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QUARTERLY

# 10-Undecynoic acid, an inhibitor of cytochrome P450 4A1, inhibits ethanolamine-specific phospholipid base exchange reaction in rat liver microsomes*°

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**1,12-Dodecanedioic acid, the end-product of ω-hydroxylation of lauric acid, stimulates in a concentration dependent manner, phosphatidylethanolamine synthesis *via* ethanolamine-specific phospholipid base exchange reaction in rat liver endoplasmic reticulum. On the other hand, administration to rats of 10-undecynoic acid, a specific inhibitor of ω-hydroxylation reaction catalyzed by cytochrome P450 4A1, inhibits the ethanolamine-specific phospholipid base exchange activity by 30%. This is accompanied by a small but significant decrease in phosphatidylethanolamine content in the endoplasmic reticulum and inhibition of cytochrome P450 4A1. On the basis of these results it can be proposed that a functional relationship between cytochrome P450 4A1 and phosphatidylethanolamine synthesis exists in rat liver. Cytochrome P450 4A1 modulates the cellular level of lauric acid, an inhibitor of phospholipid synthesis. In turn, ethanolamine-specific phospholipid base exchange reaction provides molecular species of phospholipids, containing mainly long-chain polyunsaturated fatty acid moieties, required for the optimal activity of cytochrome P450 4A1.**

The cytochrome P450 (CYP) 4A subfamily of cytochromes P450 comprises proteins involved in lipid metabolism. These proteins are capable of hydroxylating the terminal ω-carbon, and to a lesser extent the ω-1 carbon, of saturated and unsaturated fatty acids [1, 2]

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**Abbreviations:** CYP, cytochrome P450; ER, endoplasmic reticulum; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLBE, phospholipid base exchange; PS, phosphatidylserine; 10-UDYA, 10-undecynoic acid.

(Fig. 1). For CYP4A1, one of the most extensively studied isoforms of the CYP4A subfamily, physiological substrates are lauric, palmitic and arachidonic acids, as well as related prostaglandins and leukotrienes [3]. The reactions catalyzed by CYP4A lead to the formation of biologically active substances. $\omega$-Hydroxylation of fatty acids produces primary alcohols which are converted to dicarboxylic acids by aldehyde and alcohol dehydrogenases, while $\omega$-1 hydroxylation results in the production of secondary alcohols, such as $\omega$-1 oxo-fatty acids [4].

CYP4A1 represents 1–2% of total cytochrome P450 protein in endoplasmic reticulum (ER) isolated from normal rat liver [5]. Similar to other cytochromes P450, its level and activity are influenced by a variety of factors, including diet, environmental pollutants and xenobiotics [6]. After induction with clofibrate, a hypolipidaemic drug and peroxisome proliferator, the level of CYP4A1 rises to 16–30% of the total cytochrome P450 level in rat liver [5, 7, 8]. It is accompanied by peroxisome and ER proliferation and selective induction of other cytochromes P450 catalyzing fatty acid oxidation [3]. CYP4A1 induction is probably an adaptive response to fatty acid overload. In addition, the metabolites of arachidonic acid may be involved in the pathology of hypertension. CYP4A1 has been found to oxidize terminal acetylenes to ketones which can modify proteins or can be metabolized to other reactive compounds [9]. Using f4A1 protein (active site of CYP4A1 fusion protein, mimicking the capabilities of purified and reconstituted rat liver CYP4A1), it has been further confirmed that indeed a wide range of chemical compounds can be metabolized by this enzyme [10, 11]. CYP4A1 is strongly inhibited by therapeutic agents, such as naproxen, ibuprofen and cimetidine [11]. Utilization of specific inhibitors of CYP4A1, synthesized from fatty acids, can help to understand the biological significance of the $\omega$-hydroxylation reaction. These highly selective and irreversible inhibitors include acetylenic fatty acid analogs: 11-dodecanedioic acid and 10-undecynoic acid ($C{\equiv}C{-}(CH_2)_8{-}COOH$, e.g. 10-UDYA) [12, 13]. Up to date more than 600 compounds, in which acetylenic bonds are present, is known. Recently, acetylenic fatty acid analogs have been found to exist in nature, in seed oils from *Chrysanthemum corybosum* [14] and *Heisteria silvanii* [15]. These substances inhibit cytochrome P450 activity both *in vivo* and *in vitro*. The detailed mechanism of inhibition of cytochrome P450 activity by acetylenic fatty acid analogs is unknown. There are two groups of hypotheses:

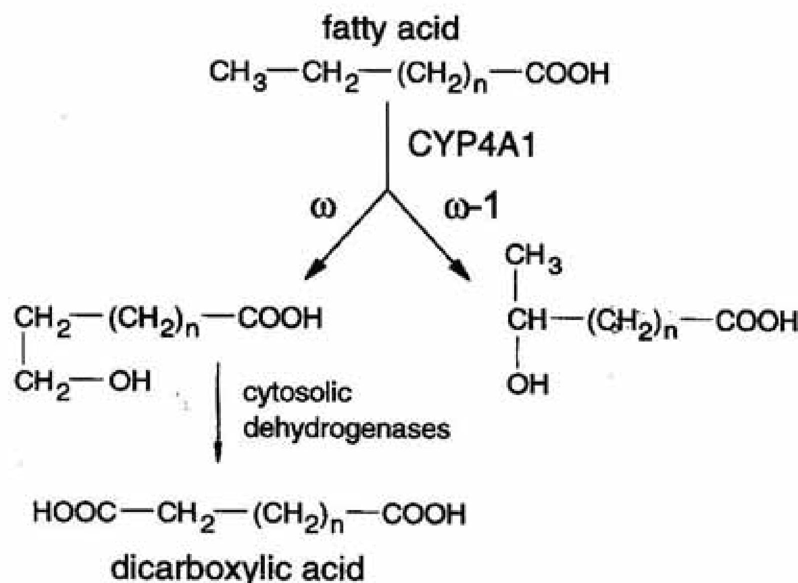


**Figure 1. Hydroxylation of the terminal $\omega$- or $\omega$-1 carbon atom of fatty acid by cytochrome P450 4A1.**

The reactions lead to the formation of hydroxylated fatty acid which is further oxidized to a dicarboxylic acid. The latter reaction is catalyzed by alcohol and aldehyde dehydrogenases.

one postulates that inactivation may involve heme alkylation within the CYP molecule, while the second one that the inhibition is due to peptide modification. Some hints are provided by the results of studies on inactivation of plant $\omega$-hydroxylase by series of radiolabeled acetylenic analogs of lauric acid. These studies revealed that, for enzyme inactivation, the position occupied by an acetylenic bond in the fatty acid molecule is essential [16].

A positive cooperation between cytochrome P450-dependent enzymes and ethanolamine-specific phospholipid base exchange (PLBE) enzyme in rat liver subjected to metabolic or oxidative stress has been previously described [17]. Furthermore, it has been found that administration of clofibrate to rats causes an increase in the amount of phosphatidylethanolamine (PE) in ER and mitochondria. This was accompanied by an enhanced expression of CYP4A1, elevation of its activity towards lauric acid and increased activity of ethanolamine-specific PLBE reaction, all without affecting serine-specific PLBE activity leading to synthesis of phosphatidylserine (PS) [7, 8]. In agreement with previous observations [18–20] these results point to the existence in ER from rat liver of a separate PLBE enzyme exclusively catalyzing synthesis of PE (Fig. 2).

In the present report the functional relationship between CYP4A1 and ethanolamine-specific PLBE activities in ER of rat liver, where both enzymes are co-localized, is further investigated. The *in vivo* effects of 10-UDYA, an inhibitor of CYP4A1 activity, on synthesis of PE *via* PLBE reaction in rat liver microsomes, in comparison to the effects evoked by clofibrate, are examined.

## MATERIALS AND METHODS

***Chemicals.*** Ethan-1-ol-2-amine hydrochloride, 2-(*p*-chlorophenoxy)-2-methylpropionate (clofibrate), lauric acid, 10-undecynoic acid (10-UDYA), 1,12-dodecanedioic acid, ATP and Triton X-100 were obtained from Sigma (St. Louis, MO, U.S.A.). [2-$^{14}$C]Ethan-1-ol-2-amine

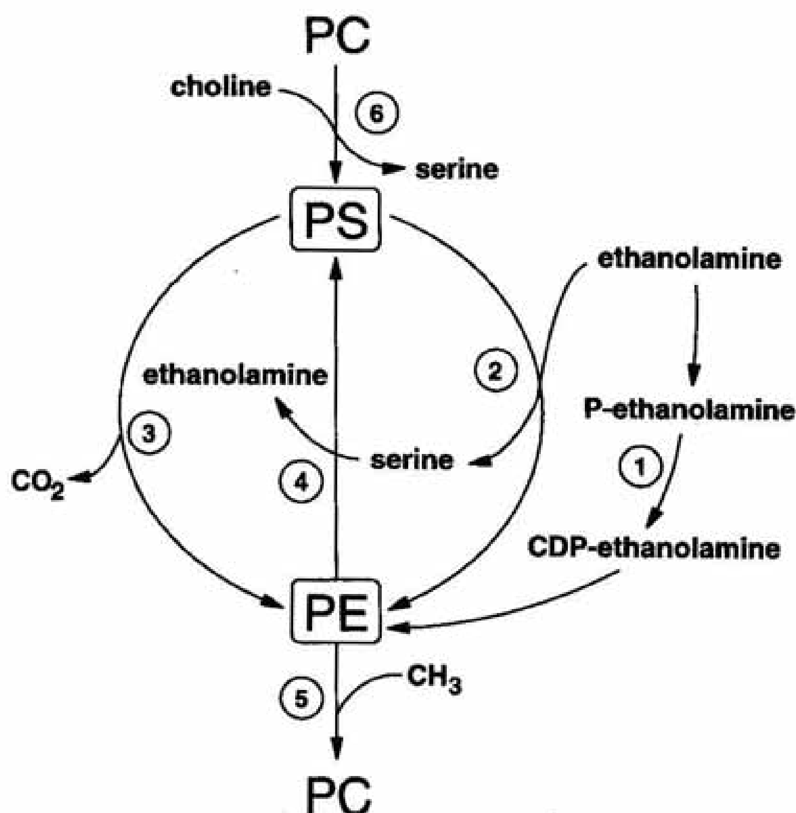


**Figure 2. Synthesis of phosphatidylethanolamine in rat hepatocytes.**

PE is synthesized mainly *via* the *de novo* pathway (1) from ethanolamine through intermediate substrates: phosphoethanolamine and CDP-ethanolamine. Ethanolamine-specific PLBE reaction (2) accounts for 8–9% of the total PE synthesis in hepatocytes. In addition, PE is formed by decarboxylation of phosphatidylserine (PS) (3). Thus, PE can be converted back to PS due to the action serine-specific PLBE enzyme, PS synthase II (4), or undergoes two rounds of methylation to phosphatidylcholine (PC). The latter reactions are catalyzed by methyltransferases I and II (5). Finally, interconversion of PC to PS is accomplished by serine-specific PLBE enzyme, PS synthase I (6).

(54 mCi/mmol), [1-$^{14}$C]lauric acid (58 mCi/mmol), and rat cytochrome P450 IVA ECL$^{TM}$ Western blotting kit were purchased from Amersham (U.K.). Silica gel 60 plates were from Merck (Darmstadt, Germany). All other chemicals were of the highest purity commercially available.

***Animals and 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 mass, or with 10-UDYA at a dose of 25 mg/kg of body mass or with 0.9% saline (control animals) [21]. Food was removed 8 h post injection and the animals were sacrificed at 24 h after the injection. ER vesicles were isolated as described in [22], and resuspended in a buffer containing 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.

***Determinations of ethanolamine-specific PLBE and CYP4A1 activities.*** PLBE activity was determined essentially as described previously [18]. 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 the unmetabolised substrate were separated using one-dimensional thin-layer chromatography in hexane/diethylether/glacial acetic acid (90:28.5:1.5, by vol.) [23]. Lauric and 1,12-dodecanedioic acids served as standards.

***Other determinations.*** Protein concentration was determined according to the method of Lowry *et al.* [24] with bovine serum albumin as a standard. Immunodetection of CYP4A1 protein by Western blotting was performed essentially as described by Lenart *et al.* [7, 8]. Phospholipids were extracted from membranes as described by Bligh & Dyer [25] and separated on silica gel by one-dimensional thin-layer chromatography in chloroform/ethanol/water/triethylamine (30:34:8:35, by vol.) [26]. The content of phospholipid phosphorus was assessed as described in [27].

## RESULTS AND DISCUSSION

The energy-independent incorporation of choline, ethanolamine or serine into phospholipids *via* PLBE reaction, in the case of PE, serves as a basic metabolic pathway for the remodeling of the preexisting membrane molecular species of this phospholipid [17]. On the other hand, the ability of PE molecules to induce local nonbilayer structures by forming hexagonal phases may be responsible for the regulation of the activity of CYP4A1. Moreover, PLBE reaction produces mainly phospholipid molecular species enriched in long-chain polyunsaturated acyl moieties derived from arachidonic (20:4), docosatetraeonic (22:4) and docosahexaenoic (22:6) acids.

It has been already shown that treatment of rats with peroxisome proliferator, clofibrate, induces hepatic CYP4A1 protein level and stimulates its activity which is concomitant to activation of ethanolamine-specific PLBE reaction [7, 8]. Clofibrate administration does not change the ethanolamine-specific PLBE enzyme affinity for ethanolamine ($K_m$ 21 $\mu$M after clofibrate administration, in comparison to $K_m$ 23 $\mu$M in control animals) but results in a 1.7-fold increase of $V_{max}$ of the enzyme [8]. As a possible explanation we have proposed that elevated activity of CYP4A1 after clofibrate treatment keeps the concentration of lauric acid (the inhibitor of PE synthesis *in vivo* [28]) at low levels and, therefore, the PLBE reaction is fully active. Among various factors modifying PLBE activity already described, one of most important is the tissue

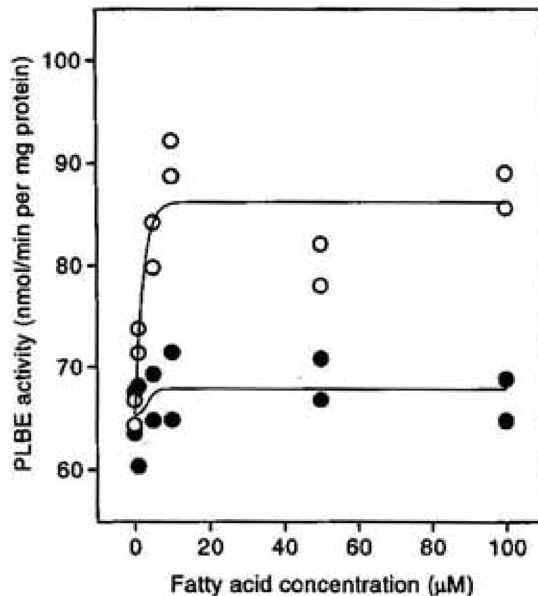


**Figure 3. Alterations of ethanolamine-specific PLBE activity by lauric acid and its derivative, 1,12-dodecanedioic acid.**

Rat liver microsomes were preincubated with lauric acid (●) or 1,12-dodecanedioic acid (○) at the concentrations indicated in the abscissa, for 10 min at room temperature, followed by the assay of enzymatic activity. Determinations were performed in duplicate. Mean values for two experiments are shown. They varied by 5%.

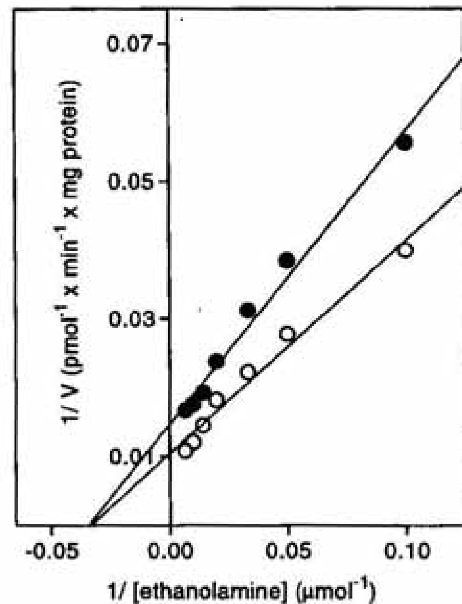


**Figure 4. The influence of administration of 10-UDYA on the kinetic parameters of the ethanolamine-specific PLBE reaction in rat liver ER membranes.**

ER vesicles (1 mg protein/ml) isolated from control (○) or 10-UDYA-treated (●) rats were incubated in duplicate with [2-$^{14}$C]ethanolamine at concentration ranging from 10 to 100 $\mu$M, in the presence of 1 mM $CaCl_2$. The results are expressed as double reciprocal plots from which values of $V_{max}$ and $K_m$ are calculated. They are given in Table 2. Mean values for three experiments are presented. They varied by less than 5%.

level of free fatty acids. Long-chain polyunsaturated fatty acids, as docosahexaneoic acid (22:6), were found to evoke a strong stimulatory effect on PE synthesis *in vivo*, whereas shorter-chain saturated fatty acids, such as lauric acid (12:0), had the opposite effect [17, 18, 28]. Other fatty acids, such as oleic acid (18:1), had a medium effect [28].

Alternatively, a stimulatory effect of the product of CYP4A1 with lauric acid as a substrate, 1,12-dodecanedioic acid, on PE synthesis *via* the PLBE reaction in ER could be taken into account. In Fig. 3 the *in vitro* effect of this compound on ethanolamine-specific PLBE activity is shown, indeed revealing a moderate stimulation of PE synthesis. Under the same conditions lauric acid did not affect the PLBE activity. This result suggests that the inhibitory effect of lauric acid on PE synthesis observed *in vivo* [28] is not a direct one and involves another, yet unidentified, mechanism.

To further examine the relationship between CYP4A1 and ethanolamine-specific PLBE activities in rat liver we have employed a specific inhibitor of cytochrome P450, 10-undecynoic acid. Administration of 10-UDYA to rats did not significantly change the affinity of the PLBE reaction for ethanolamine ($K_m$ 27 $\mu$M). However, $V_{max}$ of the enzyme decreased from 0.11 nmol/min per mg protein in control animals to 0.08 nmol/min per mg protein (Fig. 4 and Table 1) in 10-UDYA treated rats and this difference was statistically significant. The alteration in the activity of the PLBE enzyme was accompanied by inhibition of CYP4A1 activity. The activity of CYP4A1 after 10-UDYA administration de-

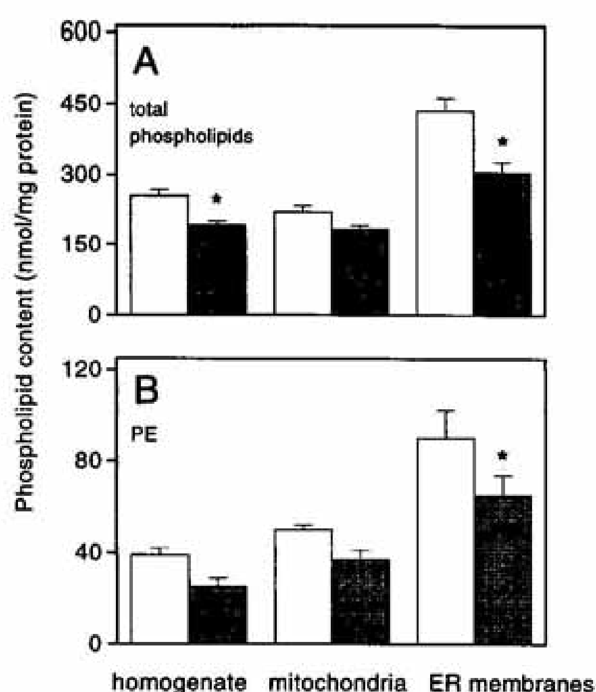


**Figure 5. Effects of 10-UDYA administration on the content of total phospholipids (A) or phosphatidylethanolamine (B) in rat liver homogenates, mitochondria, and endoplasmic reticulum (ER).**

White columns, control animals; shaded columns, rats treated with 10-UDYA. The data are means for three independent experiments ±S.D. *Significantly different from controls at $P < 0.01$.

creased from 1.59 nmol/ min per mg protein (in control rats) to 0.72 nmol/min per mg protein in inhibitor-treated animals (Table 1). The inhibition of PE synthesis *via* PLBE reaction upon administration of 10-UDYA is consistent with lauric acid accumulation in the liver due to the inhibition of CYP4A1 activity by its specific inhibitor. It is worth stating that 10-UDYA has no effect on PLBE activity *in vitro* (in concentration up to 2.5 mM) either in the presence or in the absence of NADPH. Therefore, it can be concluded that the effect of this compound on PLBE activity is mediated either by changes in the activity of CYP4A1 or by the influence on the expression of ethanolamine-specific PLBE enzyme.

As a continuation the possibility whether administration of 10-UDYA resulting in a decrease of ethanolamine-specific PLBE activity may also affect phospholipid content in rat liver has been examined. Previously it has been shown that phospholipid content in rat liver ER changes upon administration of clofibrate, with nearly a twofold increase in PE levels in comparison to control animals [7, 8] (Table 2). 10-UDYA evoked an opposite effect, diminishing the level of total phospholipids in ER by 30% (Fig. 5A). The level of total phospholipids in rat liver homogenate and mitochondria also decreased in comparison to control rats by 25% and 17%, respectively. Among phospholipid classes, PE content in ER was reduced by 29% (Fig. 5B). On the basis of these results, it can be concluded that since the level of lauric acid is regulated by CYP4A1, CYP4A1 activity may be responsible for fluctuations in PE content in the cell *via* modulation of ethanolamine-specific PLBE reaction.

In summary, these results provide further confirmation of the existence of a close functional relationship between CYP4A1 and ethanolamine-specific PLBE reaction in rat liver. The physiological significance of such a relationship awaits further examination. But

**Table 1. Kinetic parameters of ethanolamine-specific PLBE reaction in rat liver ER upon administration of 10-UDYA and clofibrate**

| Animals treated with | $V_{max}$ (nmol/min per mg protein) | Factor | $K_m$ (μM) | CYP4A1 (nmol/min per mg protein) |
|---|---|---|---|---|
| Control | 0.11 ± 0.01 | 1.0 | 21 ± 2 | 1.59 ± 0.38 |
| 10-UDYA | 0.08 ± 0.01[a] | 0.7 | 27 ± 2 | 0.72 ± 0.09 |
| Clofibrate | 0.18 ± 0.01[b] | 1.7 | 23 ± 3 | 6.32 ± 1.41[b] |

ER vesicles (1 mg/ml) were incubated in duplicate with [2-$^{14}$C]ethanolamine at a concentration range from 10 to 100 μM in the presence of 1 mM $CaCl_2$. Mean values ±S.D. for three experiments are presented. Values were calculated from double-reciprocal plots of enzyme activity *versus* ethanolamine concentration. Statistical significance with respect to control animals: [a]$P < 0.02$ and [b]$P < 0.01$.

**Table 2. Effects of 10-UDYA and clofibrate administration on the content of phospholipids in rat liver ER**

| Animals treated with | Total phospholipids (nmol/mg protein) | % | PE (nmol/mg protein) | % |
|---|---|---|---|---|
| 0.9% saline | 435 ± 28 | 100.0 | 90 ± 12 | 100.0 |
| 10-UDYA | 304 ± 23[a] | 69.9 | 65 ± 9[a] | 72.2 |
| Clofibrate | 570 ± 31[a] | 131.0 | 145 ± 11[a] | 161.1 |

Mean values ±S.D. for three experiments are shown. [a]Statistical significance: $P < 0.01$.

it can be postulated that both CYP4A1 and PLBE play a role in repairing specific molecular phospholipid species damaged under oxidative or metabolic stress.

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