

**CLOFIBRATE INCREASES HEPATIC TRIIODOTHYRONINE (T_3)-
AND THYROXINE (T_4)-GLUCURONOSYLTRANSFERASE
ACTIVITIES AND LOWERS PLASMA T_3 AND T_4 CONCENTRATIONS
IN PIGS**

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ABBREVIATIONS: ACO, acyl CoA oxidase; BCA, bicinchoninic acid; CPT-1, carnitine palmitoyl transferase-1; CYP, cytochrome 450; DTT, dithiothreitol; GAPDH, glycerinaldehyde-3-phosphate dehydrogenase; RT-PCR, reverse phase-polymerase chain reaction; pNP, p-nitrophenol; PTU, 6-propyl-2-thiouracil; PPAR α , peroxisome proliferator-activated receptor α ; TSH, thyroid stimulating hormone; UDPGA, uridine diphosphate-glucuronic acid; UGT, uridine diphosphate-glucuronosyltransferase; T₃, 3,3',5-triodothyronine; T₄, thyroxine.

ABSTRACT:

In rats clofibrate acts as a microsomal enzyme inducer and disrupts the metabolism of thyroid hormones by increasing hepatic glucuronidation of thyroxine. Whether similar effects occur in the pig has not yet been investigated. This study was performed to investigate the effect of clofibrate treatment on metabolism of thyroid hormones in pigs. To this end, an experiment with 18 pigs was performed which were assigned to two groups. One group received a control diet, the other group was fed the same diet supplemented with 5 g clofibrate/kg, for 28 days. Pigs treated with clofibrate had higher hepatic activities of T₃- and T₄-uridine diphosphate-glucuronosyltransferases (UGTs) and lower concentrations of total and free T₄ and total T₃ in plasma than control pigs ($P<0.05$). Weights and histology of the thyroid gland (epithelial height, follicle lumen diameter) did not differ between the two groups, but pigs treated with clofibrate had higher mRNA concentrations of various genes in the thyroid responsive to thyroid-stimulating hormone (TSH) such as TSH receptor, sodium iodine symporter, thyroid peroxidase and cathepsin B than control pigs ($P<0.05$). Pigs treated with clofibrate also had lower hepatic mRNA concentrations of proteins involved in plasma thyroid hormone transport [thyroxine-binding globulin ($P<0.10$), transthyretin ($P<0.05$), albumin ($P<0.05$)] and thyroid hormone receptor α_1 ($P<0.05$) than control pigs. In conclusion, this study shows that clofibrate treatment induces a strong activation of T₃- and T₄-UGTs in pigs, leading to increased glucuronidation and markedly reduced plasma concentrations of these hormones, accompanied by a moderate stimulation of thyroid function.

Introduction

Fibrates are synthetic agonists of peroxisome proliferator-activated receptor- α (PPAR α), a nuclear receptor also activated by natural ligands like free fatty acids or some eicosanoids. Activation of PPAR α leads to up-regulation of transcription of several genes involved mainly in mitochondrial and peroxisomal β -oxidation, ketogenesis and gluconeogenesis (Mandard et al., 2004). Fibrates have been in clinical use as hypolipidemic agents for several decades. Several studies in rodents and cell culture systems have shown that fibrates, like many other drugs (e.g. phenobarbital, 3-methylcholanthrene, polychlorinated biphenyl, tetrachlorobiphenyl, pregnenolone-16 α -carbonitrile or dexamethasone), induce uridine diphosphate-glucuronosyl-transferases (UGTs) (Beestra et al., 1991; Saito et al., 1991; Barter and Klaassen, 1992a, 1992b, 1994; Visser et al., 1993a, 1993b; Viollon-Abadie et al., 2000; Jemnitz et al., 2000; Vansell and Klaassen, 2002). UGTs, consisting of UGT1 and UGT2 isoforms, are localized in the endoplasmatic reticulum of hepatocytes and extrahepatic tissue and belong to the enzymes of phase II metabolism. With broad and overlapping substrate specificities, the UGT isoenzymes catalyze the glucuronidation of differential functional groups, using UDP-glucuronic acid as the cofactor (Miners and Mackenzie, 1991; Mackenzie et al., 1997). Thyroid hormones, thyroxine (T₄) and triiodothyronine (T₃), are substrates of hepatic UGTs, and glucuronidation of these hormones is the main metabolic pathway for deactivating them (Jemnitz et al., 2000). In rats several of the drugs acting as inducers of microsomal enzymes have been shown to produce hypertrophy and hyperplasia of thyroid follicular cells, most probably through increased deactivation of thyroid hormones by UGTs, leading to a reduction of serum T₄ and possibly T₃ (Beestra et al. 1991; Saito et al. 1991; Barter and Klaassen 1992a, 1994). In mice, in contrast to rats, clofibrate treatment did not alter T₃- and T₄-UGT activities and plasma concentrations of thyroid hormones (Viollon-Abadie et al. 1999). These studies show species-specific differences in the effects of clofibrate on hepatic thyroid hormone metabolism, i.e. glucuronidation of thyroid hormones.

In rodents, PPAR α agonists not only induce many genes involved in various metabolic pathways such as β -oxidation, ketogenesis and gluconeogenesis but also cause severe peroxisome proliferation in the liver, hepatomegaly and hepatocarcinogenesis (Peters et al., 2005). In contrast to rodents, PPAR α agonists do not induce peroxisome proliferation or tumor in the liver of many other species, such as guinea pigs, swine, monkeys and humans, although they retain a hypotriglyceridemic effect in these species (Holden and Tugwood, 1999). In non-proliferating species, expression of PPAR α in the liver is much lower and the response of many genes to PPAR α activation is weaker than in proliferating species (Cheon et al., 2005). It is known that PPAR α activation can modulate metabolizing enzymes of phase I and II biotransformation (Rushmore and Kong, 2002; Zhou et al., 2005). Moreover, it has been shown that some UGT isoforms (UGT1A9, UGT2B4) are PPAR α target genes (Barbier et al., 2003a; 2003b). Therefore, non-proliferating species could respond differently from proliferating species to clofibrate with respect to induction of UGTs, i.e. UGTs involved in glucuronidation of thyroid hormones. To our knowledge, the effect of clofibrate on the hepatic thyroid hormone metabolism has not yet been investigated *in vivo* in a non-proliferating species.

The aim of our study was to investigate the effects of clofibrate treatment on hepatic thyroid hormone metabolism, i.e. activities of T₃- and T₄-UGTs in pigs, representing a non-proliferating species. Therefore, as well administration determining hepatic activities of T₃- and T₄-UGTs, we also measured plasma concentrations of thyroid hormones, thyroid weights, thyroidal epithelial cell height and follicle lumen diameter and gene expression levels of several thyroidal genes involved in thyroid hormone biosynthesis (TSH receptor, sodium iodide symporter, thyroid peroxidase, dual oxidase 2, thyroglobulin, cathepsin B and type-II iodothyronine deiodinase). We also investigated the effect of clofibrate on mRNA expression of genes involved in thyroid hormone transport (transthyretin, thyroxine-binding globulin and

albumin), peripheral conversion of thyroid hormones (type-I iodothyronine deiodinase) and thyroid hormone signaling (thyroid hormone receptor α_1) in the liver.

Materials and Methods

Chemicals. Bilirubin, Brij 56, clofibrate, dithiothreitol (DTT), p-nitrophenol (pNP), 6-propyl-2-thiouracil (PTU), cholic acid, T₃, T₄, Triton X-100 and UDP-glucuronic acid (UDPGA) were obtained from Sigma (Deisenhofen, Germany), [¹²⁵I]-T₃ (3076 μ Ci/ μ g) and [¹²⁵I]-T₄ (1500 μ Ci/ μ g) were obtained from Amersham Biosciences (Freiburg, Germany) and bicinchoninic acid (BCA) protein assay reagent from Interchim (Montelucon, France).

Animals and treatments. Eighteen male 8-week-old crossbred [(German Landrace x Large White) x Pietrain] pigs, bred in the local animal facility, were used. They weighed between 11.0 and 13.5 kg. They were individually housed in a room maintained at 23°C and 50 to 60% relative humidity with light from 0600 to 1800 h. On the day before the start of the experimental feeding period all pigs were weighed and assigned to two groups with body weights of 12.0 ± 1.1 (SD) kg (control group) and 11.9 ± 0.6 (SD) kg (treatment group). Both groups of pigs received a nutritionally adequate diet (National Research Council, 1998) for growing pigs containing (in g/kg) wheat (400), soybean meal (230), wheat bran (150), barley (100), sunflower oil (90) and mineral premix including L-lysine, DL-methionine and L-threonine (30). This diet contained 14.4 MJ metabolisable energy and 185 g crude protein per kg. The diet of the treatment group was supplemented with 5 g clofibrate per kg. To standardize feed intake each pig within the experiment received 700 g of the diet daily, which was completely consumed by all animals in the experiment. The clofibrate dosage in the treated pigs was 220 mg/kg body weight/day. The pigs had free access to water via nipple drinking systems. The experimental diets were administered for 28 days. All experimental procedures described followed established guidelines for the care and use of laboratory animals and were approved by the local veterinary office.

Sample collection. After completion of the feeding period the animals were killed under a light anesthesia. Blood was collected into heparinized polyethylene tubes. Liver and thyroid gland were dissected and weighed. Plasma was obtained by centrifugation of the blood (1,100 x g; 10 min). All samples were stored at -80°C pending analysis.

Total RNA preparation and cDNA synthesis. Total RNA from liver and thyroid tissue was isolated by TRIzol reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer's protocol, resuspended in diethyl pyrocarbonate-treated water and stored at -80°C until use. The concentration of total RNA was determined by ultraviolet absorbance at 260 nm. The quality of all RNA samples was assessed by agarose gel electrophoresis. cDNA was prepared from total RNA (1.2 µg) by reverse transcription using M-MuLV Reverse Transcriptase (MBI Fermentas, St. Leon-Rot, Germany) and oligo(dT)₁₈ primers (Operon, Cologne, Germany).

Semiquantitative polymerase chain reaction (PCR). Expression analysis for semiquantitative reverse transcriptase-PCR was normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal standard. cDNA templates (2 µl) were used in a final volume of 20 µl containing 0.2 µM of the corresponding primers (Roth, Karlsruhe, Germany) (see Table 1), 1.5 mM magnesium chloride, 1 x PCR buffer, 1 U Taq polymerase (Gene Craft, Luedinghausen, Germany) and 0.2 mM deoxyribonucleoside triphosphates (Roth, Karlsruhe, Germany). Each PCR cycle comprised denaturation for 30 s at 94°C, annealing for 30 s at 60-64°C (see Table 1) and elongation for 1 min at 72°C, followed by a final extension period for 10 min at 72°C. Number of cycles for each primer pair was tested before. Cycle number was as follows: Genes of thyroid gland: GAPDH, 23; sodium iodide symporter, 25; dual oxidase 2, 42; cathepsin B, 35; TSH receptor, 28; type-II iodothyronine deiodinase, 40; thyroglobulin, 33; hepatic genes: GAPDH, 32; acyl CoA oxidase (ACO), 32; carnitine palmitoyl transferase-1 (CPT-1), 32; albumin, 20; thyroid hormone receptor α₁, 35; thyroxine-binding globulin, 42; transthyretin, 30; type-I iodothyronine deiodinase, 40. A water control was

included in all PCR reactions for detection of contamination and dilutions of the isolated total RNA corresponding to the cDNA synthesis were used as template in order to exclude impurities due to genomic DNA. A volume of 10 µl per PCR reaction was submitted to agarose gel electrophoresis (1.5%). Ethidium bromide stained gels were digitized for quantification (apparatus and software from Syngene, Cambridge, UK).

Preparation of hepatic microsomes. One g of liver was homogenized in a medium (10 ml) containing 0.25 M sucrose and 0.1 M phosphate buffer (pH 7.4) using a Potter-Elvehjem homogenizer. Homogenates were centrifuged at 1,000 x g for 10 min at 4°C, and the supernatant was centrifuged at 15,000 x g for 15 min. The microsomal pellet was obtained by centrifugation of the 15,000 g supernatant at 105,000 g for 60 min. Microsomal pellets were suspended in the homogenization medium and the protein concentration was determined with the BCA reagent according to the supplier's protocol using bovine serum albumin as standard.

Enzyme assays. The activity of pNP-UGT in hepatic microsomes was assayed by the method of Thurman et al. (1981). The assay mixture consisted of 50 mM potassium phosphate buffer (pH 7.4), 0.2 mg bovine serum albumin/ml, 1 mM magnesium chloride, 0.5 mM DTT, 0.5 mg Triton X-100/ml, 0.2 mM pNP, 0.2 mM UDPGA and 0.25 mg microsomal protein/ml. The final volume of the assay was 200 µl. A blank was incubated without UDPGA. The reaction was stopped after 30 min at 37°C by addition of 1 ml 0.1 M sodium hydroxide solution. The pNP glucuronidation was quantified by measuring the decrease of absorbance at 400 nm. The concentration of pNP glucuronide was calculated using a molar extinction coefficient of 18,300 cm⁻¹.

Activity of bilirubin-UGT in hepatic microsomes was measured in an assay mixture containing 0.1 M Tris-hydrochloride (pH 7.8), 0.1 mM bilirubin and 5 mM UDPGA in a final volume of 200 µl. Suspended microsomes were preincubated with 20 mg/ml sodium cholate (1:1, v/v) for 10 min at 4°C. The reaction was started by addition of 1 mg microsomal

protein/ml for 60 min at 37°C. The amount of bilirubin glucuronide formed during the incubation was quantified with a commercial kit (DiaSys Diagnostic Systems, Holzheim, Germany).

T₃- and T₄-UGT activities were determined in separate assays using a modified version of the method of Beetstra et al. (1991) by incubating 1µM T₃ or T₄, respectively, and 0.1 µCi of ¹²⁵I-labeled T₃ or T₄ in reaction mixture containing 75 mM Tris-hydrochloride (pH 7.8), 7.5 mM magnesium chloride, 0.25 mg Brij 56/ml, 5 mM UDPGA and 1 mM PTU. The final volume of the assay was 200 µl. Reactions were started by adding 0.5 mg microsomal protein/ml at 37°C. Blanks were performed in the absence of UDPGA. After 30 min reactions were terminated by addition of 200 µl ice-cold methanol and the mixtures were centrifuged at 3,500 x g for 8 min. 50 µl of the supernatants were injected into a high performance liquid chromatograph for separation of T₃ or T₄ glucuronides formed during the incubation by a modified version of the method of Jemnitz et al. (1999). The high performance liquid chromatography equipment consisted of a 1100 series pump (isocratic), an autosampler, a LiChrospher 100 RP 18e column (125 mm x 4 mm, 5 µm particle size) with matching guard column (4 x 4 mm, Agilent Technologies, Waldbronn, Germany). The mobile phase consisted of 50 mM potassium dihydrogen phosphate and methanol (43:57, v/v, pH 7.0). For separation of T₃-glucuronide, the flow rate was 0.8 ml/min. For separation of T₄-glucuronide, the flow rate was 1.25 ml/min. Fractions containing T₃- or T₄-glucuronide, respectively, were collected with a fraction collector 203 (Gilson International, Bad Camberg, Germany). The radioactivity of the fractions was counted to calculate T₃- and T₄-UGT activities.

Histology of thyroid gland. Samples of thyroid glands were fixed by immersion in 10% neutral buffered formalin, processed for embedding into paraffin wax and cut into 4 µm sections. For light microscopy the sections were stained with hemalum and eosin. The epithelial cell height was measured using 4 cells per follicle in 100 follicles of each thyroid. The lumen diameter was measured in 10 sections for 10 follicles per section of each thyroid.

All pictures were digitized and the parameters measured using the Lucia G (Nikon, Düsseldorf, Germany) software (release 4.81).

Analysis of plasma hormones. The plasma concentrations of free and total T₄ and total T₃ were measured with radioimmunoassay kits (MP Biomedicals, Eschwege, Germany).

Statistics. The results were analyzed using Minitab (State College, Pa, USA) statistical software (release 13). Statistical significance of differences between control group and treatment group was evaluated using Student's t-test. Mean values were considered significantly different for $p < 0.05$.

Results

Initial and final body weights after an experimental period of 28 days were similar in both groups of pigs (Table 2). Animals treated with clofibrate had heavier livers ($p < 0.05$) and higher concentrations of microsomal protein in the liver ($p < 0.05$) than control pigs (Table 2). Relative hepatic mRNA concentration of the PPAR α target genes ACO and CPT-1 were higher ($p < 0.05$) in pigs treated with clofibrate than in control pigs (ACO: 1.39 ± 0.27 vs. 1.00 ± 0.35 ; CPT-1: 1.60 ± 0.13 vs. 1.00 ± 0.12 ; means \pm SD, $n = 9$ for each group). Moreover, concentrations of total and free T₄ and total T₃ in plasma were markedly lower in pigs treated with clofibrate than in control pigs ($p < 0.05$) while the T₄/T₃ ratio did not differ between both groups of pigs (Table 2).

Pigs treated with clofibrate had a higher activity of bilirubin-UGT in the liver than control pigs (1.08 ± 0.05 vs. 0.44 ± 0.02 nmol · min⁻¹ · mg⁻¹; means \pm SD, $n = 9$ for each group; $p < 0.05$). The activity of hepatic pNP-UGT was lower in pigs treated with clofibrate than in control pigs (44 ± 5 vs. 70 ± 8 nmol · min⁻¹ · mg⁻¹; means \pm SD, $n = 9$ for each group; $p < 0.05$). Activities of hepatic T₃- and T₄-UGTs were higher in pigs treated with clofibrate than in control pigs ($p < 0.05$, Figure 1).

Weights of thyroids, diameter of follicle lumen and thyroid epithelial cell height did not differ between both groups of pigs (Table 3). Relative mRNA concentrations of TSH receptor, sodium iodide symporter, thyroid peroxidase and cathepsin B were higher in thyroids of pigs treated with clofibrate than in thyroids of control pigs ($p < 0.05$, Figure 2). mRNA concentration of type-II iodothyronine deiodinase was lower in thyroids of pigs treated with clofibrate than in thyroids of control pigs ($p < 0.05$); those of dual oxidase 2 and thyroglobulin did not differ between both groups of pigs (Figure 2).

Pigs treated with clofibrate had lower hepatic mRNA concentrations of transthyretin, albumin and thyroid hormone receptor α_1 than control pigs ($p < 0.05$; Table 4). Hepatic mRNA concentration of thyroxine-binding globulin tended to be lower in pigs treated with clofibrate compared to control pigs ($p < 0.10$), while hepatic mRNA concentration of type-I iodothyronine deiodinase did not differ between both groups of pigs.

Discussion

To our knowledge, this is the first study to investigate the effect of clofibrate on the hepatic metabolism of thyroid hormones in the pig. It is well known that pigs are a non-proliferating species, meaning that treatment with PPAR α agonist cause no or only weak peroxisome proliferation in the liver. Interestingly, in this study clofibrate treatment caused a significant increase in liver weights of pigs by about 15%. This is in disagreement with a recent study in which pigs did not show significantly increased liver weights after a 1-week treatment with a dose of clofibrate similar to those used in our study (Cheon et al., 2005). The difference in these results could be due to the longer treatment period in our study compared with that in the study of Cheon et al. (2005). Moreover, we observed a moderate up-regulation of the PPAR α target genes ACO and CPT-1 in the liver of pigs treated with clofibrate which indicates that clofibrate treatment caused PPAR α activation in these pigs. Nevertheless, increases in liver weights and hepatic ACO and CPT-1 mRNA concentration were much

DMD #11379

lower than those observed in rodents treated with clofibrate. In rats and mice, feeding PPAR α agonists increases liver weights by 50% or more and mRNA concentrations of ACO 5- to 10-fold compared with untreated controls (Kawashima et al., 1990; He et al., 2002; Li et al., 2004; Frederiksen et al., 2004).

Several families of UGT enzymes are expressed in the liver. To study the effect of clofibrate treatment on the induction of microsomal enzymes, we determined the activities of bilirubin- and pNP-UGTs. The finding that clofibrate treatment strongly increases bilirubin-UGT is in accordance with studies in rats and mice (Visser et al., 1993a; Viollon-Abadie et al., 1999). The finding that clofibrate reduces the activity of pNP-UGT is also in accordance with a study in which clofibrate significantly reduced the activity of pNP-UGT in the liver of Wistar rats (Visser et al., 1993a). These observations suggest that clofibrate stimulated the microsomal enzyme system in pigs in a similar way as in rats. This study also shows that clofibrate treatment strongly increases the activity of T₃- and T₄-UGT in the liver, which in turn leads to a dramatic reduction of plasma T₃ and T₄ concentrations. Increased activity of T₄-UGT had previously been observed in Wistar rats but not in mice treated with clofibrate (Visser et al., 1993a; Viollon-Abadie et al., 1999). The increased activity of T₃-UGT in pigs treated with clofibrate, however, is in strong contrast to rats, where clofibrate treatment did not increase T₃-UGT activity (Visser et al., 1993a).

In rats T₄ is accepted as a substrate by hepatic bilirubin-UGTs (UGT1A1) and phenol-UGTs (UGT1A6) and it was shown that increased activities of these enzymes were associated with increased glucuronidation of T₄ in the liver (Beetstra et al., 1991; Magdalou et al., 1993; Visser et al., 1993a, 1993b; Viollon-Abadie et al., 2000; Vansell and Klaassen, 2002). The enzymes involved in glucuronidation of thyroid hormones in pigs have not yet been identified. The fact that activities of both bilirubin- and T₄-UGTs were increased suggests that in pig liver T₄ was also glucuronidated by bilirubin-UGTs, as happens in rats. It is probable that enzymes other than UGT1A1 and UGT1A6 can also be induced by clofibrate in

rats and are involved in T₄ conjugation (Jemnitz et al., 2000). In rat liver glucuronidation of T₃, unlike glucuronidation of T₄, is catalyzed by androsterone-UGTs (Beetstra et al., 1991; Visser et al., 1993b). The increased T₃-UGT activity in pigs treated with clofibrate could therefore also have been due to an increased activity of androsterone-UGT, although this was not assayed in this study. UGTs in pig liver have been less extensively investigated and have not yet been phenotyped. It therefore remains unknown which specific UGTs were responsive for the increased glucuronidation of T₃ and T₄ in pigs treated with clofibrate.

It has been shown that activation of PPAR α leads to transcriptional up-regulation of the cytochrome P450 (CYP) 4A genes, which are also constituents of the microsomal biotransformation system in both proliferating and non-proliferating species (Lawrence et al., 2001; Cheon et al., 2005). It has further been shown that some UGT isoforms (UGT1A9, UGT2B4) are PPAR α target genes (Barbier et al., 2003a, b). PPAR α is naturally activated during fasting and it was shown by Visser et al. (1996) that food restriction resulted in increased bilirubin and thyroid hormone UGT activities in rats. These findings suggest that UGTs catalyzing the glucuronidation of thyroid hormones may be transcriptionally up-regulated by activation of PPAR α . It is well known that expression of PPAR α in the liver is much lower in non-proliferating species and that the response of many genes to PPAR α activation is weaker than in proliferating species. This is also true for up-regulation of microsomal CYP 4A genes by treatment with PPAR α agonists (Lawrence et al., 2001; Cheon et al., 2005). If PPAR α plays a crucial role in the activation of UGTs catalyzing thyroid hormone glucuronidation the effect of clofibrate on up-regulation of these enzymes in pigs would be expected to be much lower than in proliferating species such as rats or mice. But activation of T₃- and T₄-UGTs by clofibrate was even stronger in pigs than reported for rats or mice (Visser et al., 1993a; Viollon-Abadie et al., 1999). These findings suggest that activation of PPAR α does not play a key role in clofibrate-induced up-regulation of thyroid hormone

UGTs. Nevertheless, the role of PPAR α in the regulation of thyroid hormone glucuronidation should be further investigated.

The increased activities of T₃- and T₄-UGTs make it highly probable that the markedly reduced plasma concentrations of T₃ and T₄ in pigs treated with clofibrate are mainly due to increased glucuronidation of these hormones in the liver. As most T₃ is generated in peripheral tissues, mainly the liver, by deiodination of T₄, a reduced T₃ concentration could potentially be due to an inhibition of type-I iodothyronine deiodinase. Indeed, in the study of Visser et al. (1993a), clofibrate treatment of rats reduced the activity of that enzyme, which might be responsible for the reduced concentration of T₃ observed in their study. We did not determine the activity but only the mRNA concentration of that enzyme in the liver, which was not influenced by clofibrate treatment. Interestingly, in contrast to hepatic type-I iodothyronine deiodinase, type-II deiodinase in the thyroid showed a reduced mRNA concentration in pigs treated with clofibrate compared with control pigs. A reduced activity of type-II deiodinase, which converts T₄ to T₃ in the thyroid, might play some role in the reduced T₃ concentration in plasma. However, as the thyroid produces less than 20% of total T₃ (Findlay et al., 2000), a reduced activity of type-II deiodinase most probably plays a minor role in the reduced plasma concentration of T₃. The reduction of plasma concentrations of total T₃ (by 47% vs. control), free T₄ (by 32% vs. control) and total T₄ (by 35%) concentrations by clofibrate are also stronger than those observed in Wistar rats. In Wistar rats a dose of 800 mg clofibrate/kg body weight/day reduced plasma T₃ concentration by 27% but did not reduce plasma total and free T₄ (Visser et al., 1993a). In mice a dose of 300 mg clofibrate/kg body weight/day reduced plasma free T₄ concentration by 13% but did not significantly reduce plasma concentration of free T₃ (Viollon-Abadie et al., 1999). It is clear that different studies cannot be directly compared with each other but these data nevertheless suggest that pigs could be even more sensitive to disruptions of thyroid hormone metabolism by clofibrate than rodents.

Reduced plasma concentrations of T₃ and T₄ are expected to increase the release of TSH from the pituitary gland. It has indeed been shown that microsomal enzyme inducers elevate TSH plasma concentrations in rodents, which in turn stimulates proliferation of epithelial cells in thyroid tissue as a result of increased glucuronidation of thyroid hormones (e.g. Curran and DeGroot, 1991; DeSandro et al., 1991; Saito et al., 1991; Liu et al., 1995). As no assay was available for measuring TSH concentration in plasma of the pigs, we determined mRNA concentrations of various genes in the thyroid which are responsive to TSH treatment. The finding that mRNA concentrations of TSH receptor, sodium iodide symporter, thyroid peroxidase and cathepsin B, all genes responsive to TSH, were moderately increased by 40 to 70% suggests that the thyroid was stimulated by the increased plasma concentration of TSH. This suggestion is confirmed by a study which demonstrated that TSH plasma concentrations are increased by microsomal enzyme inducers which stimulate the glucuronidation of T₃ (Klaassen and Hood, 2001). Our study further shows that expression levels of dual oxidase 2, an hydrogen peroxide-generating system which constitutes the rate-limiting step of thyroid hormone synthesis, and of thyroglobulin, a protein involved in thyroid hormone synthesis and storage, are not altered by clofibrate treatment.

The finding that thyroid weights, epithelial cells and follicle lumen diameter were not increased by clofibrate was unexpected and suggests that stimulation of the thyroid was moderate, only increasing gene expression of TSH-responsive genes in the thyroid while histological alterations, i.e. increased epithelial cell height, may take longer than four weeks to become evident.

The action of thyroid hormones like T₃ is mediated by thyroid hormone receptors which belong to the family of nuclear hormone receptors. The present study shows that clofibrate treatment reduces gene expression of thyroid hormone receptor α_1 in the liver of rats. This finding agrees with a recent study in which bezafibrate down-regulated thyroid hormone receptors in rat liver (Bonilla et al., 2001). That study further showed that down-

regulation of thyroid hormone receptors was due to activation of PPAR α . It is therefore likely that in our study down-regulation of thyroid hormone receptor α_1 in pigs treated with PPAR α was also due to PPAR α activation by clofibrate. Down-regulation of thyroid hormone receptor implies that the biological activity of T₃ might have been reduced in pigs treated with clofibrate.

Thyroxine-binding globulin, transthyretin and albumin are the major plasma transport proteins in pigs (Janssen et al., 2002). These proteins are synthesized in the liver. We found that in our study gene expression of these proteins in the liver was reduced by clofibrate treatment of pigs. In studies by Motojima et al. (1992, 1997) the same effect of clofibrate on expression of transthyretin was observed in rats and several mouse strains, while there was no effect in PPAR α -null mice. This suggests that down-regulation of transthyretin expression was induced by PPAR α activation. Consequently, down-regulation of transthyretin and possibly also of thyroxine-binding globulin and albumin could be the result of PPAR α activation by clofibrate. Our data suggest, although we did not measure concentrations of these proteins in blood, that clofibrate treatment lowers not only concentrations of thyroid hormones in plasma but could also reduce the transport capacity for thyroid hormones.

In conclusion, this study shows for the first time that clofibrate treatment induces a strong activation of T₃- and T₄-UGTs in pigs, leading to increased glucuronidation and markedly reduced plasma concentrations of these hormones. These alterations were accompanied by moderately increased mRNA concentrations of various TSH-responsive enzymes in the thyroid gland, reduced hepatic mRNA concentrations of proteins involved in thyroid hormone transport and thyroid hormone receptors. As the pig represents a species that does not respond with peroxisome proliferation to treatment with PPAR α agonists the study shows that clofibrate treatment also disrupts the metabolism of thyroid hormones in non-proliferating species.

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DMD #11379

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DMD #11379

Footnote:

There are no footnotes

DMD #11379

Legends for Figures

FIG. 1. Activities of triiodothyronine (T_3)- and thyroxine (T_4)-uridine diphosphate glucuronosyltransferases (UGTs) in the liver of pigs fed a control diet or a diet supplemented with 5 g clofibrate per kg for 28 days. Data are reported as means \pm SD with nine animals per group. * Significantly different to control group ($p < 0.05$).

FIG. 2. Relative mRNA concentrations of TSH receptor (TSHR), sodium iodide symporter (NIS), dual oxidase 2 (DUOX2), thyroid peroxidase (TPO), thyroglobulin (TG), cathepsin B (Cat B) and type-II iodothyronine deiodinase (ID-II) in the thyroid of pigs fed a control diet or a diet supplemented with 5 g clofibrate per kg for 28 days. All mRNA concentrations were determined by semiquantitative RT-PCR and normalized by GAPDH. Data are reported as means \pm SD with nine animals per group. Data are expressed relative to mRNA concentrations of control pigs (control = 1). * Significantly different to control group ($p < 0.05$).

TABLE 1

Sequences of primers used for semiquantitative RT-PCR

Gene (NCBI GenBank)	Forward Primer	Reverse Primer	Size, bp/Annealing temperature
Acyl CoA oxidase (AF185048)	CTC GCA GAC CCA GAT GAA AT	TCC AAG CCT CGA AGA TGA GT	218/60°C
Albumin (X12422)	GCA CGA GAA GAC ACC AGT GA	CGA GTG CAG TTT GCT TCT TG	200/62°C
Carnitine palmitoyl transferase-I (AF288789)	GCA TTT GTC CCA TCT TTC GT	GCA CTG GTC CTT CTG GGA TA	198/60°C
Cathepsin B (AJ315560)	GGC CTC TAT GAC TCG CAT GT	GCA AGT TCC CCT CAA GTC TG	198/60°C
Dual oxidase 2 (AF547267)	GAC CCA GCG GCA GTT TGA ATG G	AGG GCC GCA GCT GAA CAC TCC	295/64°C
Glycerinaldehyde-3-phosphate dehydrogenase (AF017079)	AGG GGC TCT CCA GAA CAT CAT CC	TCG CGT GCT CTT GCT GGG GTT GG	446/60°C
Sodium iodide symporter (AJ487855)	AGT CAT CAG CGG CCC CCT CCT C	ACC GAT GCC GTC TGC CGT GTG	456/60°C
Thyroglobulin (AF165610)	CAG TAA GGG CTT CCG TCT TG	GGA GCT GCA CTG AGG AAT GT	198/60°C
Thyroid hormone receptor α_1 (AJ005797)	CCA GAT GGA AAG CGA AAA AG	TGG GAT GGA GAT TCT TCT GG	199/60°C
Thyroid peroxidase (X04645)	CTG GGC GCC GTG CTC GTC TG	ACG CGG GTG GCA TCT GAC TCT GAC	287/65°C
Thyroxine-binding globulin (NM214058)	GTG GCT TCT TGG GCA TGT AT	GAA CCT CCG GTA CAG GTT GA	206/62°C

Continued

TABLE 1, continued

Transthyretin (X87846)	ATG GTC AAA GTC CTG GAT GC	TGC CTT CCA GTA GGA TTT GG	207/60°C
TSH receptor (NM214297)	GCC TGC CCA TGG ACA CTG AGA C	CTG ACC CCG GTA TGC CTG AGC	422/60°C
Type-I iodothyronine deiodinase (AY533206)	CTC TGG GTG CTC TTT CAG GT	ATC GGA CCT TCA GCA CAA AC	199/62°C
Type-II iodothyronine deiodinase (NM001001626)	CTC GGT CAT TCT CCT CAA GC	TGC TTC CTT CAG GAT TGG AG	200/60°C

DMD #11379

TABLE 2

Body and liver weights, microsomal protein in the liver and plasma thyroid hormone concentrations in pigs fed a control diet or a diet supplemented with 5 g clofibrate per kg for 28 days

Data are reported as means \pm SD with nine animals per group

	Control	Clofibrate
Initial body weight (kg)	12.0 \pm 1.1	11.9 \pm 0.6
Final body weight (kg)	26.0 \pm 1.5	25.2 \pm 1.2
Liver weight (g)	673 \pm 63	779 \pm 63*
Hepatic microsomal proteins (mg/g liver)	12.4 \pm 1.8	15.4 \pm 2.4*
Plasma thyroid hormones		
Total thyroxine (T ₄ , nmol/l)	45.2 \pm 13.1	29.6 \pm 7.1*
Free thyroxine (pmol/l)	12.6 \pm 3.7	8.6 \pm 2.2*
Total triiodothyronine (T ₃ , nmol/l)	1.20 \pm 0.52	0.64 \pm 0.11*
T ₄ :T ₃ ratio	41.1 \pm 13.2	40.3 \pm 10.7

* $p < 0.05$ compared to control group

DMD #11379

TABLE 3

Thyroid weight, follicle lumen diameter and epithelial cell height in thyroid of pigs fed a control diet or a diet supplemented with 5 g clofibrate per kg for 28 days

Data are reported as means \pm SD with nine animals per group

	Control	Clofibrate
Thyroid weight (g)	2.17 \pm 0.19	2.39 \pm 0.55
Follicle lumen diameter (μm)	84.9 \pm 10.9	88.8 \pm 14.2
Epithelial cell height (μm)	7.53 \pm 0.14	7.64 \pm 0.67

DMD #11379

TABLE 4

Relative hepatic mRNA concentrations of transthyretin, thyroxine-binding globuline, albumin, thyroid hormone receptor α_1 and type-I iodothyronine deiodinase in pigs fed a control diet or a diet supplemented with 5 g clofibrate per kg for 28 days

Data are reported as means \pm SD with nine animals per group. All mRNA concentrations were determined by semiquantitative RT-PCR and normalized by glycerinaldehyde-3-phosphate dehydrogenase. Data are expressed relative to mRNA concentrations of control pigs (control = 1).

	Control	Clofibrate
Transthyretin	1.00 \pm 0.14	0.87 \pm 0.12*
Thyroxine-binding globulin	1.00 \pm 0.14	0.89 \pm 0.18 [#]
Albumin	1.00 \pm 0.11	0.87 \pm 0.11*
Thyroid hormone receptor α_1	1.00 \pm 0.14	0.84 \pm 0.10*
Type-I iodothyronine deiodinase	1.00 \pm 0.07	0.98 \pm 0.07

Results are the mean \pm SD of nine animals per group

* $p < 0.05$ compared to control group

[#] $p < 0.10$ compared to control group

Figure 1

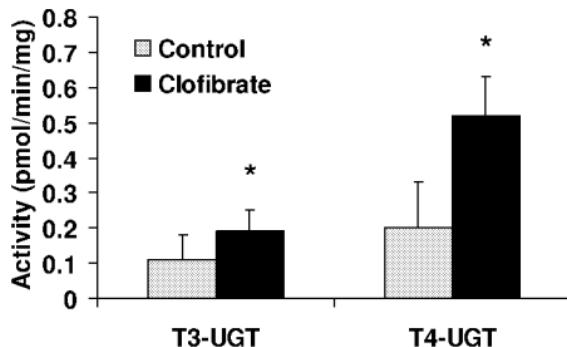


Figure 2

