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TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL ANALYSIS OF PEROXISOMAL PROTEIN ENCODING GENES FROM RAT TREATED WITH AN HYPOLIPEMIC AGENT, CIPROFIBRATE

EFFECT OF AN INTERMITTENT TREATMENT AND INFLUENCE OF OBESITY

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Abstract—The treatment of rats with ciprofibrate, a potent peroxisome proliferator, led to increased levels of the peroxisomal acyl-CoA oxidase (ACO) mRNA. How ciprofibrate functions to elevate ACO mRNA is not known. To help determine the mechanism of ciprofibrate action, *in vitro* transcription assays were performed. It was determined that ciprofibrate was responsible for a 3.5-fold stimulation of the rate of ACO transcription within 24 hr of ingestion. It was also observed that the transcription rate stimulation following a 2-week ciprofibrate treatment of Wistar rats was maintained following 4 weeks of ciprofibrate withdrawal. Re-introduction of the drug after the 4-week pause resulted in greater stimulation than was initially observed. The results demonstrate that the effect of ciprofibrate is rapid and persists at least twice as long as the initial treatment period. In Zucker rats, both lean and obese, ACO mRNA levels were examined following 2 weeks of ciprofibrate treatment (1 or 3 mg/kg body weight/day). The presence of increased blood levels of triglycerides did not increase ciprofibrate action on transcription, although basal levels of transcription of peroxisomal enzymes were higher in obese rats. The increase in the ACO mRNA level was greater than the transcription rate stimulation suggesting a post-transcriptional regulation.

Key words: acyl-CoA oxidase; ciprofibrate; obese Zucker rats; peroxisomal β-oxidation; run on transcription assay

Liver peroxisomes contain a fatty acid β-oxidation system [1] composed of three proteins: ACO†, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/Δ3-Δ2 enoyl-CoA isomerase trifunctional enzyme (HD) and KT. ACO is the rate-limiting enzyme catalysing the first step of peroxisomal β-oxidation. Substrates of peroxisomal β-oxidation are, among other things, very long chain fatty acids [2, 3], and the side chain of cholesterol whose first step involves trihydroxycopraostanoyl-CoA oxidase [4].

Over the last decade, many studies have focused on the proliferation of peroxisomes in rodent liver [5], an intriguing biological phenomenon which is triggered by various chemicals including hypolipemic drugs of the fibrate family, employed in humans to reduce the risk of coronary disease. Administration of the drug ciprofibrate to rodents has been shown to markedly induce the number and size of peroxisomes and the peroxisomal β-oxidation system [6]. We and others [7–9] have reported increased levels of the three β-oxidation enzymes mRNA under ciprofibrate treatment. Furuta *et al.* [7] have shown that the increase in β-oxidation enzyme mRNA was accompanied by an enhanced turnover of enzyme, suggesting a post-translational regulation of expression.

It has recently been proposed that peroxisome proliferators such as ciprofibrate induce the transcription of peroxisomal enzymes by activating a nuclear receptor, the PPAR [10]. The PPAR belongs to the steroid hormone receptor superfamily and binds as an heterodimer with RXR to a PPRE present in the 5′ flanking region of the ACO gene [11]. PPREs are also present in the 5′ flanking sequence of the HD gene [12, 13] and the KT gene [11]. Although no ligand for PPAR has yet been identified, it has been shown that it is activated by xenobiotics (e.g. the hypolipidemic drugs), by retinoids and steroid hormones [14–16]. PPAR is also regulated by fatty acids, particularly polyunsaturated fatty acids [17]. Interestingly, fatty acids have also been shown to regulate the expression of several genes involved in lipid metabolism, such as those encoding low density lipoprotein receptors [18], fatty acid synthase and S14 protein [19].

An alternative hypothesis for the regulation of peroxisomal protein gene expression focuses not on

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† Abbreviations: ACO, acyl-CoA oxidase; CAT, chloramphenicol acetyl transferase; HD, hydratase/dehydrogenase/isomerase trifunctional enzyme, KT, keto-acyl-CoA thiolase; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; RXR, 9-*cis* retinoic acid receptor; SD, Sprague–Dawley rat; Zl, lean Zucker rat; Zo, obese Zucker rat.

nuclear receptors, but on the involvement of lipids. According to Elcombe and Mitchell [20], some peroxisome proliferators (such as metabolite VI derived from DEHP) provoke inhibition of mitochondrial β-oxidation, leading to lipid accumulation in the liver. In order to maintain lipid homeostasis, microsomal ω-oxidation and peroxisomal β-oxidation are co-induced. These two hypotheses (receptor-mediated action response and lipid perturbation response) are not necessarily exclusive.

The purpose of the present study was to characterize further the cellular mechanisms underlying the induction of peroxisomal β-oxidation by ciprofibrate. Enzymatic activity, mRNA level and rate of transcription of ACO were investigated. Previous studies using transient co-transfection of cells with PPAR expression vector and reporter constructs containing ACO-PPRE sequence driving the CAT reporter gene demonstrated that ciprofibrate activates PPAR and that the transcription of ACO was activated by the binding of PPAR to its PPRE. We therefore chose to extend this *in vitro* analysis of the mechanism of action of ciprofibrate by utilizing the nuclear run-on technique with hepatic nuclei isolated from rats treated *in vivo*. The steady-state level of a given mRNA represents the balance of its degradation and transcription rate, the latter parameter being studied by run-on assay. *In vitro* transcription assays in isolated nuclei take into account the *in vivo* pharmacokinetics of the hypolipidemic drug and the physiological state of the animal. Despite these advantages, only a few authors have used "run on" assays in isolated nuclei to estimate the transcription rate of peroxisomal protein genes. Chatterjee *et al.* [21] demonstrated that Wy14,643, a potent peroxisome proliferator, provokes an 11-fold stimulation of the transcription rate of the peroxisomal HD trifunctional enzyme in cultured rat hepatocytes after 20 hr. Hertz *et al.* [22] showed that rats treated with nafenopine (14 days, at 7.5 mg/day/100 g body weight) exhibited a 6-fold stimulation of ACO transcription in thyroidectomized rat liver and a 10-fold stimulation in euthyroid rat liver. In our study, the effect of ciprofibrate on the rate of ACO transcription was examined at an earlier time point than has previously been reported (i.e. following intermittent treatment) and in the presence of high triglyceride blood levels by comparing lean and obese rats.

MATERIALS AND METHODS

Animals and treatments

SD and Wistar male rats weighing 160–180 g were purchased from Charles River (St. Aubin lés Elbeuf, France). Zl male rats (heterozygote Fa/fa, 350 g) and Zo male rats (homozygote fa/fa, 500 g) were purchased from CNRS, (Orléans, France). In the first and third experiments, ciprofibrate was administered by gastric intubation of the drug solution in low viscosity carboxymethylcellulose (Sigma, Saint Quentin, France) either for 24, 48 or 72 hr (1st experiment: Wistar rats, 3 mg/kg body weight/day) or 2 weeks (3rd experiment: Zucker and SD rats, 1 or 3 mg/kg/day). The same amount of carboxymethylcellulose was delivered to rats of the control groups. In the second experiment, rats were fed with food in pellets (Aliments UAR, Villemoisson/Orge, France) containing ciprofibrate [20 mg/100 g of food (200 ppm)] corresponding to the daily ingested drug of 2 mg/kg body weight. Three groups of four animals were treated as follows: (A) a 2-week ciprofibrate treatment; (B) as for A, followed by a 4-week pause from ciprofibrate treatment; (C) as for B, followed by a 2-week treatment.

Chemicals

Ciprofibrate was a gift from Sterling Winthrop (Dijon, France), [α^{32}P]UTP (400 Ci/mmol) was from Amersham (Les Ulis, France), RNases A and T1 were purchased from Sigma (Saint Quentin, France), creatine phosphokinase, phosphocreatine, proteinase K and DNase were from Boehringer-Mannheim (Meylan, France).

Nuclear run-on transcription assay

Isolation of rat liver nuclei. Nuclei were freshly prepared as described by Simonet and Ness [23]. The pelleted nuclei were resuspended in an ice-cold storage buffer as a 50% suspension (20 mM Tris–HCl pH 7.9, 140 mM KCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 14 mM β-mercaptoethanol, 30% glycerol). Nuclei were frozen in liquid nitrogen and then stored at −70° until further use.

Labeling and isolation of nascent RNA transcripts. *In vitro* elongation of nascent RNA chains was carried out using 25×10^6 nuclei per assay. Transcription reactions contained 30% (v/v) of nuclei in a storage buffer supplemented with 0.4 mM each of ATP, CTP, GTP, 10 mM phosphocreatine, 100 μg/mL creatine phosphokinase and 25 μCi of [α^{32}P]UTP (400 Ci/mmol). Reaction volumes were incubated at 26° for 45 min. The reaction was terminated on ice and RNase-free DNase was added to 100 μg/mL. An equal amount of the buffer (0.6 M NaCl, 50 mM Tris–HCl pH 7.5, 20 mM $MgCl_2$) was added and the reaction mixture incubated at room temperature for 10 min. The solution was transferred into a tube containing 2 mL of proteinase buffer (0.15 M NaCl, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1% SDS). Proteinase K (200 μg/mL) was added and incubation was continued at 37° for 30 min. Sodium acetate (pH 5) was added to a final concentration of 0.1 M, along with 200 μg of yeast tRNA. The solution was extracted once with phenol at 55° and once with phenol/chloroform (v/v) at room temperature. The aqueous phase was precipitated with 2 vol. of ethanol. The pellet was dissolved in 400 μL of 0.1 M sodium acetate (pH 7.0) and precipitated three additional times to remove non-incorporated nucleotides. The final pellet was rinsed with 70% ethanol, dried, and dissolved in 30 μL of 0.1 × SET (1 × SET = 1% SDS, 1 mM EDTA, 10 mM Tris–HCl, pH 7.5). The RNA solution was heated in a boiling water bath for 5 min and quickly chilled on ice prior to hybridization.

The *in vitro* transcription assay was optimized for the best [α-^{32}P]UTP incorporation in mRNAs ($2–15 \times 10^4$ cpm incorporated in nascent mRNAs/μL of reaction volume). This *in vitro* elongation of mRNAs initiated *in vivo* is specifically accomplished

by RNA polymerase II, as indicated by an 82% inhibition of transcription assay in the presence of α-amanitin (0.5 μg/mL). Nascent mRNAs exhibit molecular weights ranging from 0.1 to 4 kb as estimated by agarose electrophoresis (data not shown).

Transcription rate analysis by hybridization to immobilized cDNA. ACO, catalase and actin transcripts were detected by hybridization to specific cDNA sequences previously bound to nylon filters, as follows. Linear pBR322 plasmid containing rat ACO or catalase cDNAs, and recombinant pUC18 plasmid containing mouse actin cDNA were prepared as described by Sambrook *et al.* [24], and an excess of 2 μg were bound to Nylon filter (Gene Screen Plus, NEN—Dupont de Nemours). Prior to hybridization with nuclear transcripts, the filters were treated for 16–24 hr at 42° in 50% formamide, 1 × Denhardt (Sigma), 1% SDS, 5 × SSC (1 × SSC: sodium saline citrate, NaCl 0.15 M, sodium citrate 15 mM, pH 7.0), 5% dextran sulfate, 50 μg/mL heparin. Hybridizations were performed for 3–4 days at 42° in the same buffer containing the nuclear transcripts. Following hybridization, the filters were washed twice for 15 min in 2 × SSC containing 0.1% SDS at 42°, twice for 15 min in 0.5 × SSC, 0.1% SDS and twice for 15 min in 0.1 × SSC, 0.1% SDS. The filters were then treated with an RNase buffer containing 0.3 M NaCl, 40 mM EDTA, 10 mM Tris–HCl pH 7.5, 10 μg/mL of RNase A and 1 μg/mL of RNase T1 at 37° for 30 min to hydrolyse the non-hybridized mRNA. Finally, the filters were washed for 1 hr in the same buffer without the RNases. The variations of ^{32}P-radioactive mRNAs bound to the various filters were estimated by laser densitometric scanning of the autoradiograms (CS 9000, Shimadzu).

Preparation of total RNA

Total RNA was prepared from livers previously frozen in liquid nitrogen, using the LiCl method described by Auffray and Rougeon [25], and stored at −70°. Northern blots were performed as described by Cherkaoui *et al.* [26]. Peroxisomal ACO cDNA and catalase cDNA probes prepared according to Miyazawa *et al.* [27] were kindly provided by Dr. T. Osumi. Mouse β-actin cDNA probe was a gift from S. Alonso [28].

Enzyme assays

Cyanide-insensitive acyl-CoA oxidation activity used as a peroxisomal marker was assayed with palmitoyl-CoA as substrate in liver homogenate according to the established procedure of Lazarow and de Duve [1] and in isolated peroxisomes (the measurements in peroxisomes gave the same kind of results and were not shown). The ACO was not measured directly in our experiment. Indeed the procedure measured the first three steps of peroxisomal β-oxidation (ACO, hydratase, 3-hydroxyacyl-CoA dehydrogenase). Since the oxidase is considered rate-limiting, we referred to "acyl-CoA oxidase" activity instead of "acyl-CoA oxidation". Catalase was measured in liver homogenate according to the established method of Baudhuin *et al.* [29]. Proteins were measured according to the procedure of Lowry *et al.* [30].

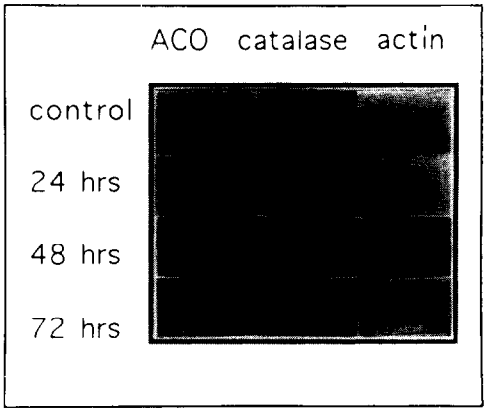

Fig. 1. Run-on analysis by slot blot hybridization of the transcription rate of genes encoding ACO, catalase and actin. Wistar rats were treated for 24, 48 or 72 hr by ciprofibrate. Data related to this figure are shown in Table 1. For experimental conditions, see Materials and Methods.

RESULTS

Analysis of the stimulation of transcription by a short ciprofibrate treatment

Peroxisomal ACO enzyme activity and mRNA levels increase in rat liver following a 2-week ciprofibrate treatment [8]. We examined whether ACO response was observed as soon as 24 hr after ciprofibrate administration (Fig. 1). We also examined the response of peroxisomal catalase activity under the same conditions. Previous studies showed only a 2-fold increase in catalase activity even after several months of treatment [31]. The ACO and catalase activities were measured 24, 48 and 72 hr after the beginning of treatment. The hepatic ACO specific activity (Table 1) showed a 3.5-fold stimulation after 24 hr of ciprofibrate treatment (3 mg/kg body weight/day) as compared to the control. Maximum elevation of ACO activity (9-fold) occurred after 72 hr. Catalase activity (Table 1) was stimulated to a lesser extent after 24 hr of ciprofibrate treatment (1.3-fold) and reached a 2-fold increase following 48 hr of treatment and was maintained at the same level after 72 hr.

Next, the transcription rate of ACO gene and catalase gene was measured using isolated rat liver nuclei. The transcription rate of the ACO gene (Table 1) was increased from 3 to 5 and then to 8 after 24, 48 and 72 hr of ciprofibrate treatment, respectively, in correlation with the elevation of enzyme activity. The rate of transcription of the catalase gene (Table 1) experienced a 3-fold stimulation after 72 hr.

Nevertheless, the transcription rate of the β-actin gene was also increased by the ciprofibrate treatment (1.4–3.1-fold induction). Indeed, the somatic index of the liver was remarkably increased as early as the first day of treatment. This important and rapid hepatic hypertrophy explains why the transcription rate of the β-actin gene experienced such stimulation. Normalized by β-actin values, the increase of the

Table 1. Specific activities of peroxisomal acyl-CoA and catalase and densitometric analysis of the transcription rate of the corresponding genes from control and short-term treated rats

		Control	24 hr	48 hr	72 hr
ACO	Specific activity (nmol/min/mg protein)	8 ± 1.6	29 ± 10 (×3.5)	58 ± 7 (×7)	79 ± 8 (×9.5)
	Transcription rate (arbitrary unit)	38 ± 4.2	124 ± 9.8 (×3.3)	192 ± 7.6 (×5)	304 ± 15.4 (×8)
Catalase	Specific activity (unit/mg protein)	24 ± 1.8	32 ± 7.3 (×1.3)	52 ± 0.3 (×2.2)	47 ± 4.2 (×2)
	Transcription rate (arbitrary unit)	16 ± 2.1	22 ± 4.8 (×1.4)	33 ± 4.3 (×2)	52 ± 3.8 (×3.3)
β-actin	Transcription rate (arbitrary unit)	14 ± 1.5	20 ± 2.1 (×1.4)	31 ± 2.7 (×2.2)	43 ± 7.5 (×3.1)
ACO/actin	Transcription rate ratio (no unit)	2.7	6.2 (×2.3)	6.2 (×2.3)	7.1 (×2.6)

Male Wistar rats were treated 24, 48 or 72 hr with 3 mg/kg body weight/day of ciprofibrate. For further experimental conditions, see Materials and Methods. Specific activities are expressed as nmol/min/mg of liver homogenate protein for acyl-CoA oxidase and as unit/mg of liver homogenate protein as described by Baudhuin *et al.* [29], for catalase. Transcription rate values correspond to arbitrary units obtained by laser densitometric scanning of the slot blots. Values are the means ± SD obtained from three different rats. Normalization of ACO values to β-actin was done by dividing ACO transcription rate by actin transcription rate for each period of treatment. In each lane, factors in brackets correspond to the fold of stimulation as compared to the average of control values.

Table 2. Specific activities of peroxisomal acyl-CoA oxidase and catalase and densitometric analysis of the transcription rate of the corresponding genes from male Wistar rats treated with ciprofibrate during three distinct periods (treatment/pause/treatment)

		Control	A	B	C
ACO	Specific activity (nmol/min/mg protein)	6 ± 1.1	284 ± 17 (×49)	114 ± 16.1 (×20)	238 ± 7 (×41)
	Transcription rate (arbitrary unit)	53 ± 4.8	247 ± 13.5 (×4.7)	167 ± 11 (×3.1)	535 ± 22 (×10)
Catalase	Specific activity (unit/mg protein)	43 ± 8.9	69 ± 2.2 (×1.6)	47 ± 10.1 (×1.1)	84 ± 0.5 (×1.9)
	Transcription rate (arbitrary unit)	48 ± 1.5	83 ± 6 (×1.7)	84 ± 8.1 (×1.7)	99 ± 6.4 (×1.9)
β-actin	Transcription rate (arbitrary unit)	30 ± 4.2	86 ± 7.1 (×2.8)	43 ± 2.1 (×1.4)	89 ± 1.8 (×3)
ACO/actin	Transcription rate ratio (no unit)	1.7	2.9 (×1.7)	3.9 (×2.3)	6 (×3.5)

The three distinct periods are called A, B, C: A correspond to rats treated daily for 2 weeks and then killed. B correspond to rats killed after a 4-week pause following the treatment. C correspond to rats killed after a 2-week treatment following the pause. For further experimental conditions, see Materials and Methods. Specific activities are expressed as nmol/min/mg of liver homogenate protein for acyl-CoA oxidase and as unit/mg of liver homogenate protein as described by Baudhuin *et al.* [29], for catalase. Transcription rate values correspond to arbitrary units obtained by laser densitometric scanning of the slot blots. Values are the means ± SD obtained from three different rats. Normalization of ACO values by actin values was done by dividing ACO transcription for each period of treatment. In each lane, factors in brackets correspond to the fold of stimulation as compared to the average of control values.

transcription rate of ACO gene is lessened (about 2-fold induction) but remains significant.

Analysis of the effect of an intermittent ciprofibrate treatment on the rate of transcription

Having determined that the effect of the ciprofibrate on transcription is rapid, we next set out to investigate the duration of the stimulation, and the effect on transcription rate of the re-introduction of the drug following withdrawal.

A 2-week treatment of Wistar rats with ciprofibrate resulted in a 50-fold increase in ACO specific activity (Table 2). When ciprofibrate was removed from the diet during the following 4 weeks, ACO activity in the treated group was still 20 times greater than that in the control group. An additional 2-week ciprofibrate treatment increased ACO activity up to 40 times the activity in the rat control group. Therefore, the effect of ciprofibrate on ACO activity in rat liver does lessen but is not abolished after 4 weeks of drug removal. At the transcriptional level, a 2-week ciprofibrate ingestion provoked a 5-fold

increase in the transcriptional rate of the ACO gene (Table 2), i.e. 10 times less than the 50-fold increase in ACO specific activity. A 3-fold increase in the rate of transcription was still observed after ciprofibrate was removed from the diet for 4 weeks. Re-administration of ciprofibrate increased the transcriptional rate of the ACO gene 10-fold as compared to control rats, which is a 2-fold greater increase than was observed following the initial 2-week ciprofibrate treatment period, indicating an additive effect of ciprofibrate. Between the first two periods of treatment, the transcription rate of the ACO gene did not vary exactly in the same way before and after normalization by β-actin values, since the variations in the hepatic somatic index and the β-actin transcription rate were more pronounced than the variation in the ACO transcription rate between those two periods.

The specific activity of catalase (Table 2) increased 1.6 times as compared to control rats following a 2-week ciprofibrate treatment. Stopping treatment for 4 weeks resulted in the return of enzyme activity to initial levels. Rats re-treated with ciprofibrate for 2 weeks displayed a 2-fold augmentation in catalase activity. The transcription rate of the catalase gene (Table 2) did not return to the initial level after treatment was stopped, but the catalase gene exhibited a less important activation of transcription than the ACO gene (only ~ a 2-fold increase).

Influence of high triglyceride blood levels on the rate of transcription of ACO following a 2-week ciprofibrate treatment

Initial experiments were carried out on Wistar rats; however, to extend the examination of the effect of ciprofibrate, the rate of transcription of ACO was compared in lean and obese Zucker rats (obese rats are studied as a model for risk assessment in hypertriglyceridaemic humans treated with ciprofibrate). SD rats were used as a reference. Ciprofibrate was administered daily over a 2-week period at two different doses of 1 and 3 mg/kg of body weight.

Peroxisomal ACO specific activity showed a remarkable increase in all three rat strains, from 4 to 14 times, as measured in the liver homogenate (Table 3); catalase activity only exhibited an average 50% augmentation (Table 3). All stimulations were dose dependent. We examined the effect of the ciprofibrate on ACO mRNA levels at the two different doses. As shown by Northern blot analysis (Fig. 2) a 3.4 kb band corresponding to ACO mRNA was detected, and the level of these transcripts was dramatically enhanced under ciprofibrate treatment in a dose-dependent manner. Densitometric analysis (Table 4) indicated a 6–7.5-fold increase in ACO mRNA level when the rats were treated at 1 mg/day/kg of body weight of ciprofibrate. When the ciprofibrate dose was three times higher (3 mg/kg of body weight/day), ACO mRNA levels increased by an average of 10-fold.

In order to determine if the elevation of ACO mRNA levels correlated with an increase in the transcription of the ACO gene, *in vitro* transcription assays were performed. The amount of labeled nascent ACO RNA was compared in Zo, Zl and SD

Table 3. Specific activities of peroxisomal ACO and catalase, and densitometric analysis of the transcription rate of the corresponding genes from various rat strains treated with ciprofibrate for 2 weeks

	Ciprofibrate dosage (mg/kg/day)	Zucker lean rat 0	Zucker lean rat 1	Zucker lean rat 3	Zucker obese rat 0	Zucker obese rat 1	Zucker obese rat 3	Sprague–Dawley rat 0	Sprague–Dawley rat 1	Sprague–Dawley rat 3
ACO	Specific activity (nmol/min/mg protein)	12 ± 2.9	106 ± 5.1 (×8.8)	165 ± 29.9 (×13.7)	14 ± 2.3	96 ± 4.6 (×6.8)	146 ± 12.5 (×10.4)	12 ± 2.8	46 ± 12 (×3.8)	124 ± 13.8 (×10.3)
	Transcription rate (arbitrary unit)	52 ± 3.2	149 ± 10.5 (×2.9)	214 ± 16.4 (×4.1)	83 ± 3.2	97 ± 6.8 (×1.2)	106 ± 5.7 (×1.3)	54 ± 3.4	81 ± 6.2 (×1.5)	154 ± 12.1 (×2.9)
Catalase	Specific activity (unit/mg protein)	44 ± 9.3	66 ± 4.6 (×1.5)	64 ± 5.7 (×1.4)	46 ± 2.3	53 ± 4.5 (×1.1)	66 ± 5.7 (×1.4)	37 ± 4.7	49 ± 2 (×1.3)	68 ± 4.8 (×1.8)
	Transcription rate (arbitrary unit)	19 ± 1.2	39 ± 2.1 (×2)	46 ± 4 (×2.4)	46 ± 3.5	45 ± 3.6 (×1)	52 ± 7.1 (×1.1)	43 ± 5.8	73 ± 6.8 (×1.7)	84 ± 5.3 (×1.9)
Actin	Transcription rate (arbitrary unit)	17 ± 1.5	40 ± 3.6 (×2.3)	46 ± 5.2 (×2.7)	26 ± 1.6	23 ± 0.4 (×0.9)	36 ± 3.2 (×1.4)	41 ± 2.7	27 ± 1.8 (×0.7)	30 ± 4.1 (×0.7)
ACO/actin	Transcription rate ratio (no unit)	3	3.7 (×1.2)	4.7 (×1.6)	3.2	4.2 (×1.3)	3 (×0.9)	1.3	3 (×2.3)	5.1 (×3.9)

Zo, Zl and SD rats were treated at 0, 1 or 3 mg/kg/day for 2 weeks, respectively. For further experimental conditions, see Materials and Methods. Specific activities are expressed as nmol/min/mg of liver homogenate protein for acyl-CoA oxidase and as unit/mg of liver homogenate protein as described by Baudhuin *et al.* [29], for catalase. Transcription rate values correspond to arbitrary units obtained by laser densitometric scanning of the slot blots. Normalization of ACO values by β-actin values was done by dividing ACO transcription rate for each period of treatment. In each lane, factors in brackets correspond to the fold of stimulation as compared to the average of control values. Values are the means ± SD obtained from three different rats.

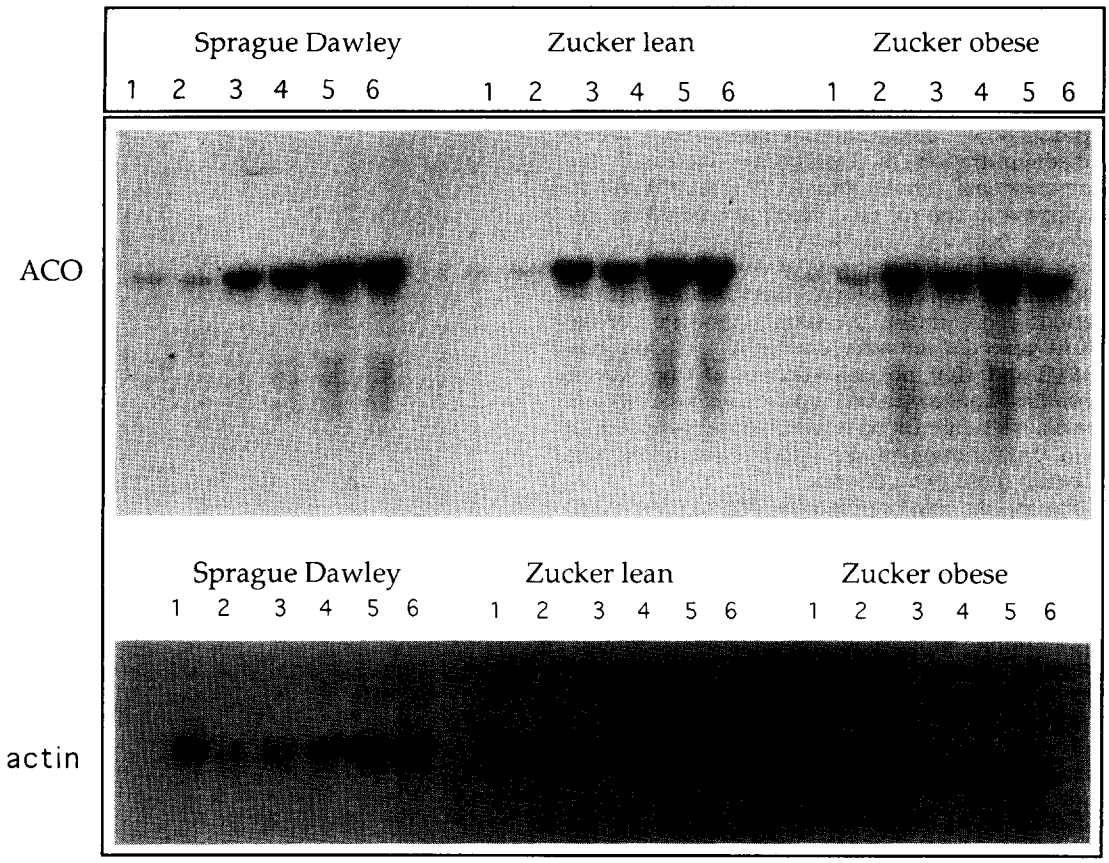

Fig. 2. Northern blot analysis of actin and peroxisomal ACO mRNA levels from liver of various rat strains treated with ciprofibrate for 2 weeks. Zo, Zl and SD rats were treated at 0, 1 or 3 mg/kg/day for 2 weeks, respectively. For experimental conditions of treatment of animals, see Materials and Methods. 1,2 = two control animals. 3,4 = two animals treated with ciprofibrate at 1 mg/kg/day p.o. for 14 days. 5,6 = two animals treated with ciprofibrate at 3 mg/kg/day p.o. for 14 days.

Table 4. Densitometric analysis of ACO mRNA level from liver of various rat strains treated with ciprofibrate for 2 weeks

	Ciprofibrate dosage (mg/kg/day)				
	0	1		3	
Rat strain	Peak area	Peak area	Fold induction	Peak area	Fold induction
Sprague–Dawley	110	663	×6	1088	×9.9
Lean Zucker	90	674	×7.5	1001	×11.1
Obese Zucker	88	641	×7.3	824	×9.3

Values shown in Table 4 correspond to arbitrary units obtained by densitometric scanning of the Northern blot. (Fig. 2) and are the means obtained from two different rats. Treatment is indicated in the legend of Fig. 2.

rat strains. The transcription rate of the ACO gene (Table 3) was induced 1.5–3 times for SD, and 3–4 times for Zl, with respect to the dose ingestion. However, the transcription rate of ACO in isolated nuclei from the liver of treated Zo rats was only slightly augmented (about 1.3-fold). Once normalized by β-actin values, the increase in the ACO transcription rate was less significant in Zucker lean treated rats but remained almost the same in the other strains. Similar transcription rates for the catalase gene (Table 3) were observed in both Zl and SD rats (a 2-fold stimulation), while no transcription rate increase was observed in Zo rats.

DISCUSSION

The purpose of our work was to examine, by

using the run-on assay, the magnitude of action of ciprofibrate, under particular experimental conditions, on the activation of acyl-CoA oxidase gene transcription in rat liver. It was of particular interest to study how quickly ciprofibrate affects ACO gene transcription. Our study confirms that the accumulation of ACO mRNA in rat liver, observed 24 hr after ciprofibrate treatment, is due mainly to a stimulation of transcription. It is important to note that administered doses of ciprofibrate were much lower (about 80-fold) than in the experiment conducted by Reddy *et al.* [32]. The effect of ciprofibrate was also observed with the housekeeping β-actin gene transcription for which we obtained a 2-fold increase (Fig. 1 and Table 1). Such an effect was already observed under thyroid hormone treatment [23].

The persistence of the ciprofibrate effect weeks after the end of treatment was not examined by Reddy *et al.* [32]. In our analyses, measurements were taken 4 weeks after the end of a 2-week ciprofibrate treatment, and again after renewal of the ciprofibrate treatment following the 4-week pause. Male Wistar rats treated for 2 weeks with ciprofibrate exhibited a 5-fold increase in the rate of transcription of the ACO gene. Following a 4-week pause in the ciprofibrate treatment, a 3-fold increase was still observed, suggesting that ciprofibrate itself or its metabolite [33] was still present in the liver. This suggestion is in accordance with the results obtained by Waddell *et al.* [34] where it was demonstrated, by using whole-body autoradiography, that [^{14}C]ciprofibrate persisted at least 27 days in murine liver following oral administration (39–41 mg/kg body weight; 0.3 μCi/g body weight). It is not unreasonable to suggest that in rat, ciprofibrate or its metabolite(s) persists in liver for 4 weeks following the cessation of treatment and continues to stimulate transcription of the ACO gene. An additional 2-week ciprofibrate treatment, following the 4-week pause, resulted in a greater stimulation of transcription than the first treatment. This is likely due to the continued presence of ciprofibrate in the liver: an additive effect of the two treatments.

In previous studies, Pacot *et al.* [9] compared the pharmacokinetics of ciprofibrate and the peroxisome proliferation parameters of the genetically obese Zucker rat (fa/fa) to the lean Zucker rat (Fa/fa) and SD rat (chosen as a more common strain). In our study, the increase in ACO gene transcription in Zucker rats was clearly not sufficient to explain the observed increase in ACO mRNA levels. We therefore suggest that ciprofibrate might also act at a post-transcriptional level to cause ACO mRNA accumulation. The obese Zucker rats presented only slight changes in their transcriptional rate, while the lean Zucker rats and SD rats exhibited a significant increase in ACO transcription rate. On the other hand, obese Zucker control rats had a basal rate of ACO transcription 50% higher than the rates observed in lean and SD rats. Godbole and York [35] showed an increase in fatty acid synthesis in obese rat liver resulting in an accumulation of fatty acid in both liver and adipose tissue. Elcombe and Mitchell [20] suggested that an increased intrahepatic lipid level might be an important factor in the genesis of peroxisome proliferation in rats. Lipid overload of mitochondrial β-oxidation leads to an increased synthesis of enzymes involved in peroxisomal fatty acid oxidation in order to maintain cellular lipid homeostasis and explains the higher basal rate of transcription of the ACO gene. Furthermore, Sorensen *et al.* [36] demonstrated that the peroxisomal acyl-CoA oxidase activity and mRNA level were increased by dexamethasone, a β-oxidation system inducer, in two hepatoma cell lines as well as in hepatocytes, and that insulin overrode the induction caused by this compound. As it is well established that obese Zucker rats are hyperinsulinaemic, this could explain why stimulation by ciprofibrate of the transcription rate of the ACO gene is weaker in Zo than in Zl, according to Sorensen's findings. The discrepancy observed both in lean and obese rats between the magnitude of increase in ACO mRNA levels and the elevation of the ACO transcription rate, leads us to suggest that the regulation of ACO gene expression is not only transcriptional.

Ciprofibrate, via an unknown mechanism, activates the PPAR (as demonstrated by *in vitro* transfection assays) which is known to bind the ACO-PPRE and activate ACO transcription (as demonstrated by run-on assays from *in vivo* treated rat). This mechanism of activation is clearly not sufficient in our experiment to explain the increase in mRNA level, suggesting a post-transcriptional (stabilization of the transcripts?) additive regulation and, according to the discrepancy between specific activities and mRNA levels, a post-translational (turnover of proteins?) regulation. Sorensen *et al.* [36] demonstrated that the increase in steady-state mRNA levels for acyl-CoA oxidase in 7800C1 hepatoma cells by TTA (tetradecylthioacetic acid, a fatty acid analogue inducing peroxisomal β-oxidation), was partly due to prolonged half-life of the transcripts. This stabilization could not fully account for the increase in steady-state mRNA levels and the data suggested that TTA also increased the transcription rate. Their and our data do not entirely agree with the data of Reddy *et al.* [32], who demonstrated by run-on assay that the elevation of ACO mRNA levels resulted from a rapid increase in the rate of transcription rather than decreased degradation of mRNA in hepatocytes treated from 1 to 16 hr with ciprofibrate. This discrepancy might be due to: (i) the fact that they used different cell lines than Sorensen [36] and (ii) differences in drug, dose and exposure time. PPAR is one regulator of peroxisomal protein gene expression, but we suggest that another mechanism, or PPAR itself, not only acts on transcription, but on the turnover of these mRNAs and proteins, as observed in other members of the steroid hormone receptor family [37, 38].

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