

Stimulatory Effect of Clofibrate and Gemfibrozil Administration on the Formation of Fatty Acid Esters of Estradiol by Rat Liver Microsomes

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Received June 28, 2000; accepted September 1, 2000 This paper is available online at <http://jpet.aspetjournals.org>

ABSTRACT

Fatty acyl-coenzyme A (CoA):estradiol acyltransferase in liver microsomes catalyzes the formation of estradiol fatty acid esters. These esters are lipophilic and have prolonged hormonal activity because they are slowly metabolized and because they slowly release estradiol. In the present study, we have shown that treatment of rats with clofibrate or gemfibrozil (peroxisome proliferators that are commonly used hypolipidemic drugs) markedly stimulate the liver microsomal esterification of estradiol. Administration of 0.15, 0.30, 0.45, or 0.60% clofibrate in an AIN-76A diet to female rats for 4 weeks stimulated fatty acyl-CoA:estradiol acyltransferase activity per milligram of microso-

mal protein by 4-, 8-, 14- and 16-fold, respectively, when estradiol was incubated with liver microsomes and a fatty acyl-CoA. Additional studies showed that incubation of ³H-labeled estradiol with liver microsomes, ATP, and coenzyme A resulted in the formation of multiple fatty acid esters of estradiol from endogenous fatty acids in liver microsomes, and the formation of these esters was stimulated manyfold by pretreatment of rats with clofibrate. This study provides the first demonstration of a stimulatory effect of an environmental agent on the esterification of an estrogen.

In 1981, Schatz and Hochberg showed that incubation of animal or human tissues with estradiol resulted in the formation of a nonpolar metabolite of estradiol (Schatz and Hochberg, 1981). Further studies indicated that incubation of ³H-labeled estradiol with bovine endometrial tissue resulted in the formation of 10 fatty acid esters of estradiol, exclusively esterified at the 17 β -hydroxyl group (Mellon-Nussbaum et al., 1982). Characterization of the enzymatic esterification of estradiol revealed that incubations of microsomes from human mammary cancer tissue with ³H-labeled estradiol and fatty acyl-coenzyme A (CoA) resulted in fatty acid esterification of estradiol (Martyn et al., 1987).

Esterified metabolites of estradiol have little or no estrogen receptor binding affinity (Janocko et al., 1984), but they possess prolonged hormonal activity in vivo. Estradiol fatty acid esters are highly lipophilic, and they have very long half-lives and may function as a reservoir, particularly in fat-rich tissues, for the prolonged release of hormonally ac-

tive estradiol (Larner and Hochberg, 1985; Larner et al., 1985; Vazquez-Alcantara et al., 1985, 1989; MacLusky et al., 1989; Hochberg et al., 1991). The formation, metabolism, and biological activity of fatty acid esters of estradiol and of other steroids were recently reviewed by Hochberg (1998). Although the functional importance of the esterification of estradiol with fatty acids is largely unclear, it is expected that changes in the metabolic formation of estradiol fatty acid esters will alter the hormonal activity of estradiol.

Earlier studies in our laboratory demonstrated that treatment of rats with drugs such as phenobarbital, certain halogenated hydrocarbon insecticides, and other inducers of the cytochrome P450 enzymes stimulated the hydroxylation of estradiol and other estrogens by liver microsomes (Levin et al., 1967, 1968; Welch et al., 1967, 1968, 1971; Suchar et al., 1996). Enhanced hydroxylation in the treated rats was associated with a decreased uterotrophic effect of estradiol and estrone (Levin et al., 1967, 1968; Welch et al., 1967, 1971). Treatment of rats with phenobarbital, dexamethasone, or 3-methylcholanthrene each stimulated the formation of a different profile of hydroxylated metabolites of estradiol (Suchar et al., 1996). Although treatment of rats with clofibrate had only a small stimulatory effect on the liver microsomal hydroxylation of estradiol (Suchar et al., 1996), preliminary

This work was supported by unrestricted donations to the Department of Chemical Biology, College of Pharmacy, Rutgers, The State University of New Jersey.

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ABBREVIATIONS: CoA, coenzyme A; E₂, estradiol; ACAT, acyl-CoA:cholesterol acyltransferase; PPAR, peroxisome proliferator activated receptor.

results indicated a manyfold stimulation in the liver microsomal esterification of estradiol with fatty acids (Xu et al., 1997).

Clofibrate and gemfibrozil (commonly prescribed hypolipidemic drugs) are two classical peroxisome proliferators which, upon administration to rodents, increase the size and number of hepatic peroxisomes (Hess et al., 1965; Reddy and Lalwani, 1983). These drugs also activate the transcription of genes for the peroxisomal β -oxidation of fatty acids (Reddy et al., 1986) and of genes for the cytochrome P450 4A family in liver microsomes (Hardwick et al., 1987; Sharma et al., 1988a,b). Clinical studies indicate that some men treated chronically with clofibrate have side effects related to disturbed sex hormone function such as decreased libido and breast tenderness or enlargement (The Coronary Drug Project Research Group, 1975). It is possible these side effects are related to clofibrate-induced changes in estradiol metabolism.

In the present study, we characterized the fatty acyl-CoA:estradiol acyltransferase in rat liver microsomes and found that treatment of rats with clofibrate or gemfibrozil markedly stimulated the formation of estradiol fatty acid esters by liver microsomes, suggesting that these drugs may prolong or enhance the hormonal action of endogenous estradiol—particularly in the mammary gland and in other lipid-rich tissues. The stimulatory effect of clofibrate on the esterification of estradiol with fatty acids may provide an explanation for the clinical side effects of clofibrate described above.

Experimental Procedures

Chemicals. [4-¹⁴C]Estradiol (~56 mCi/mmol) and [2,4,6,7,16,17-³H(N)]estradiol (110–170 Ci/mmol) were purchased from PerkinElmer (Boston, MA). Estradiol, clofibrate, gemfibrozil, palmitoyl-CoA, palmitoleoyl-CoA, stearoyl-CoA, oleoyl-CoA, linoleoyl-CoA, and arachidonoyl-CoA were purchased from the Sigma Chemical Co. (St. Louis, MO). All solvents were of HPLC grade and were purchased from Fisher Scientific (Pittsburgh, PA). The use of high grade ethyl acetate is particularly important, because the use of less pure ethyl acetate sometimes resulted in the formation of artifacts that were detected by HPLC and interfered with our assay. The purity of ethyl acetate (1999 catalog no. E195-4, Fisher Scientific, Fairlawn, NJ) used in our studies was 99.9%.

Animals and Preparation of Subcellular Fractions of Liver. Female Sprague-Dawley rats (5 or 8 weeks old) were obtained from Harlan Sprague-Dawley Laboratory (Indianapolis, IN). The animals were kept on a 12-h light/dark cycle and had free access to Purina Laboratory Chow 5001 (Ralston-Purina Co., St. Louis, MO) and water. They were allowed to acclimatize for 1 week before use, except that, in the dietary feeding experiment, animals were allowed to acclimatize for 3 days. For studies on the biochemical properties of fatty acyl-CoA:estradiol acyltransferase, liver samples from four adult female Sprague-Dawley rats (9 weeks old) were pooled and hepatic subcellular fractions (nuclei, mitochondria, microsomes, lysosomes, and cytosol) were prepared by multistep sucrose-gradient centrifugations as described by Ragab et al. (1967). In the induction studies, animals were treated with peroxisome proliferators. Female rats (9 weeks old) were injected i.p. with clofibrate (100–400 mg/kg in corn oil) or gemfibrozil (50–300 mg/kg in corn oil) once daily for 4 days, and the animals were sacrificed on the 5th day. In a dietary feeding experiment, rats (5.5 weeks old) were fed 0.15 to 0.60% clofibrate (w/w) in an AIN-76A diet (Research Diets, New Brunswick, NJ) for 4 weeks. Animals were sacrificed after treatment, and liver was removed for the preparation of microsomes as described earlier (Thomas et al., 1983). The protein concentration was determined

with the Bio-Rad (Richmond, CA) assay method according to the supplier's instructions using bovine serum albumin as a standard.

Incubation Conditions. Reaction mixtures contained 5 to 100 μ M [4-¹⁴C]estradiol (0.3–0.5 μ Ci) or ³H-labeled estradiol (1–5 μ Ci), 100 μ M fatty acyl-CoA, 5 mM magnesium chloride in 0.1 M sodium acetate buffer (pH 4.0–8.0) in a glass test tube (16-mm diameter). For the preparation of the incubation mixture, radioactive estradiol in ethanol was added first, dried under nitrogen, and then the remaining components of the incubation mixture (including nonradioactive estradiol in 5 μ l of ethanol) were added. This procedure resulted in uniform distribution of radioactive and nonradioactive estradiol throughout the incubation mixture. In some experiments, 1 nM ³H-labeled estradiol was used for the incubations. The reaction was initiated by the addition of hepatic subcellular preparations (1 mg of protein/ml for microsomes from control rats or 0.5 mg of protein/ml for microsomes from clofibrate- or gemfibrozil-treated rats). The final volume of the incubation mixture was 0.5 ml. After incubation at 37°C for 30 min, the reaction was arrested by placing the tubes on ice, followed by addition of 0.5 ml of ice-cold sodium acetate buffer (pH 5.5) and 5 ml of ethyl acetate (HPLC grade from Fisher Scientific). The samples were vortexed immediately and centrifuged for 10 min at 3000g. The organic phase was removed, and the extraction was repeated a second time. The organic solvent extracts were combined and evaporated to dryness under a stream of nitrogen. Each resulting residue was dissolved in 100 μ l of methanol and analyzed by HPLC.

HPLC Method. Measurement of esterified metabolites of estradiol was done by HPLC on a Spherisorb ODS column (5- μ m particle size, 250 \times 4.6 mm i.d.) with a modification of a previously described method (Paris and Rao, 1989). The HPLC system consisted of a Waters (Milford, MA) 600E solvent gradient programmer, a Waters Lambda-Max model 481 UV detector (set at 280 nm), and a radioactive flow detector (β -ram from IN/US, Fairfield, NJ) with a solid cell (for ¹⁴C detection) or a liquid cell (for ³H detection). The solvent system consisted of acetonitrile/H₂O with 0.1% acetic acid/methanol. The solvent gradient used for elution of the compounds from the column was as follows: 12-min isocratic at 30/6/64; 6-min with a 10 convex gradient to 60/0/40; 15-min isocratic at 60/0/40; 2-min with a 2 convex gradient to 20/0/80; 5-min isocratic at 20/0/80, and the column was then returned to initial conditions over 15 min. The flow rate was 1.2 ml/min. The retention times of the radioactive metabolites agreed exactly with corresponding UV-absorbing peaks. Metabolite quantification was based on the amount of radioactivity in the metabolite peak as compared to the total radioactivity collected from the HPLC column from each sample.

Results

Characterization of Fatty Acyl-CoA:Estriadiol Acyltransferase in Rat Liver

HPLC Assay and Liver Microsome-Catalyzed Formation of Estradiol Fatty Acid Esters. Incubation of [4-¹⁴C]estradiol with liver microsomes in the presence of arachidonoyl-CoA, palmitoleoyl-CoA, linoleoyl-CoA, oleoyl-CoA, palmitoyl-CoA, or stearoyl-CoA as a cofactor, followed by HPLC detection of the products, revealed a single radioactive peak that was less polar than estradiol (Fig. 1). No metabolites were observed when [4-¹⁴C]estradiol was incubated with liver microsomes in the absence of a fatty acyl-CoA (data not presented). Each estradiol fatty acid ester had a distinct retention time. The retention times for estradiol, estradiol-arachidonoyl ester, estradiol-palmitoleoyl ester, estradiol-linoleoyl ester, estradiol-oleoyl ester, estradiol-palmitoyl ester, and estradiol-stearoyl ester were 3, 18.4, 20.8, 22.2, 27.6, 28.1, and 34.3 min, respectively. In an additional study, rat liver microsomes were incubated with estradiol

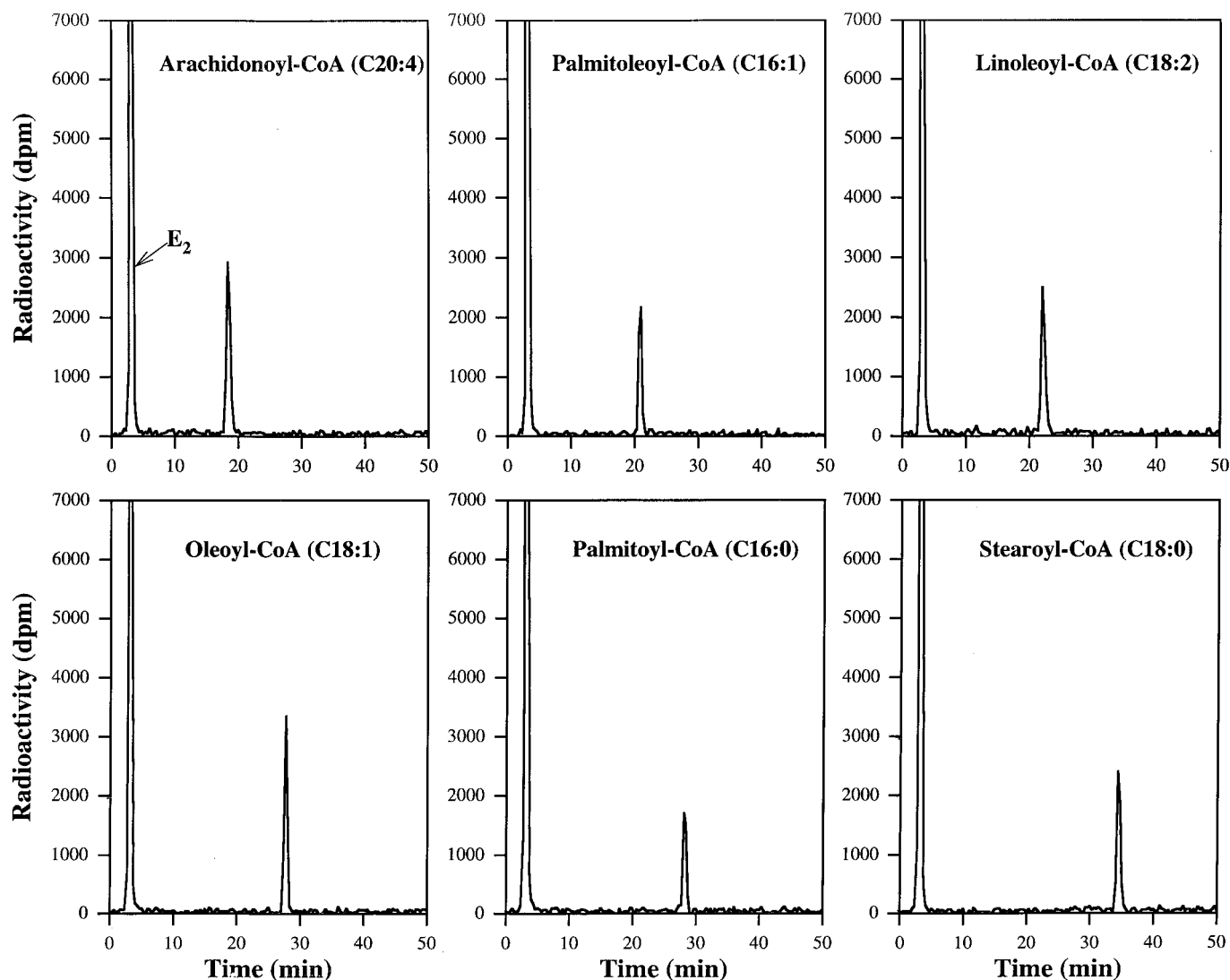


Fig. 1. HPLC analysis of the metabolic formation of estradiol fatty acid esters. Liver microsomes (1 mg of protein/ml) from adult female rats were incubated with 10 μM [$4\text{-}^{14}\text{C}$]estradiol (E_2) and 100 μM a fatty acyl-CoA (arachidonoyl-CoA, palmitoleyl-CoA, linoleoyl-CoA, oleoyl-CoA, palmitoyl-CoA, or stearoyl-CoA) at pH 5.0. The samples were extracted with ethyl acetate and evaporated to dryness under nitrogen. The resulting residues were dissolved in 100 μl of methanol and analyzed by HPLC as described under *Experimental Procedures*.

and oleoyl-CoA or stearoyl-CoA. HPLC peaks corresponding to estradiol-17 β oleoyl ester or estradiol-17 β stearoyl ester were collected from the HPLC column and analyzed by mass spectrometry. The mass spectral data agreed with that described earlier for the chemically synthesized or biosynthetically formed oleoyl or stearoyl ester of estradiol (Mellon-Nussbaum et al., 1982). The parent and fragment ions for estradiol-17 β oleoyl ester (mol. wt. = 536) were m/e 536 (equivalent to M^+), m/e 255, which is equivalent to $(\text{M} - \text{RCOO})^+$, and m/e 254, which is equivalent to $(\text{M} - \text{RCOOH})^+$. The parent and fragment ions for estradiol-17 β stearoyl ester (mol. wt. = 538) were m/e 538 (equivalent to M^+), m/e 255 which is equivalent to $(\text{M} - \text{RCOO})^+$ and m/e 254 which is equivalent to $(\text{M} - \text{RCOOH})^+$. The formation of estradiol-oleoyl ester was used as a typical fatty acyl-CoA:estradiol acyltransferase reaction and was approximately linear with time of incubation from 5 to 30 min. The rate of formation of estradiol-oleoyl ester was proportional to microsomal protein concentration from 0.25 to 1.0 mg per ml of incubation mixture. For ease of the measurements, we used

a 30-min incubation time and 0.5 to 1 mg of microsomal protein per ml for most enzyme assays. We evaluated the formation of individual estradiol fatty acid esters in the presence of different fatty acyl-CoAs, and there was about a 2-fold difference for the in vitro formation of different estradiol fatty acid esters (Table 1). Estradiol-arachidonoyl ester and estradiol-oleoyl ester were formed to the greatest extent, whereas estradiol-palmitoyl ester was formed to the least extent.

Intracellular Distribution of Fatty Acyl-CoA: Estradiol Acyltransferase. Fatty acyl-CoA:estradiol acyltransferase activity was measured in different subcellular fractions obtained from the livers of adult female Sprague-Dawley rats (Fig. 2). The liver microsomal fraction contained the highest specific activity of acyltransferase (41.1 pmol/mg of protein/min), followed by the lysosomal fraction (17.2 pmol/mg of protein/min), the nuclear fraction (16.1 pmol/mg of protein/min), and the mitochondrial fraction (13.2 pmol/mg of protein/min). Little or no fatty acyl-CoA:estradiol acyltransferase was detected in the hepatic cytosolic fraction.

TABLE 1

Effect of different fatty acyl-CoAs on the formation of estradiol fatty acid esters by rat liver microsomes

Liver microsomes (1 mg of protein/ml) from adult female rats were incubated for 30 min with 10 μM [$4\text{-}^{14}\text{C}$]estradiol and 100 μM fatty acyl-CoA in a final volume of 0.5 ml of sodium acetate buffer (0.1 M, pH 5.0). Formation of estradiol fatty acid ester was measured as described under *Experimental Procedures*. Each value is the mean \pm S.D. of triplicate determinations with the same liver microsomal preparation.

Fatty Acyl-CoA Incubated	Formation of Estradiol Fatty Acid Ester <i>pmol/mg protein/min</i>
Palmitoyl-CoA (C16:0)	30.5 \pm 0.5
Palmitoleoyl-CoA (C16:1)	41.8 \pm 0.9
Stearoyl-CoA (C18:0)	41.2 \pm 1.5
Oleoyl-CoA (C18:1)	48.0 \pm 1.0
Linoleoyl-CoA (C18:2)	42.1 \pm 2.3
Arachidonoyl-CoA (C20:4)	63.0 \pm 2.9

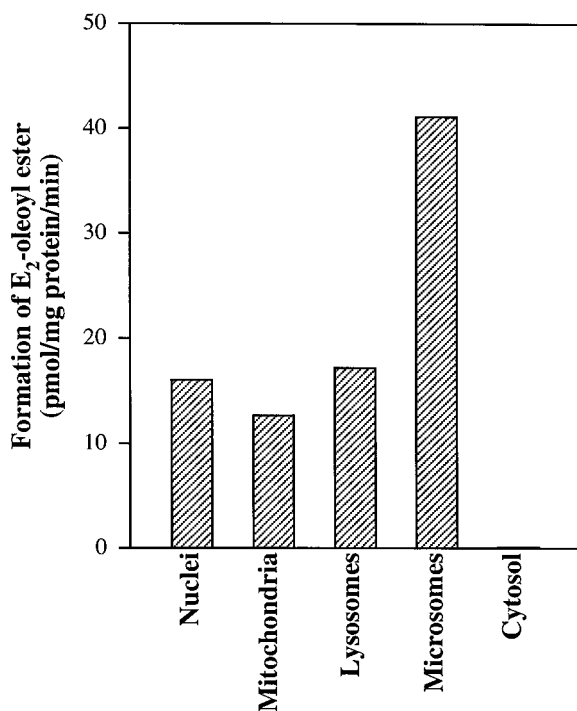


Fig. 2. Intracellular distribution of fatty acyl-CoA:estradiol acyltransferase activity in rat liver. Subcellular fractions of liver from adult female rats were isolated as described under *Experimental Procedures*. The incubation mixture consisted of 1 mg/ml subcellular protein, 10 μM [$4\text{-}^{14}\text{C}$]estradiol (E_2), and 100 μM oleoyl-CoA in a final volume of 0.5 ml of sodium acetate buffer (0.1 M, pH 5.0). Assays for the formation of E_2 -oleoyl ester were done as described under *Experimental Procedures*. The values are the mean of duplicate determinations.

pH Optimum for Fatty Acyl-CoA:Estradiol Acyltransferase. The pH dependence curve for the esterification of estradiol in the presence of either oleoyl-CoA or stearoyl-CoA were similar, and an optimum pH of 5.0 to 5.5 was observed (Fig. 3). Considerably less but measurable activity was observed at pH 7.4.

Effect of the Concentration of Estradiol and Fatty Acyl-CoA on the Formation of Estradiol Fatty Acid Esters. The rate of enzymatic conversion of estradiol (20 or 100 μM) to fatty acid esters depended on the concentration of fatty acyl-CoA. Optimum synthesis of estradiol-stearoyl ester occurred at 100 μM stearoyl-CoA, and higher concentrations of stearoyl-CoA showed an inhibitory effect on the formation of estradiol-stearoyl ester (Fig. 4). The rate of esterification as a function of increasing concentrations of estradiol is in-

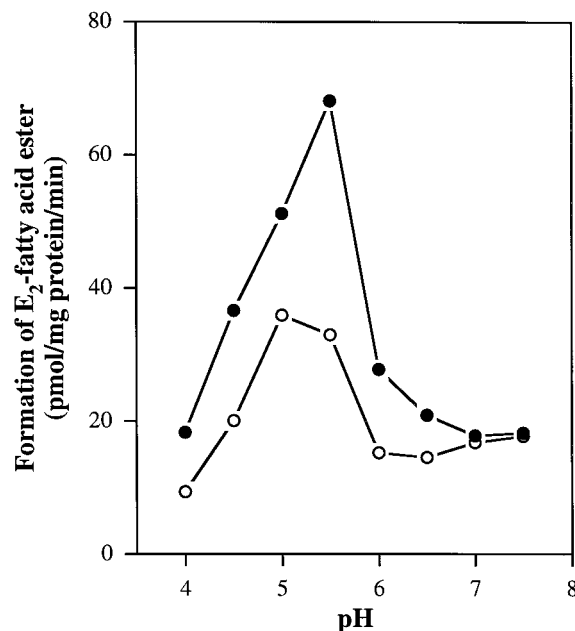


Fig. 3. The effect of different pH values on hepatic microsomal fatty acyl-CoA:estradiol acyltransferase activity. Liver microsomes (1 mg of protein/ml) from adult female rats were incubated with 10 μM [$4\text{-}^{14}\text{C}$]estradiol (E_2) and 100 μM oleoyl-CoA (●) or stearoyl-CoA (○) in a final volume of 0.5 ml of sodium acetate buffer (0.1 M, pH 4.0–7.5) at 37°C for 30 min. Assays for the formation of fatty acid esters of E_2 were done as described under *Experimental Procedures*. Each value is the mean of duplicate determinations with the same microsomal preparation.

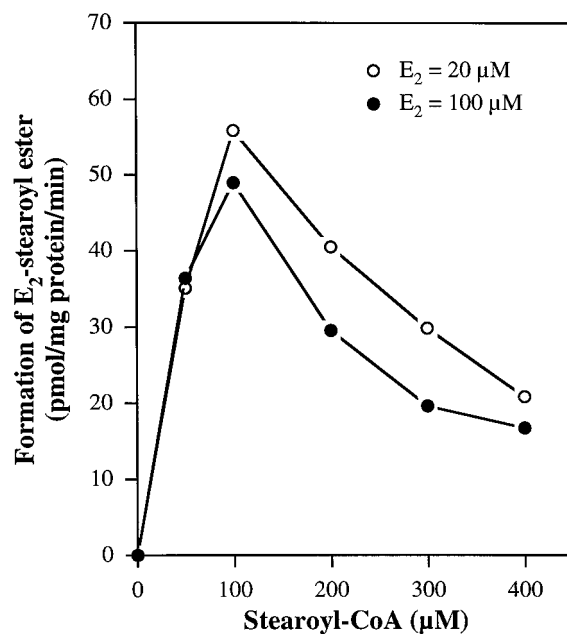


Fig. 4. The effect of different concentrations of stearoyl-CoA on the formation of estradiol-stearoyl ester by rat liver microsomes. Liver microsomes (1 mg of protein/ml) from adult female rats were incubated with 20 or 100 μM [$4\text{-}^{14}\text{C}$]estradiol (E_2) and 50 to 400 μM stearoyl-CoA in a final volume of 0.5 ml of sodium acetate buffer (0.1 M, pH 5.0) at 37°C for 30 min. Assays for the formation of E_2 -stearoyl ester were done as described under *Experimental Procedures*. Each point is the mean of duplicate determinations.

dicated in Fig. 5. Under optimal conditions for in vitro esterification (pH 5.0; fatty acyl-CoA concentration, 100 μM), the microsomal fatty acyl-CoA:estradiol acyltransferase had a K_m value for estradiol of around 4 to 6 μM in the presence of

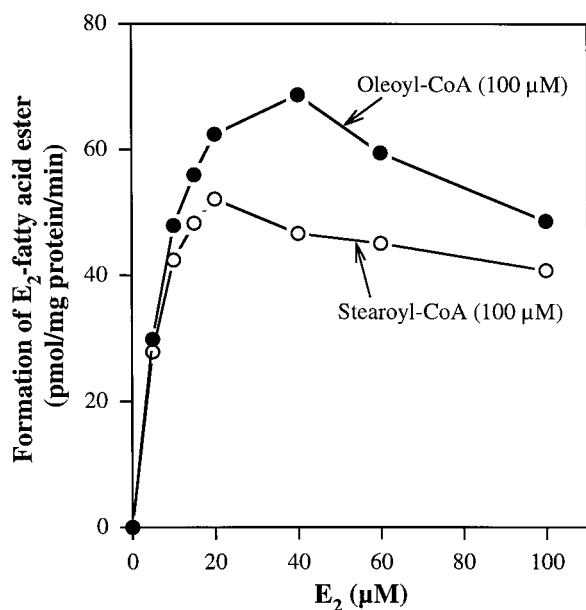


Fig. 5. Effect of different concentrations of estradiol on the liver microsomal metabolism of estradiol to fatty acid esters. Liver microsomes from adult female rats were incubated with 5 to 100 μM [$4\text{-}^{14}\text{C}$]estradiol (E_2) and 100 μM oleoyl-CoA or stearoyl-CoA in a final volume of 0.5 ml of sodium acetate buffer (0.1 M, pH 5.0) at 37°C for 30 min. Assays for the formation of fatty acid esters of E_2 were done as described under *Experimental Procedures*. Each point is the mean of duplicate determinations.

oleoyl-CoA or stearoyl-CoA. The V_{max} for estradiol esterification in the presence of oleoyl-CoA as a cofactor was slightly higher than the V_{max} for estradiol esterification in the presence of stearoyl-CoA (Fig. 5).

Stimulatory Effect of Clofibrate or Gemfibrozil Administration on Fatty Acyl-CoA: Estradiol Acyltransferase in Rat Liver Microsomes

Effect of i.p. Injections of Clofibrate and Gemfibrozil. Intraperitoneal injections of clofibrate (300 mg/kg/day in corn oil for 4 days) caused only a small increase in the NADPH-dependent metabolism of estradiol (less than a 60% increase), but administration of this compound stimulated by severalfold the activity of fatty acyl-CoA:estradiol acyltransferase in rat liver microsomes (Fig. 6). Intraperitoneal injections of clofibrate (100–400 mg/kg/day in corn oil) or gemfibrozil (50–300 mg/kg/day in corn oil) once daily for 4 days increased the rate of estradiol esterification by liver microsomes in a dose-dependent manner (Fig. 6). Maximal induction ranged from 5- to 9-fold. The magnitude of induction for estradiol esterification was similar when estradiol was incubated with palmitoyl-CoA, palmitoleoyl-CoA, stearoyl-CoA, oleoyl-CoA, linoleoyl-CoA, or arachidonoyl-CoA (Table 2). Treatment of rats with clofibrate or gemfibrozil did not influence the intracellular distribution (data not presented) or the pH dependence curve for the liver microsomal metabolism of estradiol (in the presence of oleoyl-CoA) to estradiol-oleoyl ester (Fig. 7). Treatment of rats with clofibrate or gemfibrozil had little or no effect on the apparent K_m value, but the V_{max} value was markedly increased (Fig. 8). In an additional study, treatment of adult female rats with sodium phenobarbital (i.p. injections of 75 mg/kg/day in water for 4 days), 3-methylcholanthrene (i.p. injections of 25 mg/kg/day in corn oil for 4 days), or dexamethasone (i.p. injections of 75

mg/kg/day in corn oil for 4 days) stimulated the NADPH-dependent hydroxylation of estradiol by liver microsomes, but there was a relatively small effect of these inducers on fatty acyl-CoA:estradiol acyltransferase activity (less than an 80% increase, data not presented).

Effect of Dietary Administration of Clofibrate. Administration of 0.15, 0.30, 0.45, or 0.60% clofibrate in an AIN-76A diet to female rats for 4 weeks stimulated fatty acyl-CoA:estradiol acyltransferase activity per milligram of microsomal protein by 4-, 8-, 14-, and 16-fold, respectively (Fig. 9). The liver/body weight ratios were increased 9, 19, 33, and 59%, respectively (data not presented). We determined whether pretreatment of rats with 0.60% dietary clofibrate stimulated the esterification of a low physiologically relevant 1 nM concentration of estradiol by liver microsomes. Liver microsomal fatty acyl-CoA:estradiol acyltransferase activity was increased more than 10-fold when either 1 nM or 50 μM estradiol (in the presence of 100 μM oleoyl-CoA) was incubated with liver microsomes (Table 3). In a separate study, the i.p. injection of 400 mg/kg of clofibrate once a day for 4 days stimulated fatty acyl-CoA:estradiol acyltransferase activity by 5- and 9-fold, respectively, when a 1 nM or 50 μM concentration of estradiol was used as the substrate (data not presented).

Effect of Clofibrate Administration on the Esterification of Estradiol by Endogenous Fatty Acids in Liver Microsomes. Incubation of control rat liver microsomes (1 mg of protein per ml) with ^3H -labeled estradiol, ATP, and CoA resulted in the formation of radioactive peaks corresponding to estradiol-arachidonoyl ester, estradiol-palmitoleoyl ester, estradiol-linoleoyl ester, estradiol-oleoyl ester, estradiol-palmitoyl ester, and estradiol-stearoyl ester (Fig. 10), and formation of all of these peaks were increased manyfold when estradiol was incubated with ATP, CoA, and liver microsomes from rats fed 0.60% clofibrate for 4 weeks (Fig. 10). The incubations with induced liver microsomes were done with only 0.5 mg of microsomal protein per ml. Two new peaks (D and E) not observed from incubations with control microsomes were formed during incubations with liver microsomes from clofibrate-treated rats (Fig. 10). The manyfold increase in the esterification of estradiol (pmol/mg of protein/min) by endogenous fatty acids in liver microsomes is summarized in Fig. 11. The arachidonoyl ester of estradiol was the most prominent metabolite formed (Figs. 10 and 11).

Discussion

Fatty acyl-CoA:estradiol acyltransferase in rat liver microsomes catalyzed the conjugation of estradiol with several fatty acyl-CoAs. In the presence of each of six exogenously added fatty acyl-CoAs, estradiol was esterified to the corresponding estradiol fatty acid ester, and the rate of formation of each of the estradiol fatty acid esters was similar (less than a 2-fold difference; Table 2). In contrast to these results, incubation of rat liver microsomes with ATP, CoA, and estradiol but without exogenously added fatty acids resulted in the formation of six to eight different fatty acid esters of estradiol, and the relative formation of these fatty acid esters differed by severalfold (Fig. 11). Possible reasons for differences in the relative rates of formation of the different fatty acid esters of estradiol from endogenous fatty acids in liver microsomes include: 1) differences in the content of the dif-

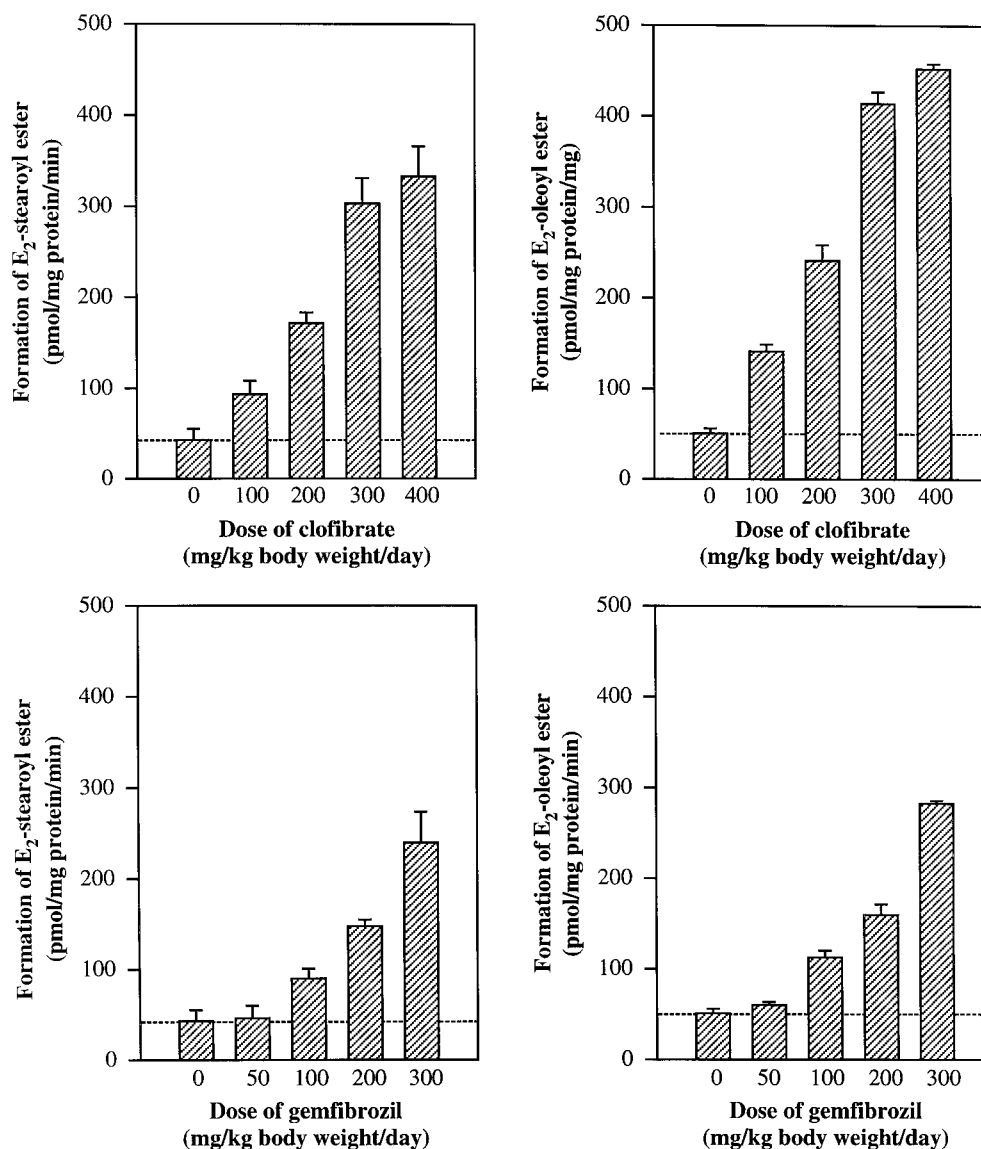


Fig. 6. Stimulatory effect of i.p. injections of clofibrate or gemfibrozil on the formation of estradiol fatty acid esters by rat liver microsomes. Adult female rats were injected i.p. with clofibrate (100–400 mg/kg in corn oil), gemfibrozil (50–300 mg/kg in corn oil), or vehicle (corn oil) once daily for 4 days. Animals were sacrificed 24 h after the last dose, and liver samples from four rats per group were pooled for the preparation of hepatic microsomes. The incubation mixture consisted of 1 mg/ml microsomal protein from control rats or 0.5 mg/ml microsomal protein from induced rats, 50 μ M [4-¹⁴C]estradiol (E₂), and 100 μ M oleoyl-CoA or stearoyl-CoA in a final volume of 0.5 ml of sodium acetate buffer (0.1 M, pH 5.0). Each value is the mean \pm S.D. obtained from triplicate determinations with the same microsomal preparation.

ferent fatty acids in liver microsomes, 2) differences in the rates of formation of different fatty acyl-CoAs in the presence of ATP and CoA, and 3) differences in the rates of esterification of estradiol by the very low amounts of fatty acyl-CoAs that are formed in liver microsomes supplemented with ATP and CoA. It is of interest that very long-chain fatty acids (over 20 carbon atoms) are oxidized predominantly by peroxisomes, whereas fatty acids with 10 to 20 carbon atoms are oxidized by both peroxisomes and mitochondria (Mannaerts and Van Veldhoven, 1993). A selective stimulatory effect of long-chain fatty acids may result in an alteration in the ratio between long- and short-chain fatty acids in cells. It is not known if clofibrate-induced β -oxidation of fatty acids can alter the cellular composition of estradiol fatty acid esters that contain long- and short-chain fatty acids.

The pH optimum for the esterification of estradiol was 5.0

to 5.5, which is consistent with what was previously observed with bovine placental microsomes (Martyn et al., 1988). In contrast to these results, the pH optimum for rat liver microsomal acyl-CoA:cholesterol acyltransferase (ACAT) was approximately 7.0 (Goodman et al., 1964), suggesting that fatty acyl-CoA:estradiol acyltransferase and ACAT are two different enzymes. Maximum conversion of 20 or 100 μ M estradiol to estradiol fatty acid esters occurred when a 100 μ M concentration of fatty acyl-CoA was used as cofactor, and the presence of a higher concentration of fatty acyl-CoA became inhibitory (Fig. 4). The inhibitory effects of high concentrations of fatty acyl-CoAs were also observed in an earlier study with human mammary cancer cells and were attributed to the detergent properties of long-chain fatty acyl-CoAs (Martyn et al., 1987).

In the present study, we found that i.p. injections of clofibrate and gemfibrozil or oral administration of clofibrate to

TABLE 2

Effect of pretreatment of rats with clofibrate or gemfibrozil on the liver microsomal esterification of estradiol by different fatty acyl-CoAs

Adult female rats were injected i.p. with gemfibrozil (300 mg/kg in corn oil), clofibrate (400 mg/kg in corn oil), or vehicle (corn oil) once daily for 4 days. The following day, the rats were sacrificed, and livers from four rats per group were pooled for the preparation of hepatic microsomes. The incubation mixture consisted of 1 mg/ml microsomal protein from control rats or 0.5 mg/ml microsomal protein from induced rats, 50 μM [$4\text{-}^{14}\text{C}$]estradiol (E_2), and 100 μM of each fatty acyl-CoA in a final volume of 0.5 ml of sodium acetate buffer (0.1 M, pH 5.0). Each value is the mean \pm S.D. obtained from triplicate determinations with the same liver microsomal preparation. The percentage of increase over control values is indicated in parentheses.

Fatty Acyl-CoA Incubated	Formation of Estradiol Fatty Acid Esters		
	Control	Gemfibrozil (300 mg/kg/day)	Clofibrate (400 mg/kg/day)
	<i>pmol/mg protein/min</i>		
Palmitoyl-CoA (C16:0)	30 \pm 4	163 \pm 17 (443% \uparrow)	190 \pm 18 (533% \uparrow)
Palmitoleoyl-CoA (C16:1)	33 \pm 5	128 \pm 11 (288% \uparrow)	170 \pm 5 (415% \uparrow)
Stearoyl-CoA (C18:0)	43 \pm 12	240 \pm 34 (458% \uparrow)	333 \pm 32 (674% \uparrow)
Oleoyl-CoA (C18:1)	51 \pm 6	283 \pm 3 (455% \uparrow)	452 \pm 6 (786% \uparrow)
Linoleoyl-CoA (C18:2)	40 \pm 6	212 \pm 14 (430% \uparrow)	297 \pm 19 (642% \uparrow)
Arachidonoyl-CoA (C20:4)	44 \pm 0.2	304 \pm 11 (591% \uparrow)	404 \pm 19 (818% \uparrow)

rats markedly stimulated the liver microsomal esterification of estradiol with fatty acids. This increase in the esterification of estradiol was observed when estradiol was incubated either with liver microsomes and a fatty acyl-CoA (Table 2) or with liver microsomes, ATP, and CoA (Fig. 11). Using the latter incubation conditions, we observed a clofibrate-in-

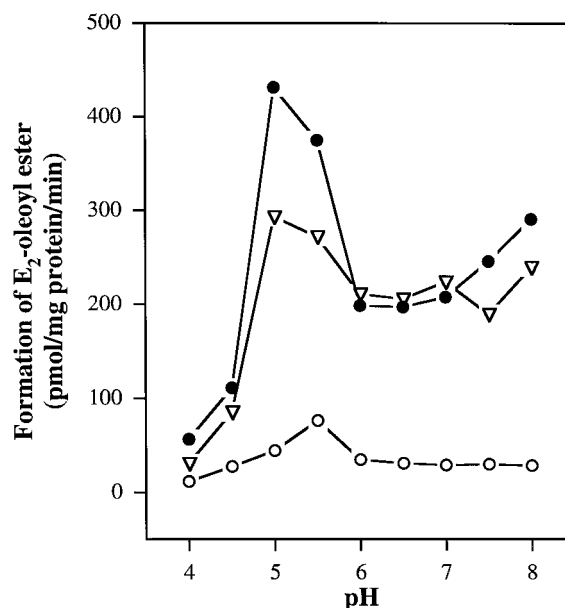


Fig. 7. Lack of effect of treatment of rats with clofibrate or gemfibrozil on the pH optimum for the liver microsomal metabolism of estradiol to estradiol-oleoyl ester. Adult female rats were injected i.p. with clofibrate (400 mg/kg in corn oil), gemfibrozil (300 mg/kg in corn oil), or vehicle (corn oil) once daily for 4 days. The incubation mixture consisted of 1 mg/ml liver microsomal protein from control rats or 0.5 mg/ml liver microsomal protein from drug-treated rats, 50 μM [$4\text{-}^{14}\text{C}$]estradiol (E_2), and 100 μM oleoyl-CoA in a final volume of 0.5 ml of sodium acetate buffer (0.1 M, pH 4.0–8.0). Each value is the mean from duplicate determinations with the same microsomal preparation. \circ , control; \bullet , clofibrate (400 mg/kg/day); ∇ , gemfibrozil (300 mg/kg/day).

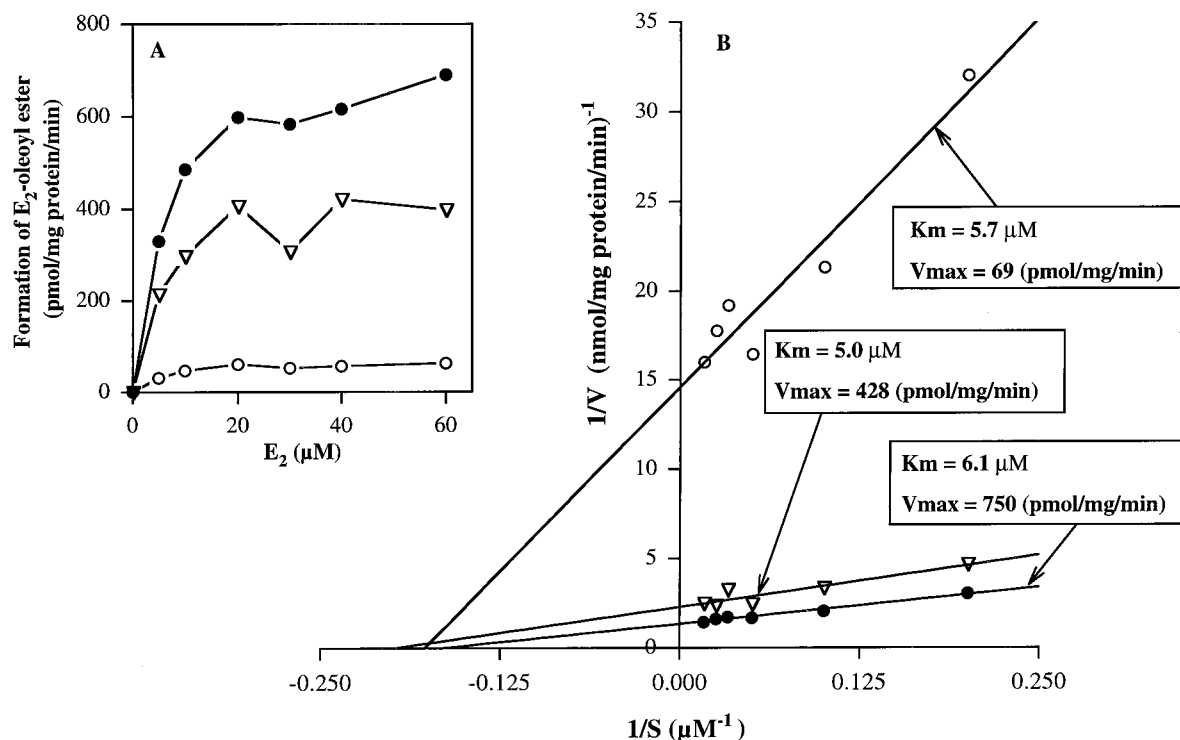


Fig. 8. Effect of treatment of rats with clofibrate or gemfibrozil on the K_m and V_{max} values for the liver microsomal metabolism of estradiol to estradiol-oleoyl ester. Adult female rats were injected i.p. with clofibrate (400 mg/kg in corn oil), gemfibrozil (300 mg/kg in corn oil), or vehicle (corn oil) once daily for 4 days. Liver microsomes were incubated with 5 to 60 μM [$4\text{-}^{14}\text{C}$]estradiol (E_2) and 100 μM oleoyl-CoA in a final volume of 0.5 ml of sodium acetate (0.1 M, pH 5.0) at 37°C for 30 min. The measurement of E_2 -oleoyl ester formed was carried out as described under *Experimental Procedures*. Each value is the mean of duplicate determinations with the same microsomal preparation. A, rate of microsomal-mediated esterification of estradiol as a function of the substrate concentration; B, Lineweaver-Burk plot for enzymatic esterification of estradiol. \circ , control; \bullet , clofibrate (400 mg/kg/day); ∇ , gemfibrozil (300 mg/kg/day).

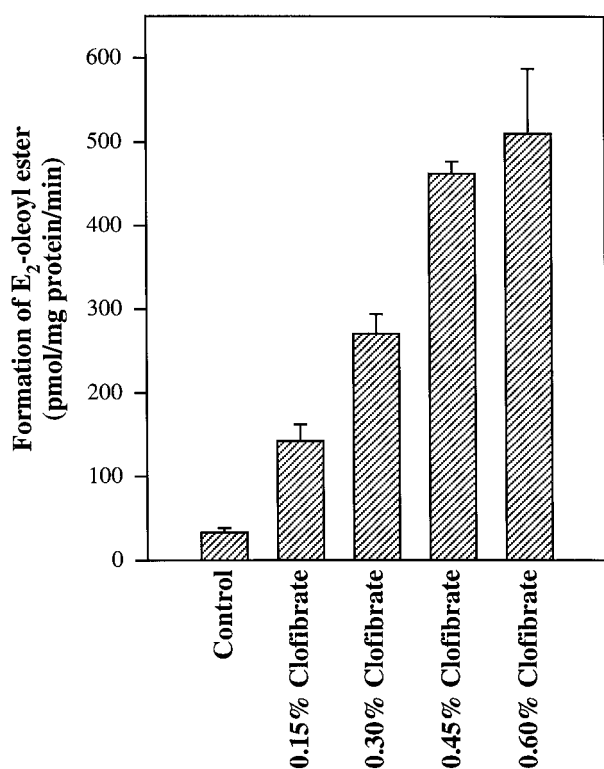


Fig. 9. Stimulatory effect of dietary clofibrate administration on the formation of estradiol-oleoyl ester by rat liver microsomes. Female rats were fed an AIN-76A diet or 0.15 to 0.60% clofibrate in an AIN-76A diet for 4 weeks. Liver was removed for the preparation of microsomes. The incubation mixture consisted of 1 mg of microsomal protein per ml from control rats or 0.5 mg of microsomal protein per ml from clofibrate-treated rats, 50 μ M [¹⁴C]estradiol (E₂), and 100 μ M oleoyl-CoA. Formation of E₂-oleoyl ester was measured as described under *Experimental Procedures*. Each value is the mean \pm S.D. obtained from liver microsomes from three to four rats.

TABLE 3

Stimulatory effect of clofibrate administration on the esterification of a low physiologically relevant 1 nM concentration of estradiol by rat liver microsomes

Female rats were fed an AIN-76A diet or 0.60% clofibrate in an AIN-76A diet for 4 weeks. [³H]Estradiol (1 nM) was incubated with 100 μ M oleoyl-CoA and 0.25 mg of protein/ml for microsomes from control rats or 0.1 mg of protein/ml for microsomes from clofibrate-treated rats at 37°C for 10 min. The data for esterification of 50 μ M estradiol were obtained from Fig. 9. Each value is the mean \pm S.D. obtained from liver microsomes from three to four rats.

Treatment	Formation of Estradiol-Oleoyl Ester	
	1 nM E ₂	50 μ M E ₂
	<i>pmol/mg protein/min</i>	
Control diet	0.02 \pm 0.01	33 \pm 6
0.60% clofibrate diet	0.23 \pm 0.01	510 \pm 77

duced manifold increase in the formation of multiple fatty acid esters of estradiol from the endogenous fatty acids present in liver microsomes (Fig. 11). To the best of our knowledge, the present study is the first demonstration of environmental modulation of the esterification of estradiol with fatty acids. The stimulatory effect of clofibrate administration on hepatic fatty acyl-CoA:estradiol acyltransferase occurred when liver microsomes and a fatty acyl-CoA were incubated with a saturating 50 μ M concentration of estradiol (~10-fold higher than the K_m ; Fig. 8) or with a low physiologically relevant 1 nM concentration of estradiol (Table 3), which may be compared with a peak plasma or serum con-

centration of estradiol during the estrus cycle of 0.3 nM in rats (Butcher et al., 1974) or 0.7 nM in humans (Mishell et al., 1971).

The results of our studies indicate that pretreatment of rats with clofibrate or gemfibrozil does not alter the pH optimum (pH 5–5.5) or K_m value for the liver microsomal esterification of estradiol (Figs. 7 and 8), but the V_{max} was increased manifold (Fig. 8). These results suggest that administration of clofibrate or gemfibrozil increased the level of the same fatty acyl-CoA:estradiol acyltransferase enzyme that is present in microsomes from untreated rats. Further studies are needed to determine whether clofibrate or gemfibrozil administration stimulates the transcription of the fatty acyl-CoA:estradiol acyltransferase gene, enhances the stability of the corresponding mRNA, facilitates the translation of the corresponding mRNA, and/or inhibits the breakdown of the fatty acyl-CoA acyltransferase protein. The induction of some enzymes (acyl-CoA oxidase and the CYP450 4A family) by peroxisome proliferators is known to result from an increased rate of gene transcription mediated by peroxisome proliferator-activated receptor alpha (PPAR α), a member of the steroid hormone receptor superfamily (Isse-mann and Green, 1990; Green and Wahli, 1994). PPAR α , when activated by peroxisome proliferators, dimerizes with retinoid X receptor α and binds to a peroxisome proliferator response element to activate gene expression (Green and Wahli, 1994; Gonzalez et al., 1998). Further studies are needed to determine whether the induction of fatty acyl-CoA:estradiol acyltransferase by clofibrate and gemfibrozil is mediated by PPAR α .

The stimulatory effect of treating rats with clofibrate on the liver microsomal esterification of low physiologically relevant concentrations of estradiol suggests that clofibrate-enhanced esterification of estradiol with fatty acids observed in vitro may also have in vivo significance. Increased formation of fatty acid esters of estradiol would be expected to enhance the hormonal activity of estradiol—particularly in fatty tissues such as the mammary gland—since these fatty acid esters are highly lipophilic and would be expected to concentrate in fatty tissues and to serve as a reservoir for slow esterase-mediated release of estradiol. Estradiol fatty acid esters have prolonged estrogenic activity. Injection of estradiol-stearoyl ester into ovariectomized mice resulted in increased estrogenic potency in the uterus and a prolonged duration of action compared with the injection of estradiol (Zielinski et al., 1991). It is expected that enhancing the metabolic formation of estradiol fatty acid esters by administration of clofibrate will prolong or enhance the hormonal activity of endogenous estradiol particularly in the mammary gland as well as in other lipid-rich tissues. Preliminary studies in our laboratory have indicated a selective stimulatory effect of clofibrate administration on the action of estradiol in the mammary gland but not in the uterus of rats (Xu et al., 1999). It has been reported that some men treated chronically with clofibrate have decreased libido and breast tenderness or enlargement (The Coronary Drug Project Research Group, 1975). The possibility that these side effects of clofibrate are related to clofibrate-induced formation of estradiol fatty acid esters requires further investigation. Epidemiological studies are needed to determine whether long-term treatment of patients with clofibrate and related hypolipidemic drugs alters the risk of breast cancer, endometrial

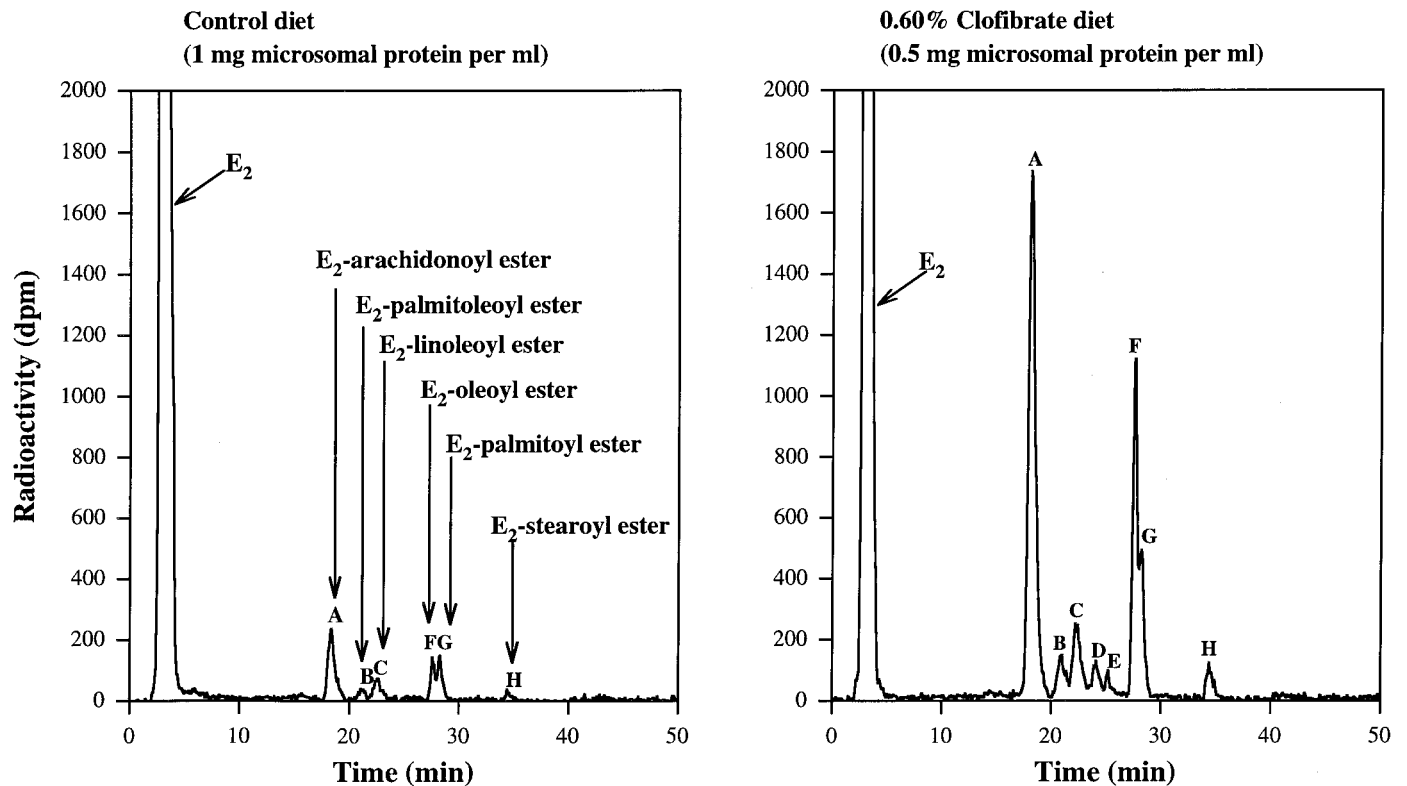


Fig. 10. Effect of dietary clofibrate administration on the formation of esterified estradiol metabolites by endogenous fatty acids in rat liver microsomes (HPLC profile). Female rats were fed an AIN-76A diet or 0.60% clofibrate in an AIN-76A diet for 4 weeks. The incubation mixture consisted of 1 mg of liver microsomal protein per ml from control rats or 0.5 mg of liver microsomal protein per ml from treated rats, 50 μ M [3 H]estradiol (E_2), 5 mM ATP, and 1 mM CoA in a final volume of 0.5 ml of sodium acetate buffer (0.1 M, pH 5.0). The products were detected by HPLC as described under *Experimental Procedures*, and the HPLC tracings are representative of those from four rats per group.

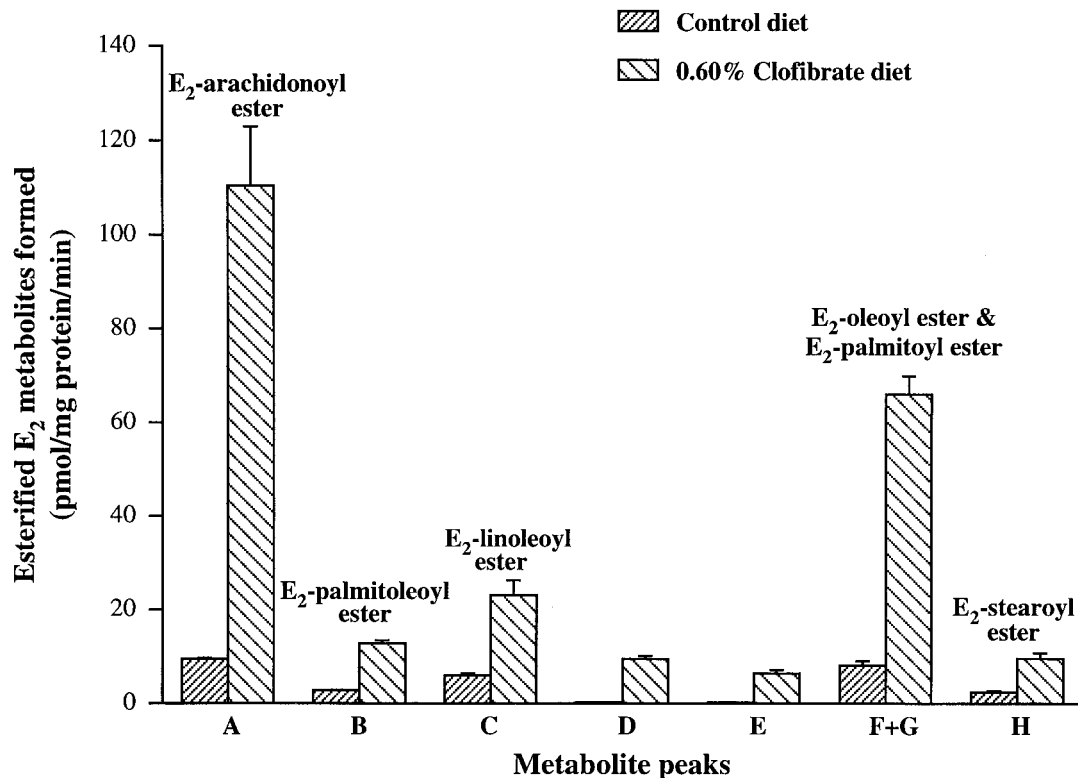


Fig. 11. Magnitude of the stimulatory effect of dietary clofibrate administration on the formation of fatty acid esters of estradiol from endogenous fatty acids in rat liver microsomes. Liver microsomes from control rats and clofibrate-treated rats were incubated with estradiol (E_2), ATP, and CoA as described in the Fig. 10 legend. Formation of fatty acid esters of E_2 was measured as described in Fig. 10. Each value is the mean \pm S.D. obtained with liver microsomes from four rats per group.

cancer, osteoporosis, or other diseases that are influenced by estrogen.

Many structurally diverse compounds in addition to the hypolipidemic drugs are peroxisome proliferators. Examples of peroxisome proliferators include the hypolipidemic drugs, herbicides (e.g., lactofen), plasticizers (e.g., phthalate esters), and solvents (e.g., trichloroethylene) (Gonzalez et al., 1998). Many of these chemicals are widely used, and they are of pharmaceutical, industrial, and environmental importance. It will be of interest to determine whether peroxisome proliferators other than clofibrate or gemfibrozil will stimulate the esterification of estradiol and alter its hormonal action.

In summary, we have studied the properties of fatty acyl-CoA:estradiol acyltransferase in rat liver microsomes. The results of our studies indicate that treatment of rats with the peroxisome proliferator, clofibrate, causes a manyfold increase in the liver microsomal esterification of estradiol with fatty acids. Additional studies are needed to evaluate the effects of clofibrate on the esterification of estradiol in the uterus, mammary gland, and in other extrahepatic tissues as well as to determine the effects of clofibrate administration on esterase activity in the liver and in extrahepatic tissues. Finally, additional studies are needed to determine the effects of clofibrate administration on the *in vivo* metabolism and action of estradiol in animals and humans.

Acknowledgments

We thank Florence Florek and Keith Williams for help in the preparation of this manuscript.

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