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Increase in hepatic expression of SREBP-2 by gemfibrozil administration to rats

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Abstract

It is well known that gemfibrozil increases the biliary output of cholesterol and phospholipids, but we have little knowledge about the impact these changes have on liver cholesterol and phospholipid biosynthetic pathways. In the present study, no changes were detected in liver lipids and CTP:phosphocholine cytidylyltransferase after gemfibrozil administration to rats. On the contrary, 3-hydroxy-3-methylglutaryl-CoA reductase mRNA (9.9-fold) and Rd activity (16.7-fold) and phosphatidate phosphohydrolase activity (1.7-fold) increased, while plasma apo B-cholesterol (40%) and triglyceride (43%) levels decreased. As a part of a compensatory homeostatic response, we report for the first time that gemfibrozil administration to rats increased the hepatic sterol regulatory element binding protein-2 (SREBP-2) mRNA (2.9-fold) and mature protein (2.2-fold) levels. An early increase in the transcriptional activity of SREBP-2 elicited by gemfibrozil administration might be responsible for the observed changes in HMG-CoA reductase, phosphatidate phosphohydrolase, and SREBP-2 expression.

Keywords: PPAR; CTP:phosphocholine cytidylyltransferase; HMG-CoA reductase; SREBP; Phosphatidate phosphohydrolase; Gemfibrozil

1. Introduction

Fibrates present a complex mechanism of action. They increase the expression of genes coding for apolipoproteins apo AI and apo AII and, as a consequence, HDL concentration rises [1, 2]. Further, they also stimulate the hepatic expression of the lipoprotein lipase gene [3] while reducing the expression of the gene coding for apo CIII [4]. Both effects result in a stimulation of the catabolism of triglyceride-rich lipoproteins, mainly VLDL and IDL. In every case, fibrates act as agonists of a nuclear receptor, the PPARα (peroxisome proliferator activated receptor), whose response element (PPRE) is present in the promoter region of the fibrate-target genes [5].

Fibrates also reduce to a variable extent plasma LDL cholesterol by a mechanism that is not so well defined. They seem to reduce intestinal cholesterol absorption, and, more important, they increase the biliary output of cholesterol and phospholipids while decreasing the biliary secretion of bile acids, thus increasing biliary lithogenicity [6, 7]. Gemfibrozil, a fibrate widely used in the treatment of hypertriglyceridemia, produce those changes in its clinical use, increasing the fecal losses of neutral sterols and phospholipids and reducing the fecal excretion of bile acids [8–10]. More recently, the change in bile composition induced by gemfibrozil and other fibrates has been related to the inhibition of hepatic cholesterol 7α-hydroxilase activity [11] and to the increased hepatic expression of the *mdr2 P-glycoprotein* gene [12]. Whether those effects are also elicited by PPARα activation is still a subject of debate.

We have little knowledge about the impact these changes in biliary cholesterol and phospholipid secretion have in the activity of the hepatic cholesterol and phospholipid biosynthetic pathways. For this reason, we studied the effect of

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Abbreviations: ACO, acyl-CoA oxidase; Apo, apolipoprotein; APRT, adenosyl phosphoribosyl transferase; CT, CTP:phosphocholine cytidylyl transferase; HDL, high-density lipoprotein; HMG-CoA Rd, 3-hydroxy-3-methyl-glutaryl coenzyme A reductase; LDL, low-density lipoprotein; PAP, phosphatidate phosphohydrolase; PPARα, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element binding protein; and VLDL, very-low-density lipoprotein.

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gemfibrozil administration to rats on plasma and hepatic lipid concentrations, and the activity and mRNA levels of key proteins controlling liver cholesterol and phospholipid synthesis, namely HMG-CoA Rd, CT, and PAP [13–15]. We treated the animals for three days with a dose of gemfibrozil known to activate PPARα [4] and to produce a hypotriglyceridemic and hypocholesterolemic effect in rats [16]. As markers of PPARα activation, we determined the activity of peroxisomal β-oxidation and the ACO mRNA levels in rat liver [17].

SREBPs are membrane-bound transcription factors that are known to transcriptionally regulate HMG-CoA Rd and other enzymes in the cholesterol biosynthetic pathway, together with the genes coding for the low-density lipoprotein receptor and the SREBP-2 itself. Hepatic cholesterol deprivation results in SREBP proteolytic activation of the precursor form present in microsomal membranes, migration of the mature SREBP form to the nucleus, and up-regulation of these target genes [18, 19]. Biliary losses of cholesterol, elicited by gemfibrozil administration, may possibly reduce hepatic cholesterol content, thus activating the SREBP pathway. For this reason, we also determined the mRNA and mature protein levels of SREBP-2 in hepatic samples, as well as the specific binding of hepatic nuclear extracts to a sterol response element (SRE) consensus oligonucleotide.

In the present work, we provide the first evidence of a stimulation of the SREBP transactivation pathway by gemfibrozil in rats. As a consequence, the expression and activity of enzymes involved in hepatic lipid synthesis are increased.

2. Materials and methods

2.1. Animals and treatments

Male Sprague–Dawley rats (Harlan) weighing 207 ± 11 g were divided at random into two groups of treatment and fed, respectively, 28 g/day of a standard diet (Panlab) or the same diet supplemented with gemfibrozil (0.3% weight/weight). After three days, animals were killed between 9–10 a.m., at the beginning of the light period. Both treatment diets were prepared as described by Alegret *et al*. [16]. Before treatment, animals were maintained with water and food *ad lib*. for 5 days, at a constant humidity and temperature and with a light/dark cycle of 12 hr. 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. Sample preparation

Livers were excised, perfused, and homogenised in a 150 mM NaCl, 1 mM dithiotreitol, 30 mM EDTA, 50 mM KH_2PO_4, pH 7.4 buffer. The postmitochondrial, microsomal, and cytosolic fractions were obtained by centrifugation [20] and frozen at −80° until needed. Protein concentration was determined by the method of Bradford [21]. Between 10–100 mg of liver tissue of each rat was immediately frozen in liquid N_2 and used for the extraction of total mRNA with the Ultraspec™ (Biotecx) reagent, following the instructions provided by the manufacturer. Blood samples were collected in heparinized tubes; plasma was obtained by centrifugation and stored at −80° until needed.

2.3. Enzyme assays

The activities of the HMG-CoA Rd (EC 1.1.1.34), CT (EC 2.7.7.15), and the peroxisomal fatty acid β-oxidation system were determined as described previously [16, 22]. The PAP (EC 3.1.3.4) activity was determined in liver postmitochondrial fraction by the method of Martin *et al*. [23], by using a [^{14}C]phosphatic acid and phosphatidylcholine emulsion as substrate, prepared as described by Aridor-Piterman *et al*. [24]. Assays *in vitro* were performed as described by Alegret *et al*. [22]. Gemfibrozil was added to the incubation medium from a stock solution adjusted to pH 8–8.5 with 0.1 mM NaOH.

2.4. Lipid analysis

Plasma total cholesterol, triglyceride, and phospholipid concentrations were measured with the Boehringer Mannheim GmbH colorimetric tests (Monotest Cholesterol CHODPAP No. 290319, Peridochrom Triglyceride GPO-PAP No. 701882, and MPR2 No 691844 phospholipid, respectively). Using reagent No. 543004, also from Boehringer Mannheim, precipitated VLDL and LDL from plasma samples, and HDL-cholesterol concentration was determined in the supernatant. Lipid in the liver was measured as described previously [25].

2.5. RT–PCR

mRNA levels were determined by reverse transcription coupled to the polymerase chain reaction (RT–PCR), basically as described by Cabrero *et al*. [26]. The sequences of the sense and antisense primers used were: 5′-GGTTTA CGGCAGCCAGCTCCT-3′ and 5′-ACGGACAATGCG GGTGATGAT-3′ for the rat *ct* gene (GenBank accession number: M36071), 5′-CCGACAAGAAACCTGCTGCCA-3′ and 5′-CAGTGCCACACACAATTCGGG-3′ for the rat *hmg-coa rd* gene (GenBank number: X55286), 5′-ACT-ATATTTGGCCAATTTTGT-3′ and 5′-TGTGGCAGTG-GTTTCCAAGCC-3′ for the rat *aco* gene (GenBank number: J02752), and 5′-CATGGACACCCTCACGGAGCT GGGCGACGA-3′ and 5′-TGCATCATCCAATAGAGG GCTTCCTGGCTC-3′ for the *srebp-2* gene (GenBank number: U12330). The rat *aprt* gene (primers 5′-AGCTTC-CCGGACTTCCCCATC-3′ and 5′-GACCACTTTCTGC-CCCGGTTC-3′) was used as internal control in the PCR

reaction to normalize the results, except for SREBP-2, where co-amplifications were performed in separate tubs and in duplicate. Amplified fragments were marked with [α^{32}P]dATP (3000 Ci/mmol) (Amersham Pharmacia Biotech). For each primer set, an increasing number of PCR cycles with otherwise fixed conditions was performed to determine the optimal cycles to be used. This was determined to be halfway through the exponential phase. The numbers of cycles were 21 for *CT*, 22 for *Hmg-CoA Rd*, 20 for *ACO*, 30 for *SREBP-2* and 23 for *APRT*. Five microliters of each PCR reaction mixture was subjected to electrophoresis in 5% polyacrylamide gel in 1 × TBE. Gels were dried, autoradiographed by using an RX-OMAT S Kodak film, and quantified by video-densitometric scanning (IMAT program, Scientific and Technical Services, University of Barcelona).

2.6. Electrophoretic mobility shift assays (EMSA)

Hepatic nuclear extracts were obtained from 0.4 g of liver tissue homogenized in 1.6 mL of homogenization buffer (0.25 M sucrose, 15 mM Tris–HCl pH 7.9, containing 60 mM KCl, 15 mM NaCl, 1 mM EGTA, 5 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, and 4 μl/mL of a protease inhibitor cocktail), essentially as described by Dignam *et al*. [27]. Twenty micrograms of nuclear extract was incubated for 10 min on ice in 10 mM Tris buffer pH 8, containing 25 mM KCl, 0.5 mM DTT, 0.1 mM EDTA, 5% glycerol, 5 mg/mL of BSA, 50 μg/mL of poly(dI-dC), and 100 μg/mL of tRNA, in a final volume of 20 μL. Approximately 100,000 cpm of a ^{32}P end-labeled double-stranded SRE-1 consensus oligonucleotide (5′-CGCGCCATCAC-CCCACGCACCG-3′) was then added and further incubated at room temperature for 15 min. Where indicated, specific competitor oligonucleotide was added before the addition of labeled probe and incubated for 10 min on ice. For the supershift assay, the specific anti-SREBP-2 antibody (Santa Cruz Biotechnology) was added before the addition of the labeled probe, and incubated for 30 min at 4°. Protein–DNA complexes were resolved by electrophoresis at 4° on a 5% acrylamide gel and subjected to autoradiography. The double-stranded oligonucleotide was end-labeled with T4 polynucleotide kinase and [γ^{32}P]ATP.

2.7. Western blot analysis

Samples of nuclear extracts (60 μg), obtained as described previously, were mixed with SDS-loading buffer and subjected to SDS–PAGE on an 10% gel [28]. Proteins were then transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore). Membrane sheets were first incubated with a goat polyclonal antibody against human SREBP-2 (N-19) (1:500 dilution) for 1 hr at room temperature. After several washes, they were incubated with horseradish peroxidase-conjugated anti-goat IgG (1:4000 dilution), according to the protocol supplied by the manufacturer. Equal loading of protein in each lane was verified by incubation with rabbit polyclonal antibody against Oct-1 (Santa Cruz Biotechnology) at a dilution 1:1000. Antibodies were diluted in Tris-buffered saline (TBS) containing 3% defatted dry milk. Immunodetection was performed by the ECL Western blotting detection system kit (Amersham Pharmacia Biotech).

Table 1
Plasma and liver lipid concentrations

		CTRL	GFB
Plasma	Total cholesterol	108 ± 12	87 ± 9**
	Free cholesterol	34 ± 5	27 ± 5*
	Cholesteryl esters	74 ± 9	60 ± 7**
	Triglycerides	117 ± 18	67 ± 18**
	Phospholipids	198 ± 26	148 ± 11**
	Cholesterol-HDL	71 ± 8	65 ± 14
	Cholesterol apoB	37 ± 11	22 ± 14***
Liver	Total cholesterol	8.6 ± 2.1	7.2 ± 0.5
	Free cholesterol	8.4 ± 1.9	7.4 ± 0.3
	Phospholipids	77.2 ± 14.4	78.6 ± 16.4
	Triglycerides	29.1 ± 20.3	23.9 ± 18.9

CTRL: Control, GFB: Gemfibrozil. Results are presented as means ± SD (N = 6), and expressed as mg/dL of plasma or 10^{-3} mg/mg of liver postnuclear supernatant protein.

* $P < 0.005$;

** $P < 0.01$; and

*** marginal statistical significance, $P < 0.07$.

2.8. Statistics

The results are the means ± standard deviation of N experiments assayed in duplicate. Statistical significance was set at $P < 0.05$. Significant differences were established by parametric (Student's *t*-test) or non-parametric (Mann–Whitney test) for unpaired data, using the computer program GraphPad InStat. Logarithms of plasma triglyceride concentration were used to calculate statistics, as the variance was not homogeneous. Linear correlation between variables was calculated by the Pearson linear correlation test, using the above-mentioned program.

3. Results

Gemfibrozil administration to rats did not modify the concentration of liver lipids. In contrast, plasma lipid levels were strongly affected by gemfibrozil treatment (Table 1). Plasma triglyceride, phospholipid, and total cholesterol were reduced by 43, 25, and 19%, respectively. The reduction in total plasma cholesterol equally affected both free (20%) and esterified (19%) plasma cholesterol fractions, and was mainly attributed to the reduction in apo B-cholesterol (40%), while HDL cholesterol was not modified.

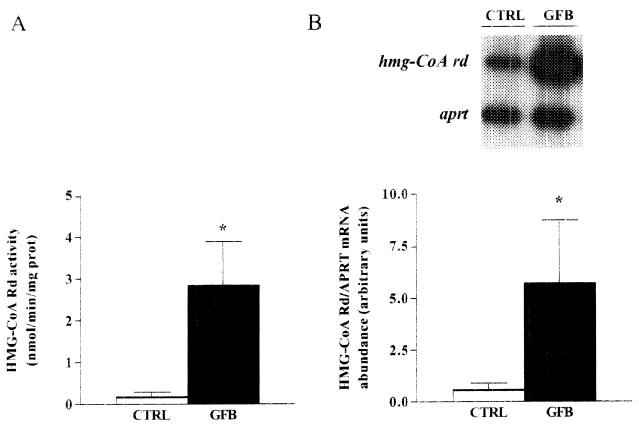

Fig. 1. (A) HMG-CoA Rd activity. Bars represent the means ± SD of the values obtained from 6 animals in each treatment group. (B) Relative levels of mRNA for HMG-CoA Rd. Bars represent the means ± SD of the values obtained from 6 animals in each treatment group. In the upper part of the figure is shown a representative autoradiography, with signals corresponding to HMG-CoA Rd and the reference gene, APRT, obtained from one control (CTRL) and one gemfibrozil (GFB)-treated rat. *$P < 0.05$.

Rat hepatic HMG-CoA Rd was strongly affected by gemfibrozil administration. Hepatic samples from treated-rats showed a 16.7-fold increase in HMG-CoA Rd activity and a 9.9-fold induction in its mRNA level, in comparison with values obtained in samples from control animals (Fig. 1). These effects were unrelated to any direct drug effect on the enzyme protein, given that HMG-CoA Rd activity was not changed upon addition of gemfibrozil to microsomal samples *in vitro* (Table 2).

Further, parallel changes in the hepatic levels of the mRNA specific for SREBP-2 (Fig. 2) and the nuclear content of the mature SREBP-2 protein (Fig. 3) were detected in gemfibrozil-treated rats, with a 2.9-fold and 2.2-fold increase, respectively, relative to control animals. EMSA gave a specific retardation band corresponding to the binding of SREBP-2 present in liver nuclear extracts to a SRE probe, as shown by competition assays and by the appearance of two super-shifted bands in the presence of an specific SREBP-2 antibody (Fig. 4B).

Table 2
Gemfibrozil effect *in vitro* on CT, PAP, and HMG-CoA Rd activities

	CTRL	GFB 0.25 mM	1 mM
CT (pmol/min/mg prot)	1810 ± 381	1873 ± 395	2149 ± 464
PAP (nmol/min/mg prot)	1.68 ± 0.29	1.67 ± 0.28	1.89 ± 0.24
HMG-CoA Rd (pmol/min/mg prot)	0.14 ± 0.05	0.13 ± 0.04	0.12 ± 0.06

CTRL: Control, GFB: Gemfibrozil, CT: CTP:phosphocholine cytidylyl transferase, PAP: phosphatidate phosphohydrolase, HMG-CoA Rd: 3-hydroxy-3-methylglutaryl-CoA reductase. Results are presented as means ± SD (N = 3).

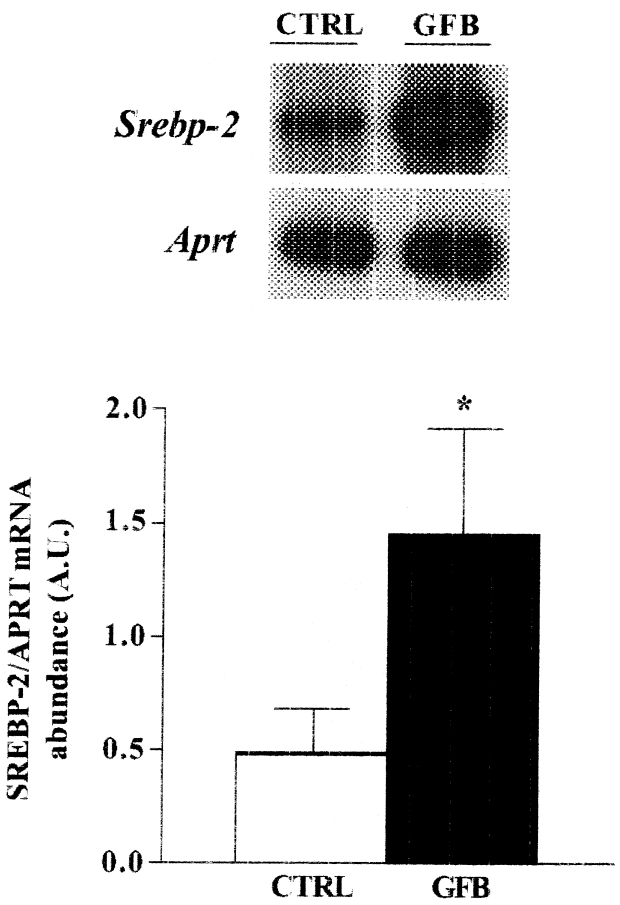

Fig. 2. Relative levels of mRNA for SREBP-2. Bars represent the means ± SD of the values obtained from 6 animals in each treatment group. In the upper part of the figure is shown a representative autoradiography, with signals corresponding to SREBP-2 and the reference gene, APRT, obtained from one control (CTRL) and one gemfibrozil (GFB)-treated rat. *$P < 0.05$.

Nevertheless, hepatic nuclear extracts from gemfibrozil-treated animals did not show any significant increase in binding to the SRE probe when compared to control animals (Fig. 4A).

Gemfibrozil induced PAP activity (1.7-fold) in hepatic samples from treated rats (Table 3), without showing any direct effect on this enzyme activity when added *in vitro* (Table 2). As cytosolic PAP has not yet been cloned or purified, it was impossible to determine the levels of its specific mRNA in liver. Further, gemfibrozil treatment did not modify CT activity, either *in vitro* (Table 2) or *in vivo*, or its mRNA level (Table 3).

As expected, gemfibrozil administration produced an activation of hepatic PPARα, as shown by a mild peroxisome proliferation response in the liver of treated rats, with increases in the activity (64%) of the hepatic peroxisomal fatty acid β-oxidation system, and in the levels of the specific mRNA for ACO (117%), the rate-limiting enzyme of this system (Fig. 5).

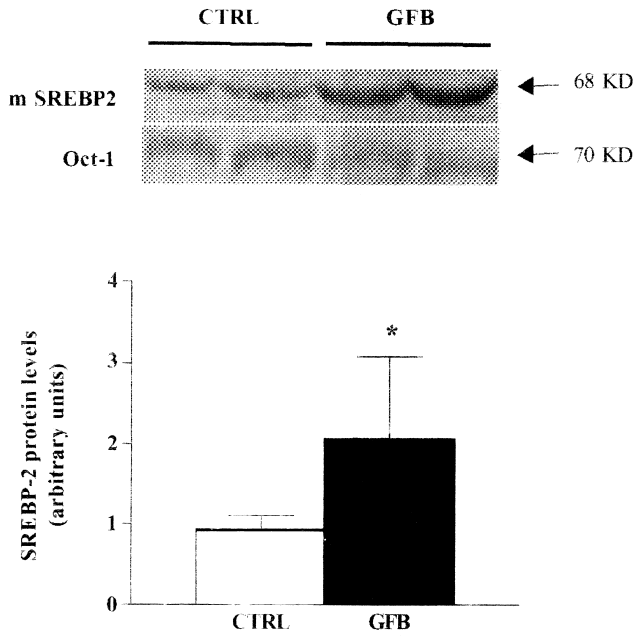

Fig. 3. Levels of the mature SREBP-2 68.000 D protein in hepatic nuclear extracts. Bars represent the means ± SD of the values obtained from 4 animals in each treatment group. In the upper part of the figure a Western blot of mature SREBP-2 protein (m SREBP-2) in nuclear extracts of two controls (CTRL) and two gemfibrozil-treated rats (GFB) is shown. Sixty micrograms of crude nuclear protein extract was loaded in each lane. OCT 1 levels are shown as a control.

4. Discussion

Gemfibrozil treatment was able to reduce plasma total cholesterol, mainly due to its effect on plasma apo B-cholesterol. This reduction was manifest despite the strong induction in HMG-CoA reductase activity detected in the liver of gemfibrozil-treated rats. The increase in reductase activity after gemfibrozil administration to rats is a well-known phenomenon [29–31]. We can discard an overexpression of HMG-CoA Rd in response to a direct inhibition of the enzyme activity by gemfibrozil, in a fashion similar to the effect elicited by statin administration to rats [32]. Although Hashimoto *et al*. [33] reported a direct inhibition of HMG-CoA Rd by gemfibrozil *in vitro*, neither data from Krause *et al*. [30] nor the data here presented support this possibility. The increase in HMG-CoA Rd activity may be part of a homeostatic response to compensate for the biliary sterol losses produced by gemfibrozil administration. Supporting this hypothesis is the fact that we have detected for the first time increased levels of SREBP-2 mRNA and mature protein in the livers of gemfibrozil-treated rats. By means of a complex proteolytic process, hepatic cholesterol deprivation results in the manifestation of SREBP transcriptional activity and up-regulation of target genes, including the *hmg-CoA rd* gene and the *srebp-2* gene itself [18, 19]. Our results do not allow us to discriminate if these changes produced by gemfibrozil are due to an increased transcrip-

Table 3
Gemfibrozil effect *in vivo* on CT and PAP

	CTRL	GFB
CT (mRNA)	1.19 ± 0.20	1.20 ± 0.26
CT (Activity)	920 ± 340	815 ± 308
PAP (Activity)	0.84 ± 0.28	1.43 ± 0.29*

CTRL: Control, GFB: Gemfibrozil, CT: CTP:phosphocholine cytidylyl transferase, PAP: phosphatidate phosphohydrolase. Results are presented as means ± SD (N = 6), and expressed as arbitrary units (CT mRNA), pmol/min/mg postnuclear supernatant protein (CT activity), or nmol/min/mg postnuclear supernatant protein (PAP activity).

* $P < 0.005$.

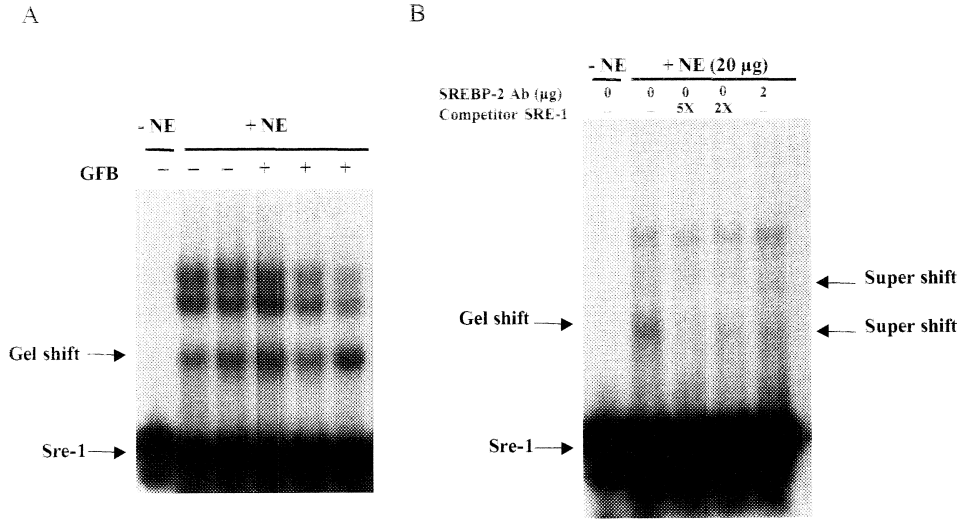

Fig. 4. Effect of gemfibrozil on the interaction of an SRE-1 probe with hepatic nuclear extracts (NE) obtained from Sprague–Dawley rats. (A) Autoradiography of a representative EMSA performed with 20 µg of liver NE obtained from control (−) and treated rats (+) after 3 days of gemfibrozil administration 0.3% (w/w), incubated with an SRE1 probe. (B) Autoradiography showing the specific competition produced by the addition of an excess-fold of the unlabeled probe (left arrow) and two supershift bands (right arrows) obtained in the presence of a polyclonal antibody against SREBP2.

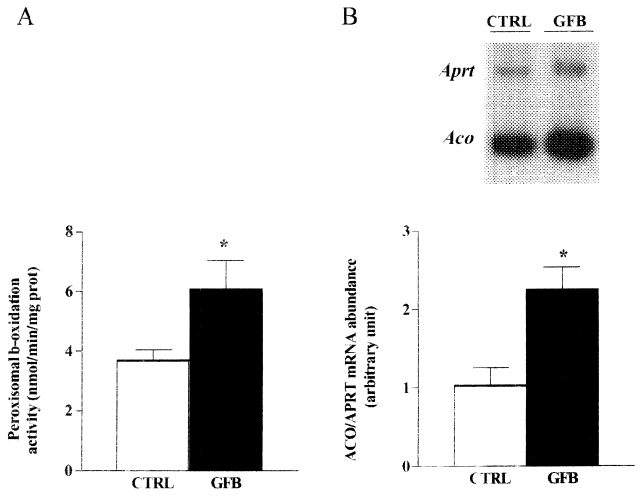

Fig. 5. (A) Peroxisomal β-oxidation activity. Bars represent the means ± SD of the values obtained from 6 animals in each treatment group. (B) Relative levels of mRNA for ACO. Bars represent the means ± SD of the values obtained from 6 animals in each treatment group. A representative autoradiography is shown in the upper part of the figure, with signals corresponding to ACO and the reference gene APRT, obtained from one control (CTRL) and one gemfibrozil (GFB)-treated rat. * $P < 0.05$.

tional activity of SREBP-2 or to its mRNA stabilization. Although gemfibrozil treatment shows a tendency to increase the binding of hepatic nuclear proteins to a specific SRE probe (see Fig. 4A), the difference did not reach statistical significance. The earliest event derived from hepatic cholesterol deprivation is the proteolytic maturation of the membrane-bound SREBP-2 already present in the liver, its migration to the nuclei, and the transcriptional activation of target genes, including the *srebp-2* gene itself. Thus, the absence of a significant increase in the DNA binding of hepatic nuclear extracts from gemfibrozil-treated rats can probably be attributed to the fact that the hepatic samples were obtained after the maximal surge of SREBP-2 activation.

Thus, as a consequence of the biliary loss of cholesterol, the hepatic cholesterol synthesis and probably uptake is increased, this latter effect contributing to the observed reduction in plasma apoB-cholesterol and triglyceride concentrations. The result of all these effects is the maintenance of the hepatic cholesterol concentration constant.

Similar to the cholesterol situation, it can be argued that, in order to counteract the drainage of hepatic phospholipids and keep its hepatic concentration constant, PAP activity is increased in gemfibrozil-treated rats. In this situation, the diacylglycerol produced by PAP activity would be diverted to phospholipid synthesis without being used to produce triglycerides. Given that statins are also able to increase PAP in rats [34], we can assume that this effect of gemfibrozil is also mediated by SREBP activation. CT expression and activity was unchanged in gemfibrozil-treated rats. Thus, we can assume that the pre-existing CT was able to maintain the concentration of liver phospholipids, but the reduction in their availability would impair the hepatic assembly of VLDL, contributing to the reduction in plasma triglyceride and phospholipid concentrations. In this sense, it is interesting to point out that we have found an almost 50% direct correlation between plasma values of phospholipids and triglycerides ($N = 12$, $r^2 = 0.484$, $P = 0.012$). Thus, two seemingly contradictory effects of gemfibrozil, i.e. the induction of PAP activity and the reduction of plasma triglyceride concentrations, could in this way be reconciled.

PAP activity does not appear to be related to peroxisome proliferation [35–37]. Similarly, there are no reports of a peroxisome proliferator response element (PPRE) in the promoter region of the *hmg-CoA rd* gene. However, both hypolipidemic and proliferative effects of fibrates are elicited by activation of the PPARα isoform present in rodent liver [5]. In fact, fibrate administration to PPARα null mice is unable to produce those effects [38, 39]. In consequence, it can be assumed that, in some unknown way, PPARα activation by gemfibrozil may be responsible for the changes in bilis composition and output, finally resulting in the activation of SREBP-2 and in the induction of PAP and of HMG-CoA Rd activities.

In summary, the present results support the idea that, at least in rats, gemfibrozil administration not only increases the catabolism of apo B-lipoproteins, but also reduces the lipid availability for VLDL assembly. This latter effect is mainly due to the well-known increase in biliary cholesterol and phospholipid losses produced by this drug. As a part of a compensatory homeostatic response to keep hepatic lipid concentrations constant, we report for the first time that gemfibrozil administration to rats increased the hepatic SREBP-2 expression and, probably, transcriptional activity. This effect is the most plausible explanation for the observed increases in HMG-CoA reductase and PAP activities detected after gemfibrozil administration.

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