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The role of proliferator-activated receptor γ coactivator–1α in the fatty-acid–dependent transcriptional control of interleukin-10 in hepatic cells of rodents

Joseane Morari[a], Adriana S. Torsoni[a], Gabriel F. Anhê[b], Erika A. Roman[a], Dennys E. Cintra[a], Laura S. Ward[a], Silvana Bordin[b], Lício A. Velloso[a,*]

[a]Department of Internal Medicine, University of Campinas, Brazil
[b]Department of Physiology and Biophysics, University of São Paulo, Brazil

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Abstract

Interleukin-10 (IL-10) is an endogenous factor that restrains hepatic insulin resistance in diet-induced steatosis. Reducing IL-10 expression increases proinflammatory activity in the steatotic liver and worsens insulin resistance. As the transcriptional coactivator proliferator-activated receptor γ coactivator–1α (PGC-1α) plays a central role in dysfunctional hepatocytic activity in diet-induced steatosis, we hypothesized that at least part of the action of PGC-1α could be mediated by reducing the transcription of the IL-10 gene. Here, we used immunoblotting, real-time polymerase chain reaction, immunocytochemistry, and chromatin immunoprecipitation assay to investigate the role of PGC-1α in the control of IL-10 expression in hepatic cells. First, we show that, in the intact steatotic liver, the expressions of IL-10 and PGC-1α are increased. Inhibiting PGC-1α expression by antisense oligonucleotide increases IL-10 expression and reduces the steatotic phenotype. In cultured hepatocytes, the treatment with saturated and unsaturated fatty acids increased IL-10 expression. This was accompanied by increased association of PGC-1α with c-Maf and p50–nuclear factor (NF) κB, 2 transcription factors known to modulate IL-10 expression. In addition, after fatty acid treatment, PGC-1α, c-Maf, and p50-NFκB migrate from the cytosol to the nuclei of hepatocytes and bind to the IL-10 promoter region. Inhibiting NFκB activation with salicylate reduces IL-10 expression and the association of PGC-1α with p50-NFκB. Thus, PGC-1α emerges as a potential transcriptional regulator of the inflammatory phenomenon taking place in the steatotic liver.

1. Introduction

Cirrhosis due to nonalcoholic steatohepatitis (NASH) is one of the major causes of disease leading to hepatic transplantation in the Western world [1,2]. The high consumption of fat-rich diets paralleled by a reduction in physical activity has fostered the increased prevalence of obesity and type 2 diabetes mellitus, both conditions known to be intimately associated with fatty liver disease [2,3]. The reasons why only some people progress from simple steatosis to the precirrhotic NASH is still a matter of intense investigation. Because the activation of proinflammatory gene expression is an important mechanism involved in NASH installation, one hypothesis proposed to explain the existence of protective phenotypes is the individual's capacity to induce an increased expression of anti-inflammatory factors in the liver [3-5].

Recent studies have shown that the anti-inflammatory cytokine interleukin (IL)-10 is expressed in the liver of diet-induced obese animal models and that reducing its activity by pharmacologic or genetic means worsens hepatic insulin resistance and increases local inflammation [6,7]. In addition, a number of studies have linked certain IL-10 gene polymorphisms to installation and progression of different inflammatory liver dysfunctions [8].

Proliferator-activated receptor γ coactivator–1α (PGC-1α) is a transcriptional coactivator known to play an important role in the control of lipid and carbohydrate metabolism and storage in the liver [9]. Restraining its

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* Corresponding author. DCM-FCM, UNICAMP, 13084 970 Campinas, SP, Brazil.
E-mail address: lavelloso@fcm.unicamp.br (L.A. Velloso).

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expression in the steatotic liver reduces the hepatic content of fatty acids while normalizing insulin signal transduction [10]. However, the mechanisms involved in this regulation are not completely understood. Here, we hypothesized that PGC-1α can regulate IL-10 gene transcription by modulating the activity of factors involved in the transcriptional regulation of the IL-10 gene. c-Maf and nuclear factor (NF)κB are 2 transcription factors that play important roles in the control of IL-10 expression [11]. Upon lipopolysaccharide and other toll-like receptor ligand stimulation, both c-Maf and NFκB modulate IL-10 gene transcription in different cell types [11,12]. In addition, recent evidence has shown that PGC-1α can physically interact with and modulate NFκB [13], which is frequently regulated by c-Maf [14,15]. Our study demonstrates that, in isolated hepatocytes, fatty acids can induce the association of PGC-1α with p50 and c-Maf, which migrate to the nucleus and bind to the promoter region of the IL-10 gene.

2. Materials and methods

2.1. Antibodies and chemicals

Antibodies against tumor necrosis factor (TNF)–α (sc-1347, goat polyclonal and sc-8301, rabbit polyclonal), IL-1β (sc-1252, goat polyclonal and sc-7884 rabbit polyclonal), IL-6 (sc-1266, goat polyclonal and sc-7920, rabbit polyclonal), IL-10 (sc-1783, goat polyclonal), PGC-1α (sc-13067, rabbit polyclonal), p50 (sc-7178, rabbit polyclonal), c-Maf (sc-7866, rabbit polyclonal), and F4/80 (sc-25830, rabbit polyclonal) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All the reagents for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were from Bio-Rad (Richmond, CA). HEPES, phenylmethylsulfonyl fluoride, aprotinin, dithiothreitol, Triton X-100, Tween 20, glycerol, collagenase, oleic acid/C18:1 (O-1383-1G), palmitic acid/C16:0 (P-5177), stearic acid/C18:0 (5376), arachidic acid/C20:0 (A-3881), behenic acid/C22:0 (B-3271), bovine serum albumin (fraction V), and fatty acid free bovine serum albumin (A-6003) were purchased from Sigma-Aldrich (St Louis, MO). Sodium thiopental was from Lilly (Indianapolis, IN). All the chemicals used in the real-time polymerase chain reaction (PCR) and diamidino-2-phenylindole (DAPI) used in immunofluorescence staining were purchased from Applied Biosystems (Foster City, CA) and Invitrogen (Carlsbad, CA).

2.2. Experimental model and treatment protocols

Male, 4-week–old, Swiss (Sw/Uni) inbred strain mice were obtained from the State University of Campinas Breeding Center. The investigation followed the university guidelines for the use of animals in experimental studies and conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (publication no. 85-23, revised 1996). The animals were maintained on a 12:12-hour artificial light-dark cycle and housed in individual cages. After random selection, 4-week–old mice were introduced to control (CD) or high-fat (HF) diets (Table 1). After 8 weeks on the HF diet, all Sw/Uni mice presented hepatic steatosis and diabetes mellitus. At this point, the biochemical and metabolic characterization of the model and the evaluation of liver histology and cytokine expression were performed. Next, Sw/Uni mice on the HF diet were randomly selected for treatment with an IL-10 neutralizing antibody (or a rabbit preimmune antiserum) or with a PGC-1α phosphorothioate modified antisense oligonucleotide (ASO) (or scrambled oligonucleotide [SCR]). The doses used and the sequences for the oligonucleotides were previously optimized and reported [6,10]. Treatments lasted for 4 days with the antibodies or 8 days with the oligonucleotides; and at the end of the respective treatment periods, mice were used for determination of histologic characteristics and/or cytokine expression.

Table 1
Macronutrient composition of the diets

	CD		HF	
	g%	kJ%	g%	kJ%
Protein	19	18	19	12
Carbohydrate	77	73	45	27
Saturated fat	4	9	36	61
kJ/g	15.8		24.5	

2.3. Metabolic and biochemical characterization of the animals

Body mass and mean daily food intake were evaluated at the end of the experimental period. Blood samples were collected for glucose and triglyceride determination by colorimetric methods [16,17] and for insulin determination by enzyme-linked immunosorbent assay [18].

2.4. Primary hepatocyte culture

Male Wistar rats (250-300 g) were anesthetized with sodium thiopental and submitted to a portal vein cannulation. An in situ liver perfusion was started with 300 mL of calcium free Hank buffer (pH 7.4) followed by 300 mL of Hank buffer (pH 7.4) containing type IV collagenase (Sigma-Aldrich) (197 U/mL) and calcium chloride (5.1 mmol/L). At the end of the perfusion, the liver was excised and gently passed through a thin net. Cells were washed in Dulbecco modified Eagle medium buffer in 3 consecutive rounds of centrifugation at 200*g* followed by 3 additional rounds of centrifugation at 50*g*. Cells were plated in 30-mm culture dishes at a final density of 6.5×10^5 cells per milliliter. After 4 hours, nonadherent cells were discarded; and adherent hepatocytes were submitted to one of the following treatments: control group, not treated; vehicle group, treated with fatty acid diluting vehicle (Dulbecco modified Eagle medium with 5% fatty acid–free albumin); unsaturated fatty acid group, treated with oleic acid at a final concentration of 22.5 μmol/L; and saturated fatty acid mixture group, treated

with a mixture containing equal amounts of palmitic, stearic, arachidic, and behenic acids at a final combined concentration of 22.5 μmol/L. Treatment lasted for 16 hours and was performed always in triplicate (exceptionally, the experiments for immunocytochemistry were carried out with 1- and 3-hour fatty acid treatment). In some experiments, cells were preincubated for 2 hours in the presence of salicylic acid (10 mmol/L) before the incubation with oleic acid, as described above. Cells treated according to these protocols were used in real-time PCR, immunoblotting, immunocytochemistry, and chromatin immunoprecipitation (ChIP) assays, as described below.

2.5. Real-time PCR

Interleukin-6, IL-10, TNF-α, IL-1β, and PGC-1α messenger RNAs (mRNAs) were measured in primarily cultivated rat hepatocytes using intron-skipping primers obtained from Applied Biosystems: TNF-α–Rn00562055_m1, IL-1β–Rn00580432_m1, IL-6–Rn00561420_m1, and IL-10–Rn00563409_m1. The PGC-1α primers were customized by Applied Biosystems encompassing the 913 to 1933 region of the *Rattus norvegicus* PGC-1α gene (NM_031347–NCBI [http://blast.ncbi.nlm.nih.gov/Blast.cgi]). Glyceraldehyde-3-phosphate dehydrogenase primers (Applied Biosystems) were used as control (4352339E). Real-time PCR analysis of gene expression was carried out in an ABI Prism 7500 sequence detection system (Applied Biosystems). The optimal concentration of complementary DNA and primers, as well as the maximum efficiency of amplification, was obtained through 5-point, 2-fold dilution curve analysis for each gene. Each PCR contained 40 ng of reverse-transcribed RNA and was run according to the manufacturer's recommendations using the TaqMan PCR master mix. Real-time data were analyzed using the Sequence Detector System 1.7 (Applied Biosystems).

2.6. Immunoprecipitation and immunoblotting

For evaluation of cytokine expression and protein/protein interaction, primarily cultivated hepatocytes or fragments of liver were homogenized in solubilization buffer at 4°C. Aliquots of the resulting protein extracts containing 0.5 mg of total protein were used for immunoprecipitation with antibodies against c-Maf, p50, and PGC-1α at 4°C overnight, followed by SDS/PAGE; transfer to nitrocellulose membranes; and blotting with anti–c-Maf, p50, and PGC-1α antibodies. In direct immunoblot experiments, 0.2 mg of protein extracts was separated by SDS-PAGE; transferred to nitrocellulose membranes; and blotted with anti–TNF-α, anti-IL-1β, anti-IL-6, anti-IL-10, anti-F4/80, and anti-PGC-1α antibodies. Specific bands were detected by chemiluminescence, and visualization was performed by exposure of the membranes to RX films.

2.7. Liver histology

Hydrated 4.0-μm sections of paraformaldehyde-fixed, paraffin-embedded liver specimens were stained by a regular hematoxylin-eosin (HE) method for evaluation of liver histology [19].

2.8. Immunocytochemistry

Isolated hepatocytes were cultivated on glass coverslips and fixed with 4% paraformaldehyde. Primary antibodies against c-Maf, p50, and PGC-1α were used in overnight incubations at +4°C. Rhodamine-conjugated secondary antibodies were used to label the target proteins, and microscopic evaluation and photodocumentations were performed on a Leica DM 4500B microscope (Wetzlar, Germany).

2.9. ChIP assay

For determination of c-Maf, p50, and PGC-1α binding to the promoter region of the IL-10 gene, a ChIP assay was performed using a commercially available kit (EZ Magna Chip) from Millipore (Billerica, MA), according to the recommendations of the manufacturer. For PCR amplification of the region of interest, 6 pairs of primers were designed spanning the 4065481 to 4066080 region of the *R norvegicus* chromosome 13 (NW_047394.1–NCBI [http://blast.ncbi.nlm.nih.gov/Blast.cgi]). The sequences of the primers were as follows: (1) sense–5′ GCC AAC AAA CCT TTC AAG G 3′, antisense–5′ ATT TCT TGC TGA CCA GGA G 3′; (2) sense–5′ GAA ATA GCG GAC ATT CAA CCC AG 3′, antisense–5′ GCA CTC GTA AGT CAC AAT G 3′; (3) sense–5′ CTC AAT CCT AAT GTG CTC TGG 3′, antisense–5′ AGC TCT GTT TTC TGC AAG GCT 3′; (4) sense–5′ TTA CGA GTG CGT GAA TGG AAC 3′, antisense–5′ CGG CTA TTT TTA GTA AGC GC 3′; (5) sense–5′ GCG CTT ACT AAA AAT AGC CG 3′, antisense–CCT CTT CTA GAC CAT GCA CTT 3′; and (6) sense–5′ AAG TGC ATG GTC TAG AAG AGG 3′, antisense–5′ AGC TCT GTT TTC TGC AAG GCT 3′. The negative control consisted of a sample containing all reagents except the primary antibody; the positive control consisted of a sample containing sonicated DNA plus reagents for DNA amplification, including primers.

2.10. Statistical analysis

Specific protein bands present in the blots were quantified by digital densitometry (ScionCorp, Frederick, MD). Mean values ± SEM obtained from densitometry scans, real-time PCR, and biochemical and metabolic determinations were compared using Tukey-Kramer test (analysis of variance) or Student *t* test, as appropriate; $P < .05$ was accepted as statistically significant.

3. Results

3.1. Inhibition of PGC-1α increases IL-10 expression in the liver and reverses hepatic steatosis

After 8 weeks on the HF diet consumption, mice become obese, hyperinsulinemic, and diabetic (Table 2). At the

Table 2
Metabolic parameters of the experimental animals

	CD	HF
Body mass (g)	33.0 ± 1.6	49.4 ± 1.9*
Food intake (g/24 h)	5.78 ± 0.69	6.28 ± 0.77
Glucose (mg/dL)	126 ± 5	301 ± 13*
Insulin (ng/mL)	5.9 ± 1.1	12.4 ± 2.4*
Triglycerides (mg/dL)	92.2 ± 12.1	94.4 ± 10.5

* $P < .05$.

macroscopic examination, the liver was enlarged and yellowish (not shown); and under histologic evaluation, a considerable increase of infiltration of the parenchyma cells with lipids was observed (Fig. 1A). This was accompanied by increased hepatic expression of IL-10, IL-6, IL-1β, and TNF-α (Fig. 1B). Reducing PGC-1α expression by inhibition of mRNA translation using a phosphorthioate modified ASO resulted in a significant increase of IL-10 expression (Fig. 1C, 2.2-fold increase vs HF, n = 5, $P < .05$), which was accompanied by an almost complete reversal of hepatic steatosis (Fig. 1D). To determine the role of IL-10 expression as an endogenous protective factor against diet-induced liver inflammation, HF-fed mice were treated with a neutralizing anti–IL-10 antibody; and the expressions of inflammatory cytokines were evaluated by immunoblot. As depicted in Fig. 1E, the neutralization of IL-10 produced a remarkable increase in the expressions of IL-6, IL-1β, and TNF-α. In addition, the immunoneutralization of IL-10 led to an increased accumulation of fat in the liver, as determined by histology (Fig. 1F), but no modification of serum triglycerides (not shown).

3.2. Fatty acids induce the expression of cytokines in isolated hepatocytes

The purity of hepatic cell primary culture was confirmed by the absence of F4/80 expression, which is expressed by Kupffer cells (Fig. 2A). The treatment of hepatic cells with both saturated and unsaturated fatty acids resulted in increased expressions of IL-10 (Fig. 2B), IL-1β (Fig. 2C), and IL-6 (Fig. 2E), but not of TNF-α (Fig. 2D). In addition, the fatty acids produced no modulatory effect on the expression of PGC-1α (Fig. 2F).

3.3. Fatty acids induce PGC-1α, p50, and c-Maf migration to the nucleus of hepatocytes

Primarily cultivated hepatocytes were treated for 1 or 3 hours with saturated or unsaturated fatty acids. The localizations of PGC-1α, p50, and c-Maf were evaluated by immunofluorescence staining. Typically, both types of fatty acids induced some migration of all 3 proteins from a clear preferential cytosolic location to the nuclear region. At 1 hour, some nuclear presence of the proteins was seen (not shown); however, at 3 hours, the nuclear expression of p50, c-Maf, and PGC-1α was considerably increased in most cells evaluated (Fig. 3A). Interestingly, in most cells examined, some c-Maf staining could be detected in the nucleus before fatty acid treatment.

3.4. Fatty acids induce the association of PGC-1α with p50 and c-Maf

Isolated hepatocytes were treated with unsaturated or saturated fatty acids, and PGC-1α was coimmunoprecipitated with p50 or c-Maf. As shown in Fig. 3B, some degree of constitutive association exists between PGC-1α and both

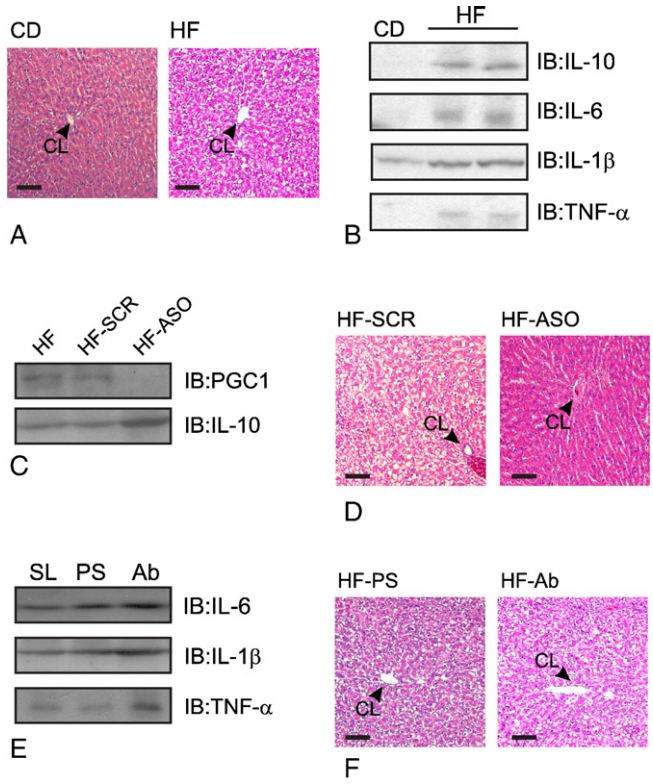

Fig. 1. Male Swiss mice were fed CD or HF diet for 8 weeks. A, Four-micrometer sections were obtained from livers and used in regular HE staining; a typical lobule is depicted (centrolobular vein). B, Total protein extracts were prepared from intact livers; separated by SDS-PAGE; transferred to nitrocellulose membranes; and blotted with anti–IL-10, anti-IL-6, anti-IL-1β, and anti-TNF-α antibodies. C and D, Mice fed HF diet for 8 weeks were treated with an intraperitoneal daily dose of scrambled (HF-SCR) or antisense (HF-ASO) oligonucleotide anti–PGC-1α for 8 days. At the end of the experimental period, livers were obtained for total protein extract preparation for typical immunoblotting as described in B, using antibodies against PGC-1α (PGC1) or IL-10 (C), or for typical HE staining of 4.0-μm liver sections, as described in A, centrolobular vein (D). E and F, Mice fed HF diet for 8 weeks were treated with a daily dose of saline, rabbit preimmune serum, or anti–IL-10 antiserum for 4 days; at the end of the experimental periods, livers were obtained for total protein extract preparation for typical immunoblotting as described in B, using antibodies against IL-6, IL-1β, or TNF-α (E), or for typical HE staining of 4.0-μm liver sections, as described in A, centrolobular vein (F). In all experiments, n = 5; photomicrographs and blots are representative of typical experiments. Black scale bars in A, D, and F correspond to 50 μm. CL indicates centrolobular vein; IB, immunoblotting; SL, saline; PS, preimmune serum; Ab, anti–IL-10 antiserum.

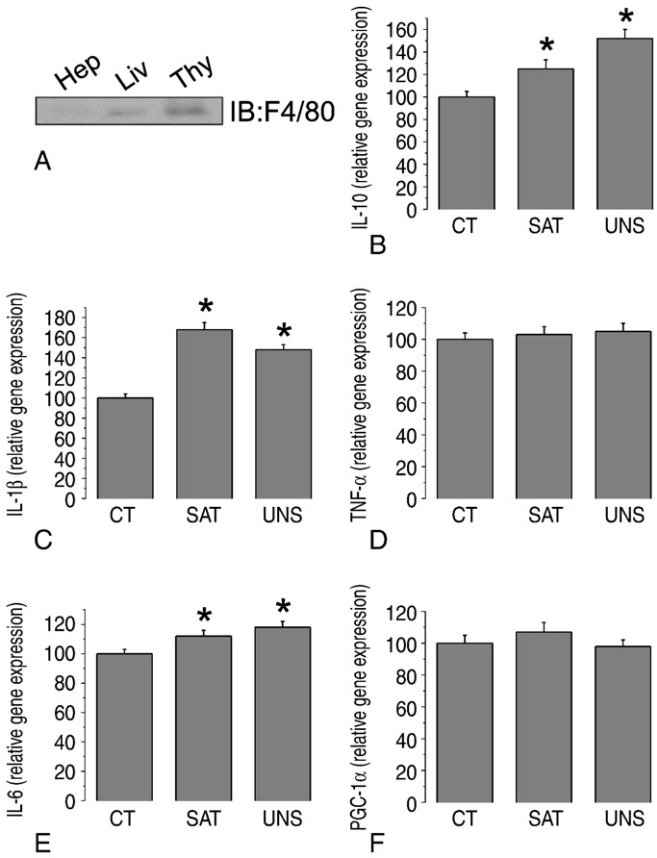

Fig. 2. A, One hundred fifty micrograms of total protein extracts from hepatocyte cell culture, intact liver, or intact thymus was separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-F4/80 antibodies. B to F, Primarily cultivated hepatocytes were treated with saline, saturated fatty acids, or oleic acid for 16 hours; and the mRNA expressions of IL-10 (B), IL-1β (C), TNF-α (D), IL-6 (E), and PGC-1α (F) were determined by real-time PCR. A, n = 5; blot is representative of typical experiment. B to F, n = 4; *P < .05 vs CT. Hep indicates hepatocyte cell culture; Liv, intact liver; Thy, intact thymus; CT, treated with saline; SAT, treated with saturated fatty acids; UNS, treated with oleic acid.

p50 and c-Maf. However, the treatment with either type of fatty acid produced a significant increase in the association of PGC-1α with p50 (2.8- and 2.9-fold for unsaturated and saturated fatty acids, respectively; n = 5; P < .05) and c-Maf (3.4- and 2.6-fold for unsaturated and saturated fatty acids, respectively; n = 5; P < .05).

3.5. PGC-1α, p50, and c-Maf bind to the IL-10 promoter region in hepatocytes

To evaluate the ability of fatty acids to induce interactions of PGC-1α, p50, and c-Maf with sequences of the promoter region of the IL-10 gene, we designed 6 pairs of primers distributed throughout the DNA region contained between nucleotides 4065481 and 4066080 of *R norvegicus* chromosome 13, which is located upstream from the starting codon of the IL-10 gene (Fig. 4A). Primers 1 to 4 were upstream, whereas primers 5 to 6 were downstream of the TATA box. In the first round of ChIP assay, some binding of all 3 proteins was detected in the regions amplified by pair of primers 2 and 4, whereas no specific binding was detected in the regions amplified by the remainder of the primers (not shown). In the second round of ChIP assay, we used the sense primer of pair 2 and the antisense primer of pair 4 to evaluate the binding of the proteins to an extended DNA region comprised between these 2 primers. As shown in Fig. 4B, under basal

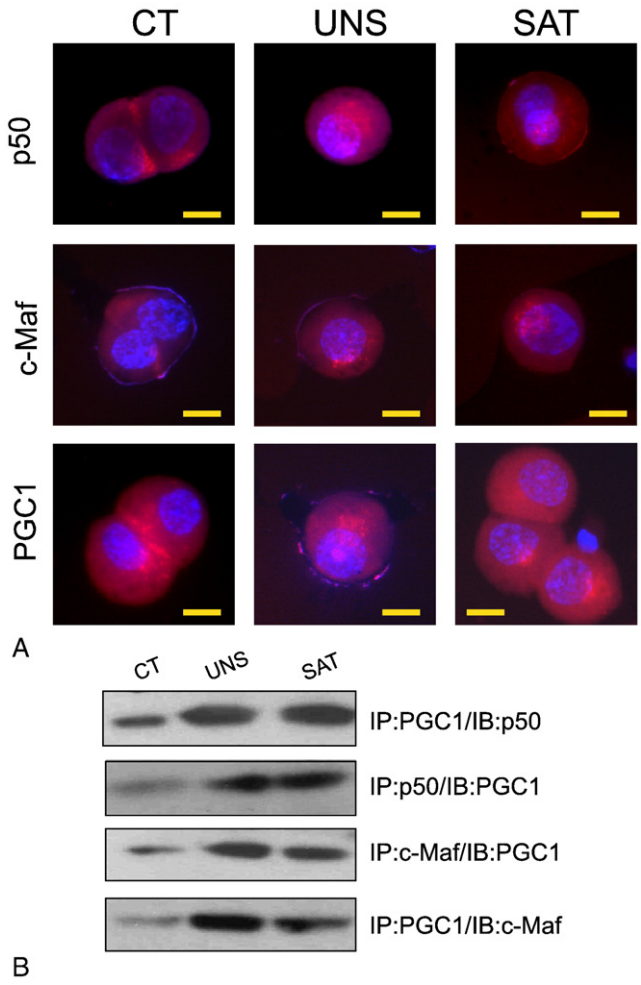

Fig. 3. A, Isolated hepatocytes were cultivated on glass slides and treated for 3 hours with saline, oleic acid, or saturated fatty acids. At the end of the experimental period, cells were fixed and used in immunofluorescence staining with anti-p50, –c-Maf, or –PGC-1α (PGC1) antibodies. The secondary antibodies were conjugated with rhodamine (red); nuclear counterstaining was performed with DAPI (blue). B, One hundred fifty micrograms of total protein extracts from primarily cultivated hepatocytes treated for 16 hours with saline, oleic acid, or saturated fatty acids was used in immunoprecipitation experiments with anti–PGC-1α (PGC1), -p50, or –c-Maf antibodies. The immunocomplexes were separated by SDS-PAGE; transferred to nitrocellulose membranes; and blotted with anti–PGC-1α (PGC1), -p50, or –c-Maf. A, Microphotographs are representative of 3 distinct experiments; yellow scale bar corresponds to 10 μm. B, Blots are representative of 5 distinct experiments. IP indicates immunoprecipitation.

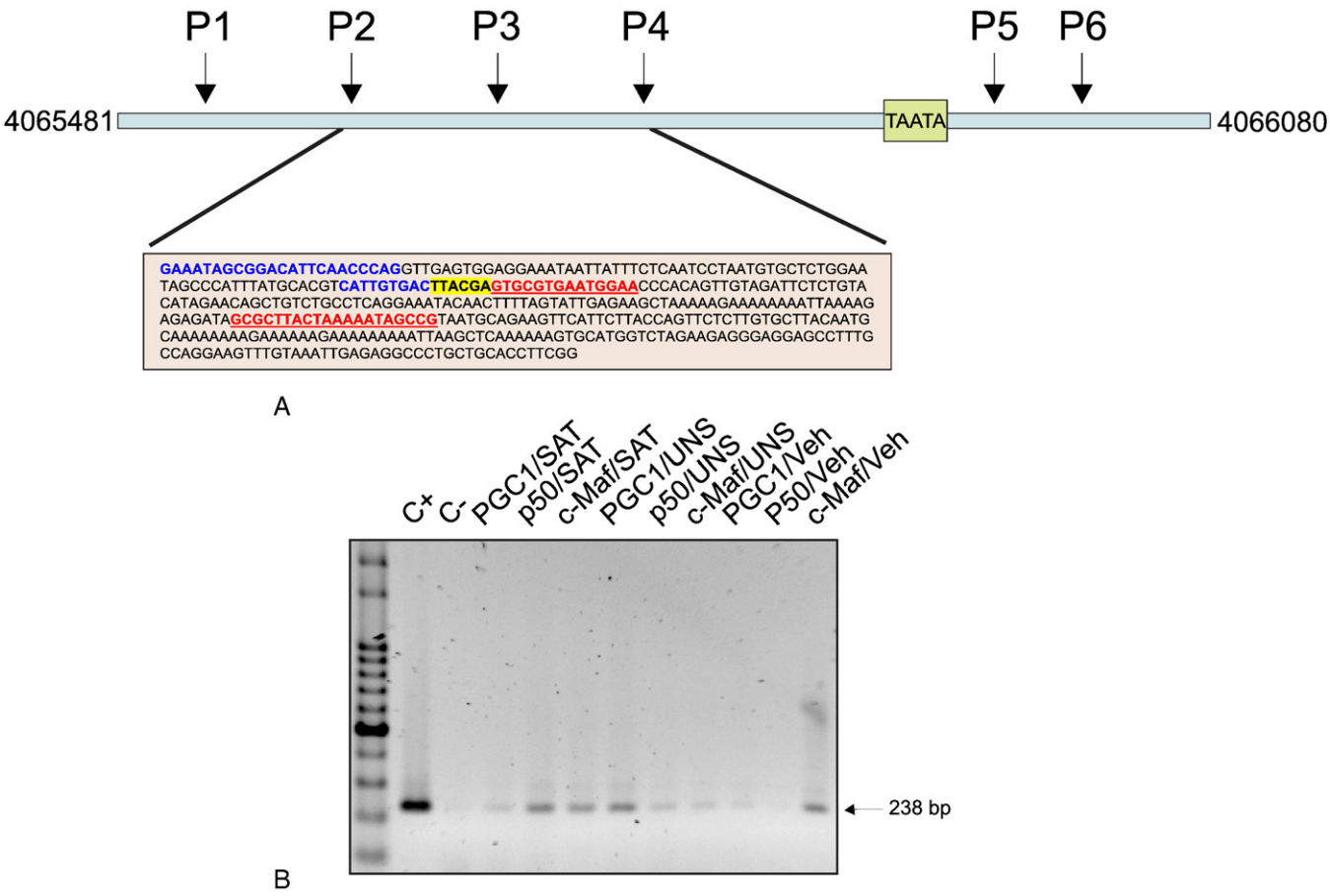

Fig. 4. A, The approximate positions of the primers (P1-6) used to screen the promoter region of the IL-10 (*R norvegicus* chromosome 13 from base 4065481 to base 4066080) by ChIP assay are depicted. The TATA box is depicted in green; pair of primer 2 is in blue and pair of primer 4 is in red underlined; bases in the yellow box are common for primers 2 and 4. B, A ChIP assay was run; and immunoprecipitated DNA amplified by primers 3 and 4 is shown (238 bp). Figure is representative of 3 independent experiments. C+ indicates positive control; C−, negative control; PGC1, immunoprecipitate anti–PGC-1α; p50, immunoprecipitate anti-p50; c-Maf, immunoprecipitate anti–c-Maf; Veh, cells treated with vehicle.

conditions, no DNA binding of PGC-1α and p50 could be detected; however, after either unsaturated or saturated fatty acid treatment, both proteins bound specifically to the DNA. Conversely, c-Maf was already bound to DNA under basal conditions; and after fatty acids treatment, the association of c-Maf with DNA was still detected.

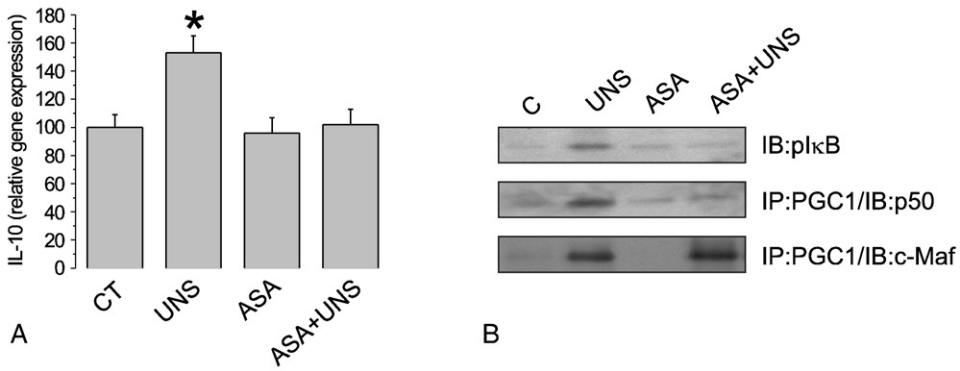

Fig. 5. Primarily cultivated hepatocytes were treated with saline, oleic acid, salicylic acid, or salicylic acid plus oleic acid. The mRNA expression of IL-10 (A) was determined by real-time PCR. The phosphorylation of IκB was determined by immunoblot, and the associations of PGC-1α with p50 or with c-Maf were determined by immunoprecipitation followed by immunoblot (B). A, n = 4; *$P < .05$ vs CT. B, Blots are representative of 5 distinct experiments. ASA indicates salicylic acid.

3.6. Inhibition of NFκB reduces fatty acid–induced IL-10 expression and impairs PGC-1α/p50 association

To inhibit NFκB, *h*epatocytes were treated with acetyl salicylic acid for 8 hours and then with the unsaturated fatty acid oleate for 16 hours. As shown in Fig. 5A, a complete inhibition of fatty acid–induced IL-10 expression was obtained with salicylate. This was accompanied by the inhibition of fatty acid–induced activation of IκB kinase (IKK), as determined by the phosphorylation of IκB (Fig. 5B, upper blot), and the association of PGC-1α with p50 (Fig. 5B, blot in the middle). The treatment with salicylate resulted in no modification of fatty acid–induced association of PGC-1α with c-Maf (Fig. 5B, lower blot).

4. Discussion

Defining the mechanisms involved in the control of inflammatory and anti-inflammatory factors expressed in the steatotic liver is believed to have an impact on the development of novel therapeutic strategies and on the capacity to predict the development of NASH and hepatic cirrhosis [2-4,20,21]. In 2 recent studies, we observed that the modulation of the expressions of IL-10 and PGC-1α has opposing effects in the installation of NASH in an animal model of diet-induced hepatic disease [6,10]. Reducing IL-10 expression worsens hepatic morphologic, inflammatory, and metabolic parameters related to NASH [6], whereas PGC-1α inhibition improves a number of metabolic parameters involved in liver function [10].

Interleukin-10 is a potent anti-inflammatory cytokine expressed, under different conditions, in most cells present in the liver such as Kupffer cells, sinusoidal endothelial cells, stellate cells, hepatic infiltrating lymphocytes, and hepatocytes [22-24]. A number of recent clinical and experimental studies have suggested that IL-10 plays an important protective role in the development of NASH and its progression to cirrhosis [6,7,25]. However, little is known about the transcriptional control of the IL-10 gene. In fact, so far, only 2 transcription factors, NFκB and c-Maf, are known to participate in the control of IL-10 expression [11,12,15]. In addition, a binding site for NF-Y has been described; but its role in the control of IL-10 expression remains elusive [26].

Here, we evaluated the hypothesis that PGC-1α could play a role in the modulation of IL-10 expression by interacting with the 2 transcription factors known to regulate the transcriptional activity of the IL-10 promoter. Initially, we demonstrated that, in the intact liver of an animal model of diet-induced NASH, the immunoneutralization of IL-10 worsens the inflammatory phenotype, whereas reducing PGC-1α increases IL-10 expression and rescues the liver from diet-induced steatosis. Although some similar results have been previously reported [6,7,10], this is the first demonstration that, in the liver environment, reducing PGC-1α expression significantly increases IL-10 levels.

Next, we established a method for inducing IL-10 expression in primarily cultivated hepatocytes by exposing the cells to fatty acids. Here, the idea was to mimic the effect of the high-fat diet, which is capable of inducing pro- and anti-inflammatory gene expression in the intact liver [6,21,27]. As previously reported, both unsaturated and saturated fatty acids significantly increase the expressions of some proinflammatory cytokines and IL-10 in the hepatic cells [28]. Differently from the intact liver of high-fat diet–treated rodents, in the isolated cell system, TNF-α and PGC-1α were not affected by the treatment with the fatty acids. Although we have no current explanation for this, we suspect that, in the absence of Kupffer cells, some of the inflammatory input generated by the fatty acids is lost, leading to a milder activation of pro- and anti-inflammatory genes. Nevertheless, even under a milder condition, both types of fatty acids induced the migration of c-Maf, p50, and PGC-1α from a preferential cytosolic distribution to an intranuclear location. The treatment with the unsaturated fatty acid was clearly superior to induce the migration as detected by the immunocytochemistry. In addition, the treatment with the fatty acids induced the physical association of both c-Maf and p50 with PGC-1α. The use of immunocytochemistry in parallel with coimmunoprecipitation offered a reproducible method, which suggests that proteins migrated as complexes to the nucleus; however, the functional outcomes of these events depend on DNA interaction of the proteins. Therefore, using ChIP assay, we evaluated whether the treatment with fatty acids would result in increased binding of c-Maf, p50, and PGC-1α to the promoter region of IL-10. For this, 6 pairs of primers, spanning 599 base pairs of the IL-10 promoter and including the TATA box, were used. Binding of all 3 proteins was detected only in the regions encompassed by pairs 2 and 4. When using the sense primer of pair 2 and the antisense primer of pair 4, we obtained the highest binding of all 3 proteins, suggesting that the DNA region located between primers 2 and 4 harbors the binding site(s) for p50 and c-Maf. In a recent study, the binding site for p50 homodimers was located −55/−46 upstream from the starting codon of the mouse IL-10 gene [29]. This region of the mouse promoter is highly similar to the region located between primers 2 and 4 of the rat promoter. Thus, we believe that all 3 proteins bind, directly or indirectly, to the region comprehended between bases −493 and −254 of the *R norvegicus* IL-10 promoter.

Finally, at least part of the specificity of the phenomenon herein described was tested by pretreating the cells with the inhibitor of IKK, salicylate. Using this strategy, we were able to abolish the fatty acid–induced IL-10 expression that was accompanied by inhibition of the formation of the p50/PGC-1α complex.

A piece of information that deserves further attention regards the fact that some of c-Maf protein is already bound to the IL-10 promoter, even in the absence of stimulus with the fatty acids. In macrophages, c-Maf can exert both stimulatory and inhibitory effect on the expression of IL-10

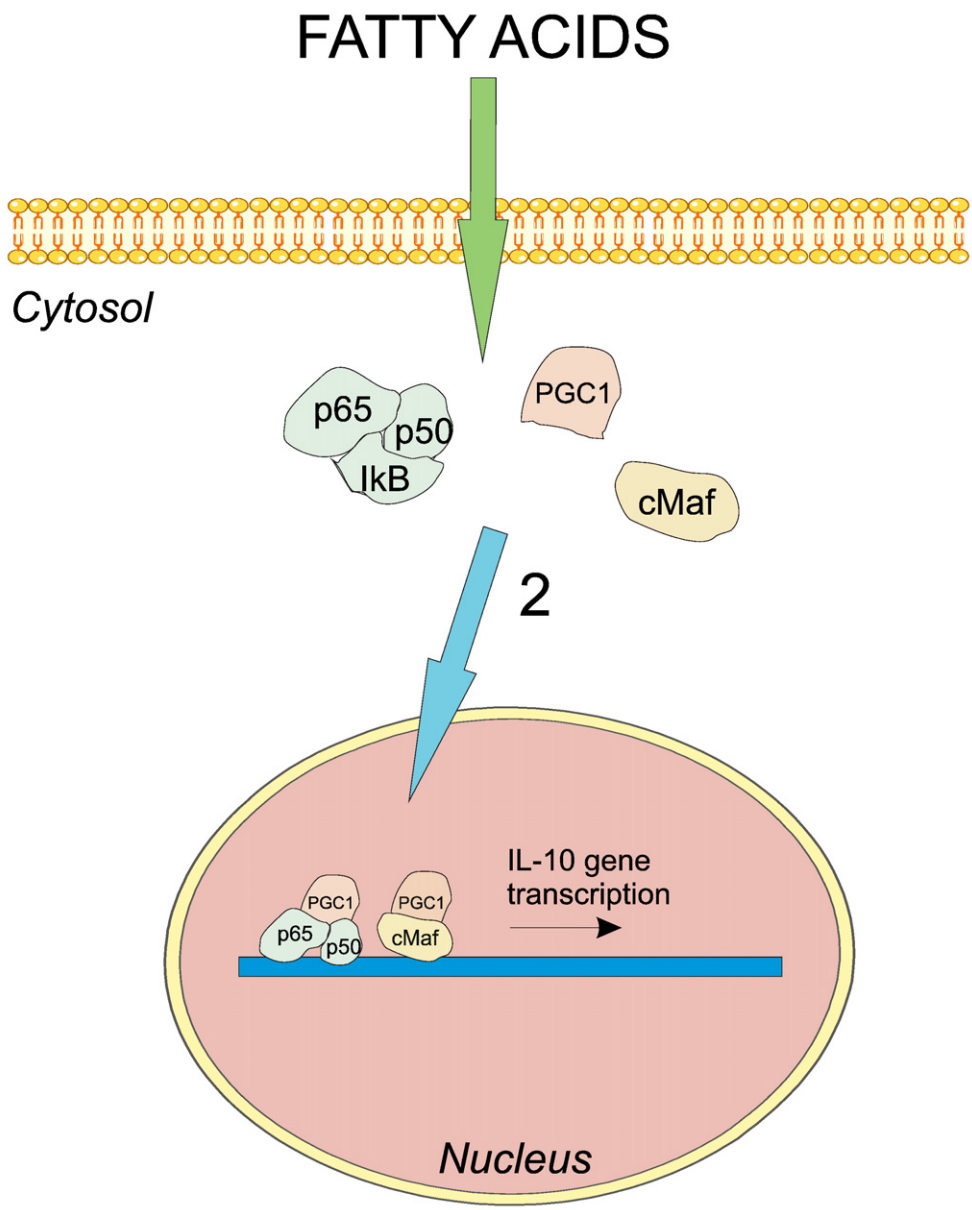

Fig. 6. Schematic model of fatty acid–induced activation of IL-10 gene transcription through the associations of PGC-1α with p50-NFκB and c-Maf. Under basal conditions, PGC-1α, p50 (complexed with p65/IκB), and c-Maf are in the cytosol (some c-Maf may be present in the nucleus, as shown in Fig. 4B). After fatty acid stimulation, complexes are formed and migrate to the nucleus. The role of PGC-1α (as coactivator or repressor) in these complexes is unknown.

and IL-12, suggesting that it possesses a dual role, dependent on different stimuli [15]. Possibly, in resting hepatocytes, c-Maf acts as a repressor of the IL-10 gene, whereas after stimulation with fatty acids, it may interact with the p50/PGC-1α complex to positively modulate the gene expression. Further experiments will be required to evaluate these hypotheses.

Recent studies have shown that the amount of PGC-1α in different tissues is crucial for optimal transcriptional control of target genes [10,30,31]. Under certain clinical or experimental conditions, overexpression of this protein may result in anomalous gene transcription leading to defective organic function. In our opinion, under discrete increase, PGC-1α can produce a mild reduction of IL-10 that has a minor impact on liver physiology; however, as steatosis progresses, so do the levels of PGC-1α, which can reach very high amounts in the liver [10]. Under this condition, the physiologic activity of the coactivator is lost; and genes under its transcriptional regulation may go out of control, as it happened with IL-10.

In conclusion, we have demonstrated that the transcriptional coactivator PGC-1α, associates with at least 2 transcription factors known to modulate IL-10 gene expression. This association may have an impact on the control of IL-10 levels in different physiologic and pathologic contexts. Fig. 6 summarizes the findings of this

study: under non–fatty-acid-stimulated condition, most PGC-1α, c-Maf, and p50 exist in a cytosolic location; however, some c-Maf may be expressed in the nucleus; after fatty acid treatment, PGC-1α associates with p50 (together with p65) and with c-MAF, binding to the IL-10 promoter and modulating IL-10 gene expression.

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