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ROLE OF THYROID STATE ON INDUCTION BY CIPROFIBRATE OF LAURATE HYDROXYLASE AND PEROXISOMAL ENZYMES IN RAT LIVER MICROSOMES

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Abstract—The effects of hypothyroidism and hyperthyroidism upon liver microsomal ω-laurate hydroxylase activity (cytochrome P450 IV A1-dependent), peroxisome proliferation marker enzyme activities and acyl CoA oxidase (AOX) expression induced by ciprofibrate (2 mg/kg/day during 8 days) were studied in the male Wistar rat so as to clarify firstly the possible involvement of thyroid hormones in the modification of peroxisomal ciprofibrate-induced enzyme activities in relation to hepatic microsomal cytochrome P450 IV A1 induction, and secondly the possible direct effect of thyroid hormones on the gene expression of specific peroxisomal enzymes. No significant change was found in the ciprofibrate-induced ω-laurate hydroxylase activity in hypothyroid rats or in rats that had received a large dose of triiodothyronine (LT_3), suggesting that the thyroid hormone does not interfere with the peroxisome proliferation process through such an indirect mechanism. The induction by ciprofibrate [2-(4-(2-2dichlorocyclopropyl)phenoxyl-2methyl-propionic acid)] of mitochondrial α-glycerolphosphate dehydrogenase and microsomal bilirubin UDPGT was decreased about 3-fold and 1.5-fold, respectively, while the induction of peroxisomal AOX, carnitine acetyl transferase and enoyl CoA hydratase enzyme activities was decreased by 36%, 34% and 22% in thyroidectomized animals, as compared to euthyroid animals. However, no significant changes in the quantity of peroxisomal proteins and in the AOX mRNA level were noted. The administration of large doses of LT3 to normal rats decreased the peroxisomal ciprofibrate AOX enzyme induction with a marked concomitant decrease in the AOX mRNA level. This suggests that high doses of LT_3 enhance the turnover of some specific mRNAs or down regulate the peroxisome proliferator receptor. Our results also do not exclude inhibition of catabolic activity towards AOX which depends on thyroid hormone.

The treatment of rats with clofibrate, or ciprofibrate [2-(4-(2-2dichlorocyclopropyl)phenoxyl-2methyl-propionic acid)] (CIP§) its structural analog, causes enlargement of the liver and proliferation of hepatic peroxisomes with a large increase (5–10-fold) in peroxisomal fatty acid β-oxidation activity [1–6]. At the same time, the induction of microsomal bilirubin glucuronidation [7] and of ω-hydroxylation of lauric acid, dependent on a special cytochrome P450 isoenzyme (cytochrome P450 IV A1), has also been observed [8]. Although it is plausible that stimulated gene transcription is primarily responsible for the increase in β-oxidation activities [9], the mechanisms leading to this induction are still unclear, particularly with regard to a possible endocrine regulation. According to Hertz *et al.* [10], thyromimetic effects are to be attributed to agents of the fibrates series. Hertz *et al.* [10] claimed to have observed an increase in both liver and peroxisomal enzyme activities and in mitochondrial α-glycerolphosphate dehydrogenase (α-GDH) (EC 1.1.9.9.5) activity in bezafibrate-treated rats.

Experiments with rats of different thyroid status have yielded conflicting data concerning the levels of peroxisomal enzyme activities [11]. The liver peroxisomal β-oxidation activity was increased 2–2.5-fold over the normal level in hyperthyroid rats [12], but while hypothyroidism has been claimed by some authors to promote an increase of the hepatic peroxisomal oxidation activity [13], others find that it brings about a decrease [12], as compared to euthyroid controls. The clofibrate-induced stimulation of peroxisomal β-oxidation has been reported to be reduced, or not to be affected, by hypothyroidism [13, 14]. We have shown a significant decrease in the CIP-induced stimulation of peroxisomal acyl CoA oxidase (AOX) activity in hypothyroid rats [15]. On the other hand, thyroid hormones are known to increase cytochrome P450 degradation through the induction of heme oxygenase, the rate limiting enzyme in the degradation of heme to bile pigments [16]. This effect results in a significant decline in hepatic P450 content, but whether only one specific cytochrome P450 isoenzyme or several are implicated has not yet been established. As a result, the exact role of cytochrome P450 IV A1 is still unclear.

The aim of the present study is to find out: first if there is an indirect involvement of thyroid hormones

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§ Abbreviations: α-GDH, α-glycerolphosphate dehydrogenase; CAT, carnitine acetyl transferase; CIP, ciprofibrate; AOX, acyl CoA oxidase; ECH, enoyl CoA hydratase; ω-LAH, ω-lauric acid hydroxylase; LT_3, 3,5,3′-triiodo-L-thyronine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SSC, saline sodium citrate; UDPGT, UDP-glucuronosyltransferase.

in the modification of peroxisomal CIP-induced enzyme activities through modulation of the hepatic microsomal cytochrome P450 IV A1 and second, the possible direct effect of thyroid hormones on the gene expression of specific peroxisomal enzymes. The expression of peroxisomal proteins was investigated by studying enzyme activities, protein by immunoblotting and mRNA with a specific cDNA probe.

MATERIALS AND METHODS

Chemicals. CIP was a gift from Sterling-Winthrop (Dijon, France). LT_3 (3,5,3′ triiodo-L-thyronine), dinitroenzoic acid, 5-bromo-4-chloro-3-indol phosphate, nitroblue tetraazolium, crotonoyl CoA, lauric acid and 12-hydroxy lauric acid were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Titanylsulfate was obtained from Reidel de Haen (Lyon, France). Acetyl CoA, palmitoyl CoA, NAD, NADPH, UDP-glucuronic acid and bovine serum albumin (fatty acid free) were purchased from Boehringer (Mannheim, Germany). [1-^{14}C]Lauric acid (52 mCi/mmol) and a multiprime DNA labeling kit were purchased from Amersham Corp (Les Ulis, France). Metrizamide was from Nycomed (Oslo, Norway).

Animals and treatment

Experiment I. Forty-eight male Wistar rats from an SPF Husbandry (IFFA CREDO, France), weighing 90–100 g on arrival, were used. They were kept at constant temperature (26°) and had free access to tap water and food (aliment control UAR). The rats were divided into six groups of eight rats each.

Groups one to three, constituting the control groups (untreated rats), were as follows: Normal group (N). Hypothyroid group (TH^-), formed by surgical thyroidectomy on each of this group's six rats. The hypothyroid state was controlled by measurement of free L-thyroxine ($F\text{-}T_4$) by radioimmunoassay. Animals with $F\text{-}T_4$ plasma levels less than 1 pM were used for subsequent treatment with CIP. Hyperthyroid group (T_3). The hyperthyroid state was obtained by i.p. administration of LT_3 at a dose of 50 μg/kg/day for 8 days.

After 4 weeks the three other groups were made up of CIP-treated rats; one of normothyroid, one of hypothyroid and one of hyperthyroid animals, as above. In the last, the daily treatment with LT_3 was continued at the same time as the CIP treatment for a period of 8 days before the animals were killed. CIP was administered at the dose of 2 mg/kg body weight/day by gastric intubation of a solution of the drug in corn oil. The same volume of corn oil (0.33 mL/kg body weight) was delivered to the rats of the control groups. The rats were killed 16 hr after the last administration of CIP. All the blood was collected in tubes containing heparin, and the plasma was separated by centrifugation at 3000 *g*. The total liver of each rat was removed, weighed and perfused with an ice-cold 0.154 M KCl solution. A piece of liver (2 g) was collected for each rat, immediately frozen with liquid nitrogen and stored at −70° for subsequent RNA isolation and mRNA hybridization studies.

A 20% (w/v) homogenate of the liver was prepared in Tris–EDTA buffer (0.154 M KCl; 0.1 M EDTA; 0.2 M Tris; pH 7.4), using a teflon Potter Elvehjem homogenizer.

An aliquot of the homogenate was successively centrifuged at 750 *g* using a Beckman centrifuge model J 21 C to eliminate incompletely homogenized cells and nuclei, at 8700 *g* for 10 min (collection of the heavy mitochondrial pellet, which was resuspended in 2.5 mL of the former Tris–KCl buffer) and at 25,000 *g* for 15 min [collection of the peroxisome-enriched pellet, which was resuspended in 2 mL of a 0.25 M sucrose; 0.2 M Tris; 0.0054 M EDTA buffer (pH 7.4)]. The supernatant was spun at 105,000 *g* for 90 min in a Beckman ultracentrifuge (model L5/50 with 42:1 rotor). The microsomal fraction was finally resuspended in the mentioned Tris–KCl buffer without EDTA.

The subcellular fractions were aliquoted and stored at −70°. The protein concentration of these fractions was determined by the method of Lowry *et al.* [17], automatized on a COBAS BIO (Roche, Switzerland), using serum albumin as standard. The rest of the homogenate was added with 10% dimethylsulfoxide (DMSO) (w/v), frozen with liquid nitrogen and stored at −70° for further preparation of the rat liver purified peroxisomes by the method of Wattiaux *et al.* [18], adapted in our laboratory by Cherkaoui Malki *et al.* [19], using a discontinuous Nycodenz gradient.

Biochemical assays. The concentration of free LT_3 was measured in the plasma using the radioimmunoassay kit from Amersham (Les Ulis, France).

The activities of mitochondrial α-GDH and succinate dehydrogenase were respectively assayed, as described by Gardner [20] and by Arrigoni and Singer [21]. The activities of catalase, acyl CoA dehydrogenase, carnitine acetyltransferase (CAT) and enoyl CoA hydratase (ECH) were measured in the peroxisomal light fraction, according to methods outlined by Hübl and Brettschneider [22], Lazarow and De Duve [23], Gray *et al.* [24] and Osumi and Hashimoto [25], respectively. The microsomal cytochrome P450 content was determined according to a procedure adopted from Omura and Sato [26]. The microsomal lauric acid ω and ω-1 hydroxylase activities, dependant on cytochrome P450 IV A1 isoenzyme, were assayed in a manner used by Parker and Orton [27]. Microsomal bilirubin UDP-glucuronosyltransferase (UDPGT) activity was evaluated according to Heirwegh *et al.* [28], using digitonin at a detergent/protein ratio of 1:4 to obtain maximal activation of the enzyme.

Protein electrophoresis and western blot. The solubilized proteins of purified peroxisomes were subjected to 10% SDS–polyacrylamide slab gel electrophoresis (SDS–PAGE) using the method of Laemmli [29], allowing western blot analysis on nitrocellulose Hybond C membrane according to Towbin *et al.* [30].

Both of the primary antibodies against total or membrane rat liver peroxisomal proteins were prepared, as by Cherkaoui Malki *et al.* [19], by

injection of these antigens to rabbits. Blood was collected by cardiac puncture of anesthetized animals. The binding of primary antibody was detected by a second antibody (goat antirabbit IgG), conjugated with rabbit alkaline phosphatase and enhanced by addition of 5-bromo-4-chloro-3-indol phosphate with nitroblue tetraazolium.

Preparation of total RNAs, northern and slot blots

Total RNAs were prepared according to the LiCl–urea method described by Auffray and Rougeon [31] from the aliquots of hepatic tissue previously frozen in liquid nitrogen and stored at −70°.

For slot blot analysis, RNAs were denatured in 50% deionized formamide, 6% formaldehyde for 1 hr at 50°, chilled on ice and then applied to the nylon filters (Gene Screen Plus ™, Dupont). After 30 min, a slight suction was applied to the manifold for 30 sec. Membranes were stored for 2 hr at −80° and prehybridized at 42° overnight in 50% formamide, 1% SDS, 1 × Denhart, 5% dextran sulfate, 5 × saline sodium citrate (SSC) and 0.005% heparin. Then, hybridization of the membranes was run overnight at 42° in a similar solution containing 25 ng of the AOX cDNA probe (gift of Dr T. Osumi) or 25 ng of chicken β-actin cDNA probe (Oncor), labeled with [α^{32}P]dCTP (50 μCi/25 ng cDNA) using the multiprimer Amersham kit.

The membranes were successively washed at 42°, twice for 15 min in 2 × SSC, 0.1% SDS, twice for 15 min in 0.5 × SSC, 0.1% SDS, and twice for 15 min in 0.1 × SSC, 0.1% SDS. The membranes were finally autoradiographed for an appropriate time at −70° using Amersham hyperfilm.

Northern blots were performed according to the method of Maniatis *et al.* [32], with AOX and β-actin cDNA probes in order to check the specificity of the hybridization.

Experiment II. Groups of 30 euthyroid, hypothyroid or hyperthyroid rats were made up in the same way as above. The animals were treated with CIP, with the exception of six animals acting as controls for each group. In every case, two groups of six animals were killed 15 and 24 hr, respectively, after a single administration of 2 mg/kg body weight. A third group was treated for 2 days and a fourth group for 7 days at the same dosage. The animals were killed 24 hr following the last administration of CIP. Peroxisome-enriched and microsomal fractions were prepared from whole livers to measure peroxisomal enzyme activities and microsomal ω-lauric acid hydroxylase (ω-LAH) activity, as described previously.

Experiment III. Sham-operated animals were compared with thyroidectomized animals to check whether surgical stress (after 4 weeks) caused any hormonal changes and influenced parameters measured in this study.

From the groups of animals (i.e. normal thyroidectomized and sham-operated, five per group), we estimated the following enzyme activities (or content): microsomal UDPGTs with 1-naphthol, 4-PNP or nopol as substrates, mitochondrial α-GDH, seric alanine amino transferase and γ-glutamyl transferase, microsomal cytochrome P450, and peroxisomal ACX. Table 1 shows there were no significant differences between the sham-operated group and the normal group, while thyroidectomy specifically effects UDPGTs and α-GDH.

RESULTS

The liver weight/body weight ratios in % (results in Table 2) showed an important hepatomegaly due to CIP administered at the dose of 2 mg/kg/day during 8 days in any group of rats. This effect was much less pronounced in hypothyroid rats.

The mitochondrial membrane bound α-GDH activity (Fig. 1), classically considered to be thyroid hormone-dependent, decreased 2-fold in hypothyroid rats compared to normal rats, but was strongly enhanced by LT_3 treatment at a pharmacological dose (6-fold). Also, CIP markedly induced this mitochondrial enzyme: this effect was however much stronger in normothyroid rats than in thyroidectomized ones (e.g. about a 10-fold increase of the enzyme activity vs a 5-fold increase). Moreover, the combination of LT_3 and CIP treatments did not result in an additive effect, as shown in Fig. 1.

The amount of total cytochrome P450 (Table 2) was increased to a small, but significant extent by

Table 1. Investigation of the non-specific effect(s) of sham operation on enzyme activities

Subcellular distribution	Enzyme activities (nmol/min/mg/protein) or content for cytochrome P450	Normal	Thyroidectomized	Sham-operated
Liver microsomes	UDPGT-1-Naphthol	104.80 ± 5.13	58.8 ± 4.31*	106.8 ± 4.48§
	UDPGT-4-PNP	75 ± 3.56	48.2 ± 4.58*	73.8 ± 3.73§
	UDPGT-Nopol	31.40 ± 1.50	48 ± 3.13	35.20 ± 3.97†§
	Cytochrome P450	0.61 ± 0.07	0.61 ± 0.07	0.56 ± 0.12
Liver mitochondria	α-GDH	2.88 ± 0.19	0.56 ± 0.14*	3.13 ± 0.5§
Liver peroxisomes	AOX	7.9 ± 2.7	5.2 ± 2	6.1 ± 1.5‡
Plasma (IU/L)	Alanine amino transferase	60.18 ± 6.6	54.25 ± 4.08	56.82 ± 3.26
	γ-Glutamyl transferase	2.17 ± 0.3	2.13 ± 0.23	1.35 ± 0.33

Results represent mean ± SD for five rats individually processed.
Statistical significance (ANOVA test): thyroidectomized vs normal: * $P < 0.001$; sham-operated vs normal: † $P < 0.05$; sham-operated vs thyroidectomized: ‡ $P < 0.01$; § $P < 0.001$.

Table 2. Effects of CIP on liver of male rats of different thyroid states

Compound	Thyroid state	Body weight (g)	Liver/body weight ratio (%)	Cytochrome P450*	ω-LAH†	ω-1-LAH†	ω-LAH/ cytochrome P450 ratio
Control group	TH⁻	201 ± 6.6**	3.3 ± 0.2¶	0.71 ± 0.05‖	1.04 ± 0.17‖	1.01 ± 0.07	1.46 ± 0.14
	N	335 ± 10	3.7 ± 0.1	0.65 ± 0.05	1.35 ± 0.12	1.19 ± 0.24	2.08 ± 0.17
	T_3	294 ± 8.1	3.5 ± 0.1	0.51 ± 0.07§	1.12 ± 0.08	1.33 ± 0.15	2.20 ± 0.19
	TH⁻	214 ± 10**	4.3 ± 0.1‡¶	0.81 ± 0.11‡‖	10.4 ± 1.90‡	2.59 ± 0.39‡	12.8 ± 1.42‡
CIP group (2 mg/kg/day)	N	314 ± 9.2	5.8 ± 0.2‡	0.99 ± 0.11‡	12.9 ± 1.87‡	2.47 ± 0.31‡	13.0 ± 1.53‡
	T_3	278 ± 8.3	5.9 ± 0.1‡	0.95 ± 0.10‡††	14.2 ± 1.32‡	2.87 ± 0.23‡	1.49 ± 1.11‡

Results represent mean ± SD for eight rats individually processed.
* Concentration expressed in nmol/mg protein.
† Specific activity expressed in nmol/min/mg/protein.
Statistical significance (ANOVA test): Treated vs control: ‡ $P < 0.001$; T_3 vs N: § $P < 0.001$; TH^- vs N: ‖ $P < 0.05$; ¶ $P < 0.01$; ** $P < 0.001$; Interaction T_3–CIP treatment: †† $P < 0.05$.

thyroidectomy. LT_3 administration, on the other hand, caused a marked decrease in the chromoprotein level (Table 2). CIP caused a significant induction of the hepatic cytochrome P450 level in normal rats (about 50%), as well as in LT_3-treated rats (about 90%) and also in hypothyroid rats, though to a much lesser extent (about 15%).

At the same time, the cytochrome P450 IV A1 linked ω-hydroxylation of lauric acid (Table 2) was increased about 10-fold by CIP. The ω-1-hydroxylation, however, showed no more than a 2-fold increase. These effects appear closely related to the fibrate series, as shown by Sharma *et al.* [8]. The thyroid state alone was not responsible for the change in both microsomal activities. The CIP-induced ω-hydroxylation was, however, lowered in the hypothyroid state (about 20%), whereas the hyperthyroid state did not influence ω-hydroxylation. It should be pointed out that the succinate dehydrase activity (measured in the peroxisomal fraction in order to evaluate mitochondrial contamination) showed a comparable 10–15% contamination in any sample analysed (results not given).

Bilirubin glucuronidation appeared to be dependent upon the thyroid state of the animals. As shown in Fig. 2, the activity of bilirubin-UDPGT in euthyroid and in hyperthyroid rats was lower by 46% and 62%, respectively, when compared to thyroidectomized animals. CIP treatment is known to promote an induction of this enzyme, but the intensity of the induction appeared to be linked to the thyroid state (Fig. 2). We noted an inducive effect of the bilirubin-UDPGT activity in hypothyroid rats (about 60%), whereas the glucuronidation of bilirubin was more strongly affected in normo- and hyperthyroid rats (about a 3-fold increase). This points to the likelihood that the inducive mechanism of fibrates on UDPGT is dependent upon thyroid hormones.

The thyroid activity did not appear to modify considerably the peroxisome marker enzyme activities such as AOX, CAT or ECH (Table 3). Only the first was significantly decreased in hypothyroid, as well as in hyperthyroid, as compared to normothyroid animals. The treatment with LT_3 in supraphysiological dosage also promoted a decrease of the ECH. CIP exhibited a very intense effect upon all three peroxisomal enzymes, indicating the peroxisomal proliferation promoted by the drug. However, after the CIP treatment, the level of the enzymatic activities remained weakly lower (respectively about 36%, 34%, 22%) in rats of hypothyroid or hyperthyroid state than in normothyroid animals. On the other hand, the treatment with LT_3 decreased the catalase activity (Table 3), but slightly stimulated the CIP-mediated induction.

Figure 3 shows the western blot analysis of peroxisomal proteins from purified peroxisomes: the western blot (A) immunolabeled with anti total peroxisomal proteins antiserum showed an equal increase in the intensity of the 80, 70 and 66 kDa bands, corresponding to the molecular mass of ECH, AOX and catalase, respectively, in all CIP-treated rats. Moreover, an increase in the intensity of

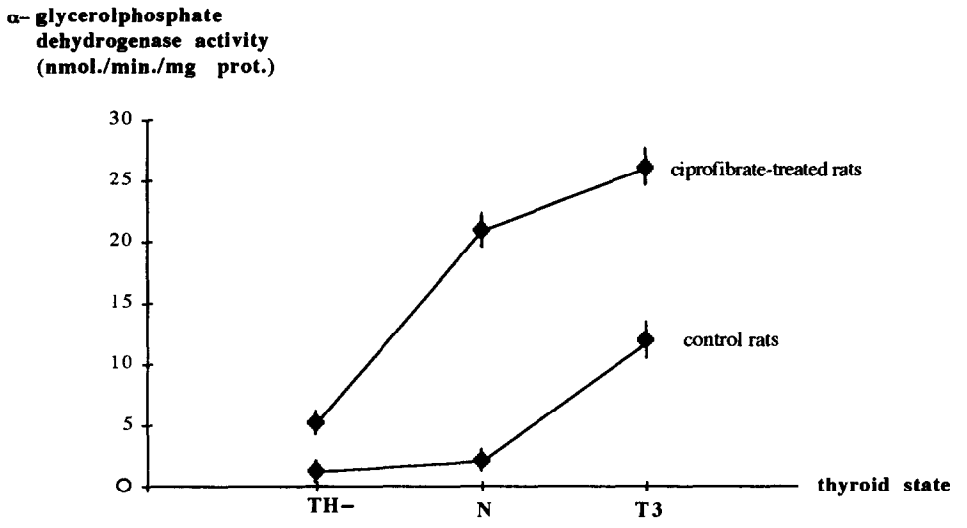

Fig. 1. Effects of CIP on liver mitochondrial α-GDH activity in male rats. Normal (N), thyroidectomized (TH^-) and LT_3-treated Wistar rats (30 μg/kg/day) received daily for 8 days either 0.33 mL of corn oil/kg body weight (control rats) or a 2 mg/kg body weight dose of CIP in the same volume of corn oil (CIP-treated rats). Each point represents a mean ± SD for N = 8 rats.

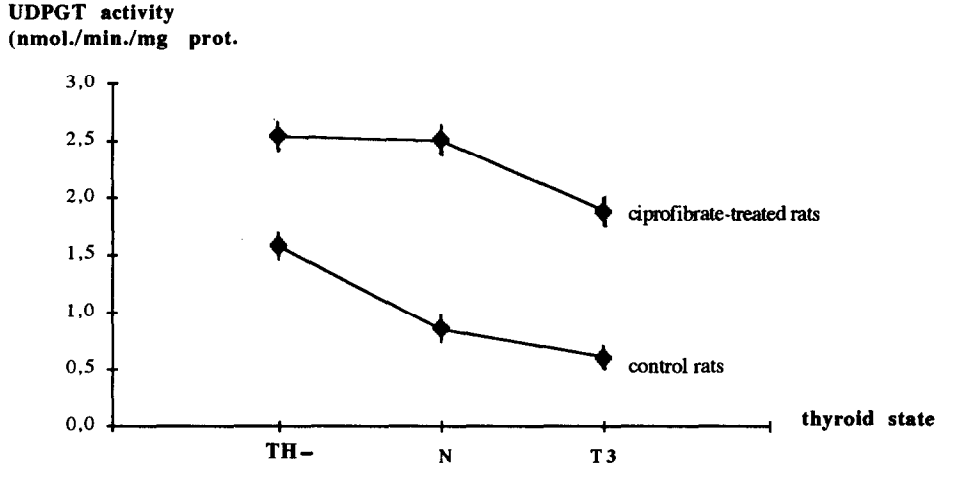

Fig. 2. Effects of CIP on liver microsomal bilirubin UDPGT activity in male rats. Normal (N), thyroidectomized (TH^-) and LT_3-treated Wistar rats (30 μg/kg/day) received daily for 8 days either 0.33 mL of corn oil/kg body weight (control rats), or a 2 mg/kg body weight dose of CIP in the same volume of corn oil (CIP-treated rats). Each point represents a mean ± SD for N = 8 rats.

peroxisomal protein bands was also noted in control hyperthyroid rats.

The western blot (B) performed with anti integral membrane peroxisomal protein antiserum showed an essentially equal increase in the intensity of the 22 and 40 kDa bands, corresponding to both of the main peroxisomal membrane proteins, in response to CIP treatment. The hyperthyroid rat, to a lesser extent, also accentuated the 22 kDa band.

Using an AOX cDNA probe, slot blot analysis (Fig. 4) with hybridized RNA produced peaks whose areas were scanned in order to quantify the effect of treatment. The results were calculated relative to those obtained with a β-actin probe, a housekeeping gene (not shown). The expression of the AOX gene was stimulated 2-fold by LT_3 administration. A marked 8-fold increase in the AOX mRNA level was found in normothyroid and hypothyroid CIP-treated rats. Thus, this increase was strongly attenuated in hyperthyroid CIP-treated animals (only a 2.4-fold increase).

The graphs A, B and C (Fig. 5) were obtained from experiment II to show the relationship between the kinetics of microsomal ω-LAH (cytochrome P450 IV A1) induction and the kinetics of peroxisomal AOX induction, promoted by a treatment with CIP.

Table 3. Effects of CIP on liver peroxisomal enzymes of male rats of different thyroid states

Compound	Thyroid state	Catalase*	AOX	ECH†	CAT‡
Control group	TH^-	24 ± 2	0.95 ± 0.12	2.03 ± 0.77	0.95 ± 0.15
	N	22 ± 3	1.64 ± 0.25	2.95 ± 0.79	0.71 ± 0.12
	T_3	10 ± 2**	0.66 ± 0.12¶	1.08 ± 0.31¶	0.96 ± 0.18
CIP group (2 mg/kg/day)	TH^-	39 ± 4§	30.9 ± 1.98§§§	43.7 ± 5.3§††	34.6 ± 2.35§‡‡
	N	42 ± 1§	48.9 ± 1.73§	56.2 ± 6.4§	52.7 ± 2.47§
	T_3	33 ± 3§¶	33.8 ± 1.92§‖,‖‖	49.5 ± 2.7§	38.2 ± 4.26§¶

Results represent mean ± SD for eight rats individually processed.

* Specific activity expressed in IU/mg protein: one unit of catalase corresponds to the amount of enzyme that destroys 90% of the substrate under described condition by Baudhuin *et al.* [37].

† Specific activity expressed in μmol/min/mg protein.

‡ Specific activity expressed in nmol/min/mg protein.

Statistical significance (ANOVA test): Treated vs control:§ $P < 0.001$; T_3 vs N: ‖ $P < 0.05$; ¶ $P < 0.01$; ** $P < 0.001$; TH^- vs N: †† $P < 0.05$; ‡‡ $P < 0.01$; §§ $P < 0.001$; Interaction T_3–CIP treatment: ‖‖ $P < 0.001$.

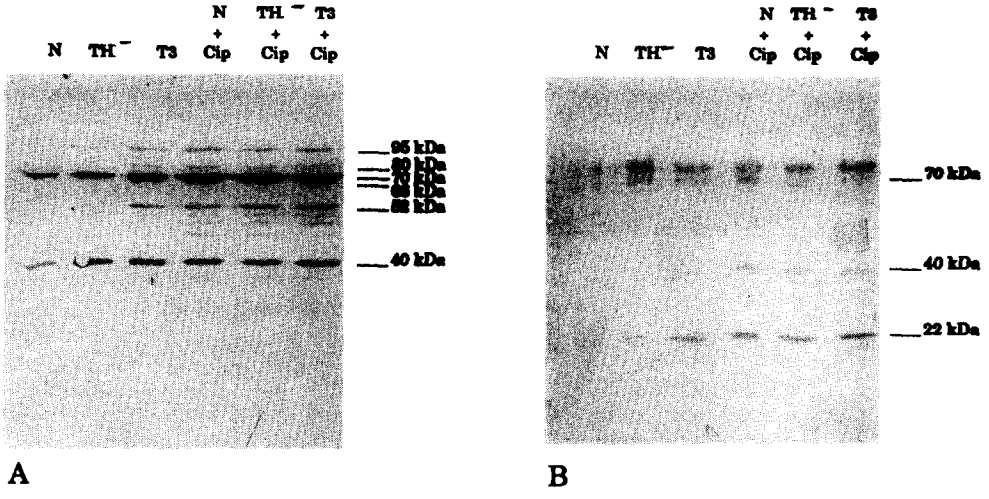

Fig. 3. Immunoblot analysis of total peroxisomal proteins (A) and peroxisomal integral membrane proteins (B). In each lane, 10 μg of peroxisomal proteins from purified peroxisomes were subjected to SDS–PAGE, then immunodecorated with the rabbit antiserum (1/200 dilution) anti-peroxisomal proteins (A) or anti-integral peroxisomal membrane proteins (B) and revealed with secondary antibody (goat anti rabbit IgG), conjugated with rabbit alkaline phosphatase. Control groups: normothyroid rats (N); hypothyroid rats (TH^-); hyperthyroid (T_3) LT_3-treated rats (50 μg/kg/day). CIP-treated groups (2 mg/kg/day): normothyroid rats (N + CIP); hypothyroid rats (TH^- + CIP); hyperthyroid, LT_3-treated rats (50 μg/kg/day) (T_3 + CIP).

In normothyroid rats, a specific inducive effect of CIP towards ω-LAH appeared very early and was detectable just 15 hr after a single administration of CIP. This effect continued to increase 24 hr after a single dose and was still accentuated at the second day after two successive doses of CIP. A full 8 days of treatment brought about a 10-fold increase of the control level activity. The induction of AOX required no less than a 2-day treatment with CIP to be detectable and so appeared noticeably delayed, compared to the induction of ω-LAH. The hypothesis of Sharma *et al.* [8] that primary induction of the ω-LAH initiates peroxisome proliferation, including the stimulation of AOX, correctly accounts for such results. The kinetics for the induction of ω-LAH or AOX did not differ significantly in LT_3-treated rats from normothyroid ones. However, in thyroidectomized animals, the inducive effect of CIP upon ω-LAH appeared markedly later than in normal rats. Moreover, the effect of even an 8-day treatment with CIP upon ω-LAH and AOX remained smaller in hypothyroid than in normothyroid rats, suggesting a possible regulatory action of thyroid hormones in the development of induction of both

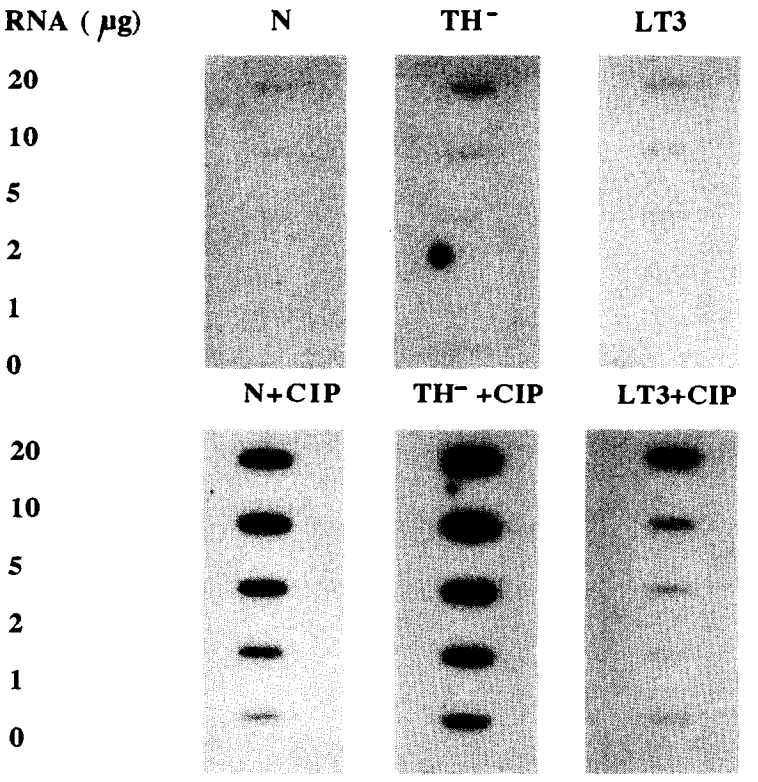

Fig. 4. Slot blot analysis of AOX mRNA level. RNA (0–20 μg) extracted from liver homogenates was applied to nylon filters and hybridized with a ^{32}P-labeled AOX cDNA probe, as described in Materials and Methods. Total RNA was applied in lane 0 as a negative control. Control groups: normothyroid rats (N); hypothyroid rats (TH^-); hyperthyroid (T_3) LT_3 treated rats (50 μg/kg/day). CIP-treated groups (2 mg/kg/day): normothyroid rats (N + CIP); hypothyroid rats (TH^- + CIP); hyperthyroid LT_3-treated rats (50 μg/kg/day) (T_3 + CIP).

of these activities and probably of the whole of peroxisomal proliferation.

DISCUSSION

Thyroid hormones obviously play a role during the development of the effects of hypolipidaemic agents belonging to the fibrate series [33]. Thus, a displacement of thyroid hormones from their transport proteins by clofibrate or fenofibrate has been evoked to explain the hypolipidaemic action of such agents in the rat [34]. It has also been observed that fibrates and other peroxisome proliferators activate the α-GDH and malate dehydrogenase genes specifically induced by thyroid hormones.

According to our results, the hepatic inducive or proliferative effects promoted by CIP in the male Wistar rat appeared to be affected by the thyroid state, particularly by hypothyroidism. Thus, in thyroidectomized animals, the effects of CIP were developed to a much lesser extent than in normal rats, as was observed with morphological parameters as well as with mitochondrial (α-GDH activity), microsomal (bilirubin UDPGT, ω-LAH) or peroxisomal (AOX, CAT or ECH) activities. The well-known slowing of growing and energy metabolism, resulting from the lack of thyroid hormone and based upon the suppression of nuclear effects promoted by LT_3 through hormonal receptor activation, must be considered to understand some of our results. Concerning α-GDH and bilirubin UDPGT, which are themselves influenced by the nuclear LT_3 receptor, the modification of the effects of CIP depending upon the thyroid status suggests that the LT_3 receptor is in some way involved in the action of the fibrate. Regarding peroxisomal enzyme expression, we found a stimulation in the expression of the AOX gene and an increase in the peroxisomal protein level by LT_3 administration to normal rats, suggesting a possible direct regulation on genetic expression. However, this stimulation appears not to be correlated with peroxisomal activity, suggesting a more complex mechanism.

Some inducive and proliferative parameters modulated in the rat by thyroid hormones, such as the induction of α-GDH, are also affected by CIP, supporting the views of Shoemaker and Yamazaki [11] that CIP acts as a transcriptional activator of thyroid hormone-dependent genes. However, other effects of CIP are opposite to those of thyroid hormone: microsomal cytochrome P450, bilirubin UDPGT, peroxisomal AOX, ECH and catalase activities were decreased by administration of LT3 to normal rats, but inversely, were strongly enhanced by CIP. Simultaneous treatment with hormonal and

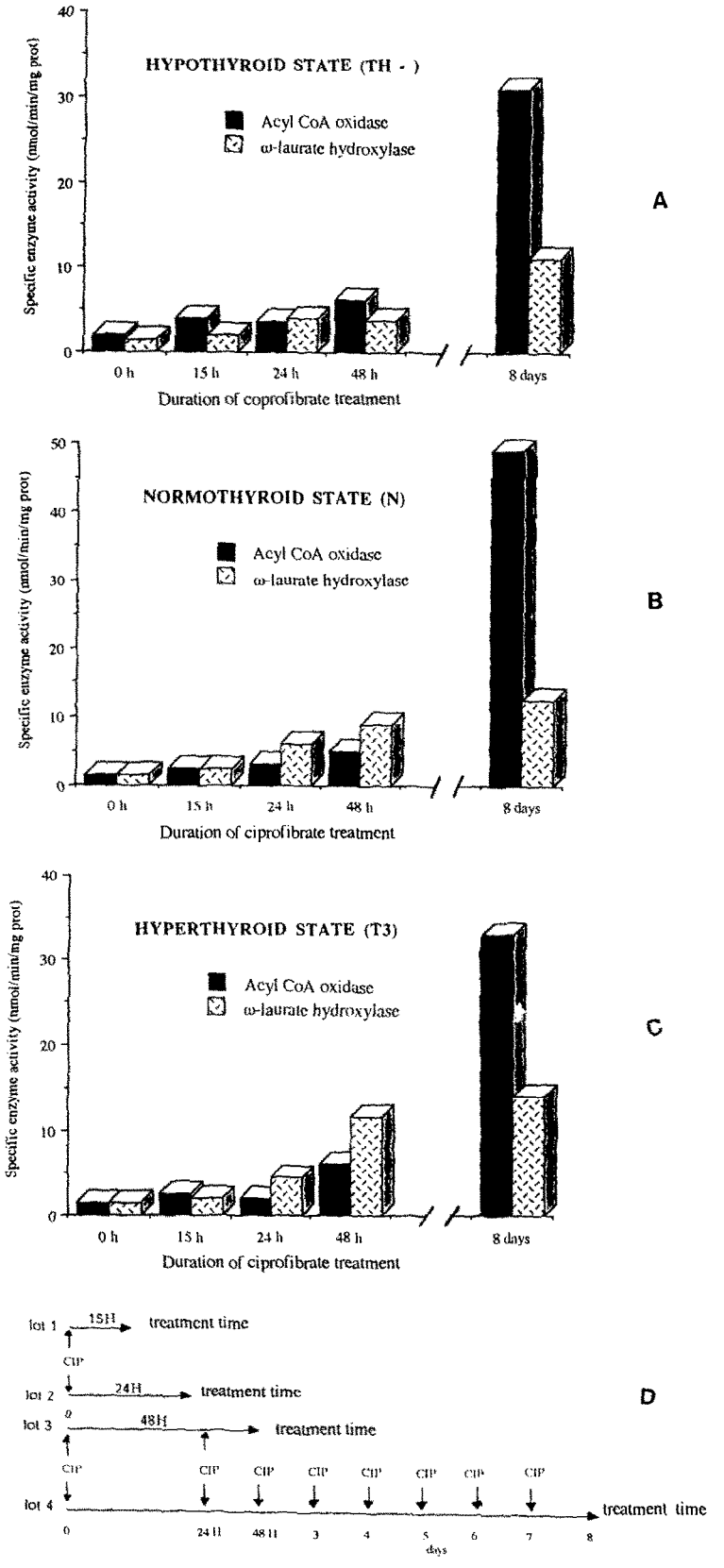

Fig. 5. Effects of treatment of varying duration with CIP at the dose of 2 mg/kg body weight on liver microsomal ω-LAH and peroxisomal AOX activities in hypothyroid (A), normothyroid (B) and hyperthyroid (C) rats. Each point represents the mean ± SD for eight rats.

hypolipidaemic agents resulted on the one hand in a decrease in the induction of peroxisomal activities, but on the other hand in a potential inductive effect of fibrate on bilirubin UDPGT and ω-LAH activity, suggesting the existence of different mechanisms of regulation. While ω-LAH induction should initiate peroxisome proliferation via dicarboxylic acid formation, according to Sharma *et al.* [8], the decrease of the effect of CIP towards peroxisome proliferation marker enzymes activities was not found to be associated in hyperthyroid rats with a similar decrease of this effect upon ω-LAH activity. This suggests that thyroid hormones do not interfere with CIP effects through Sharma's mechanism. The increase in AOX mRNA levels produced by LT_3 in normal rats accounts for a regulation of the expression of the peroxisomal AOX gene by thyroid hormones. The apparent divergence between the effects produced by hypothyroidism in CIP-treated rats on the level of AOX mRNA and on the level of the activity of the enzyme could be explained by the existence of different levels of regulation; a decrease in the stability of specific nuclear mRNA or negative regulation of the peroxisome proliferator associated receptor (recently cloned by Green *et al.* [35, 36]) in the presence of thyroid hormone can be suggested. Moreover, our results do not exclude the possibility of inhibition of the catabolic activity towards AOX by factors determined by the thyroid state of the animal. Thus, further studies of some eventual thyromimetic effects upon such receptors are now required.

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