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Distinct Responses of Mouse Hepatic CYP Enzymes to Corn Oil and Peroxisome Proliferators

Anneli Kojo, Pertti Pellinen,* Risto Juvonen,* Hannu Raunio,† Olavi Pelkonen† and Markku Pasanen*‡*

*Department of Pharmacology and Toxicology, University of Kuopio, P. O. Box 1627, FIN-70211 Kuopio, Finland and †Department of Pharmacology and Toxicology, University of Oulu, Kajaanintie 52 D, FIN-90220 Oulu, Finland

ABSTRACT. We studied the response of male DBA/2N mouse liver monooxygenases to acute (one-day) and subacute (7-day) exposure to clofibrate, gemfibrozil, and corn oil. The day following a single treatment with clofibrate (200 mg/kg), coumarin 7-hydroxylase (COH) activity decreased significantly (by 70%) with a concomitant decrease in the CYP2A4/5 protein and mRNA levels. The 7-day treatment schedule also decreased COH activity but only by 30%, though the levels of CYP2A4/5 protein and mRNA were still low. Treatment 1 and 7-day with clofibrate decreased 7-pentoxyresorufin O-dealkylase (PROD) activity by 40%. No changes were seen in testosterone 15α-hydroxylase (T15αOH) activity after 1 day of treatment with clofibrate but, after 7 days, it was decreased by 50%. Clofibrate treatment had no significant effects on testosterone 7α-hydroxylase (T7αOH), 7-ethoxyresorufin O-deethylase (EROD), or benzphetamine N-demethylase (BZDM) activities. Gemfibrozil (200 mg/kg) did not alter COH activity or CYP2A4/5 protein content after a single treatment, but a slight decrease was seen in the mRNA level. Treatment for 7 days significantly increased (2.5-fold) the activity and mRNA content but the amount of protein remained unchanged. Gemfibrozil enhanced (2–2.7-fold) PROD and EROD (2–2.5-fold) activities by both treatments, whereas T15αOH, T7αOH, or BZDM activities were not significantly affected. Treatment with corn oil for 7 days significantly decreased (65%) COH activity and CYP2A4/5 protein and mRNA levels. PROD (55%) and T15αOH (65%) activities were significantly decreased even after a single dose although injection for 7 days had no effect. Neither of the corn oil schedules had any marked effect on T7αOH, EROD, or BZDM activities. These results demonstrate: 1. a decrease in the expression of *CYP2A4/5* gene by clofibrate and corn oil; 2. substantial differences within the CYP2A subfamily in their responses to corn oil, clofibrate, and gemfibrozil; and 3. distinct responses of other xenobiotic metabolizing CYP subfamily enzymes to clofibrate and gemfibrozil. BIOCHEM PHARMACOL 51;9:1137–1143, 1996.

KEY WORDS. clofibrate; gemfibrozil; corn oil; coumarin 7-hydroxylase; mouse; liver

The multigene superfamily of CYP§ consists of different enzymes that metabolize xenobiotics and endogenous compounds, such as steroids, fatty acids, prostaglandins, and vitamins [1]. COH activity is catalyzed by the CYP2A5 gene product CYP2A5 in mouse liver [2]. CYP2A4 shows 98.3% homology with CYP2A5 at the amino acid level but has virtually no ability to catalyse COH. Instead, it is responsible for hydroxylation of testosterone at the 15α position [2, 3]. COH activity in human liver is mediated by the CYP2A6 enzyme and shows great interindividual variation [1, 4]. The third member of the 2A subfamily in the mouse, P4507α, catalyses T7αOH activity [5]. COH activity can be induced by a diverse set of chemicals, such as phenobarbital [6], pyrazole and its derivatives [7, 8], and several hepatotoxins [9]. COH activity can be inhibited *in vivo* and *in vitro* by some furanocoumarins and etomidate [10, 11].

Peroxisome proliferators are a heterogenous group of chemicals that induce several proteins and enzyme activities (e.g. peroxisomal fatty acid β-oxidation and the CYP4A subfamily) responsible for the ω-hydroxylation of fatty acids. They also cause cellular proliferation and hepatomegaly. Peroxisome proliferators are classified as nongenotoxic hepatocarcinogens in rats and mice [12, 13].

Because the expression of the *CYP2A4/5* gene complex is increased in malignancies [14] and after treatment with hepatotoxins in the mouse [9], we have extended our studies on peroxisome proliferators, which are known to be hepatocarcinogenic compounds in rodents. The effects of clofibrate and gemfibrozil were studied on the expression of

‡ Corresponding author. Tel. (358)-71-162 419; FAX (358)-71-162 424.

§ *Abbreviations:* CYP, cytochrome P450; COH, coumarin 7-hydroxylase; T15αOH, testosterone 15α-hydroxylase; T7αOH, testosterone 7α-hydroxylase; T16βOH, testosterone 16β-hydroxylase; EROD, 7-ethoxyresorufin O-deethylase; PROD, 7-pentoxyresorufin O-dealkylase; BZDM, benzphetamine N-demethylase. Nomenclature for the cytochrome P450 gene superfamily is as described by Nelson *et al.* (1993) [1]. Mouse *Cyp2a-4* encodes for a P450 form catalyzing testosterone 15α-hydroxylation and the *Cyp2a-5* gene product catalyzes coumarin 7-hydroxylation (the human counterpart is CYP2A6).

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CYP2A4/5. These drugs are widely used in the treatment of lipid disorders in humans. Because clofibrate and gemfibrozil can also affect fatty acid metabolism, we also studied whether or not corn oil alone has any effects on CYP2A or other monooxygenase activities.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the following sources: bicinchonic acid, 5-bromo-4-chloro-3-indolyl phosphate, clofibrate (2-(p-chlorophenoxy)-2-methylpropionic acid ethyl ester), coumarin, cytochrome c, gemfibrozil (5-2(2,5-dimethylphenoxy)2,2-dimethylpropionic acid), goat antirabbit IgG alkaline phosphatase, NADPH, and testosterone from Sigma Chem Co. (St. Louis, MO, U.S.A.); 7-ethoxyresorufin and 7-pentoxyresorufin from Pierce (Rockford, OR, U.S.A.); resorufin from Aldrich Co. (Milwaukee, WI, U.S.A.); and [4-^{14}C]-testosterone, ^{32}P-labelled nucleotides, Hyperfilm MP autoradiography films, and Hybond N+ nylon sheets from Amersham (Bucks, U.K.). All other reagents were of the highest grade commercially available.

Treatment of Animals

Male DBA/2N//KUO mice, 7–10 weeks old, were obtained from the National Laboratory Animal Center at the University of Kuopio and were housed in Macrolon cages in groups of 4–6 mice under standard laboratory conditions: 12 hr light/dark cycle, 22 ± 1°C temperature and 50–60% humidity. Standard rodent feed (Ewos, Sweden) and tap water were provided *ad lib*. Because we have previously shown that a single dose of corn oil can interfere with some mouse hepatic monooxygenases [9], we attempted to use other solvents that would not influence these parameters. However, either the solvents tested were too toxic (propylene glycol and dimethylsulphoxide) or too hypertonic (cyclodextrin), or the drugs did not dissolve in the solvent or the solvent was too basic (0.2 M glycine-NaOH, pH 10.5) for i.p. administration. Thus, we were unable to find another solvent for clofibrate and gemfibrozil and the drugs had to be dissolved in corn oil. The mice (4–6/group) were given clofibrate or gemfibrozil (200 mg/kg) dissolved in corn oil as a single i.p. injection (0.1 mL/10 g) for 1 or 7 consecutive days. Control animals for clofibrate and gemfibrozil were dosed with the same volume of corn oil. Because we also wished to compare the effects of corn oil to the effects of saline, another group received saline only. The animals were killed 24 hr after the last injection and the livers were divided into two parts. One part was quickly frozen in liquid nitrogen and stored at −80°C for the preparation of RNA. The other part was used for the preparation of microsomes[15].

Determination of Monooxygenase Activities

Microsomal protein concentrations were determined by the bichinchonic acid method [16]. Cytochrome P450 content was measured by the method of Omura and Sato [17]. COH activity was measured by the method of Aitio [18] as modified by Juvonen *et al.* [19] using 100 μM coumarin as a substrate. EROD and PROD activities were determined using the end-point method of Burke *et al.* [20], and BZDM activity according to the method described by Honkakoski and Lang [21]. Cytochrome c reductase determinations were carried out according to Strobel and Dignam [22] and T15αOH, T7αOH, and T16βOH activities according to the method of Waxman *et al.* [23].

Preparation of Total RNA and Slot Blot Analysis

The liver samples from mice representing the same treatment group were pooled and homogenized in guanidine thiocyanate. RNA was prepared by the method of Chomczynski and Sacchi [24]. RNA was denatured and bound to nylon filter with a slot blot apparatus. The cDNA probe p15α-15, size 1.5–2 kilobase pairs [3], was labelled using a Pharmacia oligolabelling kit. The hybridization to the probe (specific activity 10^9/dpm/μg DNA) was performed in 5 × SSC, 5 × Denhardt's, 0.1% (w/v) SDS and 0.1 mg/mL sonicated salmon sperm DNA containing 50% (v/v) formamide at 42°C. The filters were washed with 1 × SSC, 0.1% SDS at room temperature (3 × 20 min) and then exposed to X-ray films. All signals were normalized to 18S ribosomal RNA using a 20-mer oligonucleotide [25]. The cDNA probe p15α-15 recognizes both 2A4 and 2A5 mRNAs because the mRNAs are 98% homologous [3]. The intensities of the bands were quantitated with a Shimadzu CS-9000 dual wavelength scanner.

Western Blotting

The preparation and validation of anti-CYP2A4/5 antibody has been reported earlier [26]. Anti-CYP2A4/5 recognizes both CYP2A5 and the structurally similar enzyme, CYP2A4 [3]. Electrophoresis was performed on 9% (w/v) acrylamide gels with 20 μg of microsomal protein (pooled samples from the same treatment group) and 1 pmol of purified CYP2A4/5 as standard. The proteins were transferred to nitrocellulose sheets and processed as described previously [21]. The intensities of the bands were quantitated with a Shimadzu CS-9000 dual wavelength scanner.

Statistical Analysis

The values of enzyme activities are expressed as means (± SD, n = 4–6). Differences between groups were assessed initially with Kruskall-Wallis test and when significant differences ($P < 0.05$) were revealed, further analysis between treatment and control were determined with Mann-Whitney U-test.

RESULTS

Total cytochrome P450 content was not changed by 1 or 7 days of treatment with either clofibrate or gemfibrozil (data

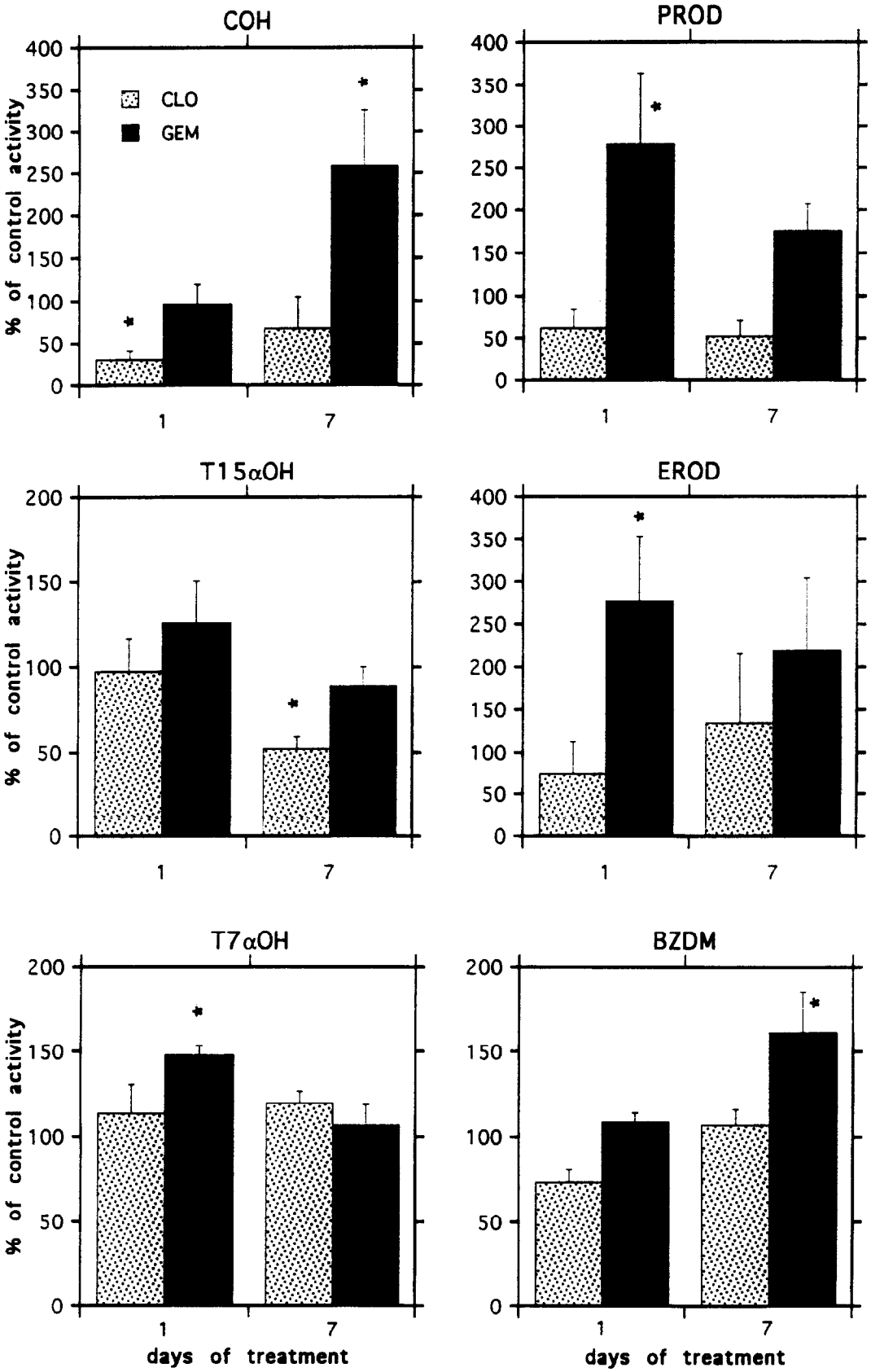

FIG. 1. Influence of 1 and 7 days' treatment with clofibrate and gemfibrozil on hepatic microsomal monooxygenase activities in male DBA/2N mice. Each bar represents mean ± SD of 4–6 animals. The values are % of residual activities from control animals receiving corn oil. Control activities (corn oil group) are shown in Table 1. Superscripts denote statistically significant differences from controls: *$P < 0.05$.

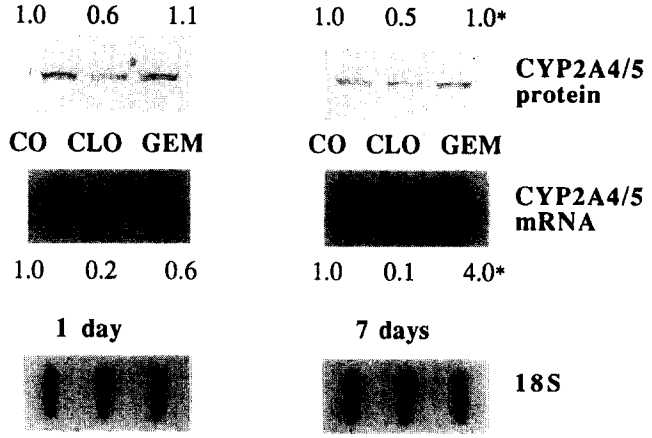

FIG. 2. The responses of CYP2A4/5 protein and mRNA to 1 and 7 days of treatment with corn oil (CO), clofibrate (CLO), and gemfibrozil (GEM). 18 S denotes a sheet reprobed with 18S probe to control for equal RNA amounts in each slot. For detection of CYP2A4/5 protein, twenty μg of microsomal protein from pooled microsomes (n = 4–6) was used. In the lower panel, each slot contains 5 μg RNA from pooled livers (n = 4–6). Serial dilutions of isolated total RNA were probed with CYP2A4/5 cDNA probe. For clarity, results from 5 μg RNA loadings are shown. *Relative levels of protein and mRNA are expressed as the ratio of the immunoblotting and hybridization signals to that of the corn oil group.

not shown). One day of treatment with clofibrate decreased COH activity by 70% (Fig. 1), accompanied by a concomitant decrease in the amounts of CYP2A4/5 protein and mRNA (Fig. 2). Treatment for 7 days had no marked effect on COH activity. The amount of CYP2A4/5 protein decreased by 50% but slot blot analysis showed a remarkable decrease (90%) in mRNA level (Figs. 1 and 2). A single dose of clofibrate did not change T15αOH activity but, after 7 daily doses, the activity of the enzyme was decreased by 50% (Fig. 1). PROD, EROD, T7αOH, and BZDM activities were not significantly changed after clofibrate treatment (Fig. 1). Cytochrome c reductase activity was increased 1.3- and 1.4-fold after 1 and 7 days of treatment with clofibrate, respectively (Table 1).

Gemfibrozil had distinct effects on mouse hepatic monooxygenases compared to those caused by clofibrate. It did not alter COH activity or the amount of CYP2A4/5 protein, but a slight decrease was seen in the mRNA level after a single dose (Figs. 1 and 2). Treatment for 7 days increased COH activity 2.5-fold with a concomitant increase in the amount of mRNA, though no changes were seen in the amount of CYP2A4/5 protein (Figs. 1 and 2). Gemfibrozil enhanced PROD and EROD activities 2.7-fold after a single dose. The activities of other enzymes were slightly elevated after gemfibrozil treatment: T7αOH activity after a single injection and BZDM activity after 7 days of treatment (Fig. 1). Neither 1 nor 7 days of treatment with gemfibrozil had any marked effects on T15αOH activity (Fig. 1). Gemfibrozil had effects similar to those of clofibrate on cytochrome c reductase activity (Table 1).

TABLE 1. The effect of 1 and 7 days of treatment with corn oil, clofibrate, and gemfibrozil on hepatic cytochrome c reductase activity in male DBA/2N mice

Treatment	1 day	7 days
Control	0.053 ± 0.006	0.058 ± 0.004
Corn oil	0.052 ± 0.002	0.059 ± 0.004
Clofibrate	0.067 ± 0.004	0.085 ± 0.009
Gemfibrozil	0.069 ± 0.001	0.081 ± 0.008

Activities are mean ± SD of 3 individuals as nmol/min/mg prot.

The comparison of the effects of corn oil and saline on mouse liver monooxygenase activities are presented in Table 2. A single dose of corn oil had no effect on COH activity but 7 days' treatment decreased this activity by 66%. Corn oil decreased CYP2A4/5 protein content by approximately 40% both after 1 and 7 days of treatment (Table 2). The mRNA content did not change after a single injection but was decreased after 7 days of treatment by 60%. There was a clear decrease (64%) in T15αOH activity after a single injection of corn oil, but this normalized during 7 days of treatment (Table 2). PROD activity was decreased by 55% after a single injection of corn oil but, once again, it returned to control levels in the 7-day schedule. No marked changes were seen in T7αOH, EROD, or BZDM activities after 1 day of treatment but after 7 daily injections of corn oil, T7αOH and BZDM activities were slightly decreased (Table 2). No changes were seen in T16βOH and cytochrome c reductase activities (Tables 1 and 2).

DISCUSSION

The present study describes the effects of 1 and 7 days of treatment with clofibrate, gemfibrozil, and corn oil on the expression of CYP2A subfamily enzymes and other monooxygenases representing CYP subfamilies 1A, 2B, and 3A in mouse liver. Clofibrate and gemfibrozil affected mouse liver xenobiotic monooxygenases in different ways, although both are peroxisome proliferators and induce hepatic CYP4A expression and peroxisomal activities in rats and mice [12].

The expression of CYP2A4/5 is increased by a diverse set of chemicals, some of which are hepatotoxic [6–9, 27–29]. Our previous studies have shown that cocaine decreases the expression of CYP2A4/5 after 5 daily injections, accompanied by inflammatory liver damage [29]. Here, we used the same dose and time of administration of clofibrate and gemfibrozil that have been reported to result in increased peroxisome proliferation [12]. Liver damage by peroxisome proliferators is seen only after long-term administration [12]. It has also been reported that peroxisome proliferation occurs after a single injection of clofibrate [30]. Here, clofibrate, when administered *in vivo*, clearly decreased the expression of CYP2A4/5. The mechanism for this suppression is unknown, but it is possible that inflammation and/or activation of peroxisome proliferator-activated receptors

TABLE 2. The effect of 1 and 7 days of treatment with corn oil on hepatic microsomal monooxygenase activities in male DBA/2N mice

	Control (1 day)	Corn oil (1 day)	%	Control (7 days)	Corn oil (7 days)	%
COH	81.19 ± 11.32	55.49 ± 21.65	68	51.97 ± 7.15	17.84 ± 4.30*	34
2A4/5 protein†	1	0.62	62	1	0.58	58
2A4/5 mRNA†	1	0.91	91	1	0.40	40
T15αOH*	0.22 ± 0.10	0.08 ± 0.02‡	36	0.12 ± 0.02	0.10 ± 0.03	83
T7αOH*	0.38 ± 0.13	0.26 ± 0.04	68	0.39 ± 0.05	0.28 ± 0.04‡	72
PROD	7.38 ± 2.27	3.29 ± 0.65§	45	3.78 ± 1.36	2.56 ± 1.23	68
EROD	74.63 ± 27.96	48.72 ± 25.98	65	72.84 ± 20.05	68.08 ± 20.99	93
BZDM*	6.44 ± 1.76	4.37 ± 0.75	68	4.21 ± 0.43	3.55 ± 0.49‡	84
T16βOH	0.17 ± 0.04	0.17 ± 0.03	99	0.14 ± 0.01	0.14 ± 0.03	98

*Activities are means ± SD of 4–6 individuals (T16βOH: $n = 3$) as pmol/mg prot × min or nmol/mg prot × min.† Relative levels of protein and mRNA are expressed as the ratio of the immunoblotting and hybridization signals to that of control group. For Western blot and slot blot analysis, the liver samples from mice representing the same treatment group were pooled. Percentages are residual activities compared to the controls. Controls vs corn oil: ‡$P < 0.05$, §$P < 0.01$.

proliferators is seen only after long-term administration [12]. It has also been reported that peroxisome proliferation occurs after a single injection of clofibrate [30]. Here, clofibrate, when administered *in vivo*, clearly decreased the expression of CYP2A4/5. The mechanism for this suppression is unknown, but it is possible that inflammation and/or activation of peroxisome proliferator-activated receptors [31] are in some way related to the expression of CYP2A4/5. Cytochrome c reductase activity was slightly increased after clofibrate treatment; thus, indicating that lack of reductase is not the rate-limiting factor in the decrease of COH activity.

T15αOH activity did not follow the decrease in COH activity seen after a single clofibrate treatment. However, a 50% decrease was seen after 7 daily doses of clofibrate. Mouse T7αOH activity, the third member of the 2A subfamily, did not respond to any of our treatments except for a slight increase after 1 day of treatment with gemfibrozil. This indicates that the expression of the two otherwise closely related P450 enzymes CYP2A4 and 2A5, and T7αOH, which has only 75% homology to CYP2A4 and 2A5 proteins [5], are regulated differently and, therefore, exhibit distinct responses to clofibrate. Salonpää *et al.* [32] have shown that more than 90% of the CYP2A4/5 mRNA in the livers of control mice was CYP2A5, and that disruption of liver function did not alter the expression profiles of CYP2A5 and CYP2A4. Thus, we conclude that changes seen in the amounts of CYP2A4/5 protein and mRNA contents are mainly of CYP2A5 origin. In rat liver, clofibrate treatment has been shown to increase several CYP activities (e.g. T16αOH, PROD mediated mainly by CYP2B, aniline hydroxylation [CYP2E], EROD [CYP1A], and T7αOH [CYP2A]) [33]. However, Sharma *et al.* [34] have reported that clofibrate can cause a decrease in rat hepatic CYP1A- and CYP2B-related activities. Our results show that, in DBA/2N mice, COH and T15αOH activities are most sensitive to inhibition by clofibrate. Thus, the induction profile of the rat differs clearly from that of the mouse.

Gemfibrozil had different effects on the expression of CYP2A4/5 compared to those caused by clofibrate: it did not have any marked effect on the expression of CYP2A4/5 after a single dose. Treatment for 7 days increased both COH activity and the amount of mRNA. However, this was not seen in the protein level, indicating that the mRNA produced may not be translated into functional protein or, alternatively, cross-reacting mRNAs and/or proteins obscure these findings. PROD and EROD activities were markedly increased after 1 day of treatment. A slight increase was seen in T7αOH activity after a single injection and in BZDM activity after 7 daily doses of gemfibrozil. Thus, gemfibrozil increased several CYP enzymes in mice, the induction profile being similar to that obtained by Wortelboer *et al.* (1991) in rats after treatment with clofibrate [33].

Polyunsaturated fatty acids are required for the optimal activity of the monooxygenase system [35–39]. A corn oil diet that is rich in polyunsaturated fatty acids has been reported to increase drug-metabolizing enzyme activities [35, 36]. Corn oil given intraperitoneally for a short period does not, however, mimic a corn oil diet. We have previously shown that a single dose of corn oil decreased mouse hepatic COH and T15αOH activities and had no effect on the other monooxygenases tested [9, 40]. Here, we also wanted to study the subchronic effects of corn oil on the expression of CYP2A4/5 as well as its effects on other monooxygenases, and to compare the effects to those of saline. After 1 day of treatment, only T15αOH and PROD activities were significantly decreased and the COH activity was only slightly decreased. However, after 7 daily administrations of corn oil, COH activity was decreased by 66%, with a concomitant decrease in the amount of CYP2A4/5 protein and mRNA. Thus, CYP2A4/5 seems to be exceptionally sensitive to inhibition by corn oil because other activities were either not changed or decreased only slightly (T7αOH and BZDM). It is evident that the effects seen after clofibrate and gemfibrozil may be partly due to an interaction with corn oil. However, other lipophilic solvents are too toxic to be used in the concentrations necessary to dissolve these agents and still keep the volume

small enough that it can be delivered intraperitoneally. However, it is our opinion that the effects of clofibrate and gemfibrozil differ so greatly from those in control mice receiving corn oil that the effects of these drugs may be considered distinct from those of the oil.

One explanation for the decreased expression of CYP2A4/5 may be related to disturbances in the fatty acyl homeostasis caused by corn oil and peroxisome proliferators such as clofibrate. Changes in fatty acyl composition alter membrane fluidity which, in turn, can influence the conformation, mobility, and function of membrane-bound proteins, including P450s [35]. However, this alone cannot explain the observed decrease in the protein and CYP2A4/5 mRNA content. Structurally, clofibrate is classified as a phenoxyacetic acid compound and gemfibrozil belongs to the alkyl-arylcarboxylic acids [12]. It is well known that the degree of peroxisome proliferation varies markedly, depending on the substance [12]. Gemfibrozil has been shown to be a more potent inhibitor of rat liver microsomal and mitochondrial palmitoyl-CoA hydrolase *in vitro* than clofibric acid [41]. Thus, it is possible that, in our mouse model, clofibrate and gemfibrozil affect the fatty acyl composition in different ways. Studies examining this are currently under way.

A pronounced species difference in chemically-induced peroxisomal proliferation has been reported by numerous groups. The higher mammalian species are considerably less sensitive to peroxisome proliferation and the carcinogenic effects of peroxisome proliferators than are rodents [12, 13]. Corn oil is a widely used solvent and one should not assume that compounds used as solvents are biologically inert. Because CYP2A enzymes are involved in the metabolism of steroids and several potent chemical carcinogens [32, 42–43], the decreased expression of CYP2A4/5 seen after corn oil may be of importance when estimating, *in vivo*, pharmacological or toxicological responses in mice of a compound dissolved in this oil.

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