DIFFERENTIAL REGULATION OF SOLUBLE EPOXIDE HYDROLASE BY CLOFIBRATE AND SEXUAL HORMONES IN THE LIVER AND KIDNEYS OF MICE

FRANCK PINOT,* DAVID F. GRANT,* JIMMY L. SPEAROW,†
ANTHONY G. PARKER* and BRUCE D. HAMMOCK*‡

Departments of *Entomology and Environmental Toxicology and †Section on Neurobiology,
Physiology and Behavior, University of California, Davis, CA 95616-8584, U.S.A.

Abstract—Soluble epoxide hydrolase (sEH) activity was measured in the liver and kidneys of male, female, and castrated male mice in order to evaluate sex- and tissue-specific differences in enzyme expression. sEH activity was found to be higher in liver than in kidneys. Activity increased with age in the liver of females, males and castrated males, but only in males did activity in the kidneys increase. There was greater activity in both the liver and kidneys of adult males than females. This sexual dimorphism was more pronounced in the kidneys (283% higher) than in the liver (55% higher). Castration of males led to a decrease in activity in both organs, but it had a greater effect on renal activity (67% decrease) than on hepatic activity (27% decrease). Treatment of castrated mice with testosterone led to an increase in sEH activity of 400% in kidneys and 49% in liver compared with surgical controls. These results suggest differential regulation of sEH by testosterone in kidneys and liver. Ovariectomized female mice had renal and hepatic activities approximately 30% greater than control females. Feeding mice with the hypolipidemic drug clofibrate produced stronger induction of sEH in liver than in kidneys. Testosterone treatment, however, caused greater induction in kidneys than in liver of females and castrated males and had no effect in either kidneys or liver in males. When given together, the effects of these two compounds appeared to be additive in both liver and kidneys. Results from western blot showed that the increase in sEH enzyme activity in kidneys is correlated with an increase in sEH protein. These results suggest that clofibrate and testosterone independently regulate sEH activity *in vivo*, and that kidneys and liver respond differently to clofibrate and testosterone.

Key words: soluble epoxide hydrolase; regulation; clofibrate; hormones; induction; peroxisome proliferators

Epoxide hydrolases (EC 3.3.2.3) are enzymes that convert epoxides to diols by the addition of water [1]. They have been found in most organs of all mammalian species tested. In mammals, the epoxide hydrolases are currently viewed as consisting of five distinct enzyme groups based on subcellular location and substrate selectivity: (1) mEH§, (2) sEH (formally called cytosolic epoxide hydrolase), (3) cholesterol epoxide hydrolase, (4) leukotriene A₄ epoxide hydrolase, and (5) hepoxilin epoxide hydrolase. Recently, epoxide hydrolases have been cloned from mammals [2–4] and from plants [5, 6]. Based on studies of sequence homology, principally to haloalkane dehalogenase (HAD) [7] which is similar to the mEH, Arand *et al.* [8] and Beetham *et al.* [9] proposed that these enzymes belong to the α/β hydrolase fold family [10]. Furthermore, the catalytic mechanisms of both mEH and HAD were shown to be similar [11, 12]. In mammals, the majority of the research has focused on the mEH and sEH, yet their physiological functions remain

unclear. Hepatic mEH is induced in rats and mice by a variety of foreign compounds and hydrolyzes a wide range of substrates [13–18]. These observations support a role for this enzyme in xenobiotic metabolism [19]. The broad spectrum of substrates of the sEH also suggests a detoxifying function [1]. However, sEH metabolizes epoxides of fatty acids more rapidly than other substrates with the epoxides of arachidonic acid being among the best substrates [20–22]. This leads to the formation and/or degradation of oxylipins, which may have biological roles. A better understanding of the physiological role of the mammalian sEH may be gained by studying its regulation. Hammock and Ota [23] were the first authors to show that sEH was induced by clofibrate and di-(2-ethylhexyl)phthalate, both of which are peroxisome proliferators. Differences in the hepatic response of male and female mice to clofibrate treatment have been investigated in our laboratory [24]. Induction of sEH activity by peroxisome proliferators has been investigated in several laboratories [25]. This class of xenobiotics is known to cause an increase in the collective volume of hepatic and proximal tubular peroxisomes and to lower serum lipid levels [26, 27]. Peroxisome proliferators are the only known exogenous compounds that increase sEH activity in mammals. Other studies, however, consistently show that sEH

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‡ Corresponding author: Tel. (916) 752-7519; FAX (916) 752-1537.

§ Abbreviations: mEH, microsomal epoxide hydrolase; sEH, soluble epoxide hydrolase; TSO, *trans*-stilbene oxide; PPAR, peroxisomal-proliferator-activated receptors; and DHEA, dehydroepiandrosterone.

activity increases with age and is higher in males than in females [28, 29]. This age dependence and the sexual dimorphism strongly suggest that sEH may be under hormonal regulation. This was confirmed by Denlinger and Vesell [30] and Inoue et al. [31], who showed the involvement of testosterone in the regulation of hepatic sEH in rat and mouse.

These previous investigations mainly addressed the study of sEH activity in the liver. Furthermore, these studies neither examined the effects of hormones and peroxisome proliferators simultaneously nor measured these effects in liver and kidneys in the same animal. In the present study we compared the regulation of sEH in kidneys and liver of male, castrated male, female and ovariectomized female mice. We examined the effects of clofibrate and testosterone administered separately or together.

MATERIALS AND METHODS

Chemicals. Clofibrate (ethyl *p*-chlorophenoxy-isobutyrate) and testosterone were purchased from the Sigma Chemical Co. (St. Louis, MO). Blotting reagents were purchased from Fisher Scientific (Pittsburgh, PA). Tritiated TSO was synthesized in our laboratory [32].

Animal experiments. 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 injection of Avertin (prepared by mixing 1.0 g of 2-methyl-1,2-butanol and 0.5 g of 2,2,2-tri-bromoethanol (Aldrich Chemical Co., Milwaukee, WI) 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 and activity measurement. Mice were killed with carbon dioxide. Livers and kidneys 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, then either frozen in liquid N_2 for later use (kidneys) or homogenized in 10 mL of the same buffer. Homogenates were centrifuged at 13,000 g for 25 min at 4°, and the supernatant fractions were centrifuged

at 100,000 g for 65 min at 4°. The cytosol was collected, divided into aliquots, and stored at −80°. Frozen kidneys were thawed at 37° and treated as above. Protein concentration was determined with bicinchoninic acid (BCA) reagent from Pierce using bovine serum albumin as a standard. sEH activity was measured using [³H]-TSO as previously described [1, 32]. In each case, the assay was shown to be linear for both protein and time.

Electrophoresis and immunoblotting. SDS–PAGE was performed using a 4% stacking and 10% resolving polyacrylamide gel (200 V constant voltage, 45 min). Immunoblotting was performed using 0.2 μm pore size nitrocellulose in a Bio-Rad Trans-Blot cell. After overnight transfer (30 V, room temperature), blots were blocked by shaking for 30 min in fetal bovine serum (FBS)/10 mM KH_2PO_4, 14 mM NaCl, 0.02% NaN_3 (pH 7.4) (PBS), 0.25% Tween-20, (50:50, v/v). Primary sEH anti-serum diluted 4000-fold was incubated for 90 min in FBS/PBS, 0.25% Tween (20:80, v/v) with shaking at room temperature. The immunoblot was washed five times for 5 min in PBS, 0.25% Tween-20. Bound immunoglobulin G (IgG) was detected by using the Amersham ECL kit as recommended by the manufacturer.

Statistical analysis. sEH activity data were analyzed by one- and two-way analysis of variance using the Super ANOVA Program (Abacus Concept, Berkeley, CA). The Tukey–Kramer Test was used for multiple range tests.

RESULTS

The first experiment measured sEH activity at 4, 8 and 16 weeks of age in females, sham-operated males, and males that had been castrated at 3 weeks of age. Figure 1 shows that activity in the liver was consistently higher than activity in the kidneys. The activity of the hepatic sEH increased with age in all three groups of animals. Castration led to a decrease in activity (27% lower at 16 weeks old). The same investigation performed with the cytosolic fraction from kidneys showed that sEH activity was higher and increased in males, but remained at the same level in females and castrated males. The decrease of the renal activity after castration (67% decrease at 16 weeks old) was stronger than that of the liver (see above). Within each age group, the difference in sEH activity between males and females was greater in kidneys than in liver.

Experiment 2 examined the effect of the duration of testosterone replacement in castrated males on hepatic and renal sEH activity. As shown in Fig. 2, after 3 days of testosterone treatment, the activity was increased by 19% in liver and 192% in kidneys compared with control animals. After 7 days of treatment the activity did not increase further in the liver, whereas it continued to increase in the kidneys. A longer treatment (28 days) led to a stimulation of the activity by 49 and 400% in liver and kidneys, respectively, over that of castrated controls.

In the next experiment, we followed the decrease of hepatic and renal sEH activity after the castration of adult male mice (Fig. 3). Like castration of young mice (Fig. 1), castration of adult mice led to a

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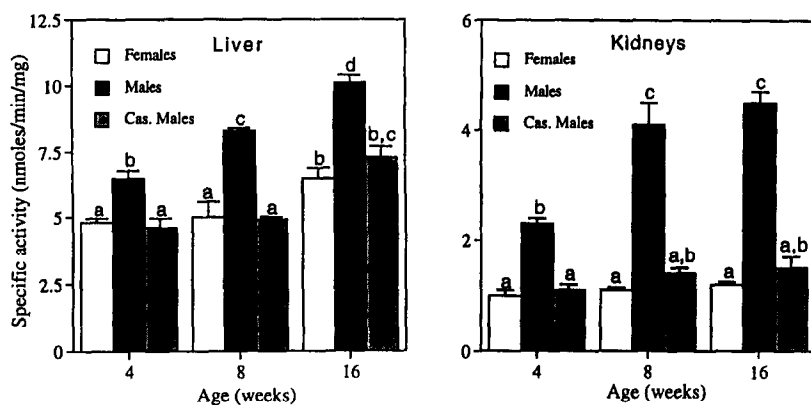* Coquelin A, personal communication. Cited with permission.

Fig. 1. sEH activity at different ages in liver and in kidneys of females, males and castrated males. Males were castrated or sham-operated at the age of 3 weeks. Unoperated females were used. For each organ, bars labeled with different letters were statistically different ($P < 0.05$). Data are the means ± SD of 3 mice.
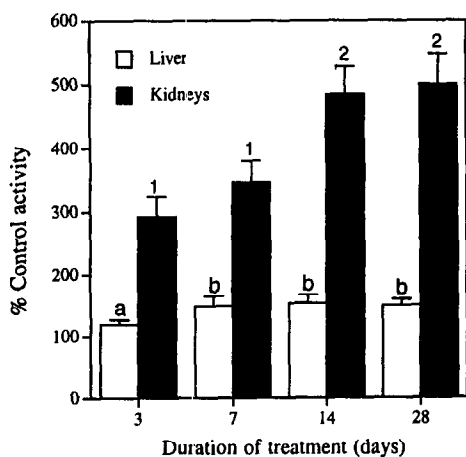

Fig. 2. Effect of testosterone replacement on sEH activity in liver and kidneys of castrated mice. Male mice were castrated at the age of 3 weeks. They received testosterone implants at the age of 11 weeks (treated 28 days), 13 weeks (treated 14 days), or 14 weeks (treated 7 or 3 days). Control mice were sham-implanted; control values were 6.7 and 1.3 nmol/min/mg in the liver and kidneys, respectively. The values at 14 and 28 days of treatment were similar to the values found in 16-week-old control males in Fig. 1. For each organ, bars labeled with different letters or different numbers were statistically different ($P < 0.05$). Data are the means ± SD of 4 mice.

decrease in activity in liver and kidneys compared with controls. However, more than 9 days of testosterone withdrawal was required for a reduction in either liver or kidney sEH activity.

To investigate the involvement of ovarian hormones on the regulation of sEH, we measured the effect of ovariectomy on the enzyme activity in both liver and kidneys. Results presented in Fig. 4 show a higher sEH activity in ovariectomized mice than in sham-operated controls with a 30 and 35%

increase in enzyme activity in liver and kidneys, respectively.

We investigated the effect of clofibrate and testosterone given separately or in combination on sEH activity (Fig. 5). In the liver of females (Fig. 5A), sham-operated males (Fig. 5B) and castrated males (Fig. 5C), clofibrate increased sEH activity more than testosterone. However, in the kidneys of females (Fig. 5D) and castrated males (Fig. 5F) testosterone treatment resulted in a greater increase in sEH activity than clofibrate administration. In kidneys of males (Fig. 5E), neither compound produced a significant effect on sEH activity. Coadministration of clofibrate and testosterone resulted in an additive increase in kidneys of females (Fig. 5D) and castrated males (Fig. 5F). Although neither clofibrate nor testosterone alone had a significant effect on sEH activity in the kidneys of males (Fig. 5E), coadministration led to an approximate doubling of activity. Coadministration of clofibrate and testosterone also resulted in an additive increase in sEH activity in the liver of castrated males (Fig. 5C). In the liver of females, the same treatment gave us data that were not statistically tight enough to rule out an additive effect (Fig. 5A). In the liver of males (Fig. 5B), we found the same activity after treatment with clofibrate only or with clofibrate plus testosterone. Thus, the additional testosterone alone had no effect.

In each experiment, data were also calculated on a per gram of tissue basis. In each case the same differences in sEH activity were found on a per milligram tissue weight basis as on a per milligram tissue weight protein basis.

Using SDS–PAGE and western blot analysis, we analyzed the amount of sEH in the renal cytosolic fraction of control mice and mice treated with clofibrate or testosterone or both (Fig. 6). Densitometric analysis indicated that changes in sEH activity were significantly correlated with changes in sEH amount ($P < 0.01$) detected by western blot.

DISCUSSION

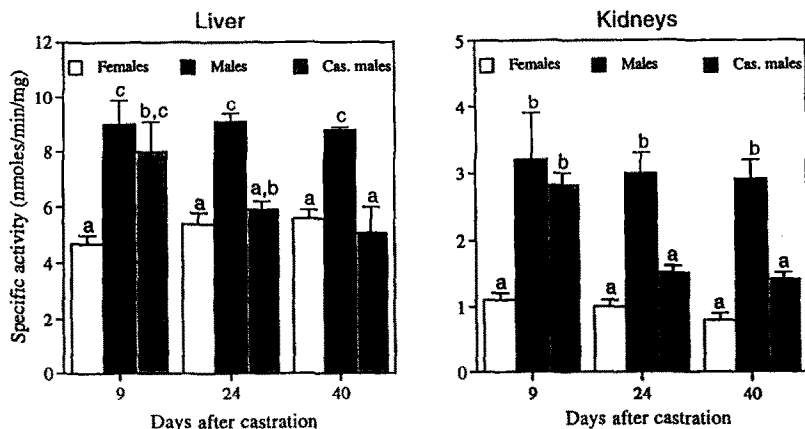The present study was undertaken to investigate

Fig. 3. Decrease of sEH activity in liver and kidneys of mice as a function of time after castration. Male mice were castrated or sham-operated at the age of 11 weeks, and killed at different times after operation. For each period, females of the same age were killed for comparison. For each organ, bars labeled with different letters were statistically different (P < 0.05). Data are the means ± SD of 3 mice.
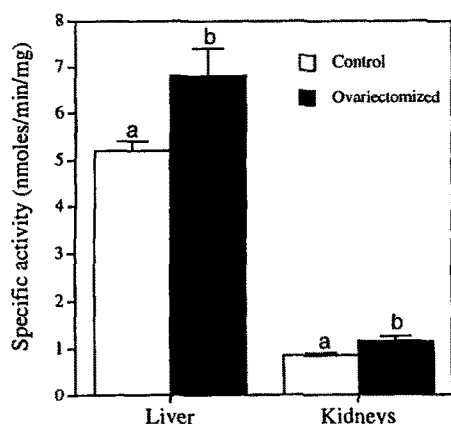

Fig. 4. Effect of ovariectomy on sEH activity in liver and kidneys of mice. Female mice were ovariectomized or sham-operated at 4 weeks of age and were killed at 11 weeks of age. Data are the means ± SD of 4 mice. For each organ, bars labeled with different letters were statistically different (P < 0.05).

the regulation of sEH by clofibrate and sexual hormones. In agreement with previous work [28–30], we found that sEH activity in the liver increased with age and was higher in males than in females. Renal activity was also higher in males than in females; however, an increase with age was only observed in males. Castration led to an alteration of activity in both organs. This observation, in addition to sex and age dependence, strongly suggests an endocrine involvement in the control of sEH activity that seems to differ between the kidneys and the liver. Sexual dimorphism was consistently greater in kidneys than in liver. This result is supported by the experiment that showed a 400% enhanced activity

in the kidneys compared with the 49% enhanced activity in the liver after 28-day testosterone treatment. The fact that the sEH activity dropped after castration of adults suggests that the effects of testosterone on sEH activity are similar in both young and adult mice. However, 9 days after castration of adult males, the sEH activity was similar to that in sham-operated animals. This may reflect the time necessary for the termination of the effects of testosterone present before castration and for the disappearance of epoxide hydrolase protein present before castration. In addition to positive regulation by testosterone, negative regulation by female hormones is suggested by the fact that in both organs sEH activity was higher in ovariectomized females than in sham-operated females. In contrast, Inoue et al. [31] have reported that ovariectomy did not affect the level of activity in mouse liver. This discrepancy may be due to the use of different strains of mice, since relative levels of sEH vary with strain [33]. An interesting experiment that is suggested by these results is whether female mice could be masculinized by both ovariectomy and testosterone replacement. The results in Fig. 5 suggest that testosterone replacement does indeed have a significant effect, but it is not clear whether ovariectomy would increase that effect.

Compounds that have the common property of causing peroxisome proliferation [34] can also induce sEH activity [25]. Fibrates (e.g. clofibrate, fenofibrate) together with other compounds (e.g. herbicides, phthalates) induce enzymes involved in fatty acid β-oxidation [35–38]. After clofibrate treatment, sEH activity was 220 and 270% of control values in male and female mice, respectively, which was in agreement with data from Moody et al. [24]. The mechanism of action of these peroxisome proliferators is unknown; however, it has been proposed that it occurs via the activation of a group of transcription factors, the PPAR [39–42]. Our results showed that clofibrate was a better inducer
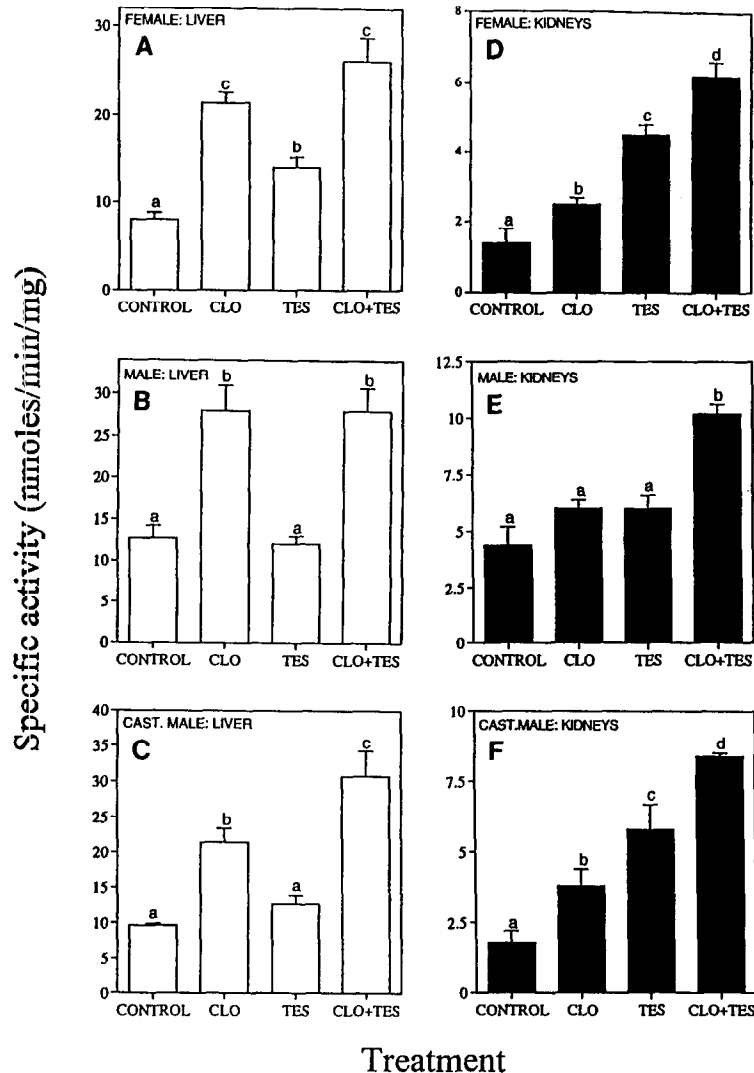
Fig. 5. Effect of clofibrate and testosterone on sEH activity in liver and kidneys of mice. Male mice were castrated or sham-operated at 3 weeks; all females were intact. At 12 weeks of age, all mice received their treatment (CLO: clofibrate; TES: testosterone; and CLO + TES: clofibrate + testosterone) for 3 weeks and were then killed as described in Materials and Methods. In each graph, bars labeled with different letters were statistically different (P < 0.05). Data are the means ± SD of 3 mice.

in the liver than in the kidneys. Interestingly, in their studies of PPAR gene expression in mice, Issemann and Green [39] demonstrated the presence of two mRNAs in the liver and only one in the kidneys. The lack of one mRNA in the kidneys may explain the weaker induction of renal sEH activity by clofibrate. Furthermore, investigation of sEH regulation in the rat showed that contrary to what occurred in the liver, sEH induction in kidneys by fenofibrate (another hypolipidemic drug) did not correlate with induction of other enzymes involved in peroxisomal β-oxidation [43]. This suggests that tissue-selective regulation of sEH can occur.

When clofibrate and testosterone were given together, the increase in sEH specific activity in kidneys was approximately equal to the sum of the

increase in activity for the two compounds given alone. This additive effect is also suggested in liver of females and castrated males but was not statistically verified. This apparent additive effect supports the hypothesis that clofibrate and testosterone act independently within each organ. The differential inducibility of sEH in these two organs may be explained by the presence of differently expressed receptors. Our results could be explained by at least two different mechanisms. Clofibrate and testosterone could act via distinct regulatory mechanisms involving different responsive elements upstream or downstream of the gene encoding sEH. Alternatively, they could share a regulation mechanism via different receptors which would be activated by compounds that belong to a different

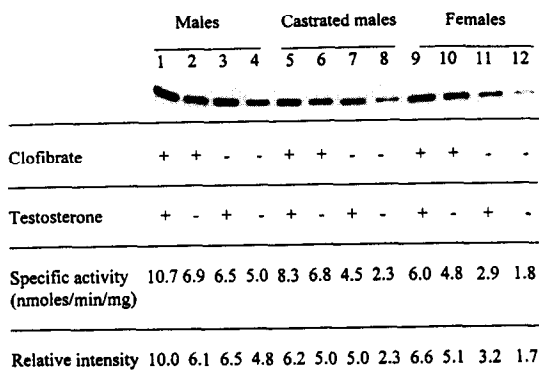|	Males				Castrated males				Females			
	1	2	3	4	5	6	7	8	9	10	11	12
Clofibrate	+	+	-	-	+	+	-	-	+	+	-	-
Testosterone	+	-	+	-	+	-	+	-	+	-	+	-
Specific activity (nmoles/min/mg)	10.7	6.9	6.5	5.0	8.3	6.8	4.5	2.3	6.0	4.8	2.9	1.8
Relative intensity	10.0	6.1	6.5	4.8	6.2	5.0	5.0	2.3	6.6	5.1	3.2	1.7

Fig. 6. Western blot and sEH activity in the kidneys of mice. Mice were 3 weeks old when castrated or sham-operated and 18 weeks old when killed. They were treated (+) with clofibrate or testosterone or were untreated controls (−). Each lane represents a single mouse. Cytosolic protein (150 ng) from kidneys was fractionated by 10% SDS–PAGE and electroblotted onto nitrocellulose. The cytosol of the same mouse was used to measure the sEH activity. We used the Amersham ECL kit, as recommended by the manufacturer, to detect bound IgG. Scanning of the film was performed using an IS-1000 Digital Imaging System from the Alpha Innotech Scientific Corp.

family (e.g. hypolipidemic drugs, fatty acids or hormones). Indeed, in rat liver DHEA induces the proliferation of peroxisomes, but this steroid hormone does not activate the receptor that has been cloned from rat liver [41, 44]. Furthermore, the presence of distinct PPAR has been established in the liver of mouse [45] and in *Xenopus* [40], suggesting the possibility, at least, that some PPAR could be activated by compounds other than those now considered as classic peroxisome proliferators.

Data from Loury *et al.* [46] suggest that the protein responsible for TSO hydrolysis in the liver and kidneys was the same with or without clofibrate treatment. Our data extend this observation to testosterone since analytical isoelectric focusing demonstrated a single peak of activity regardless of the treatment or tissue source (data not shown). Similarly, in the results from SDS–PAGE shown in Fig. 6, a single immunoreactive band was observed in each case, using an antibody prepared with affinity-purified sEH. Loury *et al.* [46] showed a 50% increase of renal sEH activity after treatment with clofibrate. The fact that the 35% of the increase that we measured in a similar experiment was not significant may be due to the low number of mice treated. This discrepancy may also be due to the use of a different strain of mouse.

Our results show that sEH is under hormonal regulation and is also regulated by a peroxisome proliferator. Previous results indicate that events related to peroxisome proliferation are linked to hormonal regulation. For instance, treatment with a steroid hormone, DHEA, leads to peroxisome proliferation and induction of peroxisomal enzymes [44]. Perfluoro-octanoic acid causes peroxisome

proliferation in male Wistar rats but not in females [47]. Induction of peroxisomal fatty acid β-oxidation in Wistar rats is dependent on testosterone [48]. Clofibrate-induced proliferation of peroxisomes is blunted in the absence of testosterone in the liver of male Sprague–Dawley rats [49]. Mice treated with fibrates can ultimately develop hepatocarcinoma [34]. Interestingly, testosterone can also promote hepatocarcinogenesis in mice [50].

Finally, it will be of great interest to analyze the 5′ flanking region of the sEH gene to look for the presence of an element similar to the motif TGACCT present on the rat acyl CoA oxidase gene promoter. This motif has been shown to be recognized by PPAR cloned from mice [51] and by nuclear hormone receptors [52–54]. Such an element could explain the regulation of the enzyme by peroxisome proliferators and by hormones like testosterone via activation of receptors.

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