Enhanced Degradation of Phospholipids by Phospholipase A2 in Liver of Carbon Tetrachloride-Treated Rat

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Stimulated phospholipid degradation was observed in liver homogenates harvested from rats who had received an injection of carbon tetrachloride (CCl_4). When the liver homogenates obtained from CCl_4-treated rats were incubated for 1 h at 37°C, approximately half of the phosphatidylethanolamine (PE) was hydrolyzed, producing a stoichiometrical amount of lysophosphatidylethanolamine (LysoPE). The homogenates obtained from control rats showed only poor hydrolytic activity toward endogenous PE. The fatty acid composition of lysoPE produced was essentially identical to that at the *sn*-1 position of PE, suggesting that A_2 type of phospholipase (PLase A_2) was involved in the degradation during incubation. The hydrolysis of endogenous PE was dependent on calcium ion. The hydrolytic activity was almost exclusively associated with the membrane fraction which was precipitated by centrifugation at $10000 \times g$, and did not require the cytosol fraction. Para-bromophenacylbromide, rabbit antibody against rat 14 kDa type II PLase A_2, and thielocin A_1, a specific inhibitor of type II PLase A_2, suppressed the hydrolysis almost completely. These results indicate that the accelerated degradation of endogenous PE observed in liver homogenates of CCl_4-treated rat may be mainly due to the activity of the enzyme closely related to type II PLase A_2.

Key words —— carbon tetrachloride, phospholipase A_2, liver injury, free radical

INTRODUCTION

Carbon tetrachloride (CCl_4) has been extensively studied as a model compound producing hepatocellular necrosis. It has been proposed that injury to the liver is a consequence of the metabolism of CCl_4 by the liver to highly reactive, free radical intermediates.[1,2] Among the toxic effects produced as a consequence of the free radical activation of CCl_4, stimulation of lipid peroxidation, the binding of the electrophilic radicals to membrane lipids and proteins, and perturbation of calcium homeostasis have been suggested to play a role in the pathogenesis of irreversible cell damage.[3–6] It has been postulated that CCl_4-induced stimulation of peroxidative breakdown of membrane lipids leads to hepatocyte death through phospholipase A_2 (PLase A_2) activation.[7–9] PLase A_2 has also been proposed to play an important role in cell injury associated with ischemia.[10–19] PLase A_2 acts on membrane phospholipids at the *sn*-2 position to generate lysophospholipids and free fatty acids.[20] The resultant PLase A_2-induced changes in phospholipid integrity and the toxic actions of free fatty acids and lysophospholipids may be critical to the altered membrane structure. The lipid peroxidation that occurs during both CCl_4-intoxication and ischemia/reperfusion may result in enhanced susceptibility of cellular membrane to PLase A_2.[21–25] Alternatively, increase in cellular calcium concentration by CCl_4-administration could lead to activation of PLase A_2 which generally requires calcium as a cofactor.[3,26,27] The potential importance of PLase A_2 activity in CCl_4-induced cell injury is supported by the findings that PLase A_2 inhibitors protected against phospholipid degradation in liver, reducing cell injury. Evidence of PLase A_2 activation was also obtained in isolated hepatocytes treated with CCl_4 by Glende and Pushpendran.[8] They showed that the production of free fatty acids and lysophosphatidylethanolamine (LysoPE) was

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enhanced in isolated rat hepatocytes exposed to CCl_4. Molecular properties of the enzyme(s) involved in the hydrolysis of liver phospholipids under the pathological conditions has not yet been elucidated. In the present study, we examined the characteristics of phospholipid hydrolysis in CCl_4-intoxicated liver.

MATERIALS AND METHODS

Animals —— Male, Wistar strain rats (7 weeks old) were obtained from Nippon Bio-Supp. Center, Tokyo.

Materials —— 1-Palmitoyl-2-[1-^{14}C]linoleoyl-*sn*-glycero-3-phosphorylethanolamine (50–60 mCi/m mol) and 1-palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphorylethanolamine (50–60 mCi/m mol) were purchased from Amersham Corp., U.K. PLase A_2 (from Naja naja venom), ethylenediaminetetraacetic acid (EDTA), ethylene bis(oxyethylenenitrilo) tetraacetic acid (EGTA), diisopropylfluorophosphate, and *p*-bromophenacylbromide were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Carbon tetrachloride was obtained from Wako Chemicals Co., Tokyo.

Unless otherwise indicated, experiments were carried out in buffer containing 10 mM Tris–HCl (pH 7.5), 145 mM NaCl, and 0.1 mM EDTA.

Treatment of Rats with CCl_4 —— In all experiments, food was withdrawn 12–14 h before CCl_4 administration. Water was available *ad libitum*. CCl_4 was given intraperitoneally as a 50% (v/v) solution in olive oil at a dose of 2 ml/kg. Control rats received the equivalent amount of olive oil intraperitoneally. Evidence of liver cells being injured by CCl_4 administration was obtained by the observation that serum levels of GOT and GPT activities increased approximately 30 and 50 fold, respectively, in the rats. The highest levels of GOT and GPT in the serum were observed between 24 and 48 h after injection. The livers obtained 24 h after injection were used for most of the experiments described below.

Preparation of Liver Homogenates —— Livers obtained from control and CCl_4-treated rat were removed rapidly, weighed and processed. Ten percent (w/v) liver homogenates in ice-cold buffer were prepared using a Potter–Erlenmeyer teflon homogenizer. The homogenates were centrifuged at $50 \times g$ for 7 min at 4°C and the supernatant fraction was used for the subsequent determinations.

Determination of PLase Activity towards Endogenous Substrate —— One milliliter of liver homogenates (20 mg protein/ml) obtained from control or CCl_4-treated rats was incubated for 1–2 h at 37°C with shaking. The reaction was stopped by the addition of 1 ml of methanol and 1 ml of chloroform. Total lipids in the reaction mixture were extracted twice by the method of Bligh and Dyer.[28] The combined chloroform phase was evaporated to dryness under reduced pressure, dissolved in a small volume of chloroform/methanol (2 : 1, v/v), and subjected to thin layer chromatography (Silica gel G, Merck). The plate was developed two dimensionally with chloroform/methanol/acetic acid (65 : 25 : 10) for the 1st dimension and with chloroform/methanol/formic acid/water (65 : 25 : 8.8 : 1.2) for the 2nd dimension. Each spot located by brief staining with iodine vapor was scraped into a glass test tube and the phosphorus was determined according to the method of Gerlach and Deuticke.[29]

Determination of PLase Activity toward Exogenous Substrate —— Radiolabeled substrate, 1-acyl-2-[1-^{14}C]linoleoyl-glycerophosphorylethanolamine or 1-acyl-2-[1-^{14}C]arachidonoyl-glycerophosphorylethanolamine was diluted with cold phosphatidylethanolamine (PE) purified from egg yolk to a specific activity of 1000 cpm/mmol and suspended in a buffer by sonication. Twenty nmol of substrate solution was added to 1 ml of the liver homogenate and incubated for 1 h at 37°C with shaking. After incubation, the reaction mixture was processed in the same way as mentioned above except that thin layer chromatography was performed with the 1st-dimensional system [chloroform/methanol/acetic acid (65 : 25 : 10)]. The spots corresponding to PE, LysoPE, and free fatty acid were scraped into liquid scintillation vials and counted in a Packard scintillation counter spectrometer.

Determination of Fatty Acid Composition —— For the determination of fatty acid compositon of PE in liver homogenate before incubation, 1 μmol of total lipid obtained from liver homogenates was subjected to 2nd-dimensional thin layer chromatography. The spot corresponding to PE was scraped and extracted by the method of Bligh and Dyer. PE thus obtained (approximately 200 nmol) was digested with 10 units of Naja naja venom PLase A_2 (Sigma) for 2 h at 25°C with vigorous shaking. Resultant free fatty acids (corresponding to the *sn*-2) and LysoPE (having the fatty acyl chain of the *sn*-1) were separated with silica gel plate in a solvent system of chloroform–methanol–water (65 : 35

: 8) and quantified as a methyl ester form by gas chromatography, using 15 : 0 methyl ester as an internal standard. The fatty acid composition of LysoPE produced after incubation was determined as described above except that PLase A_2 treatment was omitted.

Other Analytical Methods — Protein concentration was determined by the method of Lowry *et al.*[30] with bovine serum albumin as a standard.

RESULTS

Breakdown of Endogenous Phospholipids during Incubation of Rat Liver Homogenates at 37°C

Phospholipid compositions in the liver obtained from control and CCl_4-treated rat were first determined. No significant difference was observed in the composition between these two preparations (data not shown). Incubation of the liver homogenates for 1 h at 37°C, however, induced marked alterations in phospholipid composition ; significant decrease of both PE and phosphatidylserine (PS) was observed in the liver homogenates of CCl_4 treated rat. Among other phospholipids, phosphatidylinositol (PI) was slightly degraded under the same conditions. Although phosphatidylcholine (PC) content was also decreased, the decrease was not appreciably accelelated by CCl_4 treatment. Decrease of PE proceeded almost linearly in a time dependent manner with concomitant increase of LysoPE (Fig. 1). These findings suggest that A type of phospholipase is involved in the degradation observed during incubation. The hydrolysis of PE observed during incubation of liver homogenates was greatly accelerated 14 h after CCl_4 administration (Fig. 2). The maximum activity was obtained in the liver harvested at 24 h.

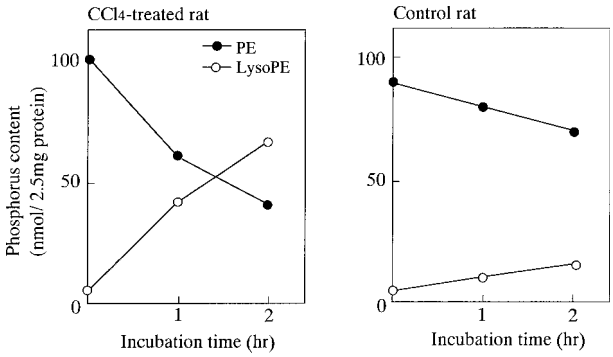

Fig. 1. Time Course of PE in Liver Homogenates Stimulated by CCl_4 Administration

Liver homogenate in 15 mM Tris–HCl (pH 7.4) containing 150 mM NaCl was incubated at 37°C for the indicated time. Thereafter, the total lipid was extracted and separated with 2-dimensional thin layer chromatography as described under Table 1. The spot corresponding to PE and LysoPE was scraped off and phosphorus content determined. Each value was a mean of triplicate experiments.

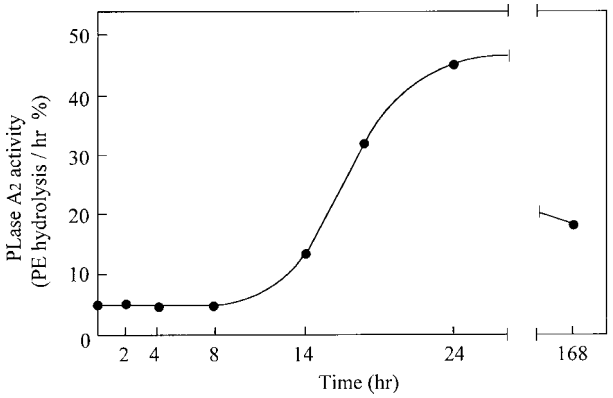

Fig. 2. Time Course of PLase A_2 Activation in Rat Liver by CCl_4

Livers were obtained at the indicated periods (0, 2, 4, 8, 14, 18, 24 or 168 h) after CCl_4 treatment. 0.5 ml of liver homogenate in 15 mM Tris–HCl (pH 7.4) containing 150 mM NaCl was incubated for 1 h at 37°C. PLase A_2 activity was determined by measuring LysoPE formed after incubation.

Involvement of PLase A_2 in the Degradation of Liver PE during Incubation of Homogenates

Next, we examined the positional specificity of the PLase A activity by determining the fatty acid compositon of LysoPE produced during incubation of the liver homogenates. Fatty acids of LysoPE were composed mostly of saturated and mono-unsaturated fatty acids such as palmitic acid, stearic acid and oleic acid (Table 1). The composition was practically the same as that of fatty acids at the *sn*-1 position of PE obtained before incubation. These results indicate that accelerated phospholipid degradation in liver homogenates of CCl_4-treated rat may be due to the action of PLase A_2 under the present conditions.

Characterization of PLase A_2-Mediated PE Breakdown during Incubation of Liver Homogenates

We examined some biochemical properties of the PLase A_2 involved in the hydrolysis of endogenous PE. The pH optimum of the hydrolysis was around 8.0 (Fig. 3). The hydrolysis could be detected in the buffer containing 0.1 mM EDTA, although addition of 5 mM EDTA or EGTA caused

Table 1. Fatty Acid Composition of PE and LysoPE Produced by Incubation in Liver Homogenates of CCl_4-Treated Rat

Species	Amounts of fatty acids (ratio %)		
	PE		Lyso PE
	Position 1	Position 2	
16 : 0	31.1	5.3	27.1
16 : 1	—	—	—
18 : 0	41.2	1.5	52.7
18 : 1	14.3	5.0	13.5
18 : 2	—	15.6	0.8
18 : 3	—	0.1	—
20 : 4	0.2	20.9	1.9
22 : 6	—	39.7	—
Unknown	13.2	11.8	4.0

To determine fatty acid compositions at the *sn*-1 and -2 positions of PE of liver homogenates, total lipids were extracted from liver homogenates and subjected to 2-dimensional thin layer chromatography. The spot corresponding to PE was scraped and then extracted by Bligh and Dyer's method. Two hundred nano moles of PE thus obtained was suspended in 50 mM Tris–HCl (pH 7.4) containing 5 mM $CaCl_2$ and hydrolyzed enzymatically using 1000 units of PLase A_2 (Naja naja). The products, LysoPE and free fatty acids were purified again with thin layer chromatography. To determine fatty acid composition of LysoPE produced by incubation of liver homogenates, the homogenates were first incubated for 1 h at 37°C. LysoPE produced were purified by 2-dimensional thin layer chromatography. The fatty acyl portion of LysoPEs and free fatty acid were methylated with sodium methoxide and diazomethane, respectively. Fatty acid compositions were determined by gas chromatography using C15 : 0 as an internal standard. Details are described in Materials and Methods.

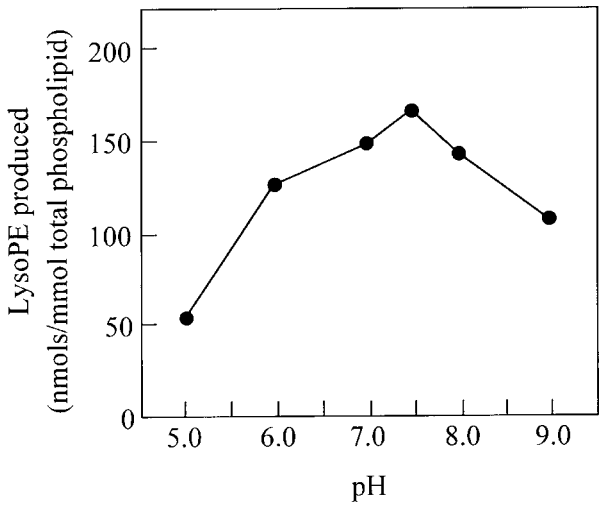

Fig. 3. pH Profile of CCl_4-Activated PLase A_2

Liver homogenate (0.5 ml) in the buffer of the indicated pH was incubated for 1 h at 37°C. PLase A_2 activity was determined by measuring LysoPE formed after incubation. The buffers used were 100 mM sodium acetate in the range from pH 5 to 6, 100 mM Tris-maleate from pH 6 to 7, and 100 mM Tris–HCl from pH 7 to 9.

Table 2. Effect of Various Treatments on PLase A_2 Activity

Treatment	PLase A_2 activity (PE hydrolysis/h %)
No addition	46.9
EDTA (5 mM)	0.1
EGTA (5 mM)	0.6
Diisopropylfluorophosphate (1 mM)	45.8
$CaCl_2$ (5 mM)	49.5
R377 (10 μg/ml)	8.3

0.5 ml of liver homogenate was first preincubated for 30 min with the indicated amounts of EDTA, EGTA, diisopropylfluorophosphate, $CaCl_2$ or an anti type II PLase A_2 antibody R377 on ice and then incubated for 1 h at 37°C. PLase A_2 activity was determined by measuring LysoPE formed after incubation.

complete inhibition of the activity (Table 2). The activity was not appreciably enhanced by the addition of extra calcium ion to the assay mixture. These results suggest that the PLase A_2 activity requires calcium ion and that endogenous (possibly membrane-associated) calcium ion is enough for the expression of the activity. The effects of various agents on the activity of liver homogenates were next examined. No inhibition was observed with diisopropylfluorophosphate, an active site-directed serine reagent. The antibody, R377, which was raised against rat platelet 14 kDa type II PLase A_2 and was shown to inhibit the enzyme activity,[31] effectively inhibited the degradation of PE in the liver homogenates. These results suggest that the enzyme responsible for the hydrolysis of endogenous substrate is immunochemically related to rat platelet type II PLase A_2. According to our unpublished observations, the activity was also blocked by a type II PLase A_2 specific inhibitor, thielocin A_1.[32]

The subcellular distribution of the PLase A_2 activity was next examined (Table 3). Total membrane fraction (100000 × *g* pellet) showed an appreciable PE hydrolysis, suggesting that not only the substrates (PE), but also the enzyme(s) are associated with the membrane fraction. Supplement of the soluble fraction (100000 × *g* supernatants) to the membranes did not have any effect on the hydrolysis (data not shown). When total membrane fraction was further fractionated by low and subsequent high speed centrifugations, the hydrolysis was only observed with the heavy membrane fraction obtained by centrifugation at 10000 × *g*. These results suggest that the enzyme was associated with the heavy membrane fraction.

Hydrolysis of Exogenously Added Substrates

PLase A_2 activity of liver homogenates was monitored with exogenous PE labeled with [^{14}C]linoleate or [^{14}C]arachidonate at the *sn*-2 position. When 2-[^{14}C]linoleoyl PE was used as a substrate, the activity of liver homogenates obtained from CCl_4 treated rat was approximately 12 pmol/mg protein/min (Table 4). This value was not significantly higher than that observed with untreated liver. Addition of calcium ion at 5 mM to the assay mixture did not appreciably enhance the hydrolysis of exogenous substrate in either preparation. The results obtained with a 2-[^{14}C]arachidonoyl PE were essentially the same as those obtained with 2-[^{14}C]linoleoyl PE (data not shown). From the data shown in Fig. 1, the specific activity toward endogenous substrates in the liver homogenates of CCl_4-treated rat was calculated to be approximately 300 pmol/min/mg. Only endogenous PE is accessible to the PLase A_2 in the homogenates of liver of CCl_4-treated rat. Exogenous PE could not be located in the same position as endogenous PE under the present conditions.

Table 3. Subcellular Localization of CCl_4-Activated PLase A_2 Activity

Sample	PLase A_2 activity (PE hydrolysis/h %)	
	Control	CCl_4-treated
Total homogenates	8.6	43.0
Total membranes	9.2	58.4
$10000 \times g$ pellet	5.7	60.3
$100000 \times g$ pellet	10.1	9.4

One milliliter of liver homogenate from either control or CCl_4-treated rat was centrifuged at $105000 \times g$ for 90 min at 4°C. The pellet was resuspended with the buffer in a final volume of 1 ml. Alternatively, 1 ml of liver homogenate was first centrifuged at $10000 \times g$ for 20 min and the resultant supernatant was further centrifuged at $105000 \times g$ for 90 min. The pellet fractions obtained from those centrifugation were resuspended with the buffer in a final volume of 1 ml. PLase A_2 activity of the membrane fractions thus obtained was determined by the hydrolysis of endogenous PE after incubation for 1 h at 37°C.

DISCUSSION

Evidence for lipolysis is found in numerous studies pertaining to haloalkane-induced oxidative stress and ischemia, where considerable phospholipid degradation is noted.[8,9,33,34] This enhanced phospholipid degradation is associated with collapse of cellular energy generation and immediately precedes cell death. PLase A_2 can be regarded as a good candidate for the hydrolysis, since in parallel with phospholipid degradation, accumulation of lysophospholipids and unsaturated free fatty acids was often observed after the oxidative tissue damage.[7–19] In liver, it is well established that PLase A_2 activity represents several distinct proteins, since the activities can be distinguished by substrate specificity, pH optimum, calcium ion requirement, and subcellular localization.[20] Biochemical properties of the enzyme(s) which is relevant to phospholipid breakdown or mechanisms of its activation, however, has been totally unknown. In only a few studies has PLase A_2 activity been measured directly. The cytosolic PLase A_2 activity in ischemic heart was measured using *in vitro* assay system and was found to be lower than that of normal heart, leading the authors and others to question whether PLase A_2 plays an important role in ischemic injury.[18,19,35]

In the present work, we observed stimulated phospholipid degradation in liver homogenates harvested from rats which had received an injection of CCl_4. It is suggested that the enhanced activity in the CCl_4-treated rat liver was not induced nonspecificaly by liver damage (for example, release of lysosomal enzymes), since the enhanced PLase activity showed a strict positional specificity for *sn*-2

Table 4. Substrate Activity of Exogenous PE for Control and CCl_4-Activated PLase A_2

Substrate	$CaCl_2$	Radioactive free fatty acid produced (% of total amount)	
		Control	CCl_4-treated
2-[^{14}C]-linoleoyl	−	1.8	1.8
	+	1.4	1.5
2-[^{14}C]-arachidonoyl	−	7.9	1.6
	+	7.8	1.6

To 1 ml of liver homogenate obtained from either control or CCl_4-treated rat, nmol of radiolabeled PE in 50 μl of the buffer was added. The reaction mixture was incubated for 1 h at 37°C in the absence or presence of 5 mM $CaCl_2$. After the incubation, the lipids were extracted, applied to silica gel plate, and developed with chloroform/methanol/acetic acid (65 : 35 : 10). The spots corresponding to free fatty acid and PE were scraped into vials to be counted.

position fatty acid residues and calcium ion requirement. These facts suggest that a certain type of PLase A_2 is accelerated in CCl_4-intoxicated rat liver. Using this assay system, we have characterized the biochemical properties of the enzyme(s) which is relevant to the process. The fact that the enhanced breakdown of endogenous phospholipids is effectively inhibited by the specific inhibitors to type II PLase A_2, *i.e.*, thielocin A_1 and the specific antibody, suggests that the enzyme involved in the process is closely related to 14 kDa type II PLase A_2.[31] The stimulated breakdown of endogenous phospholipid could be partly reconstituted using KCl-solubilized enzyme and substrate vesicles prepared from extracted membrane lipids (unpublished observations). According to the study, not only is the enzyme activity accelerated but also the susceptibility of lipid vesicles to the enzyme is increased in CCl_4-intoxicated liver. In other studies, the same type of activation of phospholipid breakdown was observed in rat ischemic heart.[36] The common features observed in CCl_4-induced liver injury and ischemia-induced heart injury are radical production and subsequent peroxidation of membrane phospholipids. These common signals may induce the activation of endogenous phospholipid breakdown. Although the mechanism of activation of the enzyme is unknown at present, several possibilities can be considered. First, PLase A_2 protein expression may be enhanced in CCl_4-intoxicated liver. Indeed, cytokine-induced synthesis of type II PLase A_2 has been reported for rat mesangial cells[37,38] and human hepatoma cells.[39] Endogenous enzyme might have been activated by the covalent or non covalent modification. Oxidative stress and haloalkanes cause a rapid and sustained rise of cytosolic calcium to supraphysiological levels, and thus may activate a variety of calcium dependent biochemical processes such as proteolysis and phosphorylation which may lead to the modification of endogenous PLase A_2. Alternatively, highly reactive substances produced under oxidative stress may covalently attach to the enzyme which in turn leads to its activation.

The other common feature of oxidative tissue damage induced by haloalkane and ischemia is peroxidation of membrane phospholipids. An association between lipid peroxidation and enhanced PLase A activity has been demonstrated in various membranes including rat liver mitochondria, lysosomes, and microsomes.[21,22,40] It has tentatively been assumed that fatty acyl hydroperoxides may be preferred substrates for PLases. Preferential hydrolysis of oxidized fatty acids was demonstrated in isolated cell membranes and artificial membrane preparations, having been documented for cellular, digestive, and venom PLases.[25,41–43] However, it should be noted that intact fatty acyl chains as well as possibly peroxidized fatty acyl chains of endogenous PE were hydrolyzed under the present conditions. PLase A_2 activity can be modified depending on the integrity of membrane phospholipids. Specific enrichment of membrane phospholipids with oxidized fatty acids is proposed to modify membrane microenvironments, thereby increasing the PLase A_2 susceptibility at regions bearing lipid discontinuity and structural imperfection. These structural alterations in membrane may present PLase(s) with substrates in more accessible physical arrangements, resulting in the enhanced hydrolysis of the membrane phospholipids. The possibility that a certain type of novel lipid produced in CCl_4-intoxicated liver may serve as a potent activator of PLase A_2 must also be considered.

Although our data support the idea that the enzyme involved in the enhanced phospholipid degradation is closely related to 14 kDa group II PLase A_2, several discrepancies exist between our observations and previously reported properties of this enzyme. For example, it should be noted that the breakdown of endogenous phospholipid is fully expressed with endogenously derived (possibly membrane associated) calcium ion. It is generally accepted that type II PLase A_2 requires mM order of calcium ion toward both exogenous and normal endogenous substrates. Thus, the enzyme involved in the process may be closely related but not the same enzyme as type II Plase A_2. Alternatively, the sensitivity of endogenous PLase A_2 for calcium ion may have changed under CCl_4-induced intoxication.

In conclusion, we have found that not only is type II-like PLase A_2 activity accelerated but also susceptibility of the membrane lipids to the enzyme is increased in rat liver intoxicated by the administration of CCl_4, although the biochemical details of the activation are unknown. Further work is needed to establish the involvement of activated PLase A_2 in liver cell injury and to identify the mechanisms linking CCl_4 metabolism to PLase A_2 activation.

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