Positive feedback between ethanolamine-specific phospholipid base exchange and cytochrome P450 activities in rat liver microsomes. The effect of clofibric acid

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Abstract The results of the present investigation relate the effects of the nutritional state and administration of clofibric acid (CLA), a hypolipidaemic drug and peroxisomal proliferator, on phosphatidylethanolamine (PE) synthesis in rat liver and fatty acid metabolism. Fasting and CLA treatment of animals causes an increase in the amount of PE in endoplasmic reticulum (ER) membranes and mitochondria, as well as in the PE/phosphatidylcholine (PC) ratio. Moreover, the activity of the ethanolamine-specific phospholipid base exchange (PLBE) enzyme in liver ER membranes of fasted animals was enhanced by 75% in comparison to that of animals fed ad libitum. The effect of CLA treatment was additive to that of starvation; PE synthesis tested in vitro via the Ca^{2+}-sensitive PLBE reaction increased 3-fold in comparison to rats fed ad libitum. This is confirmed by an increased V_{max} for the reaction, but the affinity of the enzyme for ethanolamine was not significantly changed. These effects were accompanied by an enhanced expression of cytochrome P450 CYP4A1 isoform and elevated activity of the enzyme upon CLA administration. The stimulatory effect of CLA administration on the efficiency of the ethanolamine-specific PLBE reaction can be explained by elimination of lauric acid, a known inhibitor of de novo PE synthesis, during the course of ω-hydroxylation catalysed by CYP4A1, and by increased expression of the PLBE enzyme. The products of ω-hydroxylation of lauric acid, which are then converted by dehydrogenase to 1,12-dodecanedioic acid, did not significantly affect the in vitro synthesis of PE.

Key words: Phospholipid base exchange reaction; Phosphatidylethanolamine; Clofibric acid; Hyperplasia; Starvation; Cytochrome P450; Rat liver

1. Introduction

Phospholipid synthesis in the rat liver is linked to the metabolism of fatty acids since fatty acid chains constitute the acyl moieties of phospholipid molecules. Equally important, phospholipids determine the physico-chemical properties of biological membranes, influence the activity of membrane proteins by fulfilling certain structural requirements, and serve as a resource for the generation of signalling molecules [1]. Moreover, fatty acid homeostasis is essential for the integrity of all cellular membranes. Pleiotropic effects evoked by alterations of the nutritional state of an animal in lipid composition and metabolism, as well as on the synthesis and remodelling of highly polyunsaturated molecular species of phospholipids is well documented for the liver and blood [2,3]. Fasting (the state under which gluconeogenesis occurs) accompanied by a lowering of fatty acid synthesis and their increased oxidation [4] was found to decrease the rate of synthesis and the amount of hepatic phosphatidylcholine (PC), diacyl- and triacylglycerols [5,6]. On the other hand, in rat liver after administration of clofibric acid (CLA), a correlation between the enhanced rate of phosphatidylserine (PS)-derived PC synthesis, diminution of triacylglycerols and inhibition of PC and triacylglycerol secretion into the circulation in the form of very low density lipoproteins, has been established [7]. Interestingly, starvation and treatment with CLA resulted in an elevation of the phosphatidylethanolamine (PE) level, another representative phospholipid of eukaryotic cell membranes [5–8]. Furthermore, CLA as a xenobiotic compound induced a variety of hepatic responses, amongst them rapid proliferation of peroxisomes, smooth endoplasmic reticulum (ER) membranes and mitochondria linked to alterations in the activities of lipid-metabolising enzymes [9–11]. As a result of these changes, activation of microsomal ω-hydroxylation of fatty acids by induction of cytochrome P450 isoenzymes [12,13] and subsequent induction of peroxisomal β-oxidation have been observed [11,14,15]. Stimulation of both processes in response to non-xenobiotic factors such as starvation, high fat diet, cold adaptation, oxidative stress and in diabetes mellitus or during liver regeneration was found at the same time. Each of these metabolic states is characterised by a transient accumulation of hepatic fatty acids and various lipid metabolites [16]. In addition, the positive correlation between the participation of Ca^{2+} homeostasis regulation, cytochrome P450 activity and various calcium-binding proteins (calmodulin, calreticulin) localised within the lumen of the ER compartment is well documented [17]. Since PE is a phospholipid class which fulfils the requirement of cytochrome P450 isoenzymes for optimal activity [18], it was of particular importance to examine whether its Ca^{2+}-dependent synthesis via the phospholipid base exchange (PLBE) reaction [19] is also affected by the metabolic state of the animal. In the present study we focus on verification of the hypothesis that the positive cooperation of cytochrome P450-dependent enzymes involved in fatty acid metabolism and the ethanolamine-specific PLBE enzyme exists in the rat liver under specific metabolic conditions [20]. The results obtained led us to the conclusion that the PLBE reaction is an inducible pathway of PE synthesis in hepatocytes during metabolic stress and evoked hyperplasia which is switched on when extensive remodelling of phospho-

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Abbreviations: CLA, clofibric acid; ER, endoplasmic reticulum; EP, ethanolaminephosphate; GSH, reduced glutathione; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLBE, phospholipid base exchange; PS, phosphatidylserine

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lipid molecular species or/and a process of massive synthesis of components for membrane assembly are required. Part of this work has been reported in preliminary form [21].

2. Materials and methods

2.1. Chemicals

Ethan-1-ol-2-amine hydrochloride, L-serine, D-glucose 6-phosphate monosodium salt, CLA, 1,12-dodecanedioic acid, Hoechst 33258, Triton X-100, and reduced glutathione (GSH) were obtained from Sigma (USA). [2-^{14}C]Ethan-1-ol-2-amine (54 mCi/mmol), [1(3)-^{3}H]glycerol (2–5 Ci/mmol), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-[3-^{14}C]serine (51 mCi/mmol), [1-^{14}C]lauric acid (58 mCi/mmol), and the rat cytochrome P450 IVA ECL Western blotting kit were purchased from Amersham (UK), while D-[U-^{14}C]glucose 6-phosphate (300 mCi/mmol) was from ARC (USA). Silica gel 60 plates for thin layer chromatography were from Merck (Germany), and resin AG 50W-H^{+} X8 from Bio-Rad (Austria). All other chemicals were of the highest purity commercially available.

2.2. Preparation of subcellular fractions

A group of adult male Wistar rats weighing 150–180 g were divided into three subgroups. The first was fed ad libitum, the second was starved for 16 h and injected with 0.9% saline, whereas the third was injected intraperitoneally with a single dose of CLA (250 mg/kg of body weight [11]), then fed ad libitum for 8 h and starved for another 16 h. Livers were excised, rinsed and 10% homogenates in 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, pH 7.4, 0.5 mM EGTA were prepared. The basic characteristics of biological preparations are given in Table 1. The differences in the number of rats used for various types of experiments are explained in the legend to Table 1. Mitochondria, ER membranes and the cytoplasmic fraction were isolated as described in [22], and were kept at a protein concentration of 10–20 mg/ml at −80°C for 1–2 months.

2.3. Detection of cytochrome P450 CYP4A1 by Western blotting and assay of its activity

Immunodetection of the rat CYP4A1 isoform was performed according to the instruction manual for the rat cytochrome P450 IVA ECL Western blotting kit, provided by the manufacturer. ER membranes (7.5 μg of protein per slot) isolated from various animals were subjected to SDS-polyacrylamide gel electrophoresis and electrotransferred to Hybond-ECL (nitrocellulose) membranes. The immunodetection procedure of the CYP4A1 isoform utilised an anti-cytochrome P450 primary antibody raised in sheep and an anti-sheep Ig-biotinylated species-specific secondary antibody. This was subsequently detected with a streptavidin-horseradish peroxidase conjugate which binds to the biotinylated secondary antibody. Horseradish peroxidase then catalysed the oxidation of luminol in the presence of H_2O_2 and an enhancer [23]. The emitted light was then captured on Hyperfilm-ECL film which, after processing, was converted to a computer image using a densitometer (Molecular Dynamics). The Image Quant program (version 3.3) was used for quantitative analysis of these images in order to obtain the relative amount of CYP4A1 in ER membranes. The reaction mixture for the determination of CYP4A1 activity contained a total volume of 0.4 ml, 0.1 mg/ml of ER membrane protein, 0.25 mM Tris-HCl, pH 7.4, 1 mM NADPH, and 0.1 mM [1-^{14}C]lauric acid (specific activity 2.5 mCi/mmol). The reaction was started by the addition of NADPH after preincubation of the sample for 5 min at 37°C, and prolonged for an additional 5 min. The reaction was stopped by the addition of 0.4 ml of acetonitrile:0.2% acetic acid (1:1, v/v), cooled for 10 min on ice, and centrifuged at 1500×*g* for 5 min. The product of the reaction, 11- (or 12-) hydroxylauric acid, and unmetabolised substrate were separated by one-dimensional thin-layer chromatography in hexane:diethylether:glacial acetic acid (90:28.5:1.5, v/v) [24].

2.4. Assay of activities of phospholipid synthesising enzymes in vitro and synthesis of PE in vivo

The PLBE activity was measured in an assay system containing 0.25 mg of ER membrane protein, 40 mM HEPES, pH 7.4, 1 mM $CaCl_2$ and 50 μM [2-^{14}C]ethanolamine (specific activity 1.5 mCi/mmol), as described previously [25]. Ethanolamine kinase (EC 2.7.1.82) activity was determined according to Weinhold and Rethy [26]. The reaction was carried out at 37°C for 15 min and terminated by boiling. A blank sample was boiled immediately after the addition of cytosol. After removal of proteins, the supernatant was applied to a column of resin AG 50W-H^{+} X8. Ethanolaminephosphate (EP) was separated from ethanolamine by elution with 3 ml of water followed by radiometric quantitation in dioxane scintillator. The identity of eluted EP was tested with EP and ethanolamine standards by one-dimensional thin layer chromatography in ethanol:ammonium:water (61:29:10, v/v) [27]. PS decarboxylase (EC 4.1.1.65) activity was measured in a mixture of total volume of 300 μl consisting of 100 mM K-phosphate buffer, pH 6.8, 10 mM β-mercaptoethanol, 1 mM EDTA, 80 μM [3-^{14}C]PS (1 mCi/mmol) and Triton X-100. Mitochondria (100 μg of protein, i.e. 25 nmol of phospholipids) were solubilised in the presence of Triton X-100, at a phospholipid/detergent ratio of 1:12 (w/w) [28] prior to addition to the incubation mixture. The reaction was carried out for 30 min at 37°C, i.e. for the time at which a linear dependence of PS decarboxylation versus time was observed with an efficiency not exceeding 20% of PS available in the assay medium. Then the reaction was stopped by adding 4 ml of ice-cold mixture of methanol:chloroform (1:1, v/v). Phospholipids were extracted according to Bligh and Dyer [29] and separated using one-dimensional thin layer chromatography in chloroform:methanol:water (65:25:4, v/v), which was preceded by removal of Triton X-100 with acetone. To determine the rate of PE synthesis in vivo rats were pulse-chased by a single intraperitoneal injection of 0.25 ml of [1,3-^{3}H]glycerol (40 μCi) and [2-^{14}C]ethanolamine (10 μCi) in 0.9% saline; 10 min after the injection (the short time was chosen to prevent secretion of labelled phospholipids into the circulation and their remodelling) livers were removed and subcellular fractions were isolated. Labelled phospholipids were extracted and quantified by liquid-scintillation counting in a two-channel system.

2.5. Transport of PS

Transport of PS mediated by a non-specific lipid transfer protein was determined by the PS decarboxylase assay, as described in [30]. Mitochondria isolated from livers of three subgroups of animals were used as acceptor membranes (1 mg protein, i.e. 250 nmol of phospho-

Table 1
Effect of starvation and administration of CLA on rat liver

Parameter	Animals		
	Fed ad libitum ($n=3$)	Starved ($n=10$)	CLA-treated, then starved ($n=9$)
Animal weight (g)	175.8 ±21.5	167.2 ±15.2	165.8 ±12.7
Liver weight (g)	6.6 ±0.5	4.6 ±0.4*	5.5 ±0.5
DNA (mg)[a]	1.9 ±0.1	2.6 ±0.6*	3.6 ±0.5**
Protein (mg)[a]	115.6 ±17.9	138.5 ±8.8**	139.0 ±8.6**
Phospholipids (μmol)[a]	33.9 ±2.7	26.6 ±1.4**	30.6 ±1.8*
Phospholipid/protein ratio (μmol/mg)	0.29±0.02	0.19±0.05	0.22±0.09
GSH (μmol)[a]	4.7 ±0.3	5.8 ±0.3*	6.4 ±0.5*

[a]Values are expressed in units per g of liver weight. Significantly different *$P<0.01$, **$P<0.001$ from animals fed ad libitum. The total number of animals used is given in parentheses. The differences in the number of animals used for determinations between three groups are due to the fact that rats fed ad libitum are already well described by other investigators with respect to the parameters shown here. The number of animals used for specific determinations for which results are shown in other tables and figures may vary from the total number of rats. However, these animals were the same throughout the study.

lipids). Donor vesicles consisted of [3-^{14}C]PS (0.5 mCi/mmol) and 25 nmol of bovine brain PS. Spontaneous and protein-mediated transport of PS was carried out for 30 min at 2°C in the same buffer as used for homogenisation of the liver. The cytoplasmic fraction isolated from liver homogenate of rats starved overnight served as the source of a non-specific lipid transfer protein. Spontaneous transport of PS determined without the cytoplasmic fraction (0.65 ± 0.06 nmol PS/30 min), was found to be independent of the amount of the acceptor mitochondria, and was subsequently subtracted. Mitochondria enriched in PS were reisolated, resuspended and decarboxylation of PS was carried out for 10 min at 30°C [30]. The efficiency of the reaction was determined by radiometric quantitation of PS and PE separated from extracts of total mitochondrial lipids by one-dimensional thin layer chromatography in chloroform:methanol:water (65:25:4, v/v).

2.6. Lipid peroxidation

Non-enzymatic peroxidation of ER membrane lipids in the presence of 5 μM $FeSO_4$ and 100 μM ascorbic acid was performed as described in [20]. The reaction was terminated by the addition of butylated hydroxytoluene to a final concentration of 0.1 mM. NADPH-dependent lipid peroxidation in the presence of ferric cations was achieved as described previously [20]. The level of thiobarbituric acid-reactive substances (TBARS) was measured according to Uchiyama and Mihara [31], using malondialdehyde, obtained by acidic hydrolysis of tetraethoxypropane, as a standard [20].

2.7. Other determinations

Protein concentrations were determined according to Lowry et al. [32] with bovine serum albumin as a standard. Phospholipids were extracted from membranes [29], separated by one-dimensional thin layer chromatography in chloroform:ethanol:water:triethylamine (30:34:8:35, v/v) [33], and their phosphorus content was assessed by the method of Rouser et al. [34]. DNA levels in homogenates were determined spectrofluorimetrically with the use of Hoechst 33258 [35], with excitation and emission wavelengths set at 356 nm and 492 nm, respectively. Rat liver DNA served as a standard. Glucose 6-phosphatase activity was assayed in 50 mM MOPS, pH 6.5, and 2 mM EGTA, by radiometric determination of D-[U-^{14}C]glucose hydrolysed from 0.5 mM [U-^{14}C]glucose 6-phosphate (0.16 mCi/mmol) [36]. The levels of GSH were determined in cytoplasmic fractions by using a fluorimetric method [37] with *o*-phthalaldehyde as a dye. The excitation wavelength was 350 nm and the fluorescence intensity was recorded at emission maximum of 425 nm.

3. Results and discussion

3.1. Effect of CLA administration on PE content in rat liver ER membranes and mitochondria

It is well known that different nutritional states influence liver metabolism in rodents [2,5,6]. Some of these effects, as shown below, are similar to those evoked by the administration of hypolipidaemic drugs, for example CLA [7,14], while others are opposite in effect. Overnight starvation resulted in the diminution of liver weight by about one-fourth in comparison to the liver weight of animals with free access to food [15], and was accompanied by a decrease of the content of phospholipids by 22% (Table 1). The 10% loss of the total protein content after starvation suggests that the liver used them to sustain gluconeogenesis [38]. In contrast, CLA administration led to an increase of rat liver weight by 27% and an

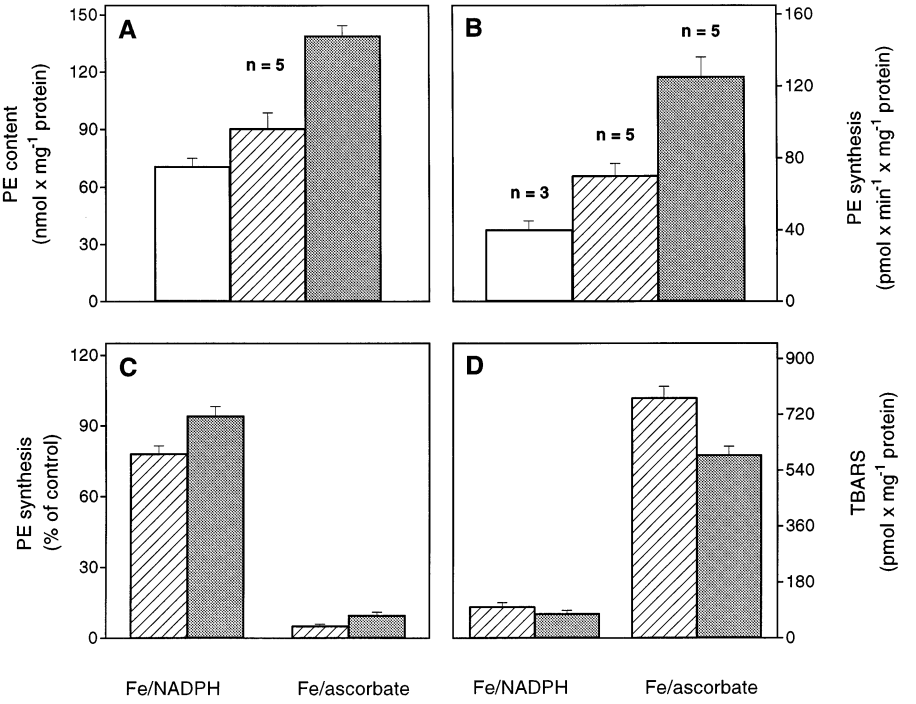

Fig. 1. Changes in PE content (A), in vitro synthesis of PE via the PLBE reaction (B), and susceptibility of the PLBE reaction to lipid peroxidation (C, D) in ER microsomes isolated from livers of animals fed ad libitum (empty bars), starved (hatched bars) and CLA-treated (filled bars). The data shown in A and B are the mean of four independent determinations; the number of animals used (*n*) is given. Phospholipids were extracted from ER membranes using a mixture of organic solvents, then separated by one-dimensional thin layer chromatopgraphy and their phosphorus content assessed as described in Section 2. The ethanolamine-specific PLBE activity (B) in ER membranes was determined in an assay system containing 0.25 mg of ER membrane protein, 1 mM $CaCl_2$ and 50 μM [2-^{14}C]ethanolamine. For the determination of susceptibility of microsomes to lipid peroxidation (C), ER membranes (1 mg protein/ml) were preincubated at 37°C with 20 μM $FeSO_4$ and 250 μM NADPH (enzymatic peroxidation) or with 5 μM $FeSO_4$ and 100 μM ascorbic acid (non-enzymatic peroxidation) for 8 min at 37°C. PE synthesis via the PLBE reaction amounted to 72 and 125 pmol/min/mg protein in microsomes from starved or CLA-treated rats, respectively. The TBARS formation (D) was measured as described in Section 2. The TBARS level in microsomes not subjected to peroxidation (initial level) amounted to 36 ± 4 pmol/mg protein (starved animals) and 29 ± 3 pmol/mg protein (CLA-treated rats). In C and D the mean values ± S.D. of three experiments are shown.

increase of the levels of phospholipids by 15% and of total protein content in comparison to starved animals. These changes should have been followed by intensive cell maturation, transcription and translation processes. We found, however, only slight variation of DNA levels in rat liver homogenate from starved animals, in contrast to an 1.9-fold increase within 24 h after administration of a single dose of CLA (Table 1). This result points to the influence of CLA on DNA replication and transcription rates within hepatocytes and to the stimulation of the cell proliferation cycle leading to hyperplasia [14]. Moreover, the increased amount of GSH in the cytoplasm, which was by 10% higher than after starvation, suggested that some mechanisms of cell protection against free radical damage are simultaneously activated [39].

The significant differences in the phospholipid content of rat liver homogenates was promising and prompted us to examine the levels of PC and PE, major classes of phospholipids, in subcellular fractions isolated from rat liver excised from three groups of animals. These groups are characterised in Table 1. The PE content in ER membranes was found to increase 1.28-fold in starved animals and 1.97-fold after CLA treatment in comparison to control, ad libitum fed rats (Fig. 1A). The content of PE in mitochondria was also elevated, but to a lesser extent (from 52.3 to 67.6 and 73.7 nmol/mg protein, respectively). Due to similar trends in PC content in examined subcellular fractions, the PE/PC ratio in ER membranes and in mitochondria differed only to a limited extent between the experimental groups, from 0.39 to 0.43 or from 0.81 to 0.92, respectively. They reached their highest values, however, after CLA administration. We hypothesise that these changes were evoked by differences in the activities of phospholipid synthesising enzymes located either in the cytoplasm or in the ER and mitochondrial membranes.

3.2. *Activities of enzymes participating in PE synthesis via different pathways*

The data published by other investigators [3,5,8] pointed to a depressed activity of enzymes (ethanolamine kinase and CTP:EP cytidylyltransferase) involved in the de novo synthesis of PE upon starvation. In our experiments (Table 2) the activity of ethanolamine kinase was decreased by one-fourth upon starvation and increased by 18% after CLA administration, although not reaching the value for ad libitum fed animals. Participation of glucagon-stimulated PE *N*-methyltransferase cannot be excluded, since PE derived from ethanolamine is the preferred substrate for methylation to PC [40,41]. It has been found, however, that de novo synthesis of PC in the hepatocytes of CLA-treated rats was stimulated with the simultaneous inhibition of PE *N*-methyltransferase [7,8]. Such fluctuations create a pool of phospholipid substrate which is used exclusively for the synthesis of PS via a calcium-dependent serine-specific PLBE reaction [7]. The pool of newly synthesised PS via this pathway can be subsequently metabolised to PE by PS decarboxylase in mitochondria [42] or via the ethanolamine-specific PLBE reaction in ER membranes [25]. PS decarboxylase activity determined in the solubilised system (Table 2) when the efficiency of the reaction was not limited by PS transport, was significantly decreased (by 21%) in starved rats in comparison to animals fed ad libitum and only slightly increased after CLA treatment. These changes can reflect differences in the amount of active enzymatic protein. However, different data were obtained when the amount of labelled PS transported via the protein-mediated pathway was similar to the amount of phospholipid present in native mitochondria. It was found that mitochondria from starved animals were better acceptors of protein-mediated transfer of liposomal PS (3.8 ± 0.2 nmol PS/30 min/mg protein) than mitochondria isolated from other groups of animals (2.7 ± 0.2 nmol PS/30 min/mg protein), reflecting differences in lipid-protein composition and the membrane surface charge. The participation of a non-specific lipid transfer protein in the transport of PS seems to be more pronounced in the case of starvation. Furthermore, the rate of PS decarboxylation in [3-^{14}C]PS-enriched mitochondria from starved animals was twice as high as in mitochondria from animals fed ad libitum (Table 2) presumably since the efficiency of PS decarboxylation depends on the efficiency of PS transport [30]. In this experiment CLA treatment resulted in the enhancement of PS decarboxylation, in agreement with results obtained by others [8]. It is worth noting that the level of PS decarboxylase activity measured in this assay was comparable to that of the ethanolamine-specific PLBE enzyme (Fig. 1B).

In the course of the present study we found that the activity of the ethanolamine-specific PLBE enzyme in ER membranes from starved and CLA-treated animals was higher by a factor of 1.75 and 3.13, respectively, in comparison to animals fed ad libitum (Fig. 1B). We also found that supplementation of the assay medium with 100 mM butylated hydroxytoluene had an additive effect to starvation on ethanolamine incorporation into phospholipid molecules via the PLBE reaction. This suggests that a hydrophobic radical scavenger may protect the newly synthesised molecular species of PE against peroxidation [20]. Moreover, ER membranes from CLA-treated animals were less sensitive to both non-enzymatic (Fe^{2+}, ascorbate) and enzymatic (Fe^{2+}, NADPH) peroxidation, as revealed by lower inhibition of the ethanolamine-specific PLBE reaction (Fig. 1C), and lower level of thiobarbituric acid reactive substances formed, in comparison to ER membranes from starved animals (Fig. 1D).

As was suggested by Tijburg et al. [2], the increased level of glucagon due to starvation may be important in maintaining the rate of PE synthesis and this might exert its effect on

Table 2
Effect of metabolic state of animals on the activities of selected enzymes involved in PE synthesis in ER membranes, mitochondria and the cytosol of rat liver

Enzyme activity[a]	Fed ad libitum ($n = 3$)	Starved ($n = 5$)	CLA-treated, then starved ($n = 5$)
Ethanolamine kinase[b]	722 ± 69	554 ± 18*	651 ± 32
PS decarboxylase[c]	1166 ± 73	924 ± 86*	1039 ± 79
PS decarboxylase in [3-^{14}C]PS-enriched membranes[c]	47 ± 4	91 ± 7*	58 ± 5

[a]Values are expressed in pmol of product/min/mg protein. For the determination of specific activities of enzymes listed the following subcellular fractions were used: [b]cytosol or [c]mitochondria. The data are the mean of four independent determinations; the number of animals is given in parentheses. The significance level *$P < 0.001$ in comparison to animals fed ad libitum. The details of assay conditions are described in Section 2.

phospholipid synthesis via alteration in Ca^{2+} homeostasis. The increase of PLBE activity in CLA-treated rats is rather specific since the activity of another microsomal enzyme tested, glucose 6-phosphatase, was not altered significantly in comparison to that in starved animals. However, the activity of this enzyme in animals fed ad libitum was elevated upon fasting (from 90.3 ± 1.8 to 118.2 ± 4.4 nmol of glucose/min/mg protein) due to the enhancement of gluconeogenesis [4]. As was earlier reported, a single oral dose administration of CLA structural analogue, ciprofibrate, did not change the activity of glucose 6-phosphatase but evoked inhibition of ER Ca^{2+}-ATPase and elevation of hepatic $[Ca^{2+}]_i$ [43]. This effect of ciprofibrate on Ca^{2+}-ATPase activity was reversed by GSH [43] which indicates that it was related to reduction of essential thiol groups of the enzyme. It is worth mentioning that Ca^{2+}-ATPase, by sequestration of cytosolic Ca^{2+} into ER stores, may arrest cells in the G_1 phase and thus play a role of a negative regulator of cell growth [43]. Suppression of this enzyme activity can stimulate cell proliferation and lead to hyperplasia, as in the case of CLA treatment [14].

3.3. Effect of CLA administration on the ethanolamine-specific PLBE activity in vitro

After observing the increased efficiency of the ethanolamine-specific PLBE reaction in liver ER membranes obtained from rats after starvation and CLA administration, it seemed important to test the kinetic parameters of PE synthesis via this pathway. We have shown previously that unsaturated fatty acids stimulate the ethanolamine-specific PLBE activity in vitro [20]. Fatty acids belong to a group of intracellular factors affecting membrane fluidity, such as cholesterol, lipid peroxides, ubiquinones, lipid-soluble vitamins, integral and peripheral membrane proteins, the cytoskeleton, pH and ions, which all have a similar effect as external factors such as detergents, organic solvents and anaesthetics [44]. The nutritional state of animals and CLA administration modulate the metabolism of fatty acids and steroid hormones [3,14] and, in consequence, the dynamic properties of the lipid phase of membrane controlling many, if not all, membrane enzymes [45]. Therefore, the two-fold increase in PE synthesis via the PLBE reaction in hepatic ER membranes of starved and CLA-treated rats in comparison to membranes isolated from animals fed ad libitum (Fig. 1B) can be explained by an increased fluidity of ER membranes.

To elucidate the connections between the membrane properties and ethanolamine-specific PLBE activity, we measured the kinetics of the reaction in the ER membranes of the three subgroups of animals. The reaction tested at a constant amount of membrane substrate (550 nmol of phospholipids/mg of ER membrane protein) obeys Michaelis-Menten kinetics with respect to the concentration of ethanolamine (10–100 μM), in the presence of 1 mM Ca^{2+} (Fig. 2A). After transformation of these data to Lineweaver-Burk plots (Fig. 2B) the apparent K_m and V_{max} values were calculated (Table 3). The K_m value for amino alcohol was practically the same for ER membranes of the three subgroups tested. The variability of V_{max} values points to some indirect effects, possibly changes in membrane fluidity but also enhanced expression of the PLBE enzyme correlating with an increased V_{max} of the reaction versus ethanolamine concentration. It is worth noting that CLA added to the assay medium inhibited the ethanolamine-specific PLBE reaction in a concentration-dependent manner by 40% at a concentration range of 0.5–1 mM, probably due to the reduction of essential –SH groups in the enzyme. Another possibility is that the effect of starvation and CLA administration on increased synthesis of PE could be correlated to the elevated expression of cytochrome P450 isoenzymes [9–12]. In fact, in the course of our studies we have observed that enhancement of PE content in ER membranes from starved and CLA-treated animals in comparison to rats fed ad libitum correlates well with the elevated expression of the cytochrome P450 CYP4A1 isoform, as detected using specific antibodies against rat CYP4A1 (Fig. 3A). We have not directly determined the specific content of CYP4A1 in ER membranes but it is well known that the level of constitutive expression of the CYP4A1 isoform is low and accounts for 1–2% of the total cytochrome P450 protein present in non-induced rat liver, i.e. 0.5–1.5 nmol/mg of protein [12,46,47]. We have been able, however, using laser densitometry (see Section 2), to evaluate the relative amount of CYP4A1 protein in rat liver ER membranes. We have found that the CYP4A1 expression is only slightly affected by starvation (a 1.3-fold increase in comparison to animals fed ad libitum), but it is significantly enhanced upon CLA treatment (a 3.3-fold increase) (Fig. 3A). This corresponds very well with a concomitant increase in the total CYP4A1 activity toward [1-^{14}C]lauric acid (Fig. 3B), although the specific activity of CYP4A1 does not change very much in the three groups of animals. A slight increase in the specific activity of CYP4A1

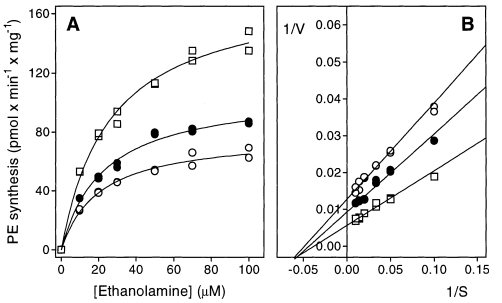

Fig. 2. The influence of clofibric acid administration on kinetic parameters of ethanolamine-specific PLBE reaction in rat liver microsomes. A: ER membranes (1 mg protein/ml) isolated from the livers of animals fed ad libitum (○), starved (●) or CLA-treated (□) were incubated in duplicate with [2-^{14}C]ethanolamine at concentrations indicated on the abscissa, in the presence of 1 mM $CaCl_2$. Mean values for three experiments are presented, they varied by less than 5%. Error bars are omitted for clarity. B: Double reciprocal plots were drawn from the results shown in A, symbols as for A. The values of V_{max} and K_m calculated with the aid of computer program based on hyperbolic regression anaysis are listed in Table 3.

Table 3
Kinetic parameters of ethanolamine-specific PLBE reaction in rat liver ER membranes

Animals	V_{max} (pmol/min/mg protein)	K_m (μM)
Fed ad libitum	78 ± 3	21 ± 2
Starved	108 ± 4	23 ± 2
CLA-treated, then starved	177 ± 7	26 ± 3

ER membranes (1 mg/ml) were incubated in duplicate with [2-^{14}C]ethanolamine at a concentration range of 10–100 μM in the presence of 1 mM $CaCl_2$. The mean values ± S.D. obtained for three experiments are presented. Values were calculated from double-reciprocal plots of enzyme activity versus ethanolamine concentration shown in Fig. 2A.

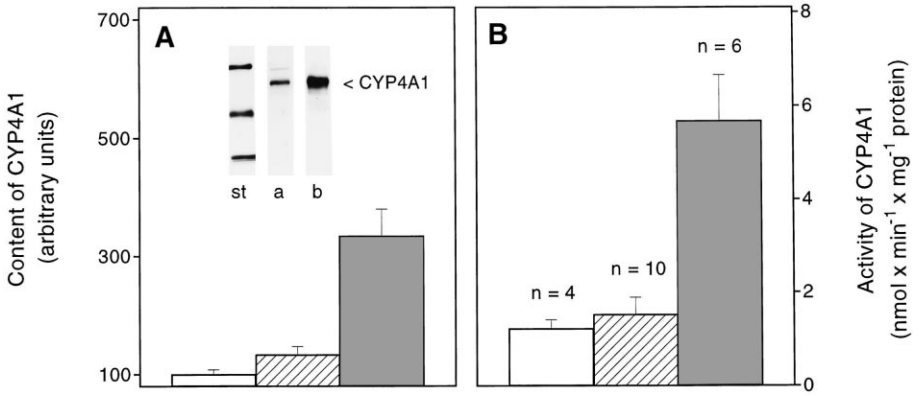

Fig. 3. Effect of CLA-administration on the expression (A) and activity (B) of cytochrome P450 CYP4A1 isoform in ER membranes of rats fed ad libitum (empty bar), starved (hatched bar) or CLA-trated (filled bar). The CYP4A1 was detected using specific antibodies and the obtained results were quantified by means of laser densitometry and the Image Quant program. The mean values ± S.D. of three experiments are shown. In lanes a and b of the insert in A 7.5 μg of membrane protein per lane was run; it was isolated from starved and CLA-treated rats, respectively. The typical result of immunodetection is shown. The following relative molecular mass standards were used: catalase from bovine liver (M_r 58 100), alcohol dehydrogenase from horse liver (M_r 39 800) and carbonic anhydrase from bovine erythrocytes (M_r 29 000). As described in a booklet provided by the manufacturer of the rat cytochrome P450 IVA ECL Western blotting kit, faint bands at molecular weights lower than CYP4A1 may be occassionally detected. This could be the result of a broader than expected specificity of antibodies, since the CYP4A family consists of highly homologous proteins, or may represent proteolytic fragments of CYP4A1. For calculations we have followed the relative amount of the major band detected with a molecular weight of approximately 53 kDa.

upon CLA administration can be explained by the stimulatory effect of some phospholipid molecular species which are synthesised via the ethanolamine-specific PLBE reaction.

1,12-Dodecanedioic acid, which is converted by specific dehydrogenase from the product of the CYP4A1 reaction, 11-(or 12-) hydroxylauric acid, when used in a concentration range from 1 μM to 1 mM, was found practically ineffective in modifying the ethanolamine-specific PLBE reaction in vitro. On the other hand, lauric acid, a substrate of CYP4A1 specialised in ω- and ω-1 hydroxylation, as well as a substrate of lauroyl-CoA oxidase, which catalyses the first step of β-oxidation in peroxisomes, is a potent inhibitor of de novo PE synthesis [48]. Moreover, CLA with a chlorine atom at position 2 of its hydrophobic backbone cannot itself undergo β-oxidation and, therefore, is a more potent inducer of peroxisomal proliferation then fasting. An increased supply of mono- and polyunsaturated fatty acids induces their enhanced metabolism by activation of peroxisomal proliferator-activated receptors and, in consequence, activates the peroxisomal β-oxidation pathway and desaturase activities [14] in both metabolic states analysed. In addition, it caused an increase of this pool of PE and PS molecular species which are preferentially interconverted via the Ca^{2+}-stimulated PLBE reaction. Moreover, higher levels of the fatty acid binding protein, a known mediator of mitogenesis, and its specific mRNA have been documented during starvation, and throughout the cell cycle, in hyperplastic hepatocytes and hepatocarcinomas after administration of peroxisomal proliferators [49].

3.4. Synthesis of PE in vivo – the effect of clofibric acid

Fig. 4 presents data reflecting the incorporation of labelled glycerol and ethanolamine into their corresponding phospholipids at a fixed time after injection. It should be taken into account that effective concentrations of injected precursors are affected to a different extent by endogenous substrates, which vary among the experimental animal subgroups. Almost all ethanolamine radioactivity was recovered in PE after labelling for a short time. Fasting caused diminution of both ethanolamine and glycerol incorporation into PE of ER membranes, in agreement with the findings that ethanolamine kinase and CTP:EP cytidylyltransferase activities are inhibited upon starvation [5,6]. However, the participation of PLBE enzyme cannot be excluded because the $^{14}C/^{3}H$ ratio was larger than one. Seven percent of labelling found in PC (at a $^{14}C/^{3}H$ ratio of 0.1) derived from ethanolamine may point to *N*-methyltransferase activities catalysing such a conversion of PE. CLA treatment stimulated an incorporation of ethanolamine into PE almost at the same level as in rats fed ad libitum and did not affect the labelling of phospholipid in the glycerol moiety. As a consequence, the highest $^{14}C/^{3}H$ ratio was found in PE from membranes of CLA-treated animals (Fig. 4B). In this case, we did not identify the radioactivity derived from ethanolamine in PC since CLA was found to inhibit the in vivo activity of PE *N*-methyltransferases [7]. The molecular species

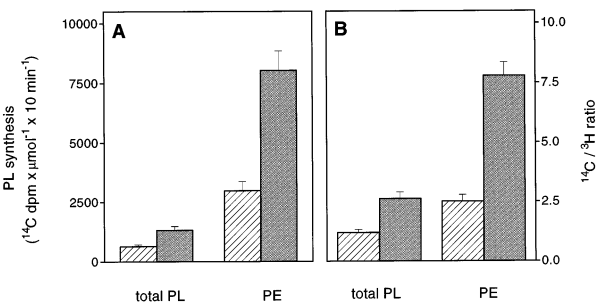

Fig. 4. The effect of CLA administration on the incorporation of radiolabelled precursors into newly synthesised phospholipids of rat liver ER membranes. A: Phospholipid precursors, ethanolamine and glycerol, were injected simultaneously into animals as a single dose containing 40 μCi of [1,3-^{3}H]glycerol and 10 μCi of [2-^{14}C]ethanolamine in 0.25 ml of 0.9% NaCl. After 10 min the livers were removed and subcellular fractions were isolated as described in Section 2. The initial ratio of [2-^{14}C]ethanolamine/[1-^{3}H]glycerol amounted to 0.25. The final $^{14}C/^{3}H$ ratios are given in B. The results represent mean values from two experiments. Starved animals (dashed bars) served as a control for CLA-treated rats (filled bars).

of PE which are synthesised via the PLBE reaction may became substrates for the serine-specific PLBE reaction [25] since ethanolamine is mainly incorporated into hexa- and tetraenoic species of phospholipids [50]. On the other hand, the cytochrome P450 CYP4A1 activity has been found to be dependent on specific phospholipid molecules, especially PS and PE unsaturated molecular species, forming a proper membrane environment for the enzyme [18].

Summarising, the data presented in this paper favour the hypothesis that positive feedback between stimulation of PE synthesis via the PLBE reaction in hepatic ER membranes and cytochrome P450 CYP4A1 activity regulated by fatty acids metabolism exists in fasted and CLA-treated rats. The possibility of co-expression of PLBE and cytochrome P450 CYP4A1 genes should be taken into consideration since target elements for peroxisomal proliferator-activated receptors have been identified upstream of the 5′-flanking regions of the genes encoding both β-oxidation enzymes and cytochrome P450 CYP4A1 [51–53].

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