



Regulation of Mouse Liver Microsomal Esterases by Clofibrate and Sexual Hormones

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ABSTRACT. Carboxylesterase activity was measured using six different substrates in microsomal preparations from female and ovariectomized female mice in order to evaluate the effects of female sex hormones on esterase expression. With three of the substrates (α -naphthyl acetate and esters 2 and 3), esterase activity was the same in both groups; however, with the others (*p*-nitrophenyl acetate and esters 1 and 4), there was a small increase in activity in ovariectomized females, compared with intact females. Castration of males followed by treatment with testosterone caused only transient increases in activity for four of the substrates (α -naphthyl acetate and esters 1, 2, and 3) and no change in activity for the other two (*p*-nitrophenyl acetate and ester 4). Treatment of male and female mice with the peroxisome proliferator clofibrate, with or without testosterone, resulted in increased hydrolysis of α -naphthyl acetate and *p*-nitrophenyl acetate, but little change for the other substrates. Clofibrate also induced α -naphthyl acetate and *p*-nitrophenyl acetate hydrolysis in castrated males, but clofibrate and testosterone administered together resulted in significant increases of activity with all substrates, which were greater than the additive effects of the two compounds administered separately. These results indicate that clofibrate causes significant alterations in the regulation of esterase activity, whereas sex hormones only cause small changes. However, it would seem that testosterone can synergize the effect of clofibrate in castrated males, resulting in higher levels of activity than with clofibrate alone. Finally, an overall increase in esterase activity might be due to a large increase in the activity of a few esterases or to a small increase in many esterases. Enzyme staining of native polyacrylamide gels reveals that the latter is true, with the majority of esterases present in mouse liver microsomes being induced to a small degree by clofibrate. *BIOCHEM PHARMACOL* 51;5:677–685, 1996.

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Mammalian species possess a number of hepatic microsomal carboxylesterases that are capable of hydrolyzing xenobiotics containing ester, thioester, and amide groups and are therefore thought to have a role in detoxication [1–4]. Some xenobiotic compounds also have an effect on the esterases themselves, causing alterations in the level of expression of these enzymes [3]. For example, peroxisome proliferators and hormones have been found to cause increases or reductions in the activity of mammalian hepatic microsomal esterases [5–11].

Peroxisome proliferators are structurally diverse compounds that cause specific effects in mammalian liver [12, 13]. Treatment with peroxisome proliferators causes liver enlargement due to an increase in the cell number and DNA content (hyperplasia) and an increase in cell size (hypertrophy) [12–15]. Hypertrophy is mainly the result of an increase in the number of peroxisomes and the amount of smooth endoplasmic reticulum in the cells, which, in turn, results in an increase in peroxisomal and microsomal enzyme activity [12–15]. If treatment with peroxisome proliferators is stopped, the liver returns to normal in a few weeks, but continued treat-

ment will lead to development of tumors [16, 17]. These effects have been seen in a number of mammalian species including hamsters, chickens, and rhesus and cynomolgus monkeys, but are most pronounced in mice and rats [12–14]. Although peroxisome proliferators have been well studied and their effects on liver physiology and biochemistry well characterized [12–15], few studies have considered the effects of these compounds on liver carboxylesterase activity [5–7]. Rats and mice treated with clofibrate have shown increases, decreases, or no alteration in esterase activity, depending on the substrate used to detect these enzymes [5–7]. There are many different esterases present in mouse and rat liver [18, 19], but previous studies have concentrated on the effect of peroxisome proliferation on esterase activity overall [5–7], so the number of liver esterase isozymes that are affected by peroxisome proliferation has not been determined. The mechanism of induction of the liver esterases is not yet understood; it may be the result of a regulatory change brought about by clofibrate or one of its metabolites [15] or it may simply be the result of an increase in surface area of smooth endoplasmic reticulum, and a proportional increase in esterase activity, brought about by peroxisome proliferation [5].

Hormones have also been found to influence the levels of

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esterase activity in the mouse and rat [8–11]. Removal of one source of hormones, the pituitary gland, in the rat resulted in alterations in esterase activity, and treatment of these hypophysectomized rats with human growth hormone restored esterase activity to normal levels [11]. Studies of the effects of sex hormones on rat liver esterases showed that activity was reduced by castration and could be restored by treatment with testosterone. Ovariectomy also caused reduction of esterase activity in rats, which could be restored by estradiol [9, 10]. However, a study examining the effect of testosterone on mouse liver esterases showed little variation in levels of activity [8]. Once again, the level and direction of alterations to esterase activity were dependent upon the substrate used to detect these enzymes.

Sex-related differences in the response of rat and mouse liver to clofibrate and other peroxisome proliferators have been reported, and the effect of clofibrate has been shown to be much more marked in males than in females [12, 20–25]. One result of this knowledge is that previous studies on the effect of clofibrate on esterase activity have concentrated on the effects in male mice and rats. Also, although sex hormones are known to alter esterase activity [8–10], no study examining the effect on esterase activity of sex and/or sex hormones combined with clofibrate treatment has been performed.

The aim of this study was to examine the effect of clofibrate and sex hormones, administered separately and together, on levels of esterase activity in mouse liver microsomes. We used a number of substrates to ascertain whether there are different effects in different subgroups of the total esterase population of mouse liver. We also used electrophoretic techniques to distinguish the mouse liver esterase isozymes and determine whether any changes in overall esterase activity were due to large increases/decreases in the activity of a small group of esterases or to smaller changes in a larger group.

MATERIALS AND METHODS

Chemicals

α -Naphthol, α -naphthyl acetate, *p*-nitrophenyl acetate, DTNB, § clofibrate (ethyl *p*-chlorophenoxyisobutyrate), and testosterone were purchased from the Sigma Chemical Co. (St. Louis, MO). *p*-Nitrophenol and fast blue RR salt were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Spectrophotometric substrates were available from previous synthesis in this laboratory [26].

Treatment of Mice

Intact, castrated, ovariectomized and sham-operated mice (strain CD2F1) were obtained from Taconic (Germantown, NY). They were housed in an environmentally controlled room: 12-hr light, 12-hr dark cycle, 22.5 to 24°, and constant humidity. They were given water and Purina rodent chow *ad lib*. The mice were held for at least 1 week after receipt prior

to treatment. Clofibrate was dissolved in corn oil and mixed into ground chow (0.5% clofibrate, 5% corn oil, w/w). Experimental animals received clofibrate for 21 days. Controls received the same diet without clofibrate. Testosterone implants were prepared by filling 10 mm long, 1.98 mm i.d., 3.18 mm o.d. silastic tubing (Dow Corning No. 602-305) with crystalline testosterone and sealing each end with 1 mm of silicone adhesive. Mice were anesthetized with an injection of Avertin (prepared by mixing 1.0 g 2-methyl 1,2-butanol and 0.5 g 2,2,2-tribromoethanol (Aldrich) in 40 mL sterile water (0.02 mL/g body weight)), and implanted subcutaneously with testosterone or control implants. This testosterone implant treatment was shown previously to maintain plasma testosterone concentrations, as well as seminal vesicle, preputial, and submandibular gland weights in castrated male mice.*

Enzyme Preparation

Mice were killed with carbon dioxide. Livers were removed, weighed, and washed briefly in cold 0.1 M sodium phosphate buffer, pH 7.4, containing 250 mM sucrose and 1 mM EDTA. Livers were homogenized in 10 mL of the same buffer on ice. The homogenates were centrifuged at 13,000 *g* for 25 min at 4°. The supernatant was then centrifuged at 100,000 *g* for 65 min at 4°. The pellet was washed by resuspending it in 10 mL buffer and again centrifuging at 100,000 *g* for 65 min at 4°. The washed pellet was resuspended in 2 mL buffer containing 0.5% Triton X-100, which was shown previously to have little effect on esterase activity from mouse liver microsomes [27]. Aliquots (1 mL) of this mouse liver microsomal preparation were stored at –80°.

Esterase Assays

The assay for measuring the rate of α -naphthyl acetate hydrolysis was performed as described previously [28]. A 0.025% (w/v) solution of fast blue RR salt in 0.1 M Tris–HCl buffer, pH 7.5, was prepared immediately before use. This fast blue solution (278 μ L) and 20 μ L of mouse liver microsomal preparation were added to wells of a microtiter plate in triplicate. For non-enzymatic controls, 20 μ L of buffer was added instead of the microsomal preparation. The reaction was initiated by addition of 2 μ L of α -naphthyl acetate (75 mM in 50% acetone) to give a final concentration in the assay of 0.5 mM. The reaction solution was mixed immediately and production of α -naphthol was monitored at 450 nm for 2 min at 23°.

Rates of *p*-nitrophenyl acetate hydrolysis were determined as described previously [28, 29]. Tris HCl buffer (278 μ L, 0.1 M), pH 7.5, and 20 μ L of mouse liver microsomal preparation were added to wells of a microtiter plate in triplicate. Buffer (20 μ L) replaced the microsomal preparation in non-enzymatic controls. The reaction was initiated by addition of 2 μ L of *p*-nitrophenyl acetate (75 mM in 50% acetone) to give a final concentration in the assay of 0.5 mM. The reaction solution

§ Abbreviation: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

*Coquelin A, personal communication. Cited with permission.

was mixed immediately and *p*-nitrophenol production was monitored at 405 nm for 2 min at 23°.

The rates of hydrolysis of various thioester substrates were determined using a modification of the Ellman method [26, 27, 30]. Tris-HCl buffer (278 μ l, 0.1 M), pH 7.5, containing 0.015% DTNB (Ellman's reagent) and 20 μ l of mouse liver microsomal preparation were added to wells of a microtiter plate in triplicate. Non-enzymatic controls contained 20 μ l of buffer in place of the microsomal preparation. The reaction was initiated by addition of 2 μ l substrate (30 mM in ethanol) to give a final concentration in the assay of 0.2 mM. The reaction solution was mixed immediately, and hydrolytic rates were monitored at 405 nm for 2 min at 23°.

In all cases, enzyme assays were performed at protein and substrate concentrations where the initial hydrolytic rates were linear with time. All rates of hydrolysis were corrected for non-enzymatic hydrolysis of the substrate. Results from the activity assays were expressed as an average specific activity for each microsomal preparation. These averages, from each organism, were used to generate a mean and standard deviation for each treatment, which was then subjected to statistical analysis.

Protein concentrations were determined using the Biorad protein assay reagent, which is based on the dye-binding method of Bradford [31]. Bovine serum albumin was used as a protein standard.

Electrophoresis

Esterases were separated by discontinuous native PAGE using the method of Vernick *et al.* [32] as adapted by Healy *et al.* [33]. Aliquots of mouse liver microsomal preparations (8.5 μ g) were applied to each lane. Electrophoresis was carried out for 2.5 hr at 15 W constant power per gel with continuous cooling. Gels were incubated in 0.1 M sodium phosphate buffer, pH 6.8, for 15–30 min and then stained for esterase activity at room temperature for 30–60 min in a solution of 0.02% (w/v) α - and β -naphthyl acetate (predissolved in acetone) and 0.05% (w/v) fast blue RR salt in 0.1 M sodium phosphate buffer, pH 6.8.

Statistical Analysis

Esterase activity data were analyzed by analysis of variance using the SuperANOVA program (Abacus Concept, Berkeley,

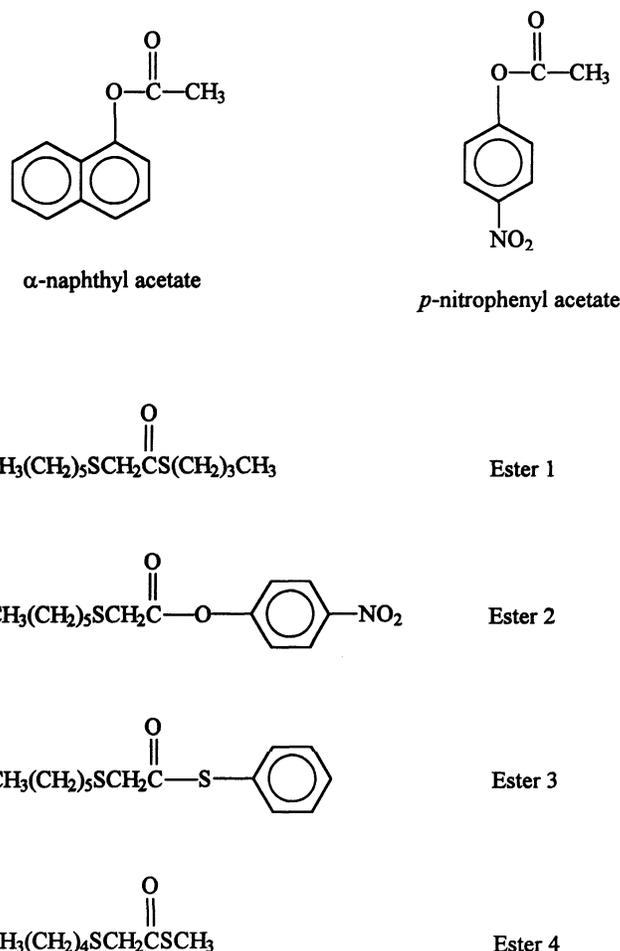


FIG. 1. Chemical structures of compounds used as esterase substrates in this study.

CA). The Tukey-Kramer test was used for multiple range tests.

RESULTS

Different esterase substrates were used to examine the activity present in liver microsomes after sex hormone and/or clofibrate treatments (Fig. 1). Some esterases are able to utilize some substrates and not others, and we wanted to optimize our

TABLE 1. Effect of ovariectomy on specific activities of liver microsomal esterases from mice*

Sex	Specific activity†‡ (nmol/min/mg)					
	α -Naphthyl acetate	<i>p</i> -Nitrophenyl acetate	Ester 1	Ester 2	Ester 3	Ester 4
Male	3940 \pm 470 ^a	3060 \pm 330 ^{a,b}	91 \pm 19 ^b	557 \pm 160 ^a	217 \pm 73 ^a	775 \pm 92 ^b
Female	3590 \pm 270 ^a	2760 \pm 320 ^a	59 \pm 15 ^a	440 \pm 130 ^a	147 \pm 44 ^a	594 \pm 62 ^a
Ovariectomy	4340 \pm 550 ^a	3320 \pm 460 ^b	83 \pm 17 ^b	507 \pm 130 ^a	219 \pm 50 ^a	690 \pm 89 ^{a,b}

* Female mice were ovariectomized or sham-operated at 4 weeks of age and were killed at 11 weeks. All mice used in this experiment were received at the same time, treated as a group, and killed together.

† Specific activity values are the means \pm SD of four mice. Assays were performed in triplicate for each mouse and were repeated once.

‡ For each substrate, means without a common superscript were significantly ($P < 0.05$) different.

TABLE 2. Effect of testosterone replacement on specific activities of liver microsomal esterases from castrated male mice*

Testosterone	Specific activity†‡ (nmol/min/mg)					
	α -Naphthyl acetate	<i>p</i> -Nitrophenyl acetate	Ester 1	Ester 2	Ester 3	Ester 4
28 days	3910 \pm 1010 ^{a,b}	1600 \pm 440 ^a	72 \pm 15 ^{a,b}	532 \pm 130 ^{a,b}	150 \pm 39 ^{a,b}	621 \pm 52 ^a
14 days	4230 \pm 860 ^b	1730 \pm 380 ^a	66 \pm 17 ^{a,b}	412 \pm 130 ^a	140 \pm 52 ^{a,b}	627 \pm 124 ^a
7 days	3340 \pm 580 ^{a,b}	1660 \pm 190 ^a	89 \pm 19 ^b	465 \pm 200 ^{a,b}	188 \pm 23 ^b	556 \pm 205 ^a
3 days	3020 \pm 1030 ^{a,b}	1760 \pm 680 ^a	55 \pm 25 ^a	756 \pm 430 ^b	148 \pm 63 ^{a,b}	784 \pm 254 ^a
None	2360 \pm 410 ^a	1270 \pm 250 ^a	42 \pm 12 ^a	360 \pm 140 ^a	91 \pm 28 ^a	504 \pm 44 ^a

* Male mice were castrated at 3 weeks of age. Testosterone implants were given at 11 weeks (28 days), 13 weeks (14 days), and 14 weeks (7 or 3 days). Control mice were sham-implanted. All mice used in this experiment were received at the same time, treated as a group, and killed together.

† Specific activity values are the means \pm SD of four mice. Assays were performed in triplicate for each mouse and were repeated once.

‡ For each substrate, means without a common superscript were significantly ($P < 0.05$) different.

chances of observing differences in esterase activity resulting from the different treatments. The substrate α -naphthyl acetate, which is a common nonspecific esterase substrate, was used to give an overall view of the effects of treatments on the whole esterase population.

To determine the effect of ovarian hormones on esterase activity, we measured activity in liver microsomal preparations of female and ovariectomized female mice (Table 1). Esterase activity in these groups was not significantly different using the substrates α -naphthyl acetate, ester 2, and ester 3. However, with the substrates *p*-nitrophenyl acetate, ester 1, and ester 4 differences could be seen. In these three cases, the level of activity in ovariectomized females was increased in comparison with intact females.

We examined the effect of testosterone on esterase activity in liver microsomes by measuring activity in males after castration followed by testosterone replacement for 3, 7, 14 and 28 days (Table 2). Hydrolysis of two of our substrates, *p*-nitrophenyl acetate and ester 4, was not altered by castration or replacement of testosterone after castration. Two others, ester 1 and ester 3, revealed an increase in activity to a peak at day 7 followed by a small decrease. Ester 2 showed an increase in activity at day 3, and then a decline back to control levels. With α -naphthyl acetate, activity increased to a peak at day 14. Overall, castrated males treated with testosterone showed no real trend of increased/decreased activity with duration of testosterone treatment.

We examined the effect of treatment with testosterone, clofibrate, and both of these chemicals combined on esterase

activity in liver microsomes from female, male, and castrated male mice (Tables 3–5). Investigation of esterase activity with the substrate α -naphthyl acetate revealed that untreated females and those treated with testosterone possessed similar levels of activity (Table 3). Clofibrate treatment caused a significant increase in activity (at least 70%) compared with the control and testosterone-treated females. The combination of testosterone and clofibrate caused a further increase in activity, an increase of over 150% compared with testosterone-treated and control organisms. A similar trend was seen with *p*-nitrophenyl acetate and ester 1, although the level of the increase was smaller in each case. With ester 2, testosterone treatment gave the lowest level of activity and clofibrate-treated and control organisms had similar levels. Once again, the combination of clofibrate and testosterone yielded the greatest level of activity. The final two substrates, ester 3 and ester 4, showed non-statistically significant differences in the levels of esterase activity between treatment groups.

Esterase activity in males, determined using the substrate α -naphthyl acetate, was lowest in control samples and testosterone-treated samples (Table 4). Clofibrate treatment caused an increase in esterase activity of 240 and 70% compared with the control and testosterone-treated samples, respectively. Treatment with a combination of testosterone and clofibrate resulted in a further increase of activity, 160 and 430% increase compared with testosterone-treated and control samples and a 50% increase over the activity seen in samples treated with clofibrate alone. A similar trend was seen when *p*-nitrophenyl acetate was used as the substrate, although the amount

TABLE 3. Effect of testosterone and clofibrate treatments on specific activities of liver microsomal esterases from female mice*

Testosterone	Clofibrate	Specific activity†‡ (nmol/min/mg)					
		α -Naphthyl acetate	<i>p</i> -Nitrophenyl acetate	Ester 1	Ester 2	Ester 3	Ester 4
+	+	9730 \pm 1730 ^b	4130 \pm 440 ^b	110 \pm 22 ^b	941 \pm 230 ^b	230 \pm 50 ^a	916 \pm 108 ^a
+	–	3930 \pm 1540 ^a	2340 \pm 810 ^a	64 \pm 14 ^a	558 \pm 140 ^a	154 \pm 40 ^a	593 \pm 101 ^a
–	+	6590 \pm 1260 ^{a,b}	3710 \pm 890 ^{a,b}	90 \pm 16 ^{a,b}	703 \pm 130 ^{a,b}	183 \pm 47 ^a	852 \pm 163 ^a
–	–	3710 \pm 440 ^a	2660 \pm 330 ^{a,b}	75 \pm 16 ^a	714 \pm 190 ^{a,b}	199 \pm 55 ^a	893 \pm 129 ^a

* Twelve-week-old female mice received their treatment for 3 weeks and were then killed as described in Materials and Methods. All mice used to obtain the data presented in Tables 3–7 were received at the same time, treated as a group, and killed together.

† Specific activity values are the means \pm SD of three mice. Assays were performed in triplicate for each mouse and were repeated once.

‡ For each substrate, means without a common superscript were significantly ($P < 0.05$) different.

TABLE 4. Effect of testosterone and clofibrate treatments on specific activities of liver microsomal esterases from male mice*

Testosterone	Clofibrate	Specific activity†‡ (nmol/min/mg)					
		α -Naphthyl acetate	<i>p</i> -Nitrophenyl acetate	Ester 1	Ester 2	Ester 3	Ester 4
+	+	11,110 \pm 4,140 ^b	5,300 \pm 1,520 ^c	138 \pm 40 ^a	1,340 \pm 540 ^a	339 \pm 130 ^a	1,340 \pm 530 ^a
+	-	4,260 \pm 1,240 ^a	2,730 \pm 740 ^{a,b}	88 \pm 23 ^a	817 \pm 240 ^a	209 \pm 56 ^a	877 \pm 170 ^a
-	+	7,250 \pm 670 ^{a,b}	4,790 \pm 760 ^{b,c}	117 \pm 14 ^a	901 \pm 260 ^a	273 \pm 71 ^a	1,200 \pm 82 ^a
-	-	2,110 \pm 420 ^a	1,640 \pm 290 ^a	79 \pm 14 ^a	637 \pm 150 ^a	166 \pm 32 ^a	650 \pm 57 ^a

* Male mice were sham-operated at 3 weeks of age. At 12 weeks of age all mice received their treatment for 3 weeks and then were killed as described in Materials and Methods. All mice used to obtain the data presented in Tables 3-7 were received at the same time, treated as a group, and killed together.

† Specific activity values are the means \pm SD of three mice. Assays were performed in triplicate for each mouse and were repeated once.

‡ For each substrate, means without a common superscript were significantly ($P < 0.05$) different.

of the increases were reduced compared with those seen with α -naphthyl acetate. Results obtained using the other four substrates, ester 1, ester 2, ester 3 and ester 4, revealed no statistically significant differences between treatments.

Esterase activity in castrated males, measured as hydrolysis of α -naphthyl acetate, was found to be lowest in control and testosterone-treated organisms (Table 5). Clofibrate treatment caused a 60% increase in esterase activity compared with both the control and testosterone-treated organisms. However, the most significant alteration in activity came as a result of clofibrate and testosterone administered in combination, an increase of over 250% compared with the control and testosterone-treated samples and 110% compared with clofibrate treatment alone. A similar trend was seen with *p*-nitrophenyl acetate, although the size of the increase was reduced with this substrate. The final four substrates, ester 1, ester 2, ester 3 and ester 4, all showed the same pattern of esterase activity, the control, testosterone-treated and clofibrate-treated samples having similar activity and the testosterone-and-clofibrate-treated samples having activity increased by at least 100% compared with the others.

The liver and body weights of each organism were also measured to determine whether any of the treatments caused physiological effects. Liver weights were expressed as percentage body weight to normalize differences seen in body and liver weights among the different sexes and castrated organisms (Table 6). In both male and female mice clofibrate and clofibrate and testosterone administered together caused similar effects, an increase in liver weight as a percentage of body

weight compared with untreated and testosterone-treated individuals. However, castrated males showed a different effect, clofibrate treatment again caused an increase of the liver/body weight ratio, but clofibrate and testosterone administered together caused an even greater increase, significantly different from clofibrate treatment alone.

Samples from each of the treatment groups were subjected to native PAGE and stained for esterase activity. Gels were also stained in the presence of paraoxon, which inhibited staining, showing that staining was specific for esterase isozymes (data not shown). In each experiment, the amount of sample loaded was such that equal amounts of protein were loaded in each lane, allowing direct comparison of activity among samples. There was little difference in the esterase pattern or activity level in the liver microsomes from male, female, and ovariectomized female mice (data not shown). A similar result was obtained in the experiment involving castration of males and testosterone replacement, with little difference in the overall banding pattern or activity of individual esterases (data not shown). There was, however, a marked difference in response to treatments with testosterone and clofibrate. Within each sex these treatments did not change the pattern of esterase occurrence, but the levels of activity of the individual esterases were altered significantly. In males and females, most of the esterase bands (10 of 14) in the clofibrate- and clofibrate-and-testosterone-treated samples showed increases in intensity compared with testosterone-treated and control samples (Fig. 2; Table 7). The results for castrated males were slightly different. Again, the clofibrate- and clofi-

TABLE 5. Effect of testosterone and clofibrate treatments on specific activities of liver microsomal esterases from castrated male mice*

Testosterone	Clofibrate	Specific activity†‡ (nmol/min/mg)					
		α -Naphthyl acetate	<i>p</i> -Nitrophenyl acetate	Ester 1	Ester 2	Ester 3	Ester 4
+	+	11,080 \pm 1,430 ^c	6,160 \pm 870 ^c	165 \pm 34 ^b	1,330 \pm 250 ^b	382 \pm 79 ^b	1,530 \pm 200 ^b
+	-	2,840 \pm 720 ^a	1,770 \pm 370 ^a	75 \pm 16 ^a	680 \pm 170 ^a	172 \pm 50 ^a	630 \pm 120 ^a
-	+	5,180 \pm 700 ^b	3,180 \pm 660 ^b	84 \pm 20 ^a	463 \pm 100 ^a	120 \pm 35 ^a	740 \pm 170 ^a
-	-	3,210 \pm 270 ^a	2,370 \pm 260 ^{a,b}	79 \pm 22 ^a	560 \pm 130 ^a	163 \pm 26 ^a	840 \pm 170 ^a

* Male mice were castrated at 3 weeks of age. At 12 weeks of age all mice received their treatment for 3 weeks and then were killed as described in Materials and Methods. All mice used to obtain the data presented in Tables 3-7 were received at the same time, treated as a group, and killed together.

† Specific activity values are the means \pm SD of three mice. Assays were performed in triplicate for each mouse and were repeated once.

‡ For each substrate, means without a common superscript were significantly ($P < 0.05$) different.

TABLE 6. Effect of testosterone and clofibrate treatments on liver weights of mice*

Sex	Testosterone	Clofibrate	Liver weight†‡ (% of body weight)	Protein concentration of microsomal preparations (mg/mL)
Female	+	+	6.45 ± 0.54 ^b	27.8 ± 1.7
Female	+	-	4.94 ± 0.01 ^a	23.6 ± 0.2
Female	-	+	6.79 ± 0.30 ^b	26.7 ± 3.5
Female	-	-	4.82 ± 0.41 ^a	22.8 ± 2.2
Male	+	+	6.51 ± 0.16 ^b	28.9 ± 0.2
Male	+	-	5.33 ± 0.17 ^a	24.6 ± 1.3
Male	-	+	6.53 ± 0.13 ^b	29.5 ± 0.5
Male	-	-	5.26 ± 0.36 ^a	26.6 ± 3.5
Castrated male	+	+	6.89 ± 0.15 ^c	28.7 ± 0.4
Castrated male	+	-	4.48 ± 0.20 ^a	24.6 ± 3.0
Castrated male	-	+	6.11 ± 0.06 ^b	25.0 ± 2.8
Castrated male	-	-	4.51 ± 0.05 ^a	21.0 ± 1.6

* Male mice were castrated or sham-operated at 3 weeks of age; all females were intact. All mice received clofibrate and/or testosterone treatment for 3 weeks at 12 weeks of age and then were killed as described in Materials and Methods. All mice used in these experiments were received at the same time, treated as a group, and killed together.

† Each value represents the mean ± SD of three mice.

‡ Means without a common superscript were significantly ($P < 0.05$) different.

brate-and-testosterone-treated samples revealed bands with increased intensity compared with testosterone-treated and control samples. However, in the control sample the esterase bands were stained more intensely than those from testosterone-treated mice (data not shown).

DISCUSSION

In our study, the hydrolysis of three esterase substrates (α -naphthyl acetate, ester 2, and ester 3) was not altered in ovariectomized females compared with intact females. A study of ovariectomized female rats showed a similar result, with no difference in esterase activity being found if α -naphthyl acetate was used as a substrate [10]. However, our results showed that there was a small increase in the hydrolysis of three other substrates (*p*-nitrophenyl acetate, ester 1, and ester 4) in ovariectomized females compared with intact females. This would seem to indicate that the ovarian hormones have an inhibitory effect on the esterases specific for these substrates. Estradiol, one of the female sex hormones, was found to decrease the activity of *p*-nitrophenyl acetate hydrolase in both male and female rats [9]. This same study also reported that *p*-nitrophenyl acetate hydrolysis was increased in ovariectomized females compared with intact females, and this activity could be returned to the level of an intact female by treatment with estradiol [9]. Further study by this group using antibodies specific for three specific carboxylesterase isozymes showed that one isozyme was at least partially regulated by estrogens [10]. Interestingly, although previous studies on mouse liver esterases have shown no differences in esterase activity for different sexes, these studies all used α -naphthyl acetate as the substrate for monitoring esterase activity [8, 34]. It is possible that differences were not found due to use of this substrate and

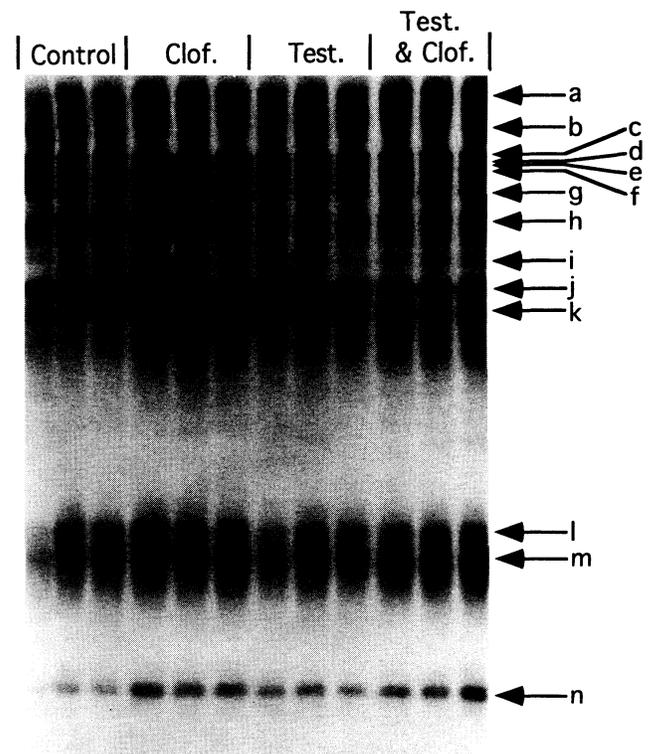


FIG. 2. Native PAGE of liver microsomal esterases from female mice treated with testosterone and/or clofibrate. Twelve-week-old female mice received their treatment for 3 weeks and were then killed as described in Materials and Methods. Individual livers were homogenized, and aliquots representing 8.5 μ g protein from each individual were subjected to discontinuous native PAGE and stained with α - and β -naphthyl acetate, as described in Materials and Methods. All mice used in this experiment were received at the same time, treated as a group, and killed together.

TABLE 7. Comparison of band density of esterases on a native polyacrylamide gel stained with α - and β -naphthyl acetate*†

Esterase	Control‡	Clofibrate	Testosterone and clofibrate	
			Testosterone	Testosterone and clofibrate
A	100 ± 15	117 ± 7	97 ± 8	110 ± 7
B	100 ± 4	102 ± 7	92 ± 4	94 ± 2
C	100 ± 11	125 ± 3	95 ± 3	122 ± 4
D	100 ± 11	131 ± 9	92 ± 5	122 ± 4
E	100 ± 9	134 ± 5	94 ± 5	122 ± 4
F	100 ± 8	130 ± 7	90 ± 3	117 ± 3
G	100 ± 9	126 ± 8	90 ± 5	111 ± 4
H	100 ± 14	119 ± 9	82 ± 6	96 ± 6
I	100 ± 15	104 ± 9	82 ± 7	96 ± 6
J	100 ± 6	107 ± 7	88 ± 3	102 ± 7
K	100 ± 3	111 ± 7	95 ± 4	116 ± 8
L	100 ± 29	124 ± 10	96 ± 7	111 ± 5
M	100 ± 21	120 ± 1	96 ± 9	118 ± 8
N	100 ± 25	164 ± 9	136 ± 7	185 ± 22

* Female mice were sham-operated at 3 weeks of age. All mice received clofibrate and/or testosterone treatment for 3 weeks at 12 weeks of age and then were killed. Individual livers were homogenized, and aliquots representing 8.5 μ g protein from each individual were subjected to discontinuous native PAGE and stained with α - and β -naphthyl acetate, as described in Materials and Methods. Densitometry was performed using an IS-1000 Digital Imaging System from the Alpha Innotech Scientific Corp.

† Each value represents the mean \pm SD of no less than three replicates of density expressed as a percentage of the control.

‡ Band densities of controls, which represent 100%, were as follows: esterase A, 0.48; esterase B, 0.59; esterase C, 0.74; esterase D, 0.71; esterase E, 0.72; esterase F, 0.66; esterase G, 0.56; esterase H, 0.55; esterase I, 0.38; esterase J, 0.59; esterase K, 0.49; esterase L, 0.41; esterase M, 0.53; and esterase N, 0.22.

that another, such as *p*-nitrophenyl acetate, may have revealed differences similar to those seen in this study.

Treatment of castrated males with testosterone resulted in a significant increase in the esterase activities determined using the substrates α -naphthyl acetate, ester 1, ester 2, and ester 3. However, in each case this increase was transient; a peak of activity occurred followed by a decline so that the esterase activity level was not significantly different from the untreated castrated males after 28 days. A study in rats showed that levels of esterase activity were altered by castration, causing a reduction in *p*-nitrophenyl acetate-hydrolyzing activity which could be restored by subsequent addition of testosterone [9]. This contrasts with our results which showed only small transient effects of testosterone replacement on esterase activity in castrated male mice.

In our study, the effect of clofibrate on esterase activity was dependent upon the substrate used to detect activity. The esterases that hydrolyze α -naphthyl acetate and *p*-nitrophenyl acetate were induced significantly in clofibrate-treated compared with untreated organisms. The esterases involved in ester 1 and ester 2 hydrolysis were induced by clofibrate in females, but not in males. Hydrolysis of ester 3 and ester 4 was not induced by clofibrate treatments in males and females. Differences in induction by clofibrate of esterases hydrolyzed by particular substrates have been reported in a number of studies. Hosokawa *et al.* [7] studied the effect of clofibrate (administered orally for 3 days at a dose of 300 mg/kg) on ester hydrolysis in liver microsomes of adult male rats using four

different substrates: *p*-nitrophenyl acetate, butanilicaine, isocarboxazid and palmitoyl CoA. Clofibrate was found to induce the enzymes hydrolyzing these compounds by 1.3-, 4.2-, 2.8- and 1.2-fold, respectively. Mentlein *et al.* [5] observed a number of different responses of microsomal esterases from male rat liver to clofibrate treatment (0.3% fed for 10 days); clofibrate hydrolysis was not induced, palmitoyl CoA/propanidid hydrolytic activity was decreased (20/40%), and acetanilide hydrolysis and decanoyl-D,L-carnitine hydrolysis were both induced greater than 3-fold. Ashour *et al.* [6] examined the effects of clofibrate (0.5% fed for 14 days) on microsomal liver esterase activities in male rats and mice, using five different substrates. In rat liver, the only significant increase in esterase activity was seen using malathion as a substrate. However, all five substrates, *p*-nitrophenyl acetate, clofibrate, malathion, diethylsuccinate and diethylphthalate, showed a significant increase in hydrolysis in mice: 2.17-, 1.50-, 1.52-, 1.35-, and 1.19-fold, respectively. Each substrate utilized in these studies possessed specificity for an individual esterase or a particular group of esterases [5–7]. Therefore, in each case the differences in esterase activity found using different substrates reflected differential induction of individual esterases by clofibrate.

With castrated males, induction of esterase activity following clofibrate treatment was seen with the substrates α -naphthyl acetate and *p*-nitrophenyl acetate. However, the combination of testosterone and clofibrate caused a more significant increase in esterase activity than either of these treatments alone. In fact, this increase was greater than the additive effect of both treatments, suggesting synergism between testosterone and clofibrate in castrated males, which is not seen to the same extent in females and males. With the other esterase substrates, esters 1, 2, 3, and 4, clofibrate treatment alone did not result in an increase of esterase activity. However, with all four substrates, there was a significant increase in esterase activity when clofibrate and testosterone were administered together.

Induction of esterase activity after treatment with clofibrate is thought to be the result of an increase in surface area of endoplasmic reticulum coupled with a change in distribution of the different esterases along the endoplasmic reticulum [5]. Our results showed an increase in specific activity after clofibrate treatment which would be expected only if there was a significant change in regulation or distribution of the carboxylesterases within the endoplasmic reticulum. If increased esterase activity was due solely to organelle induction, specific activity would remain the same and the overall esterase activity per gram liver would increase. Differences in the levels of peroxisome proliferation for different sexes and treatments may explain the differences in esterase activity seen in castrated males with testosterone and clofibrate treatment versus males and females. Sex-related differences in responsiveness of rat and mouse liver to clofibrate and other peroxisomal proliferators have been reported previously [12, 20–25]. In our study, both male and female mice treated with clofibrate, with or without testosterone, were seen to have a significant increase in liver weight as well as in esterase activity. However, with castrated males a different effect was seen, with clofibrate and testosterone interacting to produce a greater increase in

both liver weight and esterase activity than clofibrate alone. A related phenomenon was seen in rats where a number of indicators of peroxisome proliferation, including liver weight, peroxisomal β -oxidation activity, and hepatomegaly, were found to be decreased in castrated compared with intact males treated with clofibrate [24]. Treatment of castrated male rats with testosterone brought the level of peroxisome proliferation back up to that of intact males. Paul *et al.* [24] gave two possible explanations for this phenomenon: either testosterone modifies the action of clofibrate in inducing peroxisome proliferation, or testosterone has an inhibitory effect on clofibrate metabolism, keeping the levels of clofibrate in serum higher in males than in castrated males, leading to a greater degree of peroxisome proliferation. In either case, the presence of testosterone during clofibrate treatment results in an increase in peroxisome proliferation. If testosterone also increases the level of peroxisome proliferation in mice treated with clofibrate, this may explain our results for castrated males. If peroxisome proliferation in castrated males treated with clofibrate was enhanced in the presence of testosterone, the result would be an increase in both liver weight and esterase activity, higher than that seen with clofibrate treatment alone.

This effect of testosterone, however, was not seen in females, since females treated with clofibrate showed no significant differences in liver weight or esterase activity, with or without the presence of testosterone. Once again a possible explanation may be extrapolated from rats. The secretory pattern of pituitary growth hormone is sex related in adult rats and the different patterns can be mimicked by different methods of infusion of growth hormone [25]. Sugiyama *et al.* [25] found that growth hormone given to rats via the female secretory pattern had a suppressive effect on peroxisome proliferation, even in the presence of testosterone. Therefore, if we assume that growth hormone is regulated by a similar mechanism in mice, we would expect to see no differences in liver weight and esterase activity for females treated with clofibrate or clofibrate and testosterone combined.

The results obtained with the spectrophotometric assays show that overall esterase activity was induced by clofibrate treatment, but not altered significantly by ovariectomy, castration, and/or hormone treatment. Induction of overall esterase activity by clofibrate could have been due to either a large increase in the activity of a single esterase, or small group of esterases, or to small changes in the activity of a large group of esterases. Native PAGE enabled us to distinguish the mouse liver esterase isozymes and determine that the latter explanation was true in this case. Clofibrate induced 10 of the 14 esterases present on the native polyacrylamide gel, all having slightly increased activity compared with the esterases from samples not treated with clofibrate (Fig. 2 and Table 7). No esterase was induced to exceptionally high levels of activity and none had a reduction in or loss of activity, indicating that clofibrate did not cause a large increase or decrease in the regulation of any esterase, nor switch off any esterase. Therefore, the significant increase in overall esterase activity caused by clofibrate treatment is the result of activity increases in most, but not all, of the esterases present in mouse liver mi-

croosomes. Hosokawa *et al.* [10] purified three esterase isozymes from rat liver and prepared antibodies against each of them. These three enzymes were isolated by different protocols and were found to be biochemically and immunologically distinct. Using radial immunodiffusion analysis with antibodies to the three purified esterase proteins, these authors found that the amount of these three proteins was increased in the clofibrate-induced samples [7, 10]. Therefore, despite the fact that these proteins are different biochemically, they were all induced by clofibrate treatment [7], indicating that esterase induction in rats is also due to increased levels of more than one esterase.

Peroxisome proliferators cause induction of a number of different enzymes, but the mechanism by which induction occurs is unknown [12–15]. However, it has been proposed that induction occurs via activation of a group of transcription factors, the peroxisome proliferator-activated receptors (PPARs) [35–38]. PPARs are thought to activate transcription by binding particular DNA sequence elements, peroxisome proliferator response elements (PPRE), possibly as a complex with another protein, the retinoid X receptor [35, 38, 39]. PPREs have been identified upstream of a number of genes that are known to be induced by peroxisome proliferators [38, 40, 41]. The possibility exists that induction of esterases by peroxisome proliferators also occurs via this mechanism, but no data are available at this time to confirm or refute that possibility. An investigation of the regulatory sequences upstream of an esterase gene that is induced by peroxisome proliferators may shed further light upon this possibility.

Increases in esterase activity due to peroxisome proliferation will be a significant aid in affinity purification of the esterases induced by these compounds, since the starting material is enriched effectively for the enzymes of interest. Purification of one or more induced esterases may allow elucidation of the molecular mechanism of induction of these enzymes by peroxisome proliferators [5, 6, 15]. It may also be possible to isolate and identify toxicologically significant esterases since induction of esterases by peroxisome proliferators may reflect an important role for esterases in metabolism of these compounds [6, 15]. With the same set of tissues, the effects of clofibrate and steroid hormones on soluble epoxide hydrolases were examined [42]. Thus, the data in the two manuscripts can be compared directly.

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