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Lack of hypotriglyceridemic effect of gemfibrozil as a consequence of age-related changes in rat liver PPARα

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Abstract

We have investigated if changes in hepatic lipid metabolism produced by old age are related to changes in liver peroxisome proliferator-activated receptor α (PPARα). Our results indicate that 18-month-old rats showed a marked decrease in the expression and activity of liver PPARα, as shown by significant reductions in PPARα mRNA, protein and binding activity, resulting in a reduction in the relative mRNA levels of PPARα target genes, such as liver-carnitine-palmitoyl transferase-I (CPT-I) and mitochondrial medium-chain acyl-CoA dehydrogenase (MCAD). Further, in accordance with a liver PPARα deficiency in old rats, treatment of old animals with a therapeutic dose of gemfibrozil (GFB) (3 mg/kg per day, 21 days) was ineffective in reducing plasma triglyceride concentrations (TG), despite attaining a 50% reduction in TG when GFB was administered to young animals at the same dose and length of treatment. We hypothesize that the decrease in hepatic PPARα can be related to a state of leptin resistance present in old animals.

Keywords: Old age; PPARα; Rat liver; Gemfibrozil; Hypolipidemic effect; Fibrates

1. Introduction

Ageing is associated with immunosenescence, decreased hormonal secretion, decreased hepatic metabolism, lean body and bone mass, and increased fat accretion. As a consequence inflammatory diseases, dyslipidemia, atherosclerosis, obesity and type II diabetes incidence increases with age [1]. The molecular mechanisms underlying those changes in body physiology are only partly understood.

The clustering of factors such as dyslipidemia, obesity, type II diabetes and accelerated atherosclerosis steadily increases the incidence of cardiovascular diseases among elder people. Although the debate about the appropriateness of treating old people with hypolipidemic drugs seems to be progressively shifting to the appreciation of the benefits of this therapeutic measure, there is still a paucity of experimental data with respect to the effectiveness of known hypolipidemic drugs when administered to old people, specially regarding the derivatives of fibric acid or fibrates, the leading hypolipidemic drug class after statins [2,3].

Fibrates act as low affinity ligands of the nuclear receptor PPARα, increasing its transcriptional activity upon binding [3]. The nuclear receptor subfamily of PPARs consists of isoforms α (NR1C1), γ (NR1C3), and δ/β (NR1C2) [4]. Among their known functions, identification of PPAR target genes shows that PPARα mainly regulates genes implicated in lipid oxidation, being expressed in cells with high catabolic rates of fatty acids, such as hepatocytes. On the contrary, PPARγ, which was identified as an isotype highly expressed in adipose tissue, is related to body energy storage, and is indeed a key transcription factor involved in terminal differentiation of adipocytes [5].

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Abbreviations: 3-m, 3-month; 18-m, 18-month; ACO, acyl-CoA oxydase; APRT, adenosyl phosphoribosyl transferase; CTR, control; CPT-I, carnitine-palmitoyl transferase-I; EMSA, electrophoretic mobility shift assay; FAS, fatty acid synthase; GFB, gemfibrozil; MCAD, medium-chain acyl-CoA dehydrogenase; NF-κB, nuclear factor-κB; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; RT–PCR, reverse transcription–polymerase chain reaction; RXR, 9-*cis*-retinoic acid receptor; SREBP1, sterol response element binding protein-1; SCD1, stearoyl-CoA desaturase-1; UCP-2, uncoupling protein-2.

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Aged mammals, including humans, show increasing plasma concentrations of lipids [6] and reduced fatty acid oxidation. This reduction is due mainly to a decrease in the oxidative capacity of body tissues, rather than a decreased free fatty acid release, and probably is determinant in the age-related increase in adiposity and plasma lipid concentrations [7]. At present, however, the mechanisms underlying changes in fat oxidation with age are not clear. Our experimental data demonstrate that old rats have a profound reduction in the hepatic expression and activity of PPARα, thus explaining the reduced fatty acid oxidative capacity of liver tissue associated to old age. Further, in accordance with this age-dependent reduction of hepatic PPARα, we show that the administration of a well-known PPARα agonist, the fibrate drug GFB, to 18-m-old rats is ineffective in reducing the high plasma concentrations of triglycerides present in these animals. From our results and those reported previously in the literature, we hypothesize that the decrease in liver PPARα is related to a state of marked leptin resistance present in aged animals.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats, 3-m and 18-m-old, were purchased from Criffa. The animals were maintained with water and food *ad libitum* at constant humidity and temperature with a light/dark cycle of 12 hr (8.00 a.m.–8.00 p.m.) for a minimum of 5 days. After this period of acclimatization, rats were weighted and killed by decapitation under ketamine (100 mg/kg, i.p.) anesthesia between 9.00 and 10.00 a.m. Blood samples were collected at the time of death in 5% EDTA-tubes; plasma was obtained by centrifugation and stored at −80° until needed. A 10–100 mg of liver (L), skeletal muscle (M), and epididymal white adipose tissue (WAT) of each rat was immediately frozen in liquid N_2 and stored at −80° until used for the extraction of total RNA. Another sample of 500 mg of liver tissue was stored at −80° for the quantification of liver triglycerides. Further, a fresh sample of liver tissue of each rat was immediately used for obtaining nuclear extracts. For assessing the hypotriglyceridemic activity of GFB, 12 young (3 months) and 14 old rats (18 months) were randomly assigned to CTR- and GFB-treated groups. GFB was incorporated in the diet at a concentration, adjusted to the age-dependent daily food consumption, as to proportionate a daily therapeutic dose of 3 mg/kg to treated animals. From our previous experience, this dose of GFB results in a strong hypolipidemic effect without eliciting a marked peroxisome-proliferation response in the liver of treated animals, thus reproducing closely what is observed in the clinical use of GFB. Control and treated diets were prepared as described [8] and fed to the animals for 21 days; after this period, animals were sacrificed, and hepatic and plasma samples were obtained. All procedures were conducted in accordance with the principles and guidelines established by the University of Barcelona Bioethics Committee, as stated in Law 5/1995, 21st July, from the Generalitat de Catalunya.

2.2. Plasma metabolites and hormones

Plasma glucose, cholesterol, triglycerides, and NEFA were kindly determined at the Clinical Biochemistry Laboratory of the Hospital Clinic of Barcelona, by current standard procedures. The remaining plasma analytes were determined by RIA using the following commercial kits: Kl 1523 from BioLinK 2000 for growth hormone, RPA 547 and RPA 548 from Amersham Pharmacia Biotech for rat insulin and corticosterone, respectively, and RL83K from Clonagen for rat leptin.

2.3. Liver triglyceride content

Liver triglycerides were extracted and measured as described previously [9], by using the 334-UV triglyceride Sigma kit.

2.4. RNA preparation and analysis

Total RNA was isolated by using the Ultraspec reagent (Biotech). Relative levels of specific mRNAs were assessed by RT–PCR. Complementary DNA was synthesized from RNA samples by mixing 1 μg of total RNA (except in the case of WAT-PPARγ, M-SREBP1, M-FAS, L-UCP-2, SCD1, MCAD and L-SREBP1 where 0.5 μg were used), 125 ng of random hexamers as primers in the presence of 50 mM Tris–HCl buffer (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 200 U Moloney murine leukemia virus reverse transcriptase (Life Technologies), 20 U RNAsin (Life Technologies) and 0.5 mM of each dNTP (Sigma Chemical Co.) in a total volume of 20 μL. Samples were incubated at 37° for 60 min. A 5 μL aliquot of the RT reaction was then used for subsequent PCR amplification with specific primers.

Each 50-μL PCR reaction contained 5 μL of the RT reaction, 1.2 mM $MgCl_2$, 200 μM dNTPs, 1.25 μCi [^{32}P]-dATP (3000 Ci/mmol, Amersham Pharmacia Biotech), 1 unit of Taq polymerase (Life Technologies), 0.5 μg of each primer and 20 mM Tris–HCl, pH 8.5. To avoid unspecific annealing, cDNA and Taq polymerase were separated from primers and dNTPs by using a layer of paraffin (reaction components contact only when paraffin fuses, at 60°). The sequences of the sense and antisense primers used for amplification were: PPARα, 5′-GGCTCGGAGGGCTCTGTCATC-3′ and 5′-ACATGCACTGGCAGCAGTGGA-3′; L-CPT-I, 5′-TATGTGAGGATGCTGCTT-3′ and 5′-CTCGGAGAGCTAAGCTTG-3′; M-CPT-I, 5′-TTCACTGTGACCCCAGACGGG-3′ and 5′-AATGGACCAGCCCCATGGAGA; MCAD, 5′-AAATTCTTTGGGGC-

AAATGCCT-3′ and 5′-TAAATCTGATAGATCTTGGC-3′; UCP-2, 5′-AACAGTTCTACACCAAGGGC-3′ and 5′-AGCATGGTAAGGGCACAGTG-3′; ACO, 5′-ACTATATTTGGCCAATTTTGTG-3′ and 5′-TGTGGCAGTGGTTTCCAAGCC-3′; RXRα, 5′-GCTCTCCAACGGGTCGAGGCT-3′ and 5′-TGGGTGTGGTGGGTACCGACA-3′; PPARγ, 5′-TGGGGATGTCTCACAATGCCA-3′ and 5′-TTCCTGTCAAGATCGCCCTCG-3′; SREBP1, 5′-TCACAGATCCAGCAGGTCCCC-3′ and 5′-GGTCCCTCCACTCACCAGGGT-3′; SCD1, 5′-GCTCATCGCTTGTGGAGCCAC-3′ and 5′-GGACCCCAGGGAAACCAGGAT-3′; FAS, 5′-GTCTGCAGCTACCCACCCGTG-3′ and 5′-CTTCTCCAGGGTGGGGACCAG-3′; and APRT, 5′-AGCTTCCCGGACTTCCCCATC-3′ and 5′-GACCACTTTCTGCCCCGGTTC-3′. The *aprt* gene was used as internal control in the PCR reaction to normalize the results, except for L-FAS, L-PPARγ, SCD1, M-CPT-I, M-PPARγ, M-PPAR-α, WAT-PPARα, where co-amplifications for these genes and APRT were performed in separate tubes. PCR was performed in an MJ Research Thermocycler (Ecogen) equipped with a Peltier system and temperature probe. After an initial denaturation for 1 min at 94°, PCR was performed for 18 (L-FAS), 20 (SCD1), 21 (L-APRT), 22 (WAT-PPARγ), 23 (M-, L-, and WAT-UCP-2, M-CPT-I, WAT-APRT, L-ACO, L-CPT-I, L-PPARα, L-RXRα, L-, WAT- and M-SREBP1), 25 (M-FAS, MCAD, M-PPARγ, M-PPAR-α, M-APRT, L-PPARγ), or 28 (WAT-PPARα) cycles. Each cycle consisted of denaturation at 92° for 1 min, primer annealing at 60° (except 58° for ACO and 63° for SREBP1), and primer extension at 72° for 1 min and 50 s. A final 5-min extension step at 72° was performed. Five microliters of each PCR sample was electrophoresed on a 1-mm-thick 5% polyacrylamide gel. The gels were dried and subjected to autoradiography using Agfa X-ray films (Danny Commercial) to show the amplified DNA products. Amplification of each gene yielded a single band of the expected size (PPARα: 645 bp, PPARγ: 200 bp, M-CPT-I: 222 bp, L-CPT-I: 629 bp, MCAD: 824 bp, UCP-2: 471 bp, ACO: 195 bp, RXRα: 202 bp, SREBP1: 180 bp, SCD1: 521 bp, FAS: 214 bp, and APRT: 329 bp). Preliminary experiments were carried out with various amounts of cDNA to determine non-saturating conditions of PCR amplification for all the genes studied. Thus cDNA amplification was performed in comparative and semiquantitative conditions [10]. Radioactive bands were quantified by video-densitometric scanning (Vilbert Lourmat Imaging). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*aprt*).

2.5. Isolation of nuclear extracts

Nuclear extracts were isolated using the Dignam *et al.* method [11] with the modifications described by Sonnenberg *et al.* [12]. Briefly, fresh liver tissues were weighed and homogenized by a Potter Elvehjem homogenizer in 4 vol. (w/v) of buffer A containing 0.25 M sucrose, 15 mM Tris–HCl, pH 7.9, 15 mM NaCl, 60 mM KCl, 1 mM EGTA, 5 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine and a mixture of protease inhibitors (0.1 mM phenylmethylsolfonyl fluoride, 1.0 mM dithiothreitol, 5 μg/mL aprotinin, 2 μg/mL leupeptin). Homogenates were incubated for 10 min on ice and centrifuged (2000 *g*, 10 min, 4°). Pellets were resuspended in 4 vol. of buffer B (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and protease inhibitors as above) and then centrifuged at 4000 *g*, for 10 min, at 4°. Supernatants were discarded and pellets were resuspended in 2 vol. of buffer C (0.5 M HEPES, pH 7.9, 0.75 mM $MgCl_2$, 0.5 M KCl, 12.5% glycerol and the protease inhibitors). Homogenates were kept for 30 min at 4° under continuous rotary shaking, and then centrifuged at 14,000 *g* for 30 min at 4°. Finally, the resulting supernatants were dialyzed overnight at 4° with buffer D (10 mM Tris–HCl, pH 7.9, 5 mM $MgCl_2$, 10 mM KCl, 1.0 mM EDTA, 10% glycerol and the protease inhibitors). Nuclear extracts were collected in microfuge tubes and stored in aliquots at −80°. The protein concentration of the nuclear extracts was determined by the method of Bradford [13].

2.6. EMSA

The DNA sequence of double-stranded oligonucleotides used were as follows: consensus binding site of PPAR response element, 5′-CAAAACTAGGTCAAAGGTCA-3′ (Santa Cruz Biotechnology); NF-κB consensus oligonucleotide, 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (Promega); and ABS (ADD1/SREBP1 binding site), 5′-GATCCTGATCACGTGATCGAGGAG-3′ [14]. Oligonucleotides were labeled in the following reaction: 1 μL of oligonucleotide (20 ng/μL), except in the case of NF-κB where 2 μL (1.75 pmol/μL) were used, 2 μL of 5× kinase buffer, 5 U of T4 polynucleotide kinase (10 U for NF-κB), and 3 μL of [γ-^{32}P]-ATP (3000 Ci/mmol at 10 mCi/mL, Amersham) incubated at 37° for 1 hr. The reaction was stopped by adding 90 μL of TE buffer (10 mM Tris–HCl, pH 7.4 and 1 mM EDTA). To separate the labeled probe from the unbound ATP, the reaction mixture was eluted in a Nick column (Pharmacia) according to the manufacturer's instructions. Four micrograms of crude nuclear proteins (8 μg for ABS EMSA) were incubated for 10 min on ice in binding buffer (10 mM Tris–HCl, pH 8.0, 25 mM KCl, 0.5 mM DTT, 0.1 mM EDTA, pH 8.0, 5% glycerol, 5 mg/mL BSA, 100 μg/mL tRNA and 50 μg/mL poly(dI–dC)), in a final volume of 15 μL. Labeled probe (approximately 50,000 cpm) was added and the reaction was incubated for 20 min at room temperature. Where indicated, specific competitor oligonucleotide was added before the addition of labeled probe and incubated for 15 min on ice. For supershift assays, antibodies were added also before incubation with labeled probe for a further 30 min at 4°. Protein–DNA complexes were resolved by electrophoresis at 4° on a 5% acrylamide gel and subjected to autoradiography. Radioactive bands

were quantified by video-densitometric scanning (Vilbert Lourmat Imaging). Antibodies against SREBP1 (mature form), Oct-1 (octamer motif-1 transcription factor) and PPARα were from Santa Cruz Biotechnology.

2.7. Western blot analysis

Crude nuclear extract (40 μg for PPARα determination and 80 μg for SREBP1 determination) from liver were subjected to 10% SDS–PAGE. Proteins were then transferred to immobilon polyvinylidene diflouride transfer membranes (Millipore) and incubated overnight at 4° with 5% non-fat milk solution. Membranes were then incubated with the primary rabbit polyclonal antibody raised against PPARα (dilution 1:1000) [15] or against SREBP1 (dilution 1:200) (Santa Cruz Biotechnology). Detection was achieved using the SuperSignal West Dura Trial kit (Pierce). Blots were also incubated with a mouse monoclonal antibody against actin (dilution 1:5000) (Oncogene), used as a control of equal loading. Size of detected proteins was estimated using protein molecular mass standards (Life Technologies).

2.8. Statistics

The results are the mean of N values ± SD. Plasma samples were assayed in duplicate. Significant differences were established by an unpaired *t*-test, using the computer program GraphPad-InStat (GraphPad Software, V2.03). When the variance was not homogeneous, a non-parametric test was performed (Mann–Whitney test). The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Increased age in rats is associated to a marked reduction in hepatic PPARα expression and activity

We sought to investigate if increased age in rats was affecting the liver expression and/or activity of PPARα. To this purpose, we studied PPARα mRNA, protein and binding activity in liver samples from 3-m and 18-m-old rats. Liver tissue from old animals presented a reduced expression of PPARα mRNA (35.7%) and protein (62.7%) (Fig. 1A and B). In EMSA performed with a PPRE oligonucleotide, competition experiments with unlabeled probe, and the reduction in the intensity of shifted bands in the presence of a PPARα antibody, confirmed the participation of the endogenous PPARα present in hepatic nuclear extracts from 3-m-old rats in the formation of shifted bands 4 and, mainly, band 3 (Fig. 1C); we have shown previously that the interaction between a PPARα antibody and hepatic nuclear extracts can originate either true supershifted bands or almost the complete disappearance of the shifted bands containing PPARα [16], as it is the case shown in Fig. 1C. The intensity of the shifted band 3 was reduced by 69.9% in gel-shift experiments performed with hepatic nuclear extracts obtained from 18-m-old rats (Fig. 1D). Thus, the reduction in hepatic PPARα protein with old age was also reflected in a similar decrease in the intensity of the specific binding to a PPRE probe of the PPARα protein present in hepatic nuclear extracts obtained from the same aged rats.

The hepatic expression of PPARγ (1.9 ± 0.8 and 1.3 ± 0.3 mRNA arbitrary units for 3-m and 18-m-old rats, respectively), and that of the heterodimerization partner of PPARs, RXRα (3.9 ± 0.7 and 4.5 ± 0.7 mRNA arbitrary units for 3-m and 18-m-old rats, respectively), were not changed with increased age. On the contrary, the mRNA levels of the *l-cpt-I* and *mcad* genes, involved in mitochondrial fatty acid oxidation and known to be regulated by PPARα [17,18], were markedly reduced (i.e. 57.5 and 77%, respectively, in liver samples from 18-m-old rats, compared with values obtained from 3-m-old rats) (Fig. 2A and B), while the levels of ACO mRNA were not modified by age (2.8 ± 0.6 and 2.8 ± 0.3 mRNA arbitrary units for 3-m and 18-m-old rats, respectively, ns). Those changes were accompanied by a marked increase in the hepatic content of triglycerides (2.6-fold increase in 18-m-old rats vs. values obtained in 3-m-old rats) (Fig. 2C). The levels of mRNA for PPARα, PPARγ, and CPT-I were not changed by age when determined in samples from skeletal muscle and WAT (data not shown).

3.2. Hepatic PPARα changes in old rats are not accompanied by changes in other transcription factors, such as NF-κB and SREBP1

The immune system of old animals presents a high constitutive production of inflammatory mediators, such as interleukin-6 and -12, tumor necrosis factor α (TNFα), and cyclooxygenase-2 (COX-2); spontaneous hyperactivation of the oxidative stress-regulated transcription factor NF-κB seems to be at the basis of those age-related changes [19]. Poynter and Daynes [20] demonstrated that the hyperactivation state of NF-κB present in splenocytes obtained from old-female mice is related to an age-dependent decrease in the activity of PPARα. To ascertain if a similar situation was present in the liver tissue of old rats, we determined the binding of hepatic nuclear extracts to an oligonucleotide mimicking a NF-κB response element. Although we were able to detect two main retardation bands, specific for binding to the NF-κB probe, no significant increase in the intensity of those bands was detected when assaying nuclear extracts from 18-m-old rats (Fig. 3). This result indicated that the changes observed in the binding of hepatic nuclear extracts to a PPRE probe were not due to a generalized reduction in the binding properties of nuclear extracts from old animals. Moreover, the absence of an increased binding capability of hepatic nuclear extracts

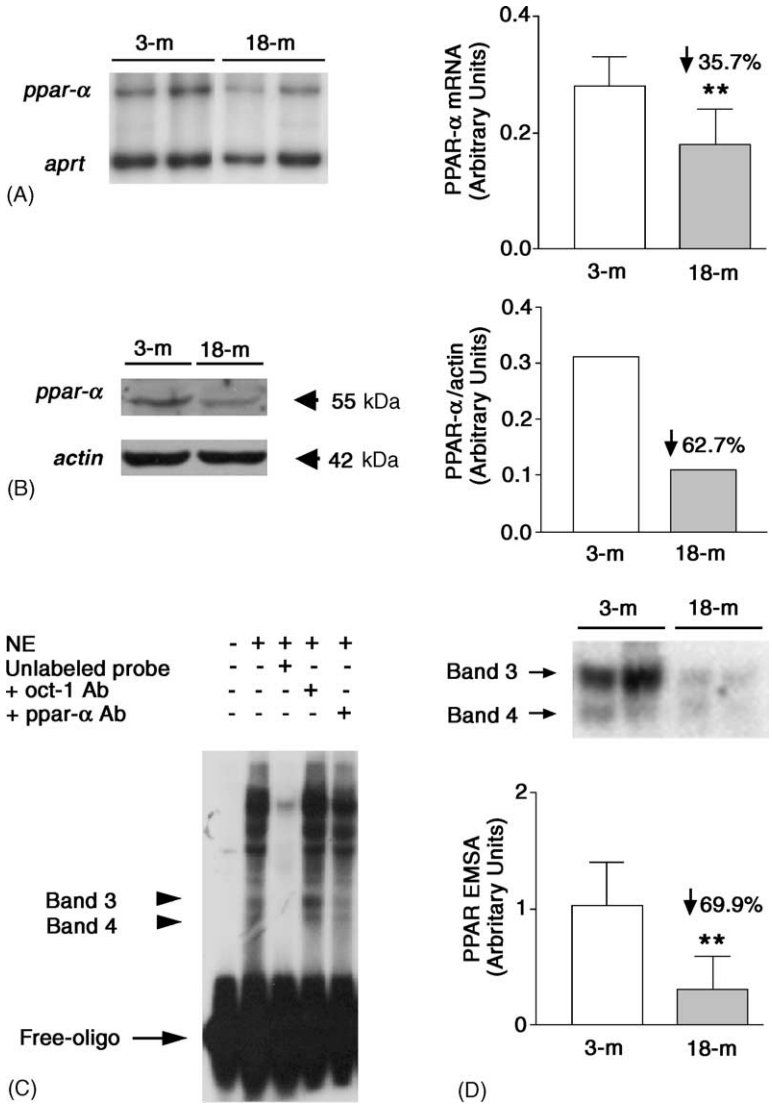

Fig. 1. (A) Relative levels of PPARα mRNA in hepatic samples from 3-m and 18-m-old animals. Each bar represents the mean ± SD of values from five animals. A representative autoradiography is presented in the left side of the figure, showing the bands corresponding to PPARα mRNA and that of the *aprt* gene, used as an internal control in the PCR reaction to normalize the results, from liver samples of two different animals from each age group. (B) Western blot of PPARα in hepatic samples from 3-m and 18-m-old animals. In each line, 40 μg of protein from a pool of liver nuclear extracts from six different animals were loaded; the amount of protein loaded was confirmed by the Bradford method, and actin was used as a control for loading equal amounts of protein. In the right side of the figure, a bar-plot representing the relative intensity of the PPARα bands is shown. (C) EMSA assay showing that the binding of hepatic nuclear extracts (NE) obtained from young rats to a PPRE oligonucleotide produces, at least, two specific bands (3 and 4) that contain PPARα protein, as they disappear in the presence of a PPARα antibody (Ab). Oct-1 Ab was used to demonstrate that the disappearance of bands 3 and 4 was not due to an unspecific interference produced by the presence in the incubation medium of immunoglobulin proteins. (D) Quantification of the intensity of shifted band 3 in EMSA performed with hepatic nuclear extracts from 3-m and 18-m-old animals (for each group, four different nuclear extracts were assayed; each nuclear extract was obtained by pooling hepatic tissue from two different animals). In the upper part of the figure is presented a representative EMSA autoradiography, showing bands 3 and 4 obtained with two different nuclear extracts from each age group. $^{**}P < 0.01$.

from aged animals to a NF-κB response element argues against the possibility that the reduction in PPARα expression and binding activity was due to a state of NF-κB hyperactivation, as reported previously in mouse tissues [20].

Eighteen-month-old male rats were overtly hypercholesterolemic and hypertriglyceridemic, presented high plasma levels of NEFA (Table 1) and, as shown in Fig. 2C, presented an accumulation of liver triglycerides. These changes could be related not only to a decreased catabolic rate of fatty acids, as a result of the reduction of hepatic PPARα activity, but also to an increase in the synthesis of hepatic lipids. Nevertheless, although old rats showed a moderate increase in the specific mRNA for SREBP1, a transcription factor known to control the expression of genes coding for lipogenic enzymes [21] (Fig. 4A), and in the amount of the mature form of the protein in hepatic nuclear extracts of 18-m-old rats (Fig. 4B), we were not able to detect the presence of SREBP1 in EMSA performed with hepatic nuclear extracts

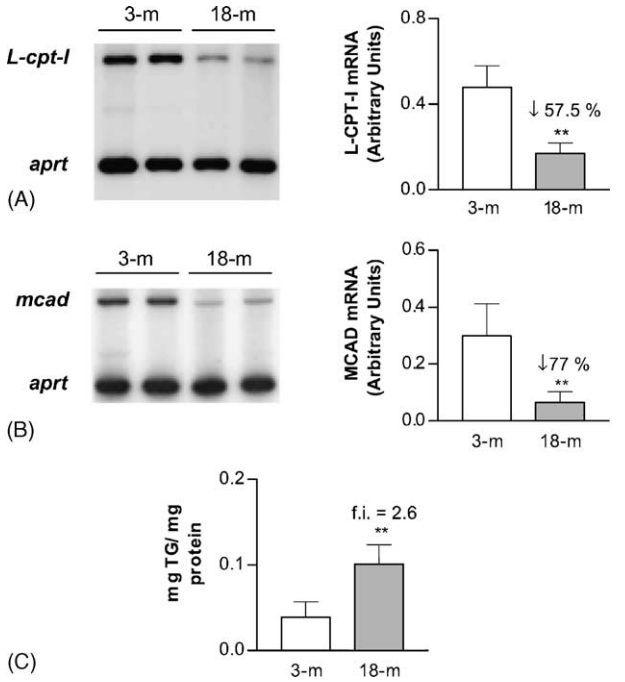

Fig. 2. (A) Relative levels of L-CPT-I mRNA in hepatic samples from 3-m and 18-m-old animals (five animals per group). A representative autoradiography is presented in the left side of the figure, showing the bands corresponding to L-CPT-I mRNA and that of the *aprt* gene, used as an internal control in the PCR reaction to normalize the results, from liver samples of two different animals from each age group. (B) Relative levels of MCAD mRNA in hepatic samples from 3-m and 18-m-old animals (five animals per group). A representative autoradiography is presented in the left side of the figure, showing the bands corresponding to MCAD mRNA and that of the *aprt* gene, used as an internal control in the PCR reaction to normalize the results, from liver samples of two different animals from each age group. (C) Liver triglyceride content, expressed as mg of triglycerides per mg of hepatic protein, of samples from 3-m and 18-m-old animals (eight animals per group). $^{**}P < 0.01$.

incubated with an ABS oligonucleotide and an specific SREBP1 antibody (data not shown). Moreover, the mild increase in hepatic FAS mRNA (1.8-fold increase vs. values obtained in samples from 3-m-old rats, Fig. 4C), and the unchanged levels of hepatic SCD1 mRNA (Fig. 4D) argues against a relevant role of an increased SREBP1 activity in the production of lipid changes observed in old rats.

Table 1
Concentrations of plasma metabolites and hormones from 3-m and 18-m-old male rats

	3-m	18-m
Cholesterol (mg/dL)	75 ± 10	144 ± 57**
Triglyceride (mg/dL)	140 ± 61	235 ± 77**
NEFA (μmol/L)	269 ± 114	527 ± 217*
Glucose (mg/dL)	175 ± 24	172 ± 22
Insulin (ng/mL)	2.4 ± 1.0	8.2 ± 5.8**
Leptin (ng/mL)	1.4 ± 1.1	22.2 ± 8.8**
Corticosterone (ng/mL)	7.7 ± 8.4	6.7 ± 5.8
Growth hormone (ng/mL)	11.1 ± 1.9	5.1 ± 3.8**

Values are means ± SD of eight animals per group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. 3-m values.

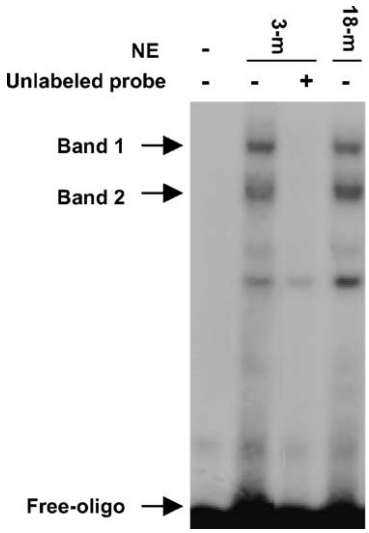

Fig. 3. EMSA assay showing the binding of pooled hepatic NE obtained from two young rats (3-m) and two old rats (18-m) to a NF-κB consensus oligonucleotide. Bands 1 and 2 are effectively competed by unlabeled oligonucleotide. The assay was repeated thrice with different nuclear extracts, obtaining similar results.

3.3. Aged-related changes in hepatic PPARα could be related to a state of leptin resistance in old rats

Concentrations of plasma metabolites and hormones from young and 18-m-old male rats are presented in Table 1. Eighteen-month-old rats were frankly hypercholesterolemic and hypertriglyceridemic, whereas plasma NEFA concentration was significantly increased by 2-fold. While plasma corticosterone concentration was not changed in old rats, a striking decrease in growth hormone was observed, with a 54% decrease in 18-m-old animals vs. plasma growth hormone concentration in 3-m-old animals. Although old rats were normoglycemic, they were overtly hyperinsulinemic (3.4-fold increase in 18-m-old animals vs. plasma insulin concentration in 3-m-old animals). These results indicate that old animals presented a state of tissue insulin resistance compensated by an increased output of this pancreatic hormone. Further, old animals were also hyperleptinemic (16-fold increase in 18-m-old animals vs. plasma leptin concentration in 3-m-old animals). Wang *et al.* [22] demonstrated very recently that old rats are markedly resistant to leptin actions. It has been demonstrated also that in the absence of leptin activity, lipogenesis is increased and PPARα-driven fatty acid oxidation is reduced, accounting for the steatosis and lipotoxicity that occur is such circumstances [23,24]. Thus, probably, the marked reduction in hepatic PPARα expression with old age could be related to a resistance of liver tissue to leptin actions. Supporting this assumption, the mRNA levels of *ucp-2*, another gene whose expression is increased by leptin activity [25], were significantly decreased ($P < 0.05$) in liver (33.8%), WAT (35.7%) and muscle (32.1%) from old animals (0.6 ± 0.1, 1.1 ± 0.2, and 0.6 ± 0.2 mRNA arbitrary units for L, WAT, and M from 3-m-old animals, respectively).

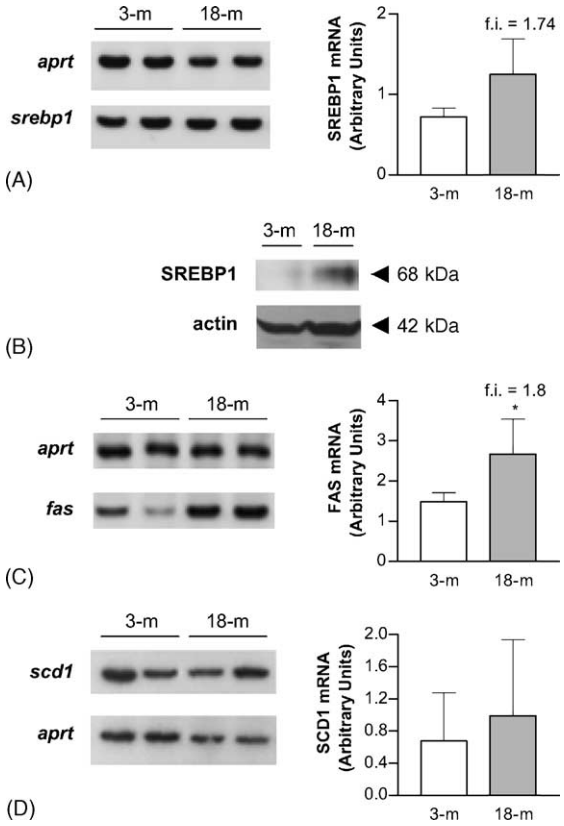

Fig. 4. (A) Relative levels of SREBP1 mRNA in hepatic samples from 3-m and 18-m-old animals (five animals per group). A representative autoradiography is presented in the left side of the figure, showing the bands corresponding to SREBP1 mRNA and that of the *aprt* gene, used as an internal control in the PCR reaction to normalize the results, from liver samples of two different animals from each age group. (B) Western blot of SREBP1 in hepatic samples from 3-m and 18-m-old animals. In each line, 80 μg of protein from a pool of liver nuclear extracts from six different animals were loaded; the amount of protein loaded was confirmed by the Bradford method, and actin was used as a control for loading equal amounts of protein. (C) Relative levels of FAS mRNA in hepatic samples from 3-m and 18-m-old animals (five animals per group). A representative autoradiography is presented in the left side of the figure, showing the bands corresponding to FAS mRNA and that of the *aprt* gene, used as an internal control in the PCR reaction to normalize the results, from liver samples of two different animals from each age group. (D) Relative levels of SCD1 mRNA in hepatic samples from 3-m and 18-m-old animals (five animals per group). A representative autoradiography is presented in the left side of the figure, showing the bands corresponding to SCD1 mRNA and that of the *aprt* gene, used as an internal control in the PCR reaction to normalize the results, from liver samples of two different animals from each age group. $^{*}P < 0.05$.

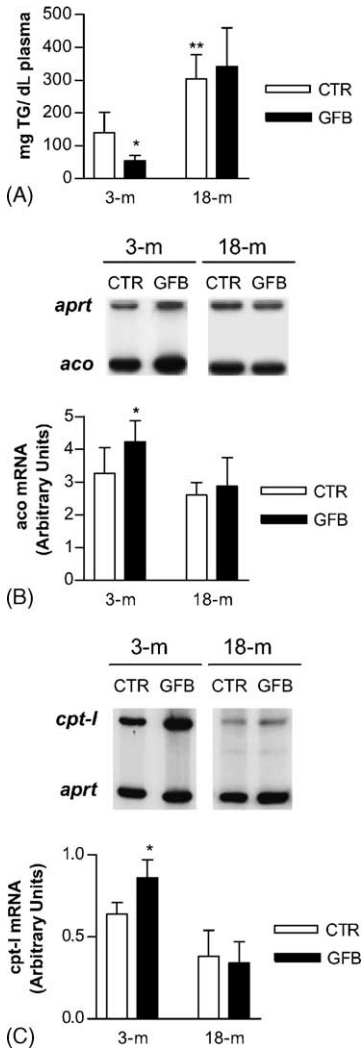

Fig. 5. (A) Plasma triglyceride concentrations in CTR- and GFB-treated (3 mg/kg per day, 21 days) 3-m and 18-m-old rats. Each bar represents the mean ± SD of values from six (3-m) or seven (18-m) animals. $^{*}P < 0.05$, GFB 3-m vs. CTR 3-m; $^{**}P < 0.01$, CTR 18-m vs. CTR 3-m. (B) Relative mRNA levels for ACO in liver samples from CTR- and GFB-treated (3 mg/kg per day, 21 days) 3-m and 18-m-old rats. Each bar represents the mean ± SD of values from six (3-m) or seven (18-m) animals. $^{*}P < 0.05$, GFB 3-m vs. CTR 3-m. In the upper part of the figure, a representative autoradiography showing the bands corresponding to ACO mRNA and that of the *aprt* gene, used as an internal control in the PCR reaction to normalize the results, from one animal of each treatment group is shown. (C) Relative mRNA levels for CPT-I in liver samples from CTR- and GFB-treated (3 mg/kg per day, 21 days) 3-m and 18-m-old rats. Each bar represents the mean ± SD of values from six (3-m) or seven (18-m) animals. $^{*}P < 0.05$, GFB 3-m vs. CTR 3-m. In the upper part of the figure, a representative autoradiography showing the bands corresponding to CPT-I mRNA and that of the *aprt* gene, used as an internal control in the PCR reaction to normalize the results, from one animal of each treatment group is shown.

3.4. GFB administration to old rats failed to reduce plasma triglyceride concentrations and did not increase hepatic ACO and CPT-I expression

GFB administration to 3-m-old rats produced a characteristic strong hypolipidemic effect, significantly reducing by 50% the plasma concentration of triglycerides with respect to the values presented in age-matched control animals (Fig. 5A). In accordance with the known activity of GFB as a PPARα activator, this hypolipidemic activity was accompanied by a significant increase (30 and 34%, respectively) in the hepatic mRNA levels of ACO (Fig. 5B) and CPT-I (Fig. 5C). ACO and CPT-I activities are the rate limiting step in the peroxisomal and mitochondrial β-oxidation system of fatty acids, respectively, and it has been extensively described that their expression and activity is increased by the administration of PPARα activators to rodents [4,5,17]. In accordance with the reduction in

hepatic PPARα by old age described above, and despite the marked hypertriglyceridemia present in 18-m-old rats (3-fold increase vs. values presented by young animals), the administration of GFB to old rats, at the same dose and length of treatment used with young rats, produced no change in plasma triglyceride concentrations (Fig. 5A). Further, in these animals, GFB administration failed to induce any significant change in the hepatic mRNA levels of ACO (Fig. 5B) and CPT-I (Fig. 5C).

4. Discussion

Old age is associated to the appearance of dislypidemia, obesity, and type II diabetes mellitus [1]. In humans, those risk factors increase the likelihood of experiencing a major cardiovascular accident in late life [26]. The Helsinki Heart Study and, more recently, the Veterans Affairs-High Density Lipoprotein Cholesterol Intervention Trial (VA-HIT) clearly demonstrate that GFB administration to humans can reduce cardiovascular mortality, specially in those patients with low HDL-cholesterol and moderately high plasma triglyceride concentrations, lipid alterations most frequently associated to type II diabetes and obesity [3]. Thus, from a theoretical point of view, as human populations grew older, more patients will present a plasma lipid profile amendable by GFB and, in general, fibrate administration.

As the majority of clinical trials have been performed in mature humans populations, between 40 and 60 years of age at baseline [2,3], related to the issue of whether to treat or not older populations with hypolipidemic drugs, it is the necessity to ascertain if these drugs maintain their therapeutical capabilities in very old people.

Old rats develop many metabolic alterations common to aged humans. Thus, the incidence of dyslipidemia, obesity associated to leptin resistance and a progressive failure of insulin-mediated glucose metabolism is common to aged rats and humans [27]. In the present work, we present evidence that old age causes a profound reduction in the expression and activity of PPARα in the liver of old rats. As a consequence, old rats become resistant to the hypotriglyceridemic effect of GFB, a well-known drug pertaining to the fibrate-class of hypolipidemic drugs. It is worth noting that GFB was used at a dose close to those used in human therapeutics, and far from the suprapharmacological doses used to induce peroxisome-proliferation and its related toxicity in rats. In our hands, bezafibrate, another fibric acid derivative, has been also unable to elicit a hypotriglyceridemic response and to increase the liver expression of ACO and CPT-I when administered to old rats (data not shown), further supporting an age-related deficit in liver PPARα, the accepted pharmacological target of fibrate drugs.

PPARα expression and/or activity is regulated by several hormone systems, being increased by corticosteroids and leptin, and decreased by growth hormone and insulin [15,22,28–30]. The fact that old rats showed no changes in plasma corticosterone concentrations and a marked reduction in plasma levels of growth hormone suggests that these hormones are probably not related to the decreased liver expression of PPARα. Although old rats presented a marked hyperinsulinemia, they were normoglycemic, indicating that the high levels of insulin were sufficient to maintain a normal insulin response in body tissues. Our old rats were also hyperleptinemic. Wang *et al.* [22] have shown that in old rats a clear resistance to the effects of leptin develops; further, these authors relate the reduction in adipose tissue sensitivity to leptin to the failure of leptin administration to old rats to increase the expression of PPARα and its target genes in WAT. Very recently, Lee *et al.* [31] have shown, by using PPARα (−/−) and PPARα (+/+) mice infused with adenovirus-leptin, that PPARα is necessary for the lipopenic action of hyperleptinemia in liver. Thus, although our results are not a direct proof of a liver state of leptin resistance in old rats, they agree with those reported previously [22,31], supporting the hypothesis that leptin resistance is directly related to lipid metabolic disturbances in old age.

In any case, whatever the reason for the decrease in hepatic PPARα expression, this situation can be a major factor explaining lipid changes detected in our 18-m-old rats. Thus, liver triglyceride accumulation by old age in rats is probably directly linked to a deficit in hepatic PPARα activity. Absence of PPARα expression in PPARα-null mice [32] or inhibition of PPARα transcriptional activity [33] consistently associates to hepatic accretion of triglycerides, and that may be due in part to impaired mitochondrial oxidation of fatty acids [32]. Thus, livers of old rats showed a marked decrease in L-CPT-I and MCAD mRNA levels. L-CPT-I and MCAD expression is under transcriptional control by PPARα, and the enzyme activity of their products regulates mitochondrial fatty acid oxidation [17,18]. Although ACO is another gene directly related to peroxisomal fatty acid oxidation and with a classical PPRE in its promoter region, its basal expression is not regulated by PPARα [34], explaining the fact that ACO mRNA levels were unmodified in old rats, despite a clear reduction in PPARα activity. Similarly, basal expression of ACO is not modified in PPARα-null mice [32,34].

PPARα deficiency is also consistently related to increases in plasma cholesterol, triglyceride and NEFA concentrations [35,36]. Accordingly, old male rats were hypercholesterolemic and hypertriglyceridemic, and they showed a clear significant increase in plasma NEFA concentrations. Further, old rats showed a marked increase in visceral white fat (2.6 ± 0.2 g vs. 6.9 ± 2.4 g of epididymal fat pad for 3-m and 18-m-old rats, respectively, $P < 0.01$), a well-reported characteristic of ageing rodents [1,22,37], substantially contributing to the gain in body weight of old animals (387 ± 25 g vs. 678 ± 75 g of body weight for 3-m and 18-m-old rats, respectively). As it is

well known that a deficiency in leptin activity causes adipocyte hypertrophy [22,38], it can be argued that, in our old animals, there was an increased flux and accumulation of fatty acids on WAT, thus explaining the detected changes in plasma NEFA concentration and the increased adiposity observed in these animals. Probably also, the increased flux of fatty acids was directed not only to WAT, but to other peripheral tissues, as skeletal muscle. Although we have not determined the triglyceride content of muscle samples, the mRNA level of FAS were reduced in these samples (66.7%, 0.7 ± 0.1 vs. 0.2 ± 0.01 mRNA arbitrary units for 3-m and 18-m-old rats, $P < 0.05$), and it is well known that polyunsaturated fatty acids down-regulate the expression of lipogenic genes [39]. Despite we found an unexpected increase in the mRNA levels for SREBP1 in skeletal muscle (76%, 1.6 ± 0.4 vs. 2.8 ± 0.3 mRNA arbitrary units for 3-m and 18-m-old rats, respectively, $P < 0.05$), it has been previously established that changes in SREBP1 mRNA abundance are not necessarily accompanied by changes in SREBP1 gene transcription [40], while changes in mRNA levels of FAS have been always associated to parallel changes in the expression of the protein and enzyme activity.

In conclusion, our results indicate that old rats are resistant to the hypolipidemic effect of fibrates due to a marked decrease in the expression of hepatic PPARα. Further, the decrease in the hepatic PPARα is probably directly related to the lipid metabolic disturbances observed in old animals. To probe if similar changes are produced in ageing humans will be a very difficult task, but it would provide an important piece of information in order to determine the appropriate clinical use of fibrates in old people.

Acknowledgments

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