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Enhancement of a Liver Form of Cytosolic Phospholipase A_2 Activity by Methylmercury

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Methylmercury (MeHg), which is widely distributed in the environment, is well known for both its acute and chronic poisoning effects on the human health; however, the precise biochemical mechanisms by which this compound elicits its toxicity in a cellular level are still poorly understood. To examine whether MeHg-induced liver injury involves activation of Phospholipase A_2 (PLA_2), the PLA_2 activity of control and MeHg-administrated livers was measured. MeHg stably enhanced a liver form of cytosolic PLA_2 activity, which exhibited several biochemical properties similar to those of the 100 kDa $cPLA_2$, except in its elution profile of a DEAE-5PW HPLC, and it migrated as a molecular weight of 80 kDa in Western blot analysis. This blotting analysis also indicated that the MeHg-induced enhancement of the activity could be due to the increase in the amount of the enzyme protein rather than a stable modification of the enzyme such as phosphorylation. Our data also showed the higher myeloperoxidase activity in MeHg-administrated liver than in the control, suggesting that this increase in the amounts of the 80 kDa PLA_2 and its activity may be resulted from infiltration of neutrophils into the liver during a hepatic injury process such as MeHg-induced inflammation. Taken together, these data suggest that MeHg-induced liver injury may be mediated by activation of the 80 kDa form of liver cytosolic PLA_2.

Keywords: Arachidonic acid, Cytosolic phospholipase A_2, liver, Methylmercury, Toxicity.

Introduction

Mercury is widely distributed in the environment (Vostal and Clarkson, 1973), arising from natural sources as well as from human activities such as industrial discharge. On the basis of chemical speciation, there are three forms of mercury, *viz.*, elemental, inorganic, and organic compounds, each of which has characteristic toxic effects on human health. Most of methylmercury (MeHg) is converted from inorganic mercury by microorganisms, present in water sediments, or derived from industrial discharges (Clarkson, 1972) and is the most important form of mercury in terms of toxicity and health effects from environmental exposures. MeHg is accumulated in a high level in the aquatic food chain reaching the highest concentration in predatory fish (Clarkson, 1977). Thus, this form of mercury is found exclusively in seafood and freshwater fish.

Although the toxic effects of MeHg on human health are well known as eliciting both acute and chronic poisoning, the precise biochemical mechanisms by which this compound elicits its toxicity on a cellular level are still poorly understood. It is known that absorbed MeHg accumulates in many organs including the brain, liver, and kidney, and it has a prolonged half-life in each tissue. It is also known that mercury accumulates in each tissue directly by binding the sulfhydryl groups of proteins in cell membranes or intracellular enzymes (Costa, 1988), enhancing the formation of reactive oxygen species (ROS) (Yee and Choi, 1996), and increasing the intracellular concentrations of Ca^{2+} (Atchison and Hare, 1994). It was

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also reported that MeHg causes a direct injury to many cells and tissues such as epithelial cells (Bohme *et al.*, 1992), kidney (Li *et al.*, 1996), liver (Slotkin *et al.*, 1986; Wester and Canton, 1992), and brain (Sarafian *et al.*, 1994; Watanabe and Satoh, 1996; Yee and Choi 1996).

Phospholipase A_2 (PLA_2) is a family of enzymes that hydrolyzes the acyl bond at the *sn*-2 position of phospholipids to generate free fatty acids and lysophospholipids (Bonventre, 1992; Dennis, 1994). The released arachidonic acid is known to produce ROS in an oxidation process called "arachidonic acid cascade", further metabolizing it to eicosanoids such as prostaglandins and leukotrienes (Krauss and Eling 1984). Lysophospholipid, the other product of PLA_2 action, not only has cytolytic action due to its fusogenic effect on the cell membranes, but also becomes the precursor of platelet activating factor, which could also be destructive with its inflammatory action.

While only small molecular mass (14–20 kDa) forms of secretory PLA_2 have been well characterized among the many forms of mammalian PLA_2s, a cytosolic form of PLA_2 ($cPLA_2$) has recently been purified, cloned, and characterized (Clark *et al.*, 1991; Kim and Bonventre, 1993). This $cPLA_2$ is active at physiological concentrations of Ca^{2+} (Gronich *et al.*, 1990; Kim *et al.*, 1991), is specific for substrates with arachidonic acid at the *sn*-2 position, and is regulated by mitogen-activated protein kinase (MAP kinase), indicating that the enzyme is implicated in the release of arachidonic acid via a signal transduction pathway. Recent evidences suggest that activation of the $cPLA_2$ may cause tumor-necrosis factor (TNF)-induced (Hayakawa *et al.*, 1993) and hydrogen peroxide (H_2O_2)-induced (Sapirstein *et al.*, 1996) cytotoxicity. Thus, it has been postulated that PLA_2 could be implicated in cellular injury induced by a variety of stimuli including MeHg. It was firstly reported that in primary prelabeled cultures of cerebellar granule cells, MeHg induced a concentration- and time-dependent release of arachidonic acid, suggesting that MeHg may regulate a neuronal form of PLA_2 (Verity *et al.*, 1994). However, the precise mechanism through which this compound activates the PLA_2 enzyme is not fully understood.

In the present study, we examined whether PLA_2 is involved in MeHg-induced hepatotoxicity and the biochemical mechanism by which the compound regulates the PLA_2 activity.

Materials and Methods

Materials MeHg chloride was purchased from Tokyo Kasei (Tokyo, Japan). 1-Stearoyl-2-[l-^{14}C]arachidonyl-*sn*-glycerol-3-phosphorycholine (2-[1-^{14}C]AA-GPC) (55 mCi/mmol) and 1-acyl-2-[l-^{14}C]arachidonyi-*sn*-glycerol-3-phosphorylethanolamine (2-[1-^{14}C]AA-GPE) (55 mCi/mmol) were purchased from the radio-chemical center, Amersham (U.K.). DEAE-5PW column was purchased from Tosoh (Japan). All other chemicals were of the highest purity available from commercial sources.

MeHg Administration Male Sprague-Dawley rats were given a single intraperitoneal dose (10 μmol/kg) per day of MeHg chloride (CH_3HgCl) suspended in 0.9% NaCl solution for 10 days. Liver samples were collected after perfusion in anesthesia. The samples were stored at −70°C until assayed.

Measurement of total mercury Total amounts of mercury in the liver tissues were determined according to the quartz tube combustion gold amalgamation method using the MV-250, MV-253 mercury analyzer (Beckman-Toshiba, Japan). Briefly, powdered calcium hydroxide (approximately 100 mg) was added to the samples (30 mg each), freezed at –70°C, and combusted at approximately 800°C for 5 min under oxygen stream at a flow rate of 1 liter/min to remove any organic acid. Mercury was captured as a form of gold-amalgam into trapping agents, and rapid cooling of the samples to 500°C released the mercury, which was determined by measuring an absorbance at 253.7 nm in a nonflammable atomic spectrophotometer. Finally, quantification of the mercury was performed from an appropriate calibration curve obtained in an independent experiment under the same conditions.

Preparation of enzyme sources Each liver tissue was homogenized with 6 volumes of 50 mM Tris/HC1 buffer (pH 7.5) containing 150 mM KCl, 3 mM $MgC1_2$, 0.5 mM EDTA using a tissue tearor (Biospec Products, Inc. model 985-370, type 2). The debris and unlyzed tissues were removed by centrifuging the homogenates at 2000 × *g* at 4°C for 40 min. The supernatants were then ultracentrifuged at 100,000 × *g* at 4°C for 1 h. The resulting supernatants were designated the "cytosolic" fractions and used as an enzyme source for a cytosolic form of PLA_2 activity, and the resulting pellets were resuspended with the homogenizing buffer of the half volumes of the supernatants with a brief sonication and used as an enzyme source for membrane-associated PLA_2 activity.

Assay for PLA_2 activity 1-Stearoyl-2-[l-^{14}C]arachidonyl-*sn*-glycerol-3-phosphorylcholine (2-[1-^{14}C]AA-GPC) was dried under nitrogen stream and resuspended in absolute ethanol. The standard system (100 μl) for assay of PLA_2 activity consisted of 75 mM Tris/HCI (pH 7.5), 5 mM $CaC1_2$, and 3.85 μM of radioactive phospholipids (approximately 55,000 cpm). The reactions were carried out at 37°C for 60 min and stopped by adding 560 μl of Dole's reagent (n-heptane/isopropylalcohol/1.0 NH_2SO_4; 400/390/10, by volume), vortex-mixed, and centrifuged. Then, the upper phase was transferred to a new tube, to which silica gel and 0.8 ml of n-heptane were added. The samples were vortex-mixed and centrifuged again for 1 min each, after which 0.8 ml of supernatant was added to counting vials containing 2.5 ml of scintillation solution (Ecoscint), and counted for radioactivity in a Packard Tri-carb liquid scintillation counter.

DEAE-5PW anionic exchange high performance liquid chromatography The 100,000 × *g* supernatant of the rat liver was loaded onto a DEAE-5PW column (7.5 cm × 7.5 mm; Tosoh Co., Tokyo, Japan) pre-equilibrated with 50 mM Tris/HC1 (pH 7.5), 1 mM EDTA. The column was eluted at a flow rate of 1 ml/min with a 20 min-linear gradient of 0.0–1.0 M NaCl. One milliliter fractions were collected.

Immunoprecipitation of PLA_2 Rabbit anti-(porcine spleen 100 kDa $cPLA_2$) antiserum made against 100 kDa cytosolic PLA_2 was prepared as previously described (Kim and Bonventre, 1993). Rabbit pre-immune serum was mixed with packed Protein A-Sepharose beads pre-equilibrated with 20 mM Tris/HCl (pH 7.5) buffer containing 5 mg/ml bovine serum albumin (BSA) (2:1, v/v) and incubated for 24 h at 4°C with constant shaking. The beads were washed 5 times with 20 mM Tris/HCl (pH 7.5) containing 1 mM EDTA and 5 mg/ml BSA. Each Protein A-Sepharose bead (30 μl bed volume) saturated with each serum was incubated with an aliquot of the active fractions obtained from a DEAE-5PW HPLC column of MeHg-treated liver at 4°C with constant shaking. After pelleting the beads, the resulting supernatants were assayed for PLA_2 activity.

Immunoblotting analysis Proteins were separated by SDS-PAGE (10% gel) (Laemmli, 1970) and then electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell) in 25 mM Tris/HCl (pH 8.3)/190 mM glycine/20% methanol. Nonspecific binding of antisera to nitrocellulose was prevented by pre-incubation of the nitrocellulose in 5% skim milk in PBS containing 0.1% Tween 20 for 12 h at room temperature. The blocked nitrocellulose membrane was incubated with diluted anti-(porcine spleen 100 kDa $cPLA_2$) antiserum for 4 h at room temperature with constant shaking. Unbound antibodies were removed with three washes of PBS, and the site of antibody binding was detected using peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and the chromatogenic substrate 4-chloro-1-naphthol.

Measurement of myeloperoxidase activity Myeloperoxidase (MPO) was extracted from the liver tissue homogenates by suspending the materials in 0.5% hexadecyltrimethylammonium bromide (HTAB) (Sigma Chemical Co., St. Louis, USA) in 50 mM potassium phosphate buffer (pH 6.0). The specimens were freeze-thawed three times. Suspensions were then centrifuged at 40,000 × g for 15 min at 4°C and the resulting supernatants assayed.

MPO was assayed spectrophotometrically; 0.1 ml of the materials to be measured was mixed with 2.9 ml of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/ml *O*-dianisidine dihydrochloride (Sigma Chemical Co., St. Louis, USA) and 0.0005% hydrogen peroxide. The change in absorbances at 460 nm was measured with a spectrophotometer (UV-160A, Shimadzu City, Japan) (Bradley *et al.*, 1982).

Measurement of protein amounts Protein concentrations were determined with Bradford reagent (Bio-Rad) using bovine serum albumin as a standard (Bradford, 1976).

Results

Total mercury concentration Total amounts of mercury in rat liver tissues were measured. Whereas mercury was not detected in the control group administrated with saline, the mercury concentration of the MeHg-treated group was determined as 16.1 ± 1.9 ppm (Table 1).

PLA_2 activity of 100,000 × g supernatants and 100,000 × g pellets of liver tissues PLA_2 activity in the 100,000 × g supernatants was assayed using 2-[1-^{14}C]AA-GPC as substrate. As shown in Fig. 1, Ca^{2+}-dependent (not Ca^{2+}-independent) PLA_2 activity from MeHg-administrated rat liver was stably enhanced by approximately two-fold compared with that of the control group. In the same experiments, both the Ca^{2+}-dependent and Ca^{2+}-independent PLA_2 activities of the 100,000 × g pellets were not changed by treatment of MeHg (Fig. 2).

Table 1. Total mercury concentration. Male Sprague-Dawley rats were given a single intraperitoneal dose (10 μmol/kg) per day of methylmercury chloride (CH_3HgCl) suspended in 0.9% NaCl solution for 10 days. Total amounts of mercury of rat liver tissues were measured according to the quartz tube combustion gold amalgamation method as described in Materials and Methods.

	Total mercury concentration (ppm)
Control	N.D.
MeHg-treated	16.1±1.9

DEAE-5PW anionic exchange HPLC To examine whether there exists a cytosolic form of PLA_2 in the 100,000 × g supernatants of homogenates of liver tissues, the supenatants were loaded onto a DEAE-5PW anionic exchange HPLC column pre-equilibrated with 50 mM Tris/HCl (pH 7.5) buffer containing 1 mM EDTA. The major activity of PLA_2 was efficiently bound to the column and the peak activity eluted at fraction number 11 (0.55 M NaCl) in both the control and MeHg-treated groups, where the activity was approximately two-folds higher in the

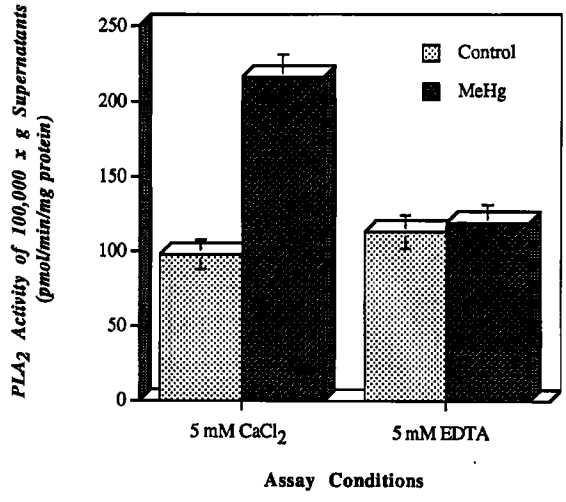

Fig. 1. PLA_2 activity in 100,000 × g supernatants of liver tissues. The 100,000 × g supernatants of control and MeHg-treated liver were prepared as described in Materials and Methods. Each sample was assayed for PLA_2 activity using 2-[1-^{14}C]AA-GPC as a substrate in the presence of 5 mM $CaCl_2$ and 5 mM EDTA for Ca^{2+}-dependent and -independent activities, respectively, as in Materials and Methods. Each value represents the mean ±SEM of three independent experiments.

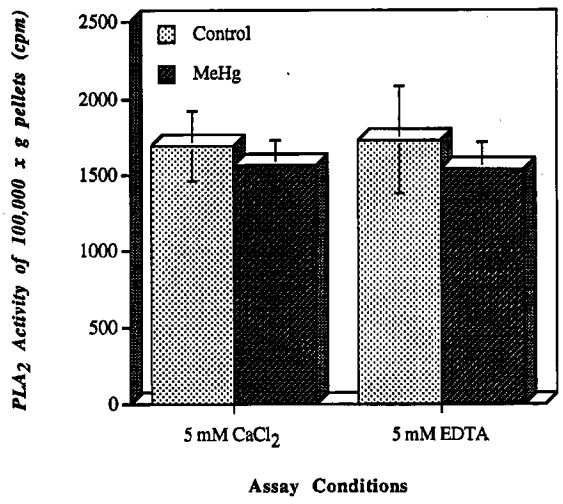

Fig. 2. PLA_2 activity in 100,000 × *g* pellets of liver tissues. The 100,000 × *g* pellets of control and MeHg-treated liver were prepared as described in Materials and Methods. Each sample was assayed for PLA_2 activity using 2-[1-^{14}C]AA-GPC as substrate in the presence of 5 mM $CaCl_2$ and 5 mM EDTA for Ca^{2+}-dependent and -independent activities, respectively, as in Materials and Methods. Each value represents the mean±SEM of three independent experiments.

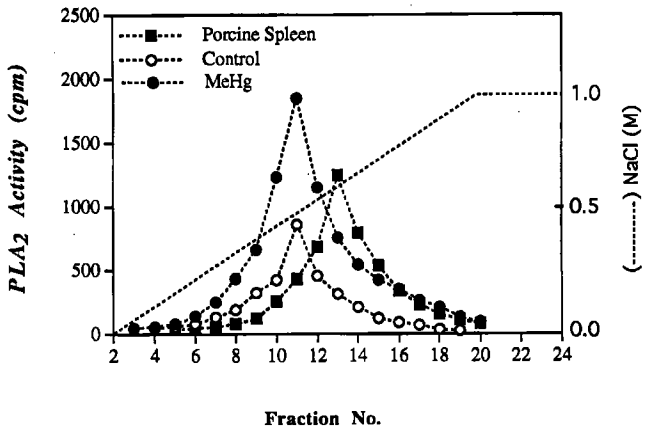

Fig. 3. DEAE-5PW anionic exchange HPLC of a liver form of cytosolic PLA_2 activity. The 100,000 × *g* supernatants were prepared from control and MeHg-treated rat livers as described in Materials and Methods. Each sample was loaded onto a DEAE-5PW anionic exchange HPLC column (0.75 cm × 7.5 cm, Tosoh Co., City, Japan) pre-equilibrated with 50 mM Tris/HCl (pH 7.5) buffer containing 1 mM EDTA. After washing the column until no protein was eluted, the column was eluted at a flow rate of 1 ml/min with 20 min-linear gradient of 0.0–1.0 M NaCl. The 100 kDa $cPLA_2$ standard was partially purified from porcine spleen as previously described (Kim and Bonventre, 1993). An liquot of active pool from a Butyl-Toyopearl hydrophobic column was applied onto the DEAE-5PW HPLC column under the same conditions. The presented data are representative results from three independent experiments.

MeHg-treated group than in the control group (Fig. 3). In contrast, when partially purified 100 kDa cytosolic porcine spleen PLA_2 (100 kDa $cPLA_2$) was loaded onto the DEAE-5PW HPLC column under the same conditions, the peak activity of $cPLA_2$ was eluted at fraction number 13 (0.65 M NaCl).

Sensitivity of the liver PLA_2 to dithiothreitol (DTT) Dithiothreitol has been used to differentiate the $cPLA_2$ activity from small molecular and secretory forms of PLA_2, the activities of which are sensitively inhibited by DTT. Each aliquot of active fractions obtained from a DEAE-5PW column of control and MeHg-treated liver cytosols, and an aliquot of group II PLA_2 partially purified from rat platelets (Kim *et al.*, 1991), was assayed using 2-[1-^{14}C]AA-GPE. Each enzyme source was pre-incubated with 2 mM DTT for 10 min at 37°C followed by addition of the substrate. As shown in Fig. 3, the liver form of PLA_2 activity obtained from a DEAE-5PW column was not affected by DTT, while the group II PLA_2 activity was inhibited under the same condition (Fig. 4).

Immunoprecipitation of rat liver cytosolic PLA_2 To examine a structural similarity of the liver cytosolic form of PLA_2 to the 100 kDa $cPLA_2$, immunoprecipitation experiments were performed using anti-100 kDa $cPLA_2$ antiserum. The effect of anti-100 kDa $cPLA_2$ antiserum on the liver form of PLA_2 enzymatic activity was evaluated by measuring 2-[1-^{14}C]AA-GPC-hydrolyzing activity in the presence of the antiserum. Incubation of active fractions from the DEAE-5PW column with antiserum against the 100 kDa $cPLA_2$ immunoprecipitated the PLA_2 activity in a time-dependent manner (Fig. 5).

Immunoblot analysis of rat liver cytosolic PLA_2 Western-blot analysis was performed for the partially purified PLA_2 from rat liver. As shown in Fig. 6, the antiserum reacted with the 80 kDa band from each source and there was more protein observed in the MeHg-treated sample compared with that in the control sample.

Myeloperoxidase (MPO) activity of rat liver MPO is a marker enzyme of the neutrophil. In the MeHg-administrated group, the MPO activity was higher by two-folds than in the control group (Fig. 7).

Discussion

MeHg stably enhanced a liver form of PLA_2 activity, which was Ca^{2+}-dependent and localized in the cytosolic fractions. The liver form of PLA_2 activity was insensitive to dithiothreitol, which has been used to differentiate a high molecular form of cytosolic PLA_2 from small molecular mass forms of PLA_2 such as group I and II enzymes (Kim and Bonventre, 1993). The liver form of

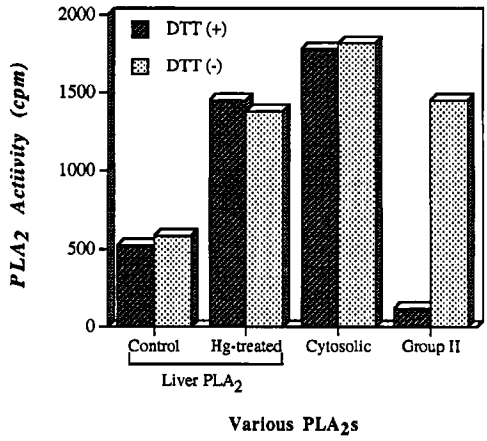

Fig. 4. Sensitivity of the liver PLA_2 to dithiothreitol (DTT). The liver PLA_2 was partially purified from control and MeHg-treated livers with a DEAE-5PW anionic exchange HPLC column as described in Fig. 3, respectively. The 100 kDa $cPLA_2$ standard was partially purified from porcine spleen as previously described (Kim and Bonventre, 1993). To compare the cytosolic forms of PLA_2 activity with a DTT-sensitive group II PLA_2 activity, rat platelet group II PLA_2 was partially purified as previously described (Kim *et al.*, 1991). Dithiothreitol (2 mM final concentration) was added to the assay system containing the PLA_2 enzymes derived from the various sources and pre-incubated at 37°C for 10 min. Following addition of the substrate, each sample was further incubated for the PLA_2 activity at 37°C for 30-min assays. The present data is a representative result from three independent experiments.

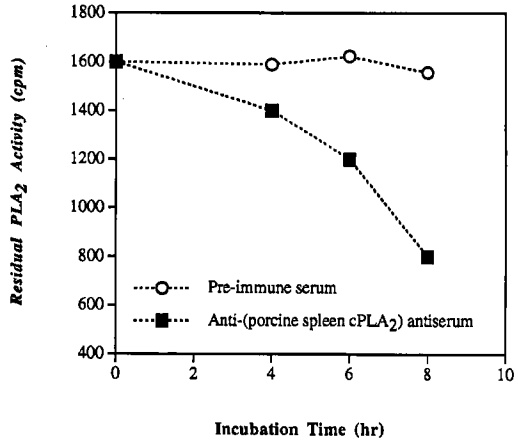

Fig. 5. Immunoprecipitation of rat liver cytosolic PLA_2. Protein A-Sepharose beads (30 μl bed volume) saturated with rabbit anti-(porcine spleen 100 kDa $cPLA_2$) serum or pre-immune serum, as described in Materials and Methods, were incubated for the indicated times with an aliquot of the active fractions obtained from a DEAE-5PW HPLC column of MeHg-treated liver at 4°C with constant shaking. After pelleting the beads, the resulting supernatants were assayed for PLA_2 activity as described in Materials and Methods. The present data is a representative result from three independent experiments.

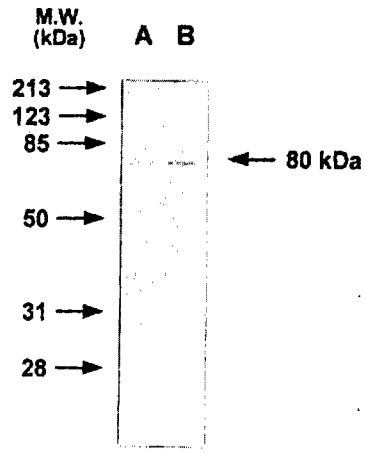

Fig. 6. Immunoblot analysis of a liver form of cytosolic PLA_2 by polyclonal anti-porcine spleen 100 kDa PLA_2 polyclonal antibody. The active fractions from the DEAE-5PW HPLC columns of control and MeHg-treated rat livers were separated by SDS-PAGE (10% gels) and blotted with 1/2000-diluted antiserum as described in Materials and Methods. Samples of the same volume (100 μl) were loaded on the gel. Positions of protein molecular-weight kDa standards are marked.

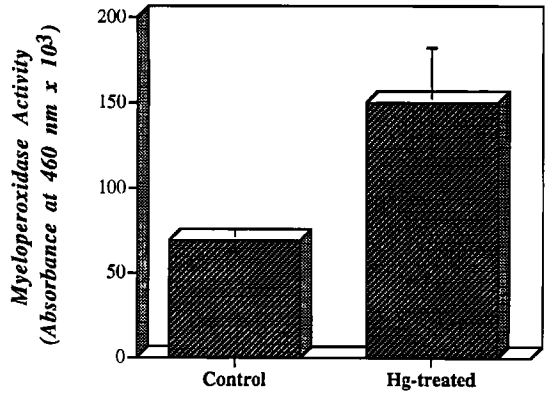

Fig. 7. Myeloperoxidase (MPO) activity of rat liver. Myeloperoxidase (MPO) was extracted from the homogenates of control and MeHg-treated livers with 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mM potassium phosphate buffer (pH 6.0) as described in Materials and Methods. The MPO activity of the samples was measured as described in Materials and Methods. Each value represents the mean±SEM of three independent experiments.

cytosolic PLA_2 activity was not affected by addition of MeHg up to 16 μM, suggesting that the enhancement of the PLA_2 activity may not be due to its direct effect (data not shown). These data suggest that activation of the liver cytosolic form of PLA_2, showing biochemical properties very similar to those of the 100 kDa $cPLA_2$, may cause MeHg-induced liver injury. This prompted us to postulate several possible mechanisms for this stable enhancement of the PLA_2 activity; *viz.*, a direct activation of the PLA_2

activity by MeHg, a covalent modification of the PLA_2 enzyme such as MeHg-induced phosphorylation, and the increase in the amount of the enzyme protein via its expression. These possibilities could be preliminarily elucidated by Western blotting analysis if the liver cytosolic PLA_2 relates to the 100 kDa $cPLA_2$ immunochemically.

Figure 5 showed that the liver PLA_2 activity was immunoprecipitated with anti-porcine spleen 100 kDa $cPLA_2$ antiserum, suggesting an immunochemical similarity to the 100 kDa $cPLA_2$. Furthermore, the liver cytosolic PLA_2 was Ca^{2+}-dependent and insensitive to dithiothreitol. These data strongly suggest that the liver cytosolic PLA_2 may be a cytosolic 100 kDa form of PLA_2. To further examine this finding, the 100,000 × *g* supernatant of the control or MeHg-treated liver tissue was applied to a DEAE-5PW HPLC column. As shown in Fig. 3, the liver cytosolic PLA_2 activity eluted in different concentrations of NaCl to the 100 kDa cytosolic form of PLA_2 partially purified from the porcine spleen. Furthermore, Western blotting analysis demonstrated that the liver cytosolic form of PLA_2 migrated as a single band of a molecular weight of 80 kDa, where that from MeHg-administrated liver was thicker than the band from the control liver (Fig. 6). These results suggested that the liver cytosolic PLA_2 activity enhanced by MeHg is due to the higher amount of the 80 kDa protein. To our knowledge, this is the first such finding that there exists a $cPLA_2$-like enzyme of a molecular mass of 80 kDa in rat liver and that the MeHg is responsible for the increase in the amount of the protein. Although the precise mechanism through which both the increased PLA_2 protein levels and the higher activity are generated in MeHg-treated liver is, at present, unknown, it appears that the long-term exposure by MeHg either induces the PLA_2 protein or increases infiltration of inflammatory cells such as neutrophils into the damaged liver tissues. The possibility of the latter could be partly proposed by an increase in myeloperoxidase (MPO) activity of MeHg-treated liver (Fig. 7), as well as by MeHg-induced enhancements of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) activities in serum — used as measures of the extent of liver damage — by 1.5- and 1.6-folds, respectively, compared with controls. On the other hand, many lines of evidence were reported that MeHg manifested its toxicity through an inhibition of protein synthesis (Brookes *et al.*, 1989; Ponce *et al.*, 1994). However, it was also reported that the exposure of MeHg elevated production of the nerve growth factor protein in the 3T3 fibroblast cell line (Soderstrom and Ebendal, 1995) and a significant stimulation of mitochondrial protein synthesis by intraperitoneal administration to mouse (Kuznetsov, 1987). Thus, at present, the former possibility cannot be ruled out that both the higher PLA_2 activity and increased amount of 80 kDa PLA_2 protein induced by MeHg may be due to the protein synthesis.

In summary, MeHg stably enhanced a liver form of cytosolic PLA_2 activity, which exhibited biochemical properties similar to those of the 100 kDa $cPLA_2$, except in its DEAE-5PW HPLC elution profile, but migrated as a molecular mass of 80 kDa in Western blotting analysis. It is suggested that, as indicated in Western blotting analysis, the MeHg-induced enhancement of the activity may be due to the increase in the amount of the enzyme protein rather than a stable modification of the enzyme such as phosphorylation. Taken together, these data suggest that MeHg-induced liver injury may be mediated by activation of the 80 kDa form of liver cytosolic PLA_2.

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