

Up-Regulation of PPAR- γ mRNA Expression in the Liver of Obese Patients: an Additional Reinforcing Lipogenic Mechanism to SREBP-1c Induction

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Introduction: Triglyceride accumulation in the liver is an early feature in the development of nonalcoholic fatty liver disease (NAFLD) associated with human obesity, which is a multifactorial syndrome and whose underlying mechanisms are beginning to be understood.

Objectives: Liver peroxisome proliferator-activated receptor- γ (PPAR- γ) mRNA expression was measured as a signaling mechanism related to steatosis in obese patients with NAFLD.

Methods: Liver PPAR- γ and sterol receptor element-binding protein 1c (SREBP-1c) mRNA (real-time RT-PCR), serum total adiponectin (RIA), and high molecular weight (HMW)-adiponectin (ELISA) levels, and insulin resistance (IR) evolution (homeostasis model assessment-IR) were determined in 22 obese NAFLD patients (16 with steatosis and six with steatohepatitis) who underwent subtotal gastrectomy with gastrojejunal anastomosis in Roux-en-Y and 16 nonobese subjects who underwent laparoscopic cholecystectomy (controls).

Results: Liver PPAR- γ mRNA levels were 112 and 188% higher ($P < 0.05$) than control values in obese patients with steatosis and steatohepatitis, respectively, who also exhibited 70 and 62% increases in those of SREBP-1c, concomitantly with IR and lower levels of serum total adiponectin and HMW-adiponectin ($P < 0.05$). Liver PPAR- γ expression showed positive associations with SREBP-1c mRNA levels ($r = 0.86$; $P < 0.0001$), serum insulin levels ($r = 0.39$; $P < 0.01$), and homeostasis model assessment-IR ($r = 0.60$; $P < 0.0001$), and negative correlations with total adiponectin ($r = -0.37$; $P < 0.01$) and HMW-adiponectin ($r = -0.51$; $P < 0.001$) levels in serum.

Conclusions: PPAR- γ is up-regulated in the liver of obese patients with NAFLD, representing an additional reinforcing lipogenic mechanism to SREBP-1c induction in the development of hepatic steatosis. (*J Clin Endocrinol Metab* 96: 1424–1430, 2011)

Triglyceride accumulation in the liver, or steatosis, is an early feature in the development of nonalcoholic fatty liver disease (NAFLD) associated with human obesity, which is a multifactorial syndrome and whose underlying mechanisms are beginning to be understood (1, 2). Recently, it was reported that the expression of transcription factors controlling lipid metabolism is deranged in the liver of obese NAFLD patients (3). This is characterized by: 1) enhancement in the mRNA expression of sterol receptor element binding

protein 1c (SREBP-1c) inducing lipogenic genes such as fatty acid synthase (FAS); 2) diminution in the mRNA expression of peroxisome proliferator-activated receptor α (PPAR- α) controlling fatty acid (FA) oxidation and secretion; with 3) consequent elevation in the SREBP-1c/PPAR- α ratio as a determinant factor favoring hepatic lipogenesis over FA oxidation leading to steatosis (3).

Although SREBP-1c has emerged as a major mediator of hepatic lipogenesis, which is under transcriptional con-

trol by insulin (4) under normal and insulin resistance (IR) conditions (5) and by liver X receptor activation (6), transcription factors such as carbohydrate responsive element binding protein (7) and PPAR- γ (8) may also play a role. In this context, carbohydrate responsive element binding protein activation is mainly achieved by glucose overload; however, its expression in the liver of NAFLD patients is significantly lower than that in the control liver (9). PPAR- γ plays a crucial role in adipogenesis and insulin sensitization (10), a transcription factor that is expressed in the liver at a level that is 9–12% that of adipose tissue in man (11). Furthermore, ribonuclease protection assay data revealed a significant enhancement in PPAR- γ mRNA expression in abdominal sc adipose tissue from obese patients compared with that in lean subjects, a process that is up-regulated synergistically by insulin and corticosteroids after *in vitro* exposure to isolated human adipocytes (11). In this study, we addressed the hypothesis that up-regulation of PPAR- γ expression occurs in the liver of obese NAFLD patients as a mechanism of hepatic steatosis. For this purpose, PPAR- γ mRNA expression in liver samples from control subjects and obese patients was assessed by real-time RT-PCR, and data obtained were correlated with the hepatic expression of SREBP-1c, IR, and the serum levels of total and high molecular weight (HMW)-adiponectin.

Patients and Methods

Patients and laboratory studies

Thirty-eight subjects were studied, including 22 obese NAFLD patients (16 with hepatic steatosis and six with steatohepatitis) who underwent subtotal gastrectomy with a gastrojejunal anastomosis in Roux-en-Y as a therapy for obesity and 16 nonobese patients who underwent laparoscopic cholecystectomy (control group). The protocol was explained in detail to the subjects, who then gave their written informed consent to participate in the study before any procedure was undertaken. Exclusion criteria included positive hepatitis B or C serology, positive antibodies (antinuclear, anti-mitochondrial, and anti-smooth muscle antibodies), smoking habits or nonsmokers with less than 1-yr cessation, and consumption of more than 40 g of ethanol per week. Nutritional and alcohol consumption histories with anthropometric measurements were obtained. IR was calculated from the fasting insulin and glucose values by homeostasis model assessment (HOMA) of IR analysis [fasting insulin (μ U/ml) \times fasting glucose (mmol/liter)/22.5] (12). Plasma adiponectin concentrations were measured in duplicate by RIA with antibody against human adiponectin and dilution of recombinant adiponectin as standard; inter- and intraassay coefficients of variation were below 7% and below 5%, respectively (Linco Research, St. Charles, MO). Plasma HMW-adiponectin levels were determined in duplicate by ELISA (EZHMWA-64K; Millipore, St. Charles MO) according to the manufacturer's specifications, with a sensitivity of 0.5 ng/ml and inter- and intraassay coefficients of variation of 7 and 3%, respectively. Laboratory tests also included serum liver parameters and lipid profile (Table 1). Both control and obese NAFLD patients were subjected to a diet

TABLE 1. Clinical and biochemical parameters in control subjects and obese patients with steatosis or steatohepatitis

Parameter (normal range)	Controls (n = 16)	Obese patients	
		Steatosis (n = 16)	Steatohepatitis (n = 6)
Age (yr)	36 \pm 2.6	38 \pm 1.8	40 \pm 3.6
Male/female ratio	5/11	6/10	2/4
Body mass index (<25 kg/m ²)	26 \pm 0.5	43 \pm 1.3 ^a	42 \pm 1.5 ^a
Waist circumference (cm)	86 \pm 4	120 \pm 2.8 ^a	120 \pm 3.9 ^a
Fasting serum glucose (<100 mg/dl)	90 \pm 2.7	96 \pm 2.3	93 \pm 4.0
Fasting serum insulin (<20 μ U/ml)	10 \pm 1.8	27 \pm 1.9 ^a	33 \pm 5.3 ^a
HOMA-IR (<2.5)	2.0 \pm 0.3	6.4 \pm 0.5 ^a	7.9 \pm 1.4 ^a
Serum total adiponectin (μ g/ml)	13.3 \pm 0.8	8.5 \pm 0.7 ^a	8.3 \pm 0.9 ^a
Serum HMW-adiponectin (μ g/ml)	7.5 \pm 0.5	3.1 \pm 0.4 ^a	2.3 \pm 0.5 ^{a,b}
Liver parameters			
Alanine aminotransferase (9–52 IU/liter)	41 \pm 4	48 \pm 4	51 \pm 9
Aspartate aminotransferase (14–36 IU/liter)	28 \pm 2	37 \pm 3	33 \pm 4
Alkaline phosphatase (38–126 IU/ml)	91 \pm 5	92 \pm 5	89 \pm 11
γ -Glutamyl transpeptidase (12–43 IU/ml)	45 \pm 7	41 \pm 7	37 \pm 6
Total bilirubin (0.2–1.3 mg/dl)	0.61 \pm 0.09	0.51 \pm 0.05	0.36 \pm 0.05
Lipid profile			
Total cholesterol (<200 mg/dl)	180 \pm 8	190 \pm 6	180 \pm 22
LDL cholesterol (<140 mg/dl)	94 \pm 9	120 \pm 4	120 \pm 16
HDL cholesterol (>40 mg/dl)	47 \pm 3	44 \pm 2	40 \pm 2
Total triglyceride (<150 mg/dl)	140 \pm 20	140 \pm 13	140 \pm 18

Values represent means \pm SE for the number of patients indicated. LDL, Low-density lipoprotein; HDL, high-density lipoprotein.

^a $P < 0.05$ compared with controls.

^b $P < 0.05$ compared with steatosis (one-way ANOVA and Newman Keuls' test).

of 25 kcal/kg body weight (where 1 kcal = 4.184 kJ), with 30% of the energy given as lipids and 15% as proteins, for at least 2 d before surgery; liver tissue of approximately 0.5 cm³ for histological diagnoses, PPAR- γ , and SREBP-1c mRNA determinations were taken during surgery. The samples were fixed in 10% formalin and paraffin embedded, and sections were stained with hematoxylin/eosin and Van Gieson's stains. Sections of each liver sample were observed in a blinded manner and evaluated for histological alterations by means of previously defined codes (3). Liver samples for biochemical determinations were frozen at –80 C. The Ethics Committee of the University of Chile Clinical Hospital and that of the Faculty of Medicine, University of Chile, approved the study protocol, which was performed in accordance with the 1975 Helsinki Declaration criteria (sixth revision, 2008).

Real-time RT-PCR assay for PPAR- γ and SREBP-1c mRNA

Total RNA was extracted from homogenized liver biopsies with Trizol reagent (Invitrogen Corp., Carlsbad, CA). Quality and quantity of RNA were checked visually before and after DNase digestion (TURBO DNase-free; Ambion, The RNA Co., Austin, TX) by denaturing gel electrophoresis and by photometric analysis (A₂₆₀ and A₂₈₀). Synthesis of cDNA was performed with 2 μ g of total RNA using SuperScript III (Invitrogen Corp.) and random hexamers according to standard procedures. Real-time RT-PCRs were performed in a LightCycler system (Roche Diagnostics, Mannheim, Germany) using SYBR Green to monitor cDNA amplification. Equal amounts of cDNA corresponding to 1/15 dilution of cDNA were used in each reaction, containing 5 μ l Platinum SYBR Green I SuperMix-UDG, 0.5 μ l BSA 20 X (Invitrogen Corp.), and 5 pmol of forward and reverse primers in a total volume of 10 μ l. The standard thermal profile used was 2 min at 50 C, 2 min at 95 C, 50 repeats of 5 sec at 95 C, 15 sec at 60 C, and a final stage of 15 sec at 72 C. Data were analyzed using LightCycler3 analysis software (Roche Diagnostics). PCR efficiency was determined for each sample and gene by LinRegPCR v7.5 (available at <http://LinRegPCR.nl>) (13). Two technical repeats were done for each combination of cDNA and primer pair, and the quality of the PCRs was checked through analysis of the dissociation and amplification curves. The products were resolved by 3% agarose gel electrophoresis to confirm the DNA fragments of expected size. Transcript levels of genes were normalized to the respective transcript level of constitutively expressed control gene, human large ribosomal protein Rp1p0 (14, 15). Normalization of transcript levels of genes by β -actin gave similar results to those obtained with Rp1p0 (data not shown). Values shown are relative transcript level (RTL) \times 1000. The PCR primers for amplification of PPAR- γ (NM-015869) were: forward, 5'-ACAGACAAAT-CACCATTCGT-3', and reverse, 5'-CTCTTTGCTCTGCTCCTG-3'; and of SREBP-1c (NM-001005291): forward, 5'-ATACCACCGCGTCTACC-3', and reverse, 5'-CAC-CAACAGCCCATTGAG-3' (Invitrogen Corp.).

Statistical analyses

Data showing Gaussian distribution using the Kolmogorov-Smirnov test are expressed as means \pm SE for the number of patients indicated. Statistical analysis of the differences between mean values from control subjects and obese NAFLD patients was assessed by either one-way ANOVA and the Newman-

Keuls' test or by Student's *t* test for unpaired data as indicated. The differences were considered statistically significant at $P < 0.05$. To analyze the association between different variables, the Spearman rank order correlation coefficient was used. All statistical analyses were computed using GraphPad Prism version 2.0 (GraphPad Software Inc., San Diego, CA).

Results

As presented in Table 1, subjects were predominantly female and exhibited comparable ages. Control patients ($n = 16$) had normal liver histology, whereas those in the obese group ($n = 22$) presented either simple macrovesicular steatosis ($n = 16$) or steatohepatitis ($n = 6$). NAFLD patients were significantly more obese than controls, as evidenced by their body mass index and waist circumference being 65 and 40% higher than controls, respectively. Fasting blood glucose levels in control and NAFLD patients were comparable, whereas fasting insulin levels in obese patients with steatosis or steatohepatitis were 170 and 230% higher than controls ($P < 0.05$), respectively, resulting in 220 and 295% enhancement ($P < 0.05$) in the HOMA index of IR. Fasting levels of total adiponectin in serum were 36 and 37.5% lower in steatosis and steatohepatitis ($P < 0.05$) than control values, respectively, whereas those of HMW-adiponectin in patients with steatosis were 31% lower than controls ($P < 0.05$), and in patients with steatohepatitis were 69 and 26% lower than controls and patients with steatosis ($P < 0.05$), respectively. The majority of patients were asymptomatic, with parameters of liver function (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ -glutamyl transpeptidase, and total bilirubin) and lipid levels (total cholesterol, high-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol, and triacylglycerols) in serum being within normal ranges in the studied groups.

Relative transcript levels of liver PPAR- γ revealed values 112 and 188% higher in steatosis and steatohepatitis over those in controls ($P < 0.05$), respectively (Fig. 1). Liver PPAR- γ mRNA expression in patients with steatosis or steatohepatitis was comparable, with a net 130% enhancement ($P < 0.05$) being observed in the joined group of obese patients with steatosis and steatohepatitis compared with control subjects (Fig. 1, *inset*).

In the studied patients, liver PPAR- γ mRNA levels were positively correlated with those of SREBP-1c ($r = 0.86$; $P < 0.0001$) (Fig. 2) also assessed by real-time RT-PCR. This correlation still holds when a steatohepatitis patient with values in the control group is not considered, suggesting that PPAR- γ and SREBP-1c are associated over critical values of expression. Furthermore, PPAR- γ and SREBP-1c mRNA levels were also correlated when the separate group of patients with steatosis ($n = 16$; $r = 0.87$;

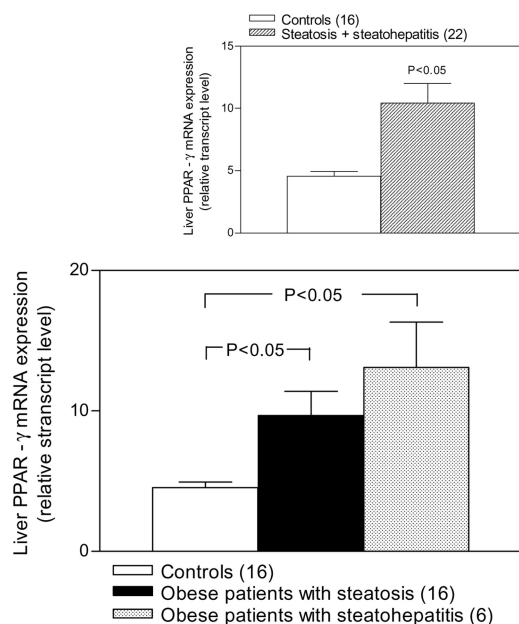


FIG. 1. Expression of PPAR- γ mRNA in the liver of control subjects ($n = 16$) and obese NAFLD patients with steatosis ($n = 16$) or steatohepatitis ($n = 6$). *Inset*, Liver PPAR- γ mRNA levels in controls and all NAFLD patients (steatosis + steatohepatitis; $n = 22$). Measurements were carried out by real-time RT-PCR, and values shown were normalized to the respective transcript level of the constitutively expressed control gene Rp1p0, expressed as means \pm SE. Significance studies were performed by one-way ANOVA and the Newman-Keuls test or by Student's t test for unpaired data (*inset*).

$P < 0.0001$) or with steatohepatitis ($n = 6$; $r = 0.85$; $P < 0.005$) are considered. Liver SREBP-1c mRNA levels exhibited net increases of 70 and 62% in obese patients with steatosis and steatohepatitis over control values ($P < 0.05$); however, expression in patients with steatosis or steatohepatitis was comparable (Fig. 2, *inset*). Levels of PPAR- γ in the liver of controls and obese patients with steatosis and steatohepatitis significantly correlated with both serum insulin levels ($r = 0.39$; $P < 0.01$) and HOMA-IR index ($r = 0.60$; $P < 0.0001$) (Fig. 3A). In the latter correlation, removal of two steatohepatitis patients with extremely high HOMA-IR values (Fig. 3A) sustained the positive association between liver PPAR- γ mRNA levels and the IR index ($r = 0.37$; $P < 0.01$). In addition, PPAR- γ exhibited a negative association with both total adiponectin ($r = -0.37$; $P < 0.01$) (Fig. 3B) and HMW-adiponectin levels in serum ($r = -0.51$; $P < 0.001$) (Fig. 3C).

Discussion

Studies addressing the expression of PPAR- γ in the human liver have established the expression of the splice variants PPAR- γ 1 and PPAR- γ 2 in lean subjects (11). Data presented indicate that hepatic PPAR- γ mRNA levels are significantly increased in obese NAFLD patients with either

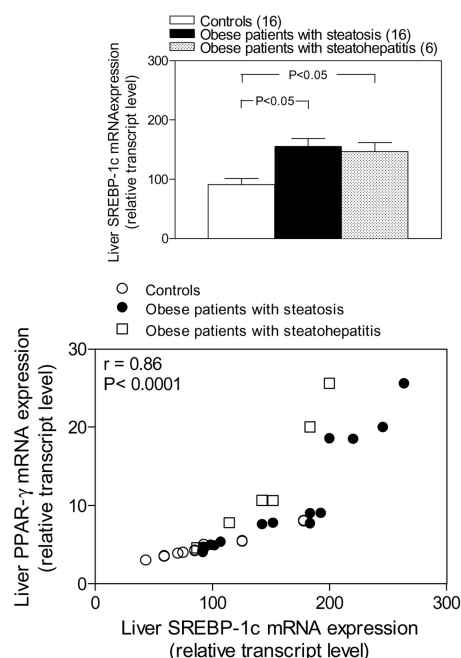


FIG. 2. Correlation between PPAR- γ mRNA expression and SREBP-1c mRNA expression in the liver of control subjects ($n = 16$) and obese NAFLD patients with steatosis ($n = 16$) or steatohepatitis ($n = 6$). *Inset*, Liver SREBP-1c mRNA expression assessed by real-time RT-PCR, normalized to the respective transcript level of the constitutively expressed control gene Rp1p0 and expressed as means \pm SE. The Spearman rank order correlation coefficient was used to assess the association between variables. Differences in average values were analyzed by one-way ANOVA and the Newman-Keuls test (*inset*).

steatosis or steatohepatitis, over lean control values, in agreement with data assessing the PPAR- γ 2 isoform (15). However, assessment of PPAR- γ expression in the liver of NAFLD patients has produced conflicting results, considering the studies showing either comparable levels in NAFLD patients and control subjects (16) or decreased PPAR- γ 1 and PPAR- γ 2 values with comparable levels of SREBP-1c expression (17). These findings are difficult to interpret considering that, in the former study, clinical and metabolic status of the studied patients was not reported (16), and in the latter study, control and NAFLD patients were lean subjects, as evidenced by their body mass indexes being below 25 kg/m² (17).

The main role of PPAR- γ in the liver is related to the regulation of glucose and lipid metabolism. Although liver PPAR- γ is not abundantly expressed under normal conditions, high expression levels are associated with induction of PPAR- γ -responsive genes related to lipid metabolism (18). These include: 1) lipoprotein lipase; 2) proteins involved in FA uptake, binding, and transport, such as FA translocase (FAT/CD36), FA transport proteins 2 and 5 (FATP2 and FATP5), and FA binding proteins 1 and 5 (FABP1 and FABP5); and 3) liver X receptor favoring both PPAR- γ and FAT/CD36 expression (18, 19). Accordingly, PPAR- γ up-regulation observed in the liver of obese

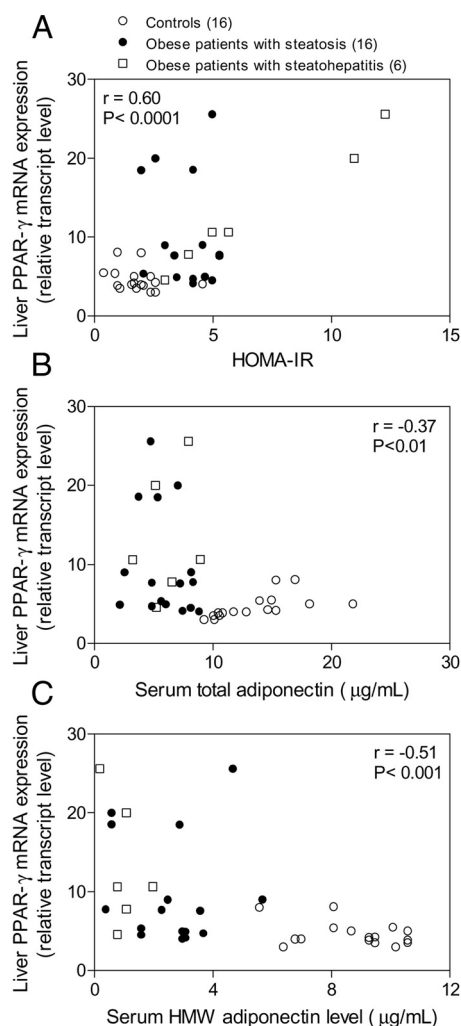


FIG. 3. Correlations between liver PPAR- γ mRNA levels and the HOMA-IR (A), the serum levels of total adiponectin (B), and the serum levels of HMW-adiponectin (C) in control subjects ($n = 16$) and obese NAFLD patients with steatosis ($n = 16$) or steatohepatitis ($n = 6$). The Spearman rank order correlation coefficient was used to assess the association between different variables.

NAFLD patients may have prosteatotic effects, considering: 1) the development of IR-dependent higher mobilization of nonesterified FAs from the adipose tissue to the liver (20, 21); and 2) up-regulation of lipoprotein lipase (22), FAT/CD36, and FATP5 (20) affording enhanced uptake and intracellular binding/transport of nonesterified FAs, thus leading to increased *de novo* FA biosynthesis (22, 23) (Fig. 4).

Up-regulation of PPAR- γ coincided with that of SREBP-1c in the liver of obese patients compared with controls, in agreement with previous data assessing SREBP-1c by RT-PCR analysis (3), parameters that were significantly correlated. It is important to note that both transcription factors are crucial in determining a lipogenic outcome because it was reported that SREBP-1c gene deletion in mice results in a 50% diminution in FA synthesis (24), indicating that SREBP-1c activity alone is not suffi-

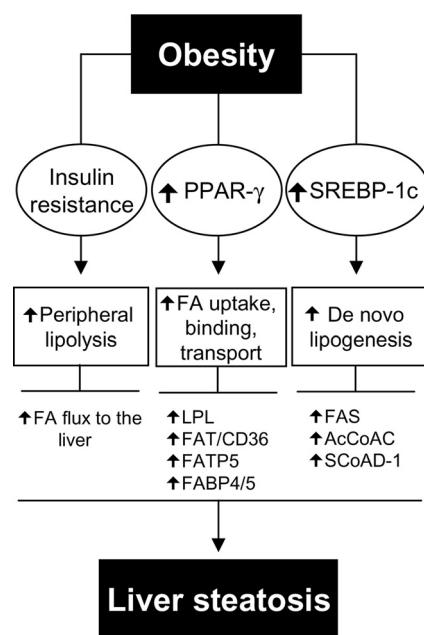


FIG. 4. Obesity-induced changes in insulin resistance and in the transcription factors PPAR- γ and SREBP-1c, leading to hepatic steatosis in NAFLD. AcCoAC, Acetyl-CoA carboxylase; LPL, lipoprotein lipase; SCoAD-1, stearoyl-CoA desaturase-1.

cient to account for the activation of lipogenic gene expression in response to carbohydrate. Furthermore, the lipogenic gene expression pattern controlled by both transcription factors is complementary rather than similar, considering that SREBP-1c activates the expression of most genes required for hepatic lipogenesis, such as acetyl-CoA carboxylase, FAS, and stearoyl-CoA desaturase-1, whereas PPAR- γ mainly induces proteins related to FA uptake, binding, and transport (18, 19, 25). These observations suggest that high expression of liver PPAR- γ in obesity is an important prolipogenic factor, which may reinforce the lipogenic actions associated with SREBP-1c up-regulation, thus representing key alterations in liver cell signaling leading to hepatic steatosis (Fig. 4). Liver PPAR- γ expression is significantly associated with the serum levels of insulin and the HOMA-IR index established in controls and obese patients with steatosis and steatohepatitis, supporting the contention that insulin signaling is involved in PPAR- γ up-regulation in obesity, as reported under normal conditions and in IR states (26, 27). Finally, up-regulation of liver PPAR- γ in obese patients occurred concomitantly with lower serum levels of total adiponectin and HMW-adiponectin, molecular forms of the adipokine positively correlating with insulin sensitivity (28, 29). Hypoadiponectinemia in obesity may be considered as an additional prolipogenic factor, due to derangement in the PPAR- α /AMP-activated protein kinase pathway inhibiting the rate-limiting enzyme in FA synthesis acetyl-CoA carboxylase (30). This proposal is supported by the diminished levels of hepatic PPAR- α mRNA expression

observed in obese patients compared with controls (3) and by the negative correlation between liver PPAR- γ mRNA levels and those of serum total adiponectin and HMW-adiponectin established.

Collectively, data presented indicate that the expression of PPAR- γ is enhanced in the liver of obese NAFLD patients in association with IR, which may represent additional mechanism up-regulating genes that encode for lipogenic proteins leading to hepatic steatosis. The prosteatotic action of PPAR- γ up-regulation is likely to reinforce that ascribed to SREBP-1c induction, a condition that may be contributed by 1) enhanced liver X receptor expression (9) activating that of SREBP-1c and PPAR- γ ; 2) PPAR- α down-regulation diminishing FA oxidation (3); 3) stimulation of FA synthesis by the associated hypoadiponectinemia; and/or 4) *n*-3 long-chain polyunsaturated FA depletion favoring lipogenesis over FA oxidation (31). The latter effect has been ascribed to consumption of hepatic *n*-3 long-chain polyunsaturated FA due to oxidative stress underlying NAFLD (2, 27), dietary imbalance (31), and/or FA desaturation derangement (32).

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