovered and radioactivity determined by scintillation counting. To assess the hepatic lipid biosynthetic capacity, primary rat hepatocytes were incubated in 2 mL of DMEM containing 10 μCi [^3H]acetic acid dissolved in 250 mM non-radiolabeled acetic acid. After 4-h incubation, cells and media were collected for analysis. Lipids were extracted, resolved by TLC, and analyzed as described above.

2.8. β-oxidation in primary hepatocytes

Primary rat hepatocytes were seeded in DMEM supplemented with 10% FBS for 4H. Media were then switched to serum-free DMEM for overnight incubation. Hepatocytes were then incubated for 4 h in 2 mL of DMEM containing 2 μCi [^3H]oleic acid dissolved in 0.4 mM OA/ 0.5% FA-free BSA. Media were collected and 300 μL was transferred to a 0.5 μL Eppendorf tube and this was placed into scintillation vials containing 500 μL water. The vials were tightly capped and incubated for 24 h at 50 °C. Then vials were placed for 24 h at 4 °C. Finally, the tubes were removed from the vials and radioactivity in water was measured by scintillation counting.

2.9. Tissue homogenate preparation and western blotting

Whole liver homogenates were prepared in homogenization buffer (250 mM sucrose, 20 mM Tris–HCl, 5 mM EDTA, pH 7.4) containing phosphatase and protease inhibitors.

For western blotting, equal amounts of proteins were resolved on 10% or 5% SDS-polyacrilamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (PerkinElmer Life Sciences Inc., Boston, MA, USA). Membranes were blocked with T-PBS 10% skimmed milk for 60 min, washed and incubated overnight at 4 °C with primary antibodies. Finally, membranes were incubated with peroxidase-conjugated secondary antibodies and bands were detected by the ECL™ detection system and quantified by densitometry using the

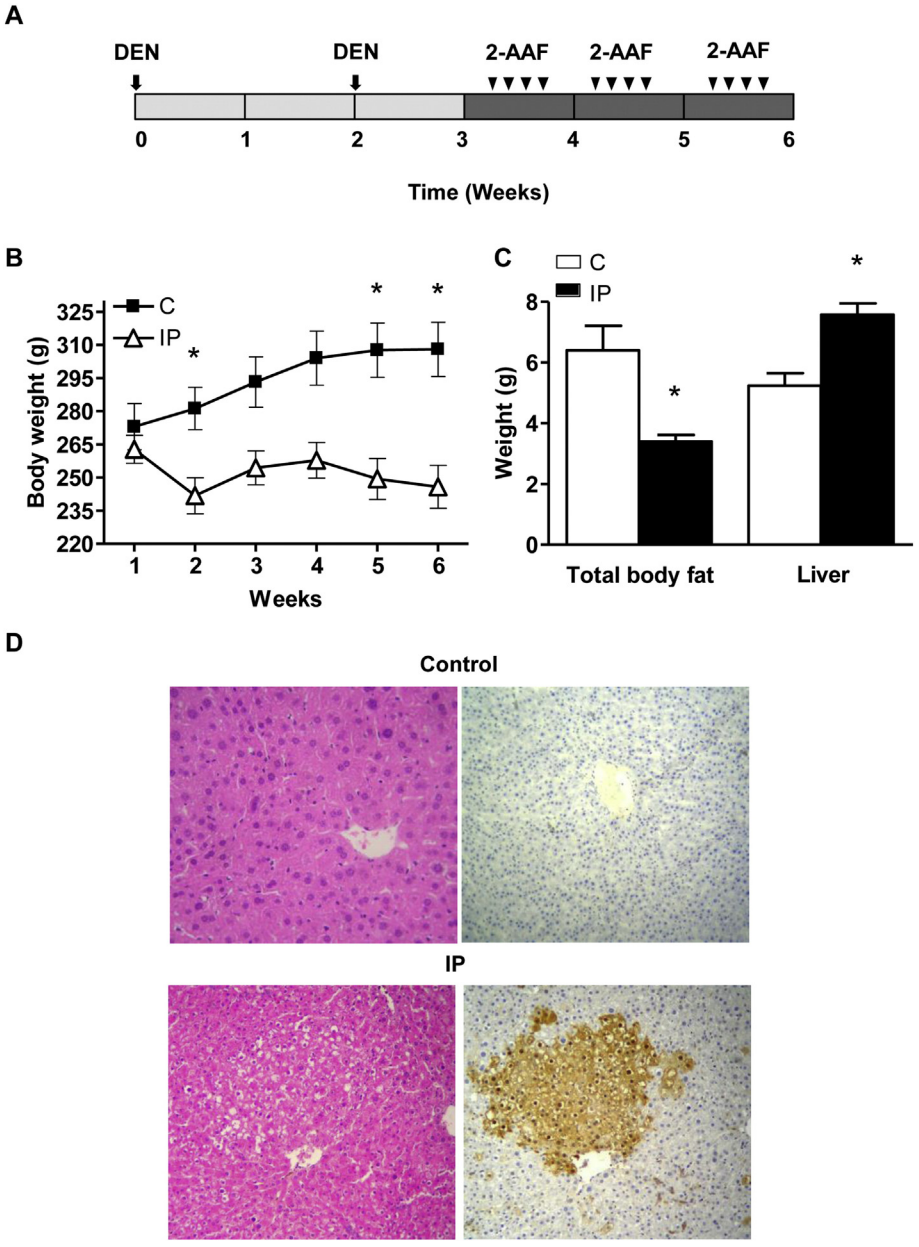

Fig. 1. Altered body, fat and liver weight in IP rats. A) Experimental protocol used to induce liver preneoplasia. Male Wistar rats were subjected to a two-stage model of hepatocarcinogenesis. IP group received two intraperitoneal doses of DEN (150 mg/kg body weight) 2 weeks apart. One week after the last injection, the animals received 20 mg/kg body weight of 2-AAF by *gavage* during 4 consecutive days per week for 3 weeks. Animals were sacrificed at the end of the sixth week. B) Body weight curves on a chow diet for C and IP rats for the 6-week treatment ($n = 5$). C) Epididymal and inguinal fat pads weights and liver weight (n = 5). Results represent means ± SEM. $^*p < 0.05$. D) H&E staining (100×) and immunostaining for r-GST P (100×) of livers from C and IP rats.

Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA) software. Both, equal loading and protein transfer for each membrane were checked by incubations with the proper antibodies and by Ponceau S staining.

2.10. Immunohistochemistry

rGST P has been described as the most effective single marker of hepatic preneoplasia in the rat [13]. Thus, immunohistochemical detection of rGST P is the most widely used method for identification, quantization and assessment of rat altered hepatic foci (AHF) [14]. Liver slices from different lobes were fixed in 10% v/v formalin solution and embedded in low melting paraffin. Immunohistochemical staining was performed using the antibody raised against rGST P (Abcam). The slices were incubated with biotinilated goat anti-rabbit secondary antibody and then with horseradish-peroxidase-conjugated streptavidin (HRP CytoScan Detection Kit, Cell Marque). Signals were detected with DAB Sustrate Kit (Cell Marque) followed by hematoxylin counterstaining. A representative number of field sections (usually 1 to 1.5 cm^2 of tissue per animal was evaluated) from each group were collected using a CCD color video camera (Sony SSC-c370) attached to a Zeiss Axiolab microscope. Images were processed using a NIH imaging analysis system (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). The number of AHF per liver and AHF as percentage of liver were calculated according to the modified Saltykov's method [10] by using the digitized images.

For analysis of Ces3/Tgh expression in and out the AHF, consecutive-section slides were examined by immunohistochemical staining with anti-rGST P and anti-Ces3/Tgh antibodies.

2.11. Fish oil administration

Adult male Wistar rats weighing 250–280 g were *gavaged* with 500 μL/day fish oil (Good N′ Natural, Argentina) two weeks before the treatment start point and along the hepatocarcinogenic treatment described above. Body weight was monitored twice a week. At the end of the treatment, animals were either studied for VLDL secretion or sacrificed by cardiac puncture and tissues (including plasma) were flash-frozen in liquid nitrogen or processed for histology.

2.12. Statistical analysis

Data are presented as means ± SEM. Statistical significance was evaluated by unpaired two-tailed Student's test. Time course studies were evaluated by two-way ANOVA followed by Bonferroni post-test (GraphPad PRISM® 4 software). *P* value <0.05 was interpreted as a significant difference.

3. Results

3.1. Animals subjected to the initiation-promotion treatment appear to be unhealthy and hypertriglyceridemic

Both C and IP rats were fed a chow diet along the development of hepatic preneoplasia. Body weight at the end of the preneoplastic treatment was 23% lower in IP than in C rats (Fig. 1B, Table 1). Also, food consumption was slightly lesser in IP rats, and no signs of dehydration were observed along the treatment (data not shown). Decreased body weight of IP rats was concomitant with a decrease in white adipose tissue (WAT) weight. Although liver size was similar in both groups, liver weight was significantly increased in IP rats (Fig. 1C), which makes the ratio liver/body weight significantly increased in the animals subjected to the hepatocarcinogenic treatment.

Fasted IP rats presented with increased plasma neutral lipid concentrations (37% and 25% for TG and CE, respectively) as well as NEFA plasma concentration, with no changes in plasma FC (Table 1). Hepatic content of TG and CE was increased in IP rats; while FC hepatic content was similar in both groups. IP rats had a modest, non-significant, increase in the activity of the hepatic enzymes AST and ALT in plasma, indicating a trend for liver damage. As usual for the IP model, H&E staining and further examination of the liver showed basically no differences between livers from C or IP rats (Fig. 1D) with no signs of acute or chronic inflammation in spite of the AHF development.

Table 1
Plasma and liver biochemical parameters in rats with hepatic preneoplasia.*

	Control	IP
Body weight (g)	305.18 ± 10.01	245.21 ± 15*
Plasma TG (mg/dl)	53.31 ± 6.70	73.03 ± 5.27*
Plasma CE (mg/dl)	45.61 ± 3.67	57.29 ± 2.55*
Plasma C (mg/dl)	47.31 ± 7.17	45.19 ± 9.91
NEFA (mEq/L)	0.91 ± 0.06	1.34 ± 0.08*
AST (U/L)	71.21 ± 3.71	80.12 ± 2.21
ALT (U/L)	40.10 ± 1.29	50.15 ± 1.90
Liver TG (μg/mg protein)	285 ± 19.36	319 ± 15.17*
Liver CE (μg/mg protein)	10.23 ± 1.25	13.91 ± 2.01*
Liver FC (μg/mg protein)	17.71 ± 2.25	16.17 ± 3.33
Glucose (mmol/L)	4.27 ± 0.11	5.81 ± 0.13*
Insulin (mg/mL)	0.31 ± 0.03	0.42 ± 0.01*
Plasma ketone bodies (μmol/mL)	0.81 ± 0.03	1.29 ± 0.09*

* $p < 0.05$ vs C.

3.2. Increased VLDL secretion in IP rats

During the fasting period, plasma lipid levels primarily reflect hepatic VLDL metabolism. Fasting concentrations of plasma VLDL-TG and VLDL-cholesterol (due to increased CE) were increased in IP rats (Table 1). VLDL secretion in vivo (measured following injection of P-407) was increased in IP rats compared to control rats (Fig. 2A). Plasma apoB100 concentration was augmented in IP group, reflecting increased hepatic secretion of VLDL from these animals (Fig. 2B). To differentiate newly secreted apoB from circulating apoB we injected rats i.v. with [^{35}S]Met/Cys together with intraperitoneal administration of P-407, and followed the secretion of newly-synthesized radiolabeled apoB. Secretion of newly synthesized apoB100 was increased in IP rats compared to control rats (Fig. 2C), which is a characteristic of VLDL over secretion in vivo. Relative lipid composition of isolated VLDL particles was similar for both genotypes (Fig. 2D). As expected, the abundance of hepatic microsomal triglyceride transfer protein (MTP) was higher in IP group respect to C (Fig. 2E).

3.3. Impaired energy homeostasis in IP rats

In spite of lower body weight and less corporal fat mass, IP rats were observed to have higher fasting concentration of glucose (5.81 ± 0.13 mmol/L versus 4.27 ± 0.11 mmol/L for control rats) and higher plasma insulin concentration (0.42 ± 0.05 ng/mL versus 0.31 ± 0.03 ng/mL for control rats) compared to control rats (Table 1), indicating an altered energy homeostasis upon the hepatocarcinogenic treatment.

3.4. Altered metabolic gene expression in IP rats

To further understand the mechanism of action on how IP treatment might contribute to hepatic steatosis, we assessed the expression of genes involved in FA synthesis, oxidation and secretion by quantitative PCR. As shown in Fig. 3A, expressions of the master transcription factors SREBP-1c, SREBP-2, fatty acid synthase (FAS), and stearoyl-CoA desaturase 1 (SCD-1) were not different between groups; however, expression of acyltransferases such as acyl-CoA:diacylglycerol acyltransferase 1 and 2 (DGAT-1 and -2) was significantly increased in IP rats compared to C rats. Also, IP rats showed increased hepatic expression of mRNA encoding FA oxidation enzymes/transporters and lipoprotein secretion compared to C rats (Fig. 3A).

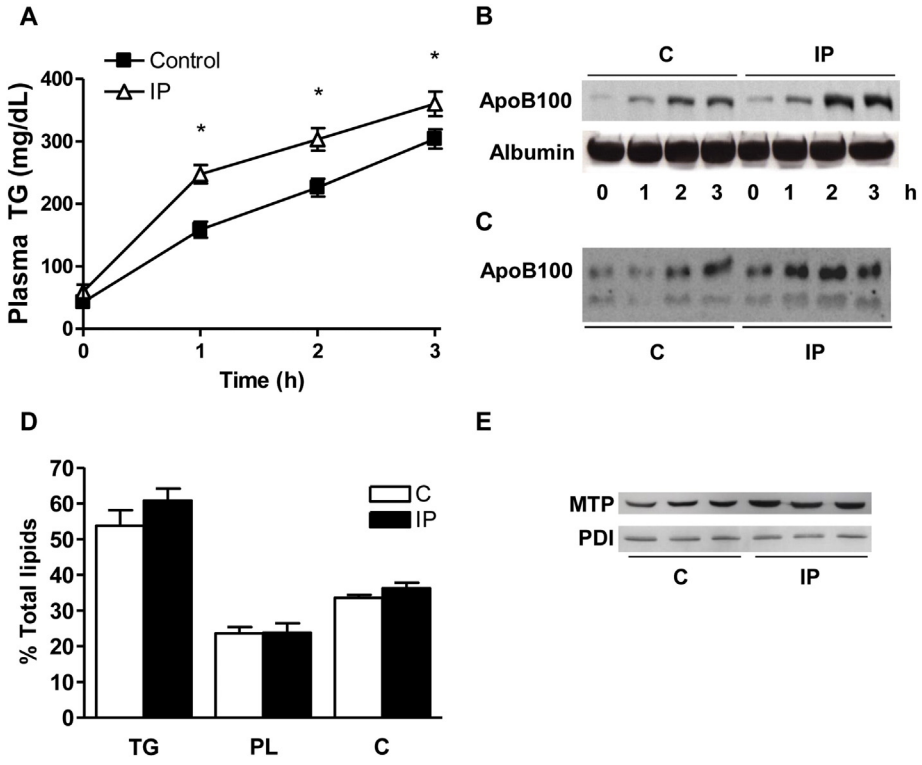

Fig. 2. Increased VLDL secretion in IP rats. (A) In vivo VLDL secretion. (n = 5). Statistical analysis was performed by repeated two-way ANOVA. (B) VLDL apoB protein composition before (time 0) and 3 h after injection of P-407. (C) Increased apoB secretion in vivo in IP rats. (D) Lipid composition of VLDL particles. E) MTP protein expression in total liver homogenates. *$p < 0.05$ vs. C.

3.5. Hepatocytes from IP rats present with increased acyltranseferase activity and normal de novo lipogenesis

To appreciate the contribution by which IP treatment leads to increased lipid accumulation and further steatosis, rat primary hepatocytes were isolated, and metabolic lipid labeling studies were performed. Incubation of hepatocytes from IP rats with OA resulted in increased number of lipid droplets compared to control wild-type hepatocytes (Fig. 3B). Accordingly, the incorporation of exogenously-supplied radiolabeled OA into different lipid species was increased in hepatocytes from IP rats (Fig. 3C), as a general measure of acyltransferase activity. Besides, de novo lipogenesis (as determined by the incorporation of radiolabeled acetate into lipids) was similar in hepatocytes from both groups (Fig. 3D).

3.6. Increased FA oxidation in IP hepatocytes

To support the results observed in gene expression studies, we measured cellular fatty acid oxidation by incubation of hepatocytes with radiolabeled OA, followed by quantitation of radiolabeled H_2O. Hepatocytes from IP rats showed increased OA oxidation compared to C hepatocytes (Fig. 3E).

3.7. Ces3/Tgh has a centrilobular distribution and is associated to cytosolic lipid droplets in normal livers

Ces3/Tgh distribution in normal rat liver is shown in Fig. 4A. Hepatic Ces3/Tgh was expressed in hepatocytes around the central vein (dotted arrows) with a cytoplasmic reticular distribution. Virtually, no immunoreactivity was observed in the periportal areas (straight arrows). At larger magnification, it is visible that Ces3/Tgh is located around the cytosolic lipid droplets, in line with previous unpublished findings from Dr. Lehner's laboratory and with Wang et al. [15].

3.8. Hepatic Ces3/Tgh expression is decreased in rats subjected to the two-phase model of hepatocarcinogenesis

It has been shown that human CES1 (the human orthologue for Ces3/Tgh) was remarkably down-regulated in tumor tissues [8]. In order to determine the relative expression of Ces3/Tgh in rats subjected to the IP treatment, we evaluated its hepatic protein levels and mRNA expression by immunoblotting and RT-PCR, respectively, and also by immunohistochemistry. Ces3/Tgh expression was decreased both at protein and mRNA levels, relative to their respective control animals (Fig. 4B). Interestingly, the immunohistochemical staining showed a significant decrease in the expression of Ces3/Tgh inside the foci in consecutive stained slides (Fig. 4C).

3.9. Dietary fish oil supplementation improves the general metabolic status in IP rats

Based on previous results from Lehner's Laboratory [9], we decided to assess whether PUFA supplementation reverses the lipogenic phenotype in IP rats in vivo. For that purpose, animals were diet supplemented with fish oil 2 weeks before and during the IP protocol, making a total of 8 weeks of fish oil consumption (Fig. 5A). Upon fish oil supplementation, hepatic Ces3/Tgh protein levels and mRNA expression were restored in IP livers to levels comparable to the C animals (Fig. 5B). Also, fish oil supplementation prevented body weight loss in IP rats compared to IP rats without fish oil supplementation (Fig. 5C, Table 2). Fish oil supplementation also had an improving effect on blood glucose disposal, as well as in plasma TG, and CE concentrations in IP rats compared to C rats (Table 2 vs. Table 1). Accordingly, upon fish oil supplementation, hepatic lipid mass was comparable between the two genotypes (Table 2 vs. Table 1). Also, VLDL-apoB100, and VLDL-TG secretions were normalized in IP rats upon fish oil supplementation (Fig. 5D). Besides, it had no effects in the activity of the hepatic enzymes AST and ALT in plasma (Table 2 vs. Table 1).

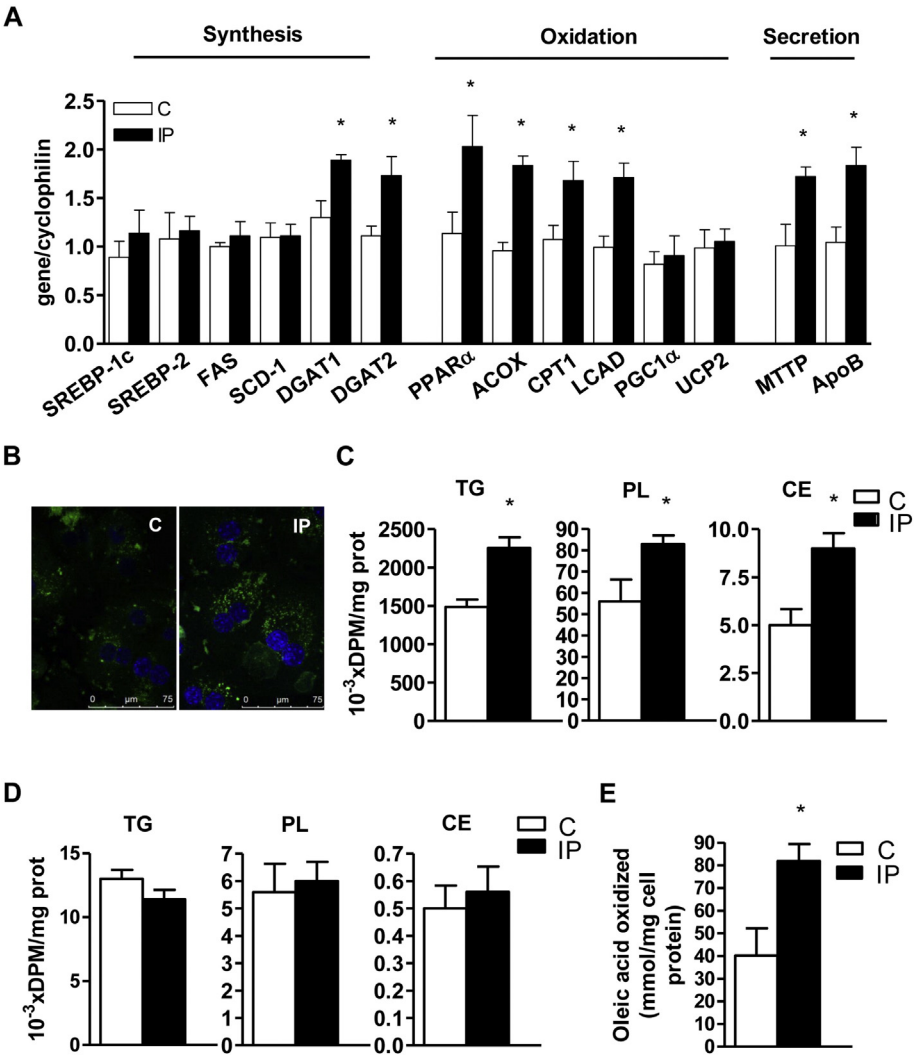

Fig. 3. Altered lipid metabolism in hepatocytes from IP rats. (A) Hepatic expression of lipid synthesis, oxidation and secretion genes in hepatocytes from IP rats by qPCR. (B) BODIPY staining of cultured hepatocytes. C) Incorporation of radiolabeled oleic acid into lipids (n = 3 dishes, 3 rats). D) De novo lipogenesis: Incorporation of radiolabeled acetic acid into lipids (n = 3 dishes, 3 rats). E) Fatty acid oxidation (n = 3 dishes, 3 rats). Bars represent mean ± SEM. $^{*}p < 0.05$.

3.10. Dietary fish oil supplementation reduces the number and volume of AHF

Changes in number and volume percentage of liver rGST P-positive foci are shown in Fig. 6. The number and volume percentages of AHF per liver in the IP rats given fish oil were significantly decreased as compared with the values from the IP group with no supplementation. Fish oil treatment reduced ~61% the total number of initiated cells capable of developing into clones of AHF respect to IP group. Fish oil decreased the growth rate and total cellular population of AHF, measured as the volume fraction of the liver occupied by foci (56% decreased respect to IP group). It is interesting to note that in fish oil supplemented IP group Ces3/Tgh expression was decreased inside some foci, as observed in IP animals without FO supplementation, but it was also completely restored in others.

4. Discussion

The earliest emerging types of AHF are composed by differentiated hepatocytes that show particular metabolic and molecular aberrations that gradually progress to the poorly differentiated neoplastic phenotype. Tumor cells show an increased protein, DNA, and FA synthesis [16,17]. In agreement, lipogenic pathways have been described to be upregulated in diverse types of human cancers [18–20]. In about two-thirds of HCC, a prominent change in lipid metabolism has been described, known by abundant cellular lipid droplets predominantly enriched in neutral lipids. In particular, early HCC may present with diffuse fatty change in contrast to other usually not showing any visible lipid accumulation [20]. This makes clear how important the metabolic change is in the transformed cells from the very early stages of cancer development. Until now, the lipid accumulation during the development of HCC was mainly attributed to increased fatty acid synthesis [3,16]. This, is mainly regulated by the cytoplasmic enzyme fatty acid synthase (FAS) which gene is, in turn, a target for the sterol regulatory element binding protein class 1(SREBP-1) (for excellent reviews on lipid synthesis and regulation see [21,22]). However, in the present study, we described a novel metabolic feature of cancer development in which fatty acid acetylation is increased rather than de novo lipogenesis commonly observed [16,20].

Here we analyzed for the first time the metabolic profile of rats subjected to a chemical model of early liver cancer development known as preneoplasia. There is a vast literature regarding the metabolic changes in cancer [4,23–26]; however, none of them are equally conclusive, since there are many controversies among the different studies

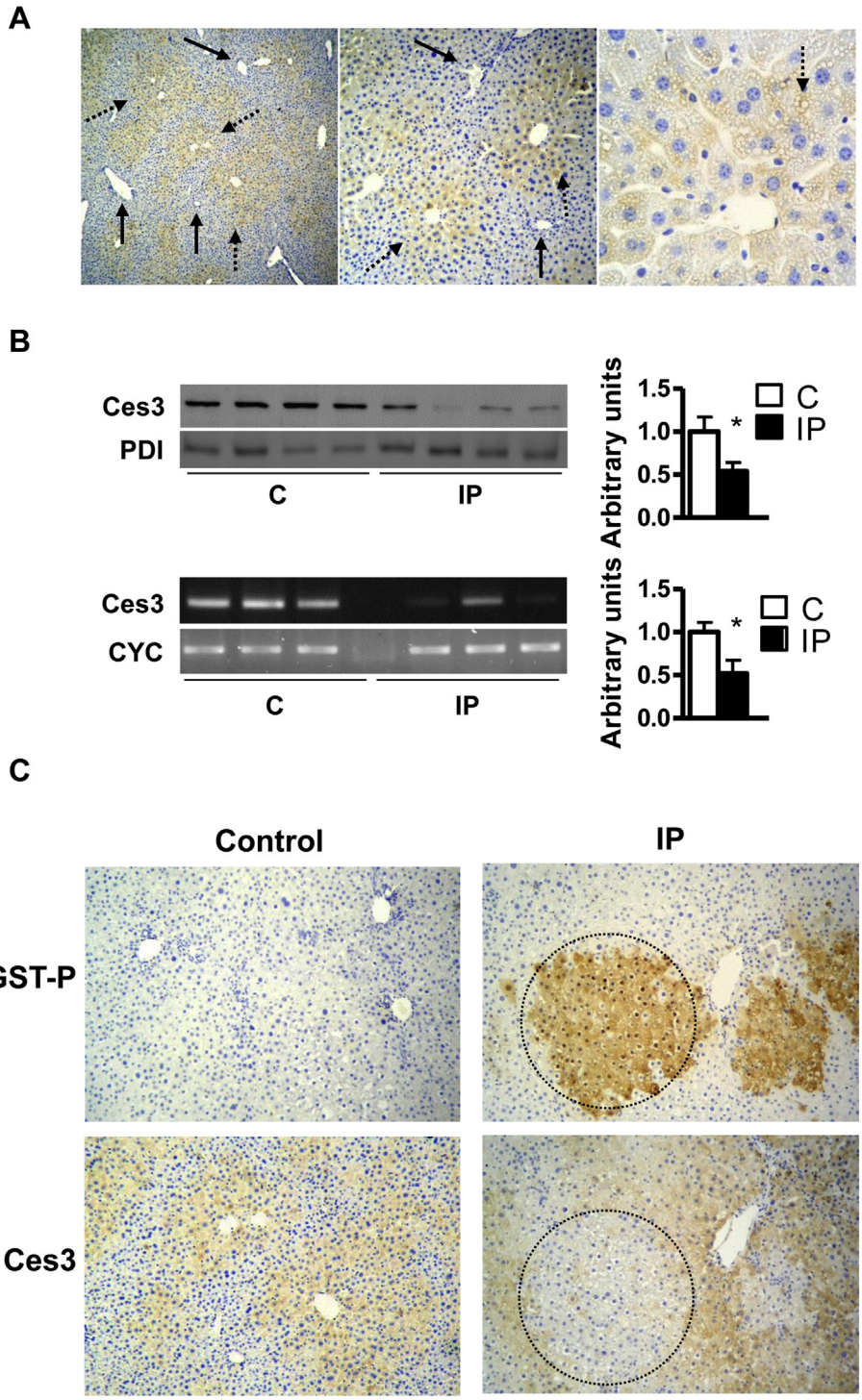

Fig. 4. Decreased Ces3/Tgh expression in AHF of IP rats. A) Representative images of liver sections showing Ces3/Tgh distribution. In the left (40×) and center (100×) panels, straight arrows show the portal areas and dotted arrows show pericentral areas. In the right panel (400×) dotted arrow shows Ces3/Tgh localization around the lipid droplets. B) Immunoblot and RT-PCR showing expression of Ces3/Tgh in liver homogenates. The bars on the right represent the densitometric analysis of the bands. Bars represent mean ± SEM. $^{*}p < 0.05$. C) Microphotographs showing rGST P and Ces3/Tgh expression in liver slides. The dotted circle shows the area occupied by foci (100×).

depending on each cancer model/system used. Thus, the knowledge of the metabolic changes in the stages of preneoplasia may pave the way to the development of new prevention strategies. Fig. 7 shows a schematic summary of the results in this study. We found several interesting and key metabolic differences between IP and C rats. As expected, most of the differences are almost phenotypically undetectable with respect to control rats; however, major changes upon the IP treatment occur at the molecular level, and they appear to be enough to induce the further progress of cancer. It is clear that we cannot exclude the possibility that some of the results of this study are due in part to the damage induced by the chemical carcinogens and not entirely to the cancer development. In this regard, at the end of the IP treatment, rats presented with decreased body weight and a slight and non-significant decrease in food intake. However, we found that the mild weight loss

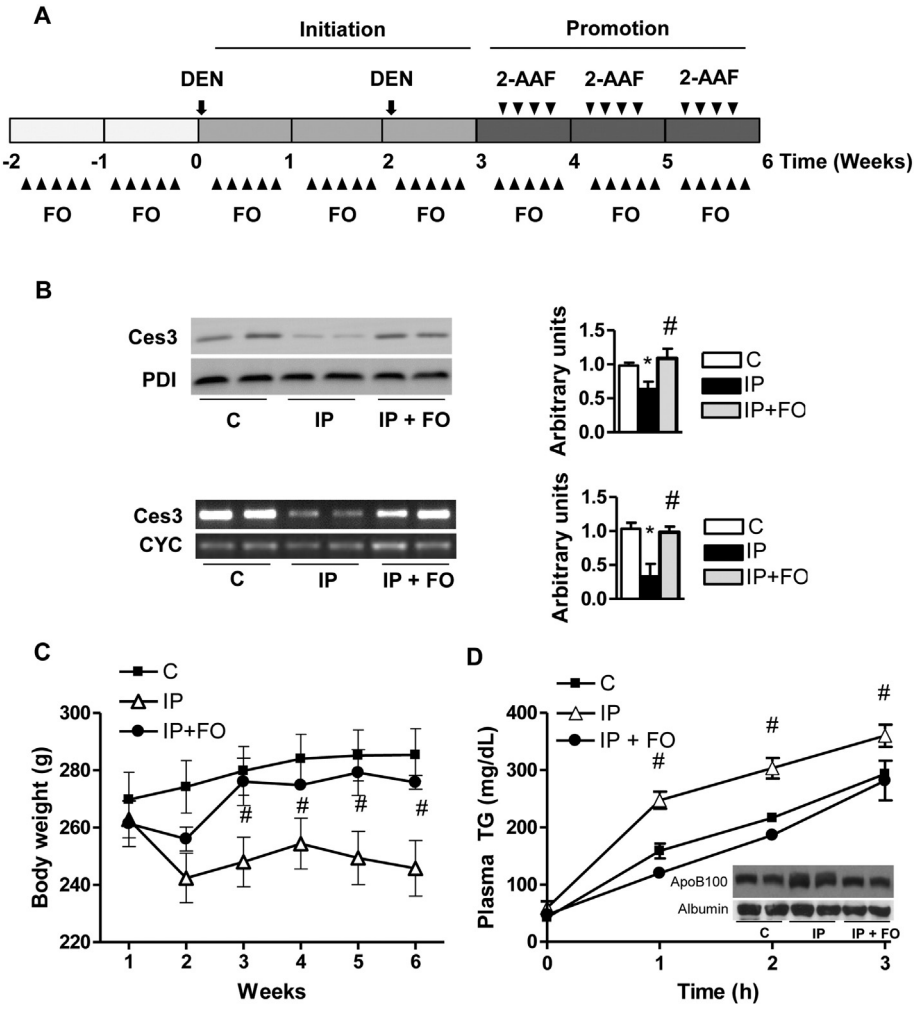

Fig. 5. Fish oil supplementation restores Ces3/Tgh expression in livers of IP rats. A) Scheme showing the protocol followed for FO administration. B) Ces3/Tgh expression in rat livers measured by immunoblotting and RT-PCR (upper and lower panels, respectively). The bars on the right represent the densitometric analysis of the bands. Bars represent means ± SEM. $^{*}p < 0.05$ vs. C; $^{\#}p < 0.05$ vs. IP. C) Body weight curves during FO administration. D) In vivo VLDL secretion upon FO administration. Inset shows the immunoblotting for ApoB100 in the same samples where TG were analyzed.

is predominantly a result of enhanced WAT lipolytical activity. This may be the physiological response from a generous WAT to a hungry liver that requires energy for the survival of the early transformed cells. It seems clear that weight loss is a hallmark of established cancers, and this is the result of the active metabolism and the combined interaction of different tissues from the very early stages of cancer development. In this connection, we found that plasma NEFA concentrations are significantly higher in IP rats with respect to control rats, and these are likely to be the building blocks for TG and CE through activation of the acyltransferase machinery in the liver. Indeed, liver weight was considerably increased in fasted IP rats, in line with liver neutral lipid accumulation. As stated above, it is interesting to note that neutral lipids accumulated in the cytosolic lipid droplets from fasting hepatocytes come from an exacerbated acyltransferase activity rather than an increase in lipogenesis. This became evident after both gene expression and labeling studies. This is novel since most studies to date show increased lipogenic phenotypes [16,20].

Table 2
Body weight and plasma and liver biochemical parameters upon fish oil supplementation.

	IP + FO
Body weight (g)	291.32 ± 15.11[#]
Plasma TG (mg/dl)	50.15 ± 5.12[#]
Plasma CE (mg/dl)	42.28 ± 3.75[#]
Plasma FC (mg/dl)	45.23 ± 2.85
NEFA (mEq/l)	1.37 ± 0.11
AST (U/l)	67.28 ± 3.11
ALT (U/l)	45 ± 2.21
Liver TG (μg/mg protein)	274.26 ± 11.12[#]
Liver CE (μg/mg protein)	11.33 ± 4.95[#]
Liver FC (μg/mg protein)	15.44 ± 1.97
Glucose (mmol/L)	4.01 ± 0.72[#]
Insulin (mg/mL)	0.37 ± 0.24[#]
Plasma ketone bodies (μmol/mL)	1.75 ± 1.47[#]

[#] $p < 0.05$ vs. IP in Table 1.

Increased fasting plasma lipid levels are a reflection of lipoprotein secretion from the liver. VLDL secretion, as evaluated as plasma TG and ApoB100 concentrations upon P-407 administration, was considerably increased in IP rats. MTP, the rate-limiting protein in VLDL secretion, showed significantly increased mRNA expression and protein abundance. It is interesting that insulin inhibits VLDL secretion and promotes apoB proteosomal degradation. On the other hand, insulin modulates MTP through a balance between ERK and p38 MAPK expression [27,28], where p38 MAPK favors MTP inhibition. In our study we

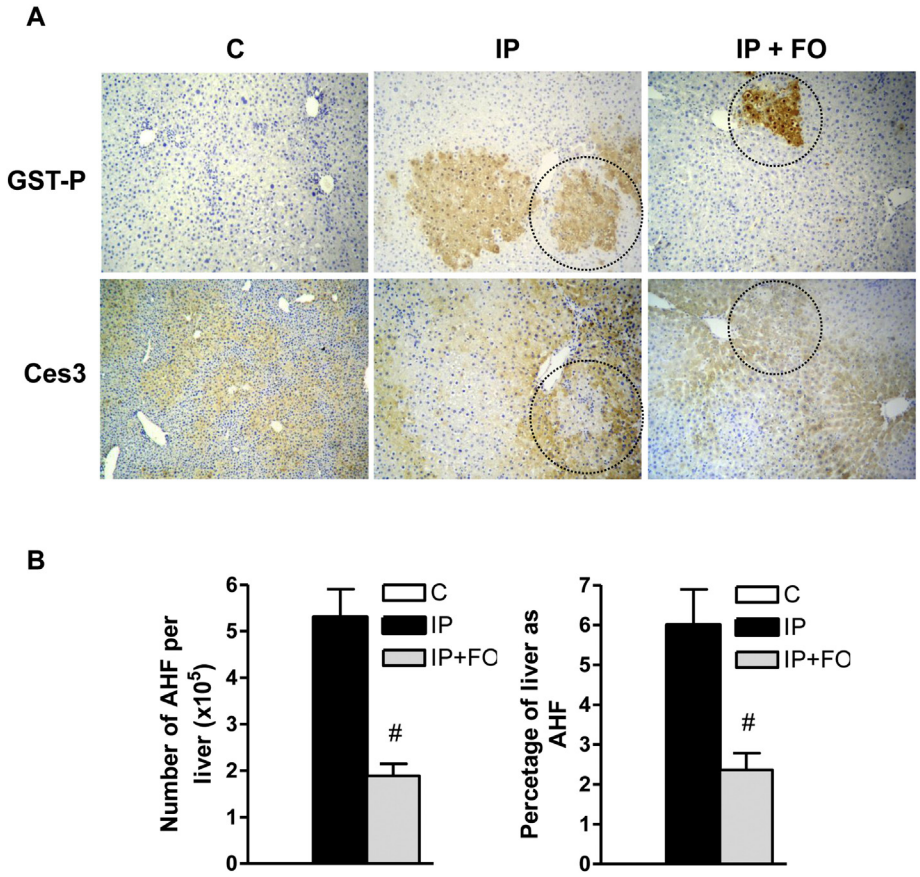

Fig. 6. FO reduces the number and volume of AHF together with Ces3/Tgh expression restoration in IP rats. A) rGST P immunostaining (100×). B) Ces3/Tgh immunostaining (100×). Dotted circles show the foci area. C) Densitometric analysis of AHF. Bars represent mean ± SEM. $^{\#}p < 0.05$ vs. IP.

showed that IP rats presented with hyperinsulinemia, and in a previous report we demonstrated that p38 MAPK protein expression is increased in IP rats. Altogether, this shows the dynamic fine-tuning of VLDL secretion through insulin and p38 MAPK balance even from the early stages of liver carcinogenesis. However, hepatocytes from IP livers keep extruding formed lipids to the extracellular compartment in order to maintain the cellular energy status, while free FA keep entering the liver from the peripheral fat pads. Concomitantly, and to the same end, FA oxidation plays a similar role in this phase of cancer development in order to decrease cellular lipid levels. Together, it appears to be a complex balance between the physiological (normal cells) and the physiopatological (cancer cells) mechanisms in order to decrease cellular lipid levels and to gain energy for survival at the very early stages of chemical hepatocarcinogenesis.

It is accepted that global metabolism and lipid metabolism, particularly, is reprogrammed in most cancers and plays a critical role in the pathogenesis of human diseases, and lipid metabolic aberrations are present in most types of hematological and solid tumors [4,18,25,26]. Excessive lipid supply to skeletal muscle, liver, and pancreas is believed to be one of the main causes of insulin resistance and is the likely contributor to the impaired insulin tolerance in IP rats.

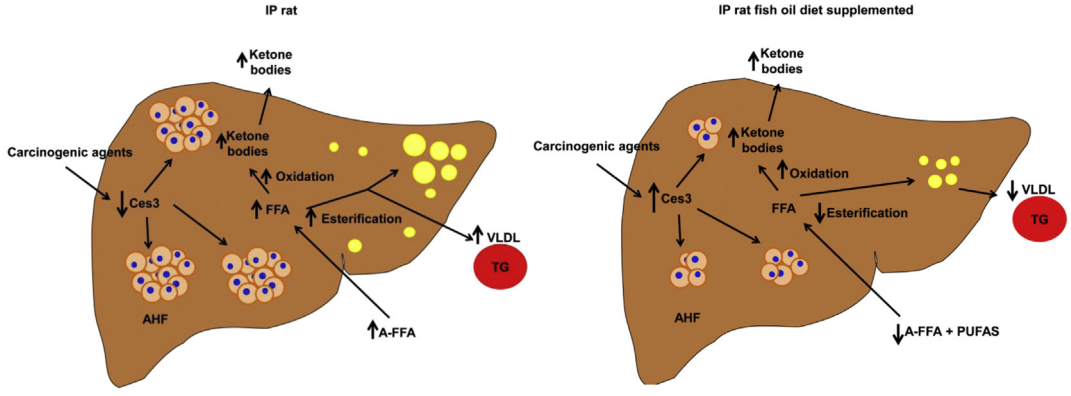

Fig. 7. Proposed model for IP animal before and after FO administration. The left panel indicates the lipid metabolic status in IP rats. The right panel shows the lipid metabolic status in IP rats after administration of FO. Adipose tissue-derived free fatty acids (A-FFA).

The most interesting finding in this work is the fact that Ces3/Tgh, an enzyme from the endoplasmic reticulum with carboxylesterase and lipase activities, is almost undetectable inside the AHF in IP rats. This magnifies the importance of regulation of this enzyme for cancer development. In a very interesting work, Maki et al. [29] showed a remarkable decrease in liver carboxylesterase activities using the Solt–Farber method in rats. They claimed that hepatic carboxylesterase activity decrease resulted in cellular accumulation of diacylglycerol, which then activates protein kinase C (PKC), which is directly related to cancer progression and metastasis. Nowadays it is accepted that PKC participates in many different cellular processes and its activation can lead to several disorders including cancer. We believe that Ces3/Tgh downregulation is not only modulating PKC expression, but also leads to changes in the cellular lipid metabolism in order to favor cancer cell survival.

It is important to ask whether Ces3/Tgh downregulation is a consequence of the chemical treatment or the transformed cells induce the decrease in enzyme expression, and probably activity as well, in order to favor their own growth. We can answer part of the question based in our own experience and in the literature. First, protein expressions of Ces3/Tgh, and other structurally related carboxylesterase, Ces1/Es-x are reduced in liver of wild-type mice exposed to DEN and 2-AAF, and it is also downregulated both at the protein and mRNA levels in human HCC samples [8]. Second, Ces3/Tgh protein expression is also decreased in regenerating livers after partial hepatectomy [29]; and finally, the hepatoma cell lines McArdle RH-77777 (rat) and HepG2 (human) lack these enzymes (tested in our laboratory, not shown).

Experiments using virus expressing Ces3/Tgh might shed some light into the original question. Nevertheless, a close approach using fish oil as a source of PUFA in order to regulate Ces3/Tgh expression, allowed us to demonstrate that upon Ces3/Tgh upregulation the general cancerous phenotype was restored back to a phenotype similar to control rats. More interesting, upon fish oil supplementation the number and volume of the AHF was significantly reduced in IP animals. These observations might explain in part many of the beneficial effects of PUFA in human diseases including cancer. In this regard, inhibition of lipid synthesis makes cancer cells more susceptible to oxidative stress-cell death. Since mammalian cells cannot produce PUFA, high rates of de novo lipid synthesis in cancer cells raises the cellular amount of saturated and monounsaturated FA, thus protecting cells from oxidative stress [30]. This occurs because PUFA are more susceptible to oxidation and generation of peroxyradicals.

To our knowledge this is the first work showing Ces3/Tgh downregulation, which finally favors AHF growth. Now it is clear that in the early stages of cancer development the whole body metabolism shifts to a more liver-centered metabolism, which is evidently different from the metabolism in more advanced stages. This is not only important from the therapeutics, but also from a preventive point of view, since changing and modeling alimentary habits could be extremely beneficial and this will have repercussions on health and economic matters. In this connection, it is interesting to note how versatile caroboxylesterases are, since global ablation of Ces3/Tgh in mice resulted in diminished VLDL secretion, improved plasma lipid profile and insulin sensitivity and decreased lipogenesis [31,32], but on the other hand, ablation of Ces1/Es-x in mice resulted in obesity, hepatic steatosis, increased hepatic lipogenesis, insulin resistance and decreased energy expenditure [9,33]. In a recent study, Lehner and collaborators have published a very interesting article on the role of Ces3/Tgh in Bcr/Abl-transformed precursor B cells skin tumor growth. They showed that the skin xenoimplants are able to disrupt VLDL production and turnover, leading to a mild hyperlipidemia in mice. Interestingly, this hyperlipemic phenotype is indirectly attenuated by genetic ablation of Ces3/Tgh, resulting in diminished tumor growth by a reduced supply of cholesterol-rich lipoproteins to the tumor [34]. Here, instead, we focused on the direct role of Ces3/Tgh on hepatocyte transformation and growth. This is different from what Lehner et al. have observed since loss of Ces3/Tgh in mice decreases blood lipids, due to impaired VLDL secretion and improves glucose tolerance [31], and the resulting low-lipid phenotype directly impacts on the skin xeoimplant growth. Apparently contradictory, both studies conclude that the lipid environment is responsible for the tumor growth.

5. Conclusions

Dysregulated lipid metabolism is one of the most recognized trademarks of cancer. Lipids are important for maintaining cellular structure, basically forming the membrane functional framework of protein complexes. Lipids also serve as fat storage depots and signaling molecules. The last decades of work began to reveal the importance of lipids for cancer biology. Here, we described a novel role for Ces3/Tgh in the metabolic shift occuring in a chemically induced liver cancer model. Ces3/Tgh is an ER enzyme which abundance was found to be strongly decreased in the early stages of liver cancer development, leading to cachexia with liver and plasma lipid accumulation. The restoration of Ces3/Tgh expression by PUFA reverted the metabolic phenotype and, more importantly, led to decrease in number and volume of the AHF, showing a preventive role of Ces3/Tgh in liver cancer development. The research connecting the molecular mechanisms that lead to HCC with its development and progression is required in order to recognize and categorize new targets for the early diagnosis, chemoprevention, and treatment of this disease.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2016.08.006.

Funding

This work was supported by research grant PICT 2013 No 1440 (to A.D. Quiroga) from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT).

Conflicts of interest

None

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

We would like to thank Dr. Richard Lehner for the generous anti-Ces3/Tgh antibody gift and for the constant support and care. We also thank Wiener Lab for the generous gift of critical reagents.

References

[1] B.Q. Starley, C.J. Calcagno, S.A. Harrison, Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection, Hepatology 51 (2010) 1820–1832.
[2] M. Stotz, A. Gerger, J. Haybaeck, T. Kiesslich, M.D. Bullock, M. Pichler, Molecular targeted therapies in hepatocellular carcinoma: past, present and future, Anticancer Res 35 (2015) 5737–5744.
[3] E. Currie, A. Schulze, R. Zechner, T.C. Walther, R.V. Farese Jr., Cellular fatty acid metabolism and cancer, Cell Metab. 18 (2013) 153–161.
[4] C.R. Santos, A. Schulze, Lipid metabolism in cancer, FEBS J. 279 (2012) 2610–2623.
[5] S.H. Caldwell, D.M. Crespo, H.S. Kang, A.M. Al-Osaimi, Obesity and hepatocellular carcinoma, Gastroenterology 127 (2004) S97–103.
[6] S.A. Harrison, Liver disease in patients with diabetes mellitus, J. Clin. Gastroenterol. 40 (2006) 68–76.
[7] R.S. Holmes, M.W. Wright, S.J. Laulederkind, L.A. Cox, M. Hosokawa, T. Imai, S. Ishibashi, R. Lehner, M. Miyazaki, E.J. Perkins, P.M. Potter, M.R. Redinbo, J. Robert, T. Satoh, T. Yamashita, B. Yan, T. Yokoi, R. Zechner, L.J. Maltais, Recommended nomenclature for five mammalian carboxylesterase gene families: human, mouse, and rat genes and proteins, Mamm. Genome 21 (2010) 427–441.
[8] K. Na, E.Y. Lee, H.J. Lee, K.Y. Kim, H. Lee, S.K. Jeong, A.S. Jeong, S.Y. Cho, S.A. Kim, S.Y. Song, K.S. Kim, S.W. Cho, H. Kim, Y.K. Paik, Human plasma carboxylesterase 1, a novel serologic biomarker candidate for hepatocellular carcinoma, Proteomics 9 (2009) 3989–3999.

[9] A.D. Quiroga, L. Li, M. Trotzmuller, R. Nelson, S.D. Proctor, H. Kofeler, R. Lehner, Deficiency of carboxylesterase 1/esterase-x results in obesity, hepatic steatosis, and hyperlipidemia, Hepatology 56 (2012) 2188–2198.
[10] M. de Lujan Alvarez, J.P. Cerliani, J. Monti, C. Carnovale, M.T. Ronco, G. Pisani, M.C. Lugano, M.C. Carrillo, The in vivo apoptotic effect of interferon alfa-2b on rat preneoplastic liver involves Bax protein, Hepatology 35 (2002) 824–833.
[11] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[12] M.L. Casella, J.P. Parody, M.P. Ceballos, A.D. Quiroga, M.T. Ronco, D.E. Frances, J.A. Monti, G.B. Pisani, C.E. Carnovale, M.C. Carrillo, M. de Lujan Alvarez, Quercetin prevents liver carcinogenesis by inducing cell cycle arrest, decreasing cell proliferation and enhancing apoptosis, Mol. Nutr. Food Res. 58 (2014) 289–300.
[13] T. Imai, T. Masui, M. Ichinose, H. Nakanishi, T. Yanai, T. Masegi, M. Muramatsu, M. Tatematsu, Reduction of glutathione S-transferase P-form mRNA expression in remodeling nodules in rat liver revealed by in situ hybridization, Carcinogenesis 18 (1997) 545–551.
[14] H.C. Pitot, Altered hepatic foci: their role in murine hepatocarcinogenesis, Annu. Rev. Pharmacol. Toxicol. 30 (1990) 465–500.
[15] H. Wang, E. Wei, A.D. Quiroga, X. Sun, N. Touret, R. Lehner, Altered lipid droplet dynamics in hepatocytes lacking triacylglycerol hydrolase expression, Mol. Biol. Cell 21 (2010) 1991–2000.
[16] J.A. Menendez, R. Lupu, Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis, Nat. Rev. Cancer 7 (2007) 763–777.
[17] N. Yahagi, H. Shimano, K. Hasegawa, K. Ohashi, T. Matsuzaka, Y. Najima, M. Sekiya, S. Tomita, H. Okazaki, Y. Tamura, Y. Iizuka, R. Nagai, S. Ishibashi, T. Kadowaki, M. Makuuchi, S. Ohnishi, J. Osuga, N. Yamada, Co-ordinate activation of lipogenic enzymes in hepatocellular carcinoma, Eur. J. Cancer 41 (2005) 1316–1322.
[18] D.A. Tennant, R.V. Duran, H. Boulahbel, E. Gottlieb, Metabolic transformation in cancer, Carcinogenesis 30 (2009) 1269–1280.
[19] J.V. Swinnen, K. Brusselmans, G. Verhoeven, Increased lipogenesis in cancer cells: new players, novel targets, Curr. Opin. Clin. Nutr. Metab. Care 9 (2006) 358–365.
[20] T. Yamashita, M. Honda, H. Takatori, R. Nishino, H. Minato, H. Takamura, T. Ohta, S. Kaneko, Activation of lipogenic pathway correlates with cell proliferation and poor prognosis in hepatocellular carcinoma, J. Hepatol. 50 (2009) 100–110.
[21] P. Ferre, F. Foufelle, Hepatic steatosis: a role for de novo lipogenesis and the transcription factor SREBP-1c, Diabetes Obes. Metab. 12 (Suppl. 2) (2010) 83–92.
[22] J. Ye, R.A. DeBose-Boyd, Regulation of cholesterol and fatty acid synthesis, Cold Spring Harb. Perspect. Biol. 3 (2011).
[23] R.J. DeBerardinis, A. Mancuso, E. Daikhin, I. Nissim, M. Yudkoff, S. Wehrli, C.B. Thompson, Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 19345–19350.
[24] O. Warburg, On the origin of cancer cells, Science 123 (1956) 309–314.
[25] P.P. Hsu, D.M. Sabatini, Cancer cell metabolism: Warburg and beyond, Cell 134 (2008) 703–707.
[26] A. Schulze, A.L. Harris, How cancer metabolism is tuned for proliferation and vulnerable to disruption, Nature 491 (2012) 364–373.
[27] W.S. Au, H.F. Kung, M.C. Lin, Regulation of microsomal triglyceride transfer protein gene by insulin in HepG2 cells: roles of MAPKerk and MAPKp38, Diabetes 52 (2003) 1073–1080.
[28] M.C. Lin, D. Gordon, J.R. Wetterau, Microsomal triglyceride transfer protein (MTP) regulation in HepG2 cells: insulin negatively regulates MTP gene expression, J. Lipid Res. 36 (1995) 1073–1081.
[29] T. Maki, M. Hosokawa, T. Satoh, K. Sato, Changes in carboxylesterase isoenzymes of rat liver microsomes during hepatocarcinogenesis, Jpn. J. Cancer Res. 82 (1991) 800–806.
[30] E. Rysman, K. Brusselmans, K. Scheys, L. Timmermans, R. Derua, S. Munck, P.P. Van Veldhoven, D. Waltregny, V.W. Daniels, J. Machiels, F. Vanderhoydonc, K. Smans, E. Waelkens, G. Verhoeven, J.V. Swinnen, De novo lipogenesis protects cancer cells from free radicals and chemotherapeutics by promoting membrane lipid saturation, Cancer Res. 70 (2010) 8117–8126.
[31] E. Wei, Y. Ben Ali, J. Lyon, H. Wang, R. Nelson, V.W. Dolinsky, J.R. Dyck, G. Mitchell, G.S. Korbutt, R. Lehner, Loss of TGH/Ces3 in mice decreases blood lipids, improves glucose tolerance, and increases energy expenditure, Cell Metab. 11 (2010) 183–193.
[32] J. Lian, A.D. Quiroga, L. Li, R. Lehner, Ces3/TGH deficiency improves dyslipidemia and reduces atherosclerosis in Ldlr(−/−) mice, Circ. Res. 111 (2012) 982–990.
[33] A.D. Quiroga, J. Lian, R. Lehner, Carboxylesterase1/esterase-x regulates chylomicron production in mice, PLoS One 7 (2012), e49515.
[34] J. Huang, L. Li, J. Lian, S. Schauer, P.W. Vesely, D. Kratky, G. Hoefler, R. Lehner, Tumor-induced hyperlipidemia contributes to tumor growth, Cell Rep. 15 (2016) 336–348.