Volume 327, number 2, 219–223 FEBS 12760 July 1993
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Transcriptional induction of the fatty acid binding protein gene in mouse liver by bezafibrate

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Received 30 April 1993; revised version received 10 June 1993

The mechanism by which hypolipidemic peroxisome proliferators of the fibrate family induce the liver fatty acid binding protein in liver of rodents is unknown. In order to delineate the level at which this protein is induced, the transcriptional activity of the specific gene encoding for liver fatty acid binding protein was measured in isolated hepatocyte nuclei obtained from male Swiss mice daily force-fed during 7 days with 400 mg/kg body weight bezafibrate. This treatment induced a 4-fold increase in the liver fatty acid binding protein transcription rate. Liver fatty acid binding protein mRNA level, measured by Northern blot analysis and cytosolic content of this protein, analyzed by immunoblotting, increased concurrently. From these results we conclude that the increase in the cytosolic liver fatty acid binding protein level by bezafibrate is due to an enhancement of the transcription rate of the liver fatty acid binding protein gene. Whether the transcriptional effect is mediated by peroxisome proliferator-receptor remains to be elucidated.

Liver fatty acid binding protein; Peroxisome proliferator hypolipidemic drug; Peroxisome proliferator activated receptor; Gene regulation

1. INTRODUCTION

Liver fatty acid binding protein (L-FABPc) is a cytosolic 14 kDa protein abundantly expressed in liver which binds with a high affinity especially to long chain free fatty acids. Several drugs that affect lipid metabolism cause modifications in the liver L-FABPc concentration (for review see [1]). In liver, it is well-established that peroxisome-proliferating hypolipidemic drugs from the fibrate family induce a rise in L-FABPc cytosolic concentration and in the abundance of its mRNA in vivo [2] and in vitro [3]. However, the level of this up-regulation is always unknown. In order to understand the origin of the high L-FABPc mRNA level found in animals treated with these xenobiotics, the measurement of relative transcription rate of L-FABPc gene was performed by run-on assay in mice chronically treated with bezafibrate. In this report, we provide the first direct evidence that the increase in L-FABPc mRNA in liver results from stimulation of L-FABPc gene transcription.

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Abbreviations: L-FABPc, liver fatty acid binding protein; I-FABPc, intestinal fatty acid binding protein; PPAR, peroxisome proliferator activated receptor; PPRE, peroxisome proliferator responsive element.

2. MATERIALS AND METHODS

2.1. *Animals*

Official French regulations (No. 87848 and No. 04727) for the care and use of laboratory animals were followed. Male Swiss mice weighing 35–38 g were purchased from CED (Centre d'Elevage Dépré) The animals were fed ad libitum a commercial diet (UAR A04-Usine d'Alimentation Rationnelle). They were daily force-fed in the morning with 400 mg/kg body weight (BW) bezafibrate (Sigma) for days; controls received in the same way the vehicle alone (200 μl of sunflower oil). Animals were sacrified in the morning after an overnight fast by aortic puncture under light ether anaesthesia. Immediately after sacrifice, one part of the liver was immediately frozen in liquid nitrogen and stored at −80°C until required for the RNA extraction. The other part was freshly used for supernatant and nuclei preparations Plasma were stored at −20°C until required for lipidic parameters determinations.

2.2. *Molecular probes*

The I- and L-FABPc cDNA probes were respectively extracted as previously described [4] from recombinant plasmids pJG19 [5] and pJG418 [6] generously provided by Dr J.I. Gordon (Washington University, St Louis, MO 63110, USA). These probes cross-react with mouse FABPc mRNA [7] Mouse β-actin is a fragment from pOB26 [8] plasmid encompassing all the sequence of β-actin mRNA (gift of S. Alonso). A 24-mer oligonucleotide specific for rat 18 S rRNA [9], but cross-reacting in the mouse, was used to ensure that equivalent amounts of RNAs were loaded and transferred.

2.3. *Northern blot analysis*

Total RNA was extracted from liver using phenol/chloroform/LiCl method [10]. Hybridization and washing conditions are described in detail elsewhere [4,9]. Autoradiograms were quantified with an automatic densitometric scanner (CS-9000, Shimadzu, Scientific Instruments).

2.4. *Nuclear run-on transcription assay*

Nuclei were freshly prepared as described by Widnell and Tata [11]. The pelleted nuclei were resuspended at 10^8/ml in ice-cold storage

buffer (20 mM Tris pH 8; 75 mM potassium acetate; 0.5 mM EDTA, pH 8; 2 mM DTT; 50% glycerol), frozen in liquid nitrogen in aliquot of 200 µl then stored at −80°C until use. In vitro elongation of nascent RNA chains was carried out using 2×10^7 nuclei according to Mezger et al. [12] with some modifications. The nuclei were incubated at 30°C during 20 min in transcription mix (20 mM Tris, pH 8; 20% glycerol; 160 mM potassium acetate; 5 mM $MgCl_2$; 2 mM DTT; plus for each reaction, 125 nmol NTP (AGC), 50 units of human placental ribonuclease inhibitor (Amersham), 2 µg creatine phosphokinase; 4 µmol phosphocreatine; 0 25 µmol $MnCl_2$ and 10 µg *E. coli* tRNA) in presence of [α-^{32}P]UTP (800 Ci/mM Amersham – 150 µCi/reaction). The reaction was stopped by addition of 40 units of DNase I (Boehringer) and 100 µg *E coli* tRNA (Boehringer) and incubated for an additional 15 min at 37°C. The mixtures were treated with 200 µg of proteinase K for 30 min at 37°C. Labeled RNA were purified as described by Linial et al [13] then denatured by alcaline hydrolysis. After their amplification by polymerase chain reaction (PCR), 1 µg of different cDNA fragments (respectively L-FABPc, I-FABPc and β-actin cDNA) was denatured and immobilized on Gene Screen membrane using an Hoeffer slot blot appartus. The filters were hybridized in presence of about 10^7 cpm of labeled RNA at 47.5°C for 3 days in rotary hybridization incubator. For each experiment, to measure accurately the volume of mixture corresponding to 10^7 cpm, differential precipitation of labeled mRNA by the trichloroacetic acid method was achieved on aliquot (5 µl) according to Sambrook et al. [14]. The filters were subjected to autoradiography after extensive high stringency washes and RNase treatment [15]. The radioactivity content of the signals was assayed in a Packard tricarb scintillation counter (Packard Prias PLD, Tricarb) L-FABPc, I-FABPc and β-actin cDNA fragments were the same as those used as labeled probes in the Northern blot analysis.

2.5. *Electrophoresis and immunoblotting procedure*

The high speed supernatants were stored at −20°C until the L-FABPc Enhanced Chemioluminescence (ECL – Amersham) Western blot analysis. The rabbit polyclonal antibody to rat L-FABPc was generously provided by Dr. J.I. Gordon as well as purified rat L-FABPc used as an internal control. After determination of the cytosolic protein concentration by BCA (Pierce), 2 µg of denatured cytosolic proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gels [14] as well as 1 µg pure rat L-FABPc in presence of rainbow coloured protein molecular weight markers (Amersham). The separated proteins were electrophoretically transferred to Hybond-ECL nitrocellulose membrane (Amersham) according to manufacturer's protocol (Biorad). The membrane was blocked with 2% bovine serum albumine then incubated for 1 h at 25°C with the anti-L-FABPc antibody at a 1:1000 dilution. Antigene–antibody complexes were detected by incubating the membrane during 1 h at 25°C with anti-rabbit IgG-horseradish peroxidase conjugate (1:1000, Amersham) and finally with Amersham's ECL reagent for 1 min

2.6. *Plasma lipids*

The concentrations of total and free cholesterol in plasma of controls and bezafibrate treated mice were performed using an automatic Cobas-bio (Roche).

2.7. *Statistical methods*

Whenever possible, the results were expressed as mean ± S.E.M. Significance of differences between groups was determined using Student's *t*-test.

3. RESULTS

3.1. *Effect of bezafibrate on body and liver weights and plasma lipids*

As shown in Table I, no modification in body weights nor in food intakes occurred in mice chronically treated with 400 mg/kg bezafibrate contrary to rats in which this protocol triggered a drastic weight loss (data not shown). For this reason, we have chosen to perform the experiment in mice rather than in rats. The efficiency of the treatment was attested by the 1.7-fold increase in liver weights and the significant decrease in plasma total and free cholesterol (Table I). Indeed, these modifications are classically found in rats subjected to fibrate treatment [16].

3.2. *Enhancement of L-FABPc expression in mouse liver under the effect of bezafibrate*

According to the results obtained in rat hepatocytes cultured in presence of bezafibrate [3], the L-FABPc mRNA abundance was clearly higher in drug treated mice (Fig. 1_1). After stripping the L-FABPc probe and reprobing with 18 S rRNA oligonucleotide, similar hybridization signals were found in the control and experimental groups, proving that the increased L-FABPc mRNA level was not due to differences in the amounts of total RNA sampled (Fig. 1_1). After densitometric scanning of autoradiograms, the L-FABPc mRNA level was expressed as ratio of hybridized signals between L-FABPc probe and 18 S rRNA control probe. In these conditions, a 3.5-fold increase in the L-FABPc mRNA level occurred in the liver of the bezafibrate treated mice as compared to controls. By contrast, no modification in β-actin mRNA level occurred after bezafibrate treatment attesting to the specificity of the L-FABPc mRNA induction (data not shown). Concurrently, the spectrodensitometric analysis of the Western blot revealed that bezafibrate caused on average a 1.7-fold rise in the cytosolic L-FABPc amounts (Fig. 1_2).

3.3. *Influence of bezafibrate on L-FABPc gene transcription rate*

To determine whether the rise in L-FABPc mRNA amounts was the consequence of an increase in the L-FABPc synthesis rate or of post-trancriptional events, nuclear run-on assays were performed on liver nuclei from control and bezafibrate treated mice. In these assays only transcripts which are already initiated are faithfully elongated giving an accurate measure of the level of transcription at the time of the nuclear isolation [13]. Two different experiments were performed in controls and 3 distinct assays in bezafibrate treated using systematically 2 animals per assay in order to verify the reproductibility of the results. Since the β-actin mRNA levels remained unchanged in mouse liver after bezafibrate treatment, β-actin cDNA was used as control. To test the specificity of hybridizations, I-FABPc cDNA was spotted in the filters because the I-FABPc is only expressed in the intestine. This negative probe allowed us to evaluate the non specific radioactivity hybridized on the membrane (= background: BG). As shown in Fig. 2, the relative transcription rate of the L-FABPc gene was substantially higher in the bezafibrate treated

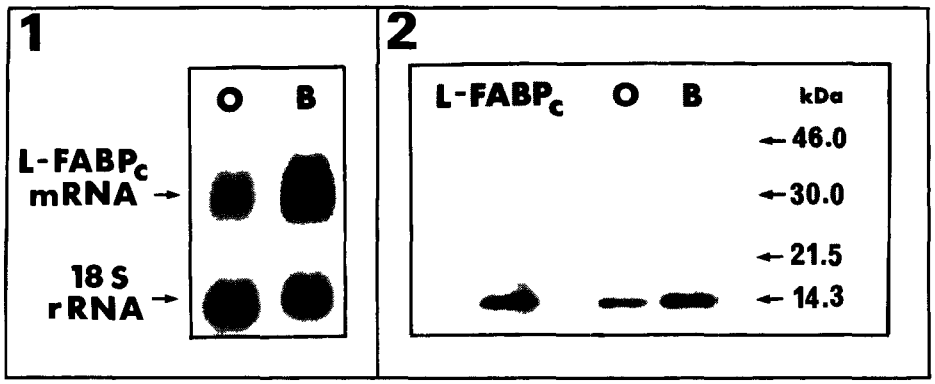

Fig. 1. Typical increase in specific L-FABPc mRNA and protein levels triggered by bezafibrate in the mouse liver. Animals were daily force-fed during 7 days with 400 mg/kg BW bezafibrate (B) in 0.2 ml of sunflower oil. Controls (O) received the vehicle alone. (1) Northern blot analysis of 20 μg of total RNA extracted from livers pooled from 4 animals. To test the amounts of total RNA really sampled, the membrane were rehybridization with 18 S rRNA oligonucleotide probe. (2) Western blot analysis of 1 μg of pure L-FABPc and 2 μg of liver cytosolic proteins from controls (O) or bezafibrate treated mice (B). Proteins were subjected to SDS-PAGE electrophoresis, transferred to nitrocellulose membrane and immunostained with anti-rat L-FABPc as described in section 2 by ECL detection. Each sample consisted of liver high-speed supernatants pooled from 4 mice. The values indicate the molecular weight relative to Rainbow coloured protein molecular weight markers.

mice than in the controls which correlated with the abundance of its message detected by Northern blot. A about 4-fold increase was found on average (Table II). The lack of any clear modification in the transcription rate of the β-actin gene attested to the validity and the specificity of the transcriptional induction of the L-FABPc gene by bezafibrate (Fig. 2).

4. DISCUSSION

We have performed a daily force-feeding with bezafibrate during 7 days in mice because: (1) this way of administration is the best to be sure that animals receive the same amount of drug; (2) bezafibrate is very efficiently absorbed [17]; (3) the maximum induced levels of cytosolic L-FABPc are reached after around 1 week of fibrate treatment in liver [18]; and (4) no weight loss and no decrease in daily food intake is observed in mice (Table I) contrary to rats subjected to the same protocol (data not shown).

Our results show that bezafibrate induces a rise in L-FABPc mRNA levels with a subsequent increase in cytosol L-FABPc content and demonstrate, to our knowledge for the first time, that this up-regulation is triggered by an enhancement of the transcription rate of the L-FABPc gene. This effect is probably due to a direct influence of the drug on liver since the bezafibrate induces a rise in L-FABPc mRNA abundance in hepatocytes in culture [3]. Several genes involved in hepatic lipid metabolism seem to be sensitive to peroxisome-proliferator hypolipidemic drugs. For instance, the transcription rate of peroxisomal fatty acyl CoA oxidase [19] and peroxisomal bifunctional enzyme [19–20] are rapidly increased after fibrate administration. By contrast, fenofibrate decreases the transcriptional activity of Apo A-I [16]. These coordinate regulations suggest the existence of a commun control mechanism.

Table I

Influence of bezafibrate treatment on body and liver weights and plasma total and free cholesterol (respectively TCS and FCS) concentrations in mice

Experiment	Body weight (g)		Liver weight (g)	Mean of the daily food intake (g)	Plasma lipidic parameters	
	Day 0	Day 7	Day 7		CS (g/l)	FCS (g/l)
O	37.2 ± 0.4 (8)	36 6 ± 0.7 (8)	1.74 ± 0.09 (8)	5.5 ± 0.3 (48)	0.81 ± 0.05 (8)	0.33 ± 0.02 (8)
B	36.6 ± 0.8 (8) ns	35.6 ± 1.5 (8) ns	2.95 ± 0.21 (8) ***	5.3 ± 1.9 (48) ns	0.58 ± 0.03 (6) **	0.25 ± 0.02 (6) *

Adult male Swiss mice were daily force-fed with 400 mg/kg body weight bezafibrate (B) during 7 days or with the vehicle alone (0 2 ml of sunflower oil: O). Liver weights were recorded at the end of the treatment period (day 7). Plasma TCS and FCS were measured as described in section 2. Values are expressed as mean ± S.E.M.; ns = non-significant results; *$P < 0.05$; **$P < 0.01$; ***$P < 0\,001$.

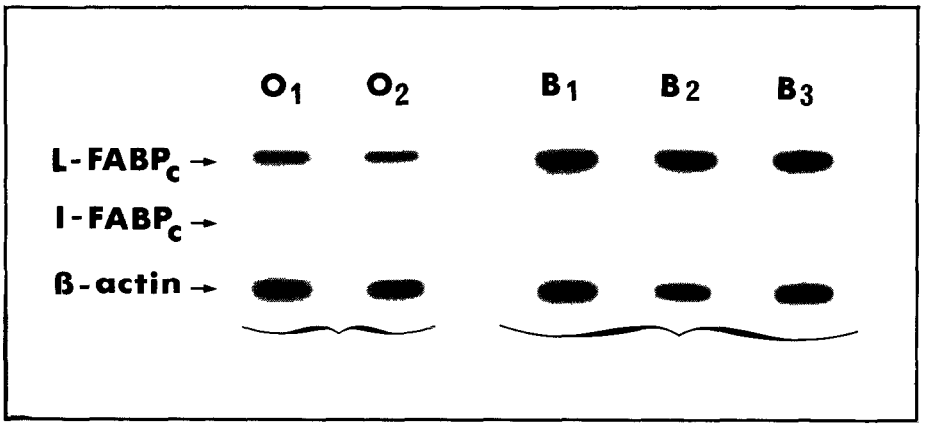

Fig 2. Effect of bezafibrate on the L-FABPc gene transcription in mouse liver. Nuclear run-on assays to evaluate transcription gene activities in livers of mice daily force-fed during 7 days with 0.2 ml of sunflower oil (controls: O_1 and O_2) or with 0.2 ml of 400 mg/kg BW bezafibrate in oil (B_1 to B_3). Liver nuclei from 2 mice were pooled for each individual assay. 1 μg of denatured cDNA fragments were bound to nylon membrane filters, hybridized to [α-^{32}P]UTP-labeled in vitro elongated transcripts and analyzed by autoradiography. Mouse β-actin was used as internal control probe and rat I-FABPc as negative probe (= background).

Recently, a transacting factor, termed peroxisome proliferator-actived receptor (PPAR), was identified in some target tissues of peroxisome proliferators including liver [21]. The activated PPAR binds a specific DNA sequence, termed peroxisome proliferator responsive element (PPRE), found in upstream of the rat acyl CoA oxidase gene [22]. It is interesting to observe that a PPRE-like sequence is contained in the promotor of the rat L-FABPc gene [23]. That might explain the reason why bezafibrate increases the transcription rate of L-FABPc gene in our experiment. However, Issemann et al., using reporter gene constructs and transfection assays in Hepa 1 cells, have recently shown that this putative responsive element is unfunctional in the context of the natural rat L-FABPc promotor but becomes active when it is placed upstream the rabbit β-globin promotor [23]. These authors suggest that the failure of the L-FABPc gene induction might be due to the high proximity of the PPRE-like sequence with the TATA box in the rat L-FABPc gene. The apparent discrepancy with the results reported here can be explain through several hypothesis: (i) the location of the PPRE sequence in the mouse L-FABPc gene is different from rat or several copies of this sequence are located at different sites largely upstream the TATA box; (ii) bezafibrate can induce the L-FABPc gene using an other way than the PPAR/PPRE complex; or (iii) a co-factor, which lacks in Hepa 1 cells but which is naturally present in mouse liver is required for PPAR activity.

Further experiments will be necessary to explore these hypothesis and understand the respective roles of peroxisome proliferators, PPAR and FABPc in the hypolipidemia triggered by fibrates.

Acknowledgements We thank S. Cereghini for advice on run-on transcription assays, T. Kanda for advice on Western blot analysis, L. Persegol from Laboratoire Central de Biochimie Médicale de Dijon (Prof. P. Gambert) for the lipids assays in plasma and M.C. Monnot for the photographic printings This work was supported by grants

Table II

Effect of bezafibrate treatment on the transcription rate of the L-FABPc gene in mouse liver

Experiment	A Input mRNA ($\times 10^6$ cpm)	B RNA hybridized L-FABPc−BG = (cpm)	B/A (% $\times 10^{-4}$) % of relative transcription of L-FABPc gene	Mean transcription rate (arbitrary units)
O_1	9.6	234 − 168 = 66	6 8	100
O_2	13.3	212 − 144 = 71	5.4	
B_1	11 4	366 − 112 = 254	22.3	430
B_2	11.4	407 − 119 = 288	25.3	
B_3	7.0	303 − 86 = 217	31.0	

Relative L-FABPc gene transcription rate in liver from mice daily force-fed during 7 days with 400 mg/kg BW bezafibrate in sunflower oil (B_1 to B_3) or with vehicle alone (0.2 ml of sunflower oil: O_1 and O_2). Each individual experiment was performed with liver nuclei pooled from 2 mice. I-FABPc cDNA bound radioactivity (= background: BG) was substracted to cpm of specific mRNA hybridized.

from the Fondation pour la Recherche Médicale, the Conseil Régional de Bourgogne, the Direction de la Recherche et des Etudes Doctorales. A.M. is a recipient of the Ministère de la Recherche et de la Technologie.

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