The induction of cytochrome P450 isoform, CYP4A1, by clofibrate coincides with activation of ethanolamine-specific phospholipid base exchange reaction in rat liver microsomes[*][○]

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Administration of a hypolipidaemic drug, clofibrate, to rats resulted, 24 h after a single intraperitoneal injection (250 mg/kg body weight), in pronounced enhancement of the rate of phosphatidylethanolamine (PE) synthesis *via* the PE-specific base exchange (PEBE) reaction in liver microsomes. This was accompanied by 3.4-fold activation of microsomal ω-hydroxylation of lauric acid by cytochrome P450 4A1 isoform (CYP4A1) and an increase in the protein content of this isoform in endoplasmic reticulum (ER) membranes. Since PE represents a class of phospholipids (PL) prerequisite for proper functioning of CYP4A1, and the PEBE reaction is an inducible pathway of PL synthesis in hepatocytes under metabolic stress, one may speculate that this reaction is switched on when extensive remodelling of PL molecular species or/and massive synthesis of lipid bilayer components for membrane assembly is required.

The cytochrome P450 gene superfamily encodes enzymes involved in lipid metabolism [1]. Particularly, the CYP4A1 isoform exhibits ω-hydroxylase activity towards laurate, palmitate and arachidonate [2]. Although hydroxylation of terminal carbon of fatty acid is a catabolic process, the conversion of arachidonate leads to a broad range of important signal metabolites [3]. Under physiological conditions the CYP4A1 protein is expressed in rat liver microsomes at a moderate level, but its expression increases several fold upon administration of hypolipidemic agents or fatty acids [4]. The PEBE reaction in ER membranes rep-

Abbreviations: CYP, cytochrome P450; EP, ethanolaminephosphate; ER, endoplasmic reticulum; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEBE, phosphatidylethanolamine-specific base exchange; PL, phospholipids; PS, phosphatidylserine; PP, peroxisome proliferators; PPAR, peroxisome proliferator-activated receptor; PPbP, peroxisome proliferator-binding protein.

resents an important anabolic pathway accounting for 8–10% of total PE synthesized in hepatocytes. The molecular species of PE which are synthesized in the PEBE reaction are important for regulation of many membraneous enzymes, including cytochrome P450 isoforms [5].

The aim of this work was to study the correlation between the induction of specific isoforms of cytochrome P450 by clofibrate administration, and changes in microsomal PL composition and activities of lipid synthesizing enzymes.

MATERIALS AND METHODS

Chemicals. Ethan-1-ol-2-amine hydrochloride, 2-(p-chlorophenoxy)-2-methylpropionate (clofibrate), lauric acid, ATP, and Triton X-100 were obtained from Sigma (U.S.A.). Resin AG 50W-H$^+$ was from Bio-Rad (Austria). 1,2-Dioleoyl-L-3-phosphatidyl-[3-^{14}C]serine (55 mCi/mmol), [2-^{14}C]ethan-1-ol-2-amine (54 mCi/mmol), [1-^{14}C]lauric acid (58 mCi/mmol) and the rat cytochrome P450 IVA ECL^{TM} Western blotting kit were purchased from Amersham (U.K.). Silica gel 60 plates were from Merck (Germany). All other chemicals were of the highest purity commercially available.

Preparation of subcellular fractions from rat liver. Adult male Wistar rats weighing 150–180 g were used throughout. Rats were injected intraperitoneally with clofibrate at a dose of 250 mg/kg of body weight or with 0.9% saline (control) [6], and killed after being starved for 16 h at 24 h after the injection. Mitochondria, ER membranes and cytosol were isolated by the conventional procedure [7]. The final pellets of mitochondria and ER membranes were resuspended in a buffer consisting of 75 mM sucrose, 225 mM mannitol and 5 mM Hepes, pH 7.4, at a protein concentration of 10–20 mg/ml, and stored at –70°C for 1–2 months.

Determinations of phosphatidylethanolamine-specific base exchange, ethanolamine kinase and phosphatidylserine decarboxylase activities. The PEBE activity was determined essentially as described by Rakowska *et al.* [8]. Ethanolamine kinase activity was determined according to Weinhold & Rethy [9]. The reaction was carried out at 37°C for 15 min and terminated by boiling. Blank samples were boiled immediately after the addition of cytosol. Denatured proteins were removed by centrifugation and supernatants were applied onto a column filled with AG 50W-H$^+$ X8 resin. Ethanolaminephosphate (EP) was separated from ethanolamine by elution with three 1-ml portions of water followed by radiometrical quantitation in 1,4-dioxane scintillator, by the method described by the manufacturer (Ubichem Ltd., U.K.). The homogeneity of eluted EP was checked chromatographically using commercial standards of EP and ethanolamine. The product of the ethanolamine kinase reaction separated from substrates migrated as a single spot on silica gel in one-dimensional system of ethanol/ammonium/water (61:29:10, by vol.) [10]. Phosphatidylserine (PS) decarboxylase activity was measured in 100 mM potassium phosphate buffer, pH 6.8, containing 10 mM β-mercaptoethanol, 1 mM EDTA, 80 μM [3-^{14}C]PS (1 mCi/mmol) and Triton X-100 (total volume 300 μl). Mitochondria (100 μg of protein corresponding to 25 nmol of total PL), prior to addition to the incubation medium, were solubilized with Triton X-100, at the total PL/detergent ratio of 1:12 (w/w) [11]. The reaction was carried out for 30 min at 37°C and stopped by adding 4 ml of cold methanol/chloroform (1:1, v/v). The reaction time of 30 min was chosen because during this period the PS decarboxylation exhibited a linear dependence on time, and decarboxylation did not exceed 20% of the PS available. Phospholipids were extracted according to Bligh & Dyer [12] and separated using one-dimensional thin-layer chromatography in a chloro-

form/methanol/water (65:25:4, by vol.) system, after removal of Triton X-100 by extraction with acetone.

Measurements of cytochrome P450 4A1 isoform activity. The reaction mixture for measurements of CYP4A1 activity contained 0.4 mg of microsomal protein in 0.4 ml of 0.25 mM Tris/HCl, pH 7.4, 1 mM NADPH and 0.1 mM [1-^{14}C]lauric acid (2.5 mCi/mmol). The reagents were preincubated for 5 min at 37°C, then NADPH was added to initiate the reaction, and the incubation continued for another 5 min. The reaction was stopped by addition of 0.4 ml of acetonitrile/0.2% acetic acid, then the samples were cooled for 10 min on ice and centrifuged at 1500 × g for 5 min. The hydroxylauric acid and unmetabolised substrate were separated using one-dimensional thin-layer chromatography in hexane/diethylether/glacial acetic acid (90:28.5:1.5, by vol.) [13].

Detection of CYP4A1 protein by Western blotting. Immunodetection of rat cytochrome P450 4A1 isoform was performed according to the instruction manual for rat cytochrome P450 IVA ECL^{TM} Western blotting kit, provided by the manufacturer.

Other determinations. Protein concentration was determined according to Lowry *et al.* [14] with bovine serum albumin as a standard. Phospholipids were extracted from membranes as described by Bligh & Dyer [12], and separated on silica gel by one-dimensional thin-layer chromatography in chloroform/ethanol/water/triethylamine (30:34:8:35, by vol.), as recommended in [15]. The content of phospholipid phosphorus was assessed as described in [16].

RESULTS

The basic observation of the presented experiments is that the phospholipid content in rat liver microsomes becomes increased upon administration of clofibrate to the animals. The increase was observed in all PL classes
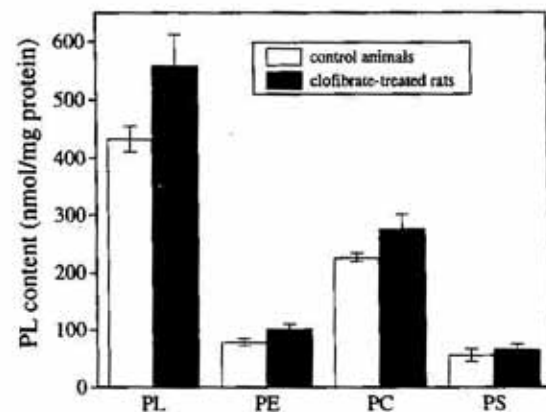

Figure 1. Effects of clofibrate administration on the content of phospholipids in liver endoplasmic reticulum membranes.

Liver lipids were extracted and determined as described in Materials and Methods.

analysed (Fig. 1), in agreement with the effect of clofibrate described by Kawashima *et al.* [17]. The most pronounced increase (by about 21%), was observed in the case of PE. To check which metabolic pathway of phospholipid synthesis in hepatocytes is affected upon administration of clofibrate, the activities of three key enzymes in PE synthesis were measured: cytosolic ethanolamine kinase (EC 2.7.1.82), mitochondrial PS decarboxylase (EC 4.1.1.65), and microsomal PEBE enzyme. No significant differences in the activity of PS de-

Table 1. The activities of selected key enzymes of phosphatidylethanolamine synthesis and CYP4A1 activity

Enzyme	Control rats	Clofibrate-treated rats
	Activity (pmol/min per mg protein)	
Ethanolamine kinase[a]	554 ± 18	651 ± 32*
PS decarboxylase[b]	924 ± 86	1039 ± 79
PEBE[c]	69.5 ± 6.7	90.3 ± 4.5*
CYP4A1[c]	1840 ± 127	6320 ± 1050

For determination of specific enzyme activity selected subcellular fractions were used: [a]cytosol, [b]mitochondria, or [c]ER membranes; the assay conditions are described in Materials and Methods. The data are means of four independent experiments ±S.D. *Significantly different from controls at P < 0.02.

carboxylase between control and clofibrate-injected rats were observed, whereas the activity of ethanolamine kinase and PEBE increased after administration of the hypolipidaemic agent. Although we found that ethanolamine kinase was significantly increased in comparison to control, this pathway did not supply those molecular species of PE, which are necessary for CYP4A1 activation [8]. Also studies *in vivo* with the labeled PL precursors (glycerol and ethanolamine) suggested that PEBE was responsible for increased PE level (not shown). The changes in PEBE activity were smaller than those of ω-hydroxylase activity of CYP4A1, which was found to be elevated in clofibrate-treated rats by a factor of 3.4 (Table 1) in comparison to control animals. In addition, by Western blot analysis, using specific antibodies against CYP4A1, a pronounced increase of this cytochrome isoform in microsomes isolated from livers of clofibrate-treated animals was detected,
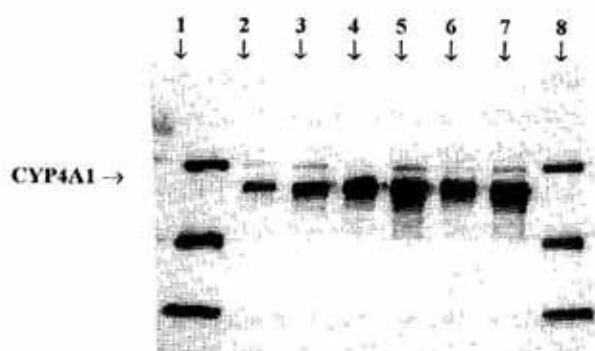

CYP4A1 →

Figure 2. Detection of CYP4A1 isoform in microsomal fractions from livers of control and clofibrate-treated rats.

The cytochrome protein was detected by the procedure described in Materials and Methods. Lanes 1 and 8, relative molecular mass standards (catalase (bovine liver) M_r 58 100; alcohol dehydrogenase (horse liver) M_r 39 800; carbonic anhydrase (bovine erythrocytes) M_r 29000); lanes 2 and 3, liver microsomes from control rats, 7.5 μg and 15 μg of protein, respectively; lanes 4 and 5, liver microsomes from clofibrate-treated rats (first animal), 7.5 μg and 15 μg of protein, respectively; lanes 6 and 7, liver microsomes from clofibrate-treated rats (second animal), 7.5 μg and 15 μg of protein, respectively.

which well correlated with the elevated activity of this isoform of cytochrome P450 (Fig. 2). On the basis of these results, one may conclude that elevated activity of CYP4A1 is a result of both overexpression of CYP4A1 protein and activation of the enzyme by specific molecular classes of PE synthesized *via* the PEBE reaction. A possible effect of enhanced expression of CYP 4A1 on the PEBE reaction is described in the Discussion section of this paper.

DISCUSSION

There is no experimental evidence for the transport of clofibrate into the cell. On the basis of physico-chemical properties of this compound, one may assume that it can easily diffuse through the membrane. Lalwani *et al.* [18] purified a peroxisome proliferator-binding protein (PPbP), and postulated that a part of the cellular response evoked by clofibrate is mediated by other specific clofibrate-binding proteins. It is possible that various other peroxisome proliferators (PP) bind to the same protein or that more than one type of PPbP exists in hepatocytes [18].

What is the mechanism of intracellular action of clofibrate? It is postulated that some metabolic effects induced by clofibrate are due to its activation to acyl-CoA thioesters, in the reaction catalysed by acyl-CoA synthase [19]. Acyl-CoA thioesters of hypolipidaemic drugs, as analogs of natural compounds, could interfere with various reactions in the cell that utilize acyl-CoA as substrates. Another explanation for the changes observed in hepatocyte metabolism after clofibrate administration was proposed by Kozuka *et al.* [20]. These investigators examined fifteen drugs belonging either to phenoxyacetic acid derivatives or to perfluorinated compounds, and found a relationship between the structure of the drug and its biological activity. Similar morphological and biochemical changes in the liver were induced by some drugs which, although struc-

turally unrelated to clofibrate, exhibited some similarity to saturated fatty acids, i.e. poor susceptibility to oxidation, and high lipophilicity [20].

Some effects evoked by clofibrate may be mediated by the calcium-dependent cellular signal transduction pathway including calcium-binding proteins (calmodulin), protein kinases, cyclic nucleotides and phosphodiesterases. It has been shown that calcium channel agonists (nicardipine, nifedipine, and dithiazem) suppress the induction by clofibrate of hepatic peroxisomal enzymes [21]. Trifluoperazine, a calmodulin agonist, and H-7, a potent inhibitor of protein kinase C, also suppressed the induction of peroxisomal enzymes by clofibrate and other PP (benzofibrate, Wy 14,643) [22]. Direct influence of clofibrate on calcium homeostasis in freshly isolated hepatocytes has been described by Shackleton et al. [23]. In these cells clofibrate caused a transient increase in intracellular Ca^{2+} concentration. This increase was mediated by Ca^{2+} release from the intracellular inositol 1,4,5-triphosphate-sensitive pool. The results of experiments performed on rat thymocytes, Ehrlich ascites tumor cells, and human platelets [24] suggest that cytochrome P450 may affect both the intracellular Ca^{2+} stores and plasma membrane Ca^{2+} permeability. Calmodulin in a form deprived of Ca^{2+} was found to stimulate the activity of microsomal cytochrome P450 which directly or indirectly (via 5,6-epoxyeicosatrienoic acid) modulated the opening of plasma membrane calcium channels. On the other hand, calmodulin with bound Ca^{2+} inhibited cytochrome P450 activity [24]. This inhibition seems to be in
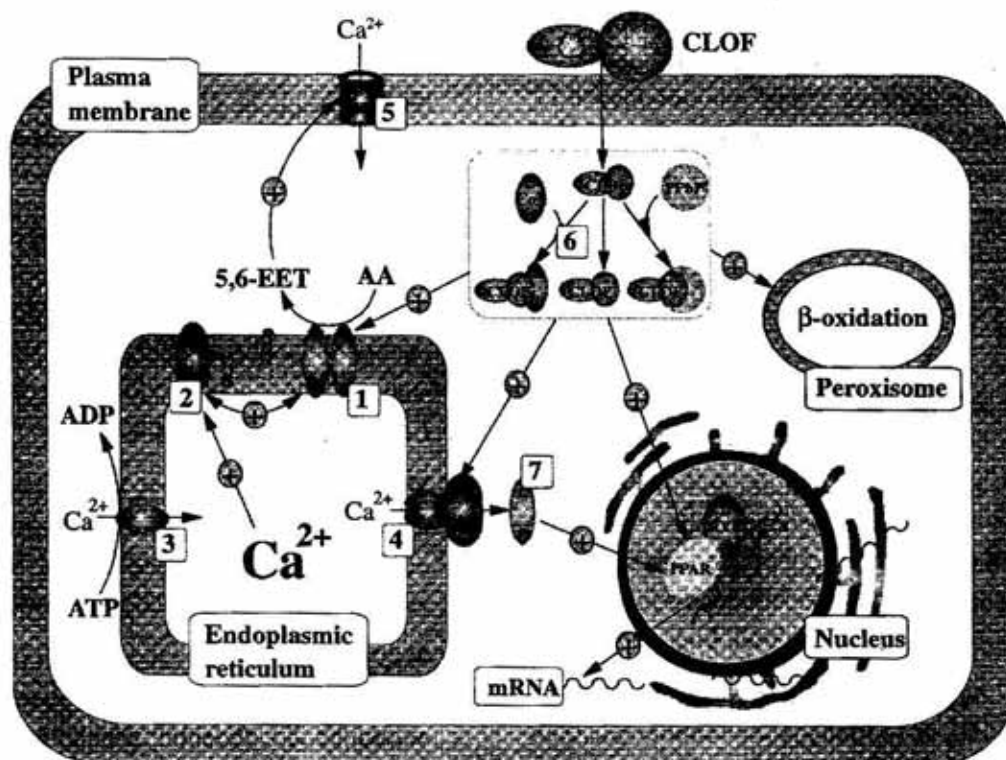

Figure 3. The targets for clofibrate within a hepatocyte.

Clofibrate after diffusion into the cell can bind to the PPbP, can be activated to acyl-CoA thioesters by acyl-CoA synthase, or activate the PPARα receptor. One of these three forms of the drug, or perhaps free clofibrate, is able to bring about changes in calcium homeostasis, peroxisome proliferation, activation of many enzymes and transcription of genes. 1, CYP4A1; 2, PEBE; 3, Ca^{2+} ATPase; 4, receptor of inositol 1,4,5-triphosphate (IP$_3$R); 5, Ca^{2+} channel; 6, acylo CoA synthase; 7, protein kinase C; CLOF, clofibrate; 5,6-EET, 5,6-epoxyeicosatrienoic acid; RXR, retinoid X receptor; AA, arachidonic acid.

agreement with the observation that the PEBE reaction is calcium-dependent [25, 26]. This mechanism may form a positive feedback mechanism between cytochrome P450 and the PEBE reaction.

Moreover, some cellular effects of clofibrate may be propagated *via* the peroxisome proliferator-activated receptor (PPAR) [27]. This receptor belongs to the steroid/thyroid/retinoid receptor superfamily, which includes also receptors for substances involved in cell growth, cell cycle regulation and cell differentiation: glucocorticoids, minerallocorticoids, estrogens, progesterone, androgens, retinoic acid, vitamin D and thyroid hormones [28]. The PPAR family comprises three distinct subtypes: PPARα, PPARβ or δ, and PPARγ. They bind to DNA as heterodimers with the retinoid X receptor [29], and act as proliferator response elements, activating transcription of specific genes [30]. Clofibrate induces PPARα receptor, and target genes affected by this class of receptors are *CYP4A1* and *CYP4A6* [31]. It can be postulated that the PEBE enzyme gene is induced by a similar mechanism. The details of the proposed clofibrate metabolism in a hepatocyte are summarized in Fig. 3.

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