Rat liver mitochondrial phospholipase A_2 is an endotoxin-stimulated membrane-associated enzyme of Kupffer cells which is released during liver perfusion

Grant M. HATCH,*‡ Dennis E. VANCE* and David C. WILTON†§

*Lipid and Lipoprotein Research Group and Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2S2, Canada and †Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, U.K.

A novel fluorescence assay for phospholipase A_2 [Wilton (1990) Biochem. J. **266**, 435–439] has been used to study the Group-II rat liver mitochondrial enzyme, and a number of novel properties of this enzyme were identified. (1) The enzyme activity was located in the liver macrophages (Kupffer cells) while negligible activity was associated with hepatocytes. (2) Although subcellular fractionation of whole liver confirmed the predominantly mitochondrial location of this enzyme activity, the analysis of the hepatocyte-free Kupffer-cell-enriched fraction revealed a different enzyme distribution, with the majority of activity being associated with the microsomal membrane fraction. (3) Bacterial endotoxin has been previously shown to be scavenged by Kupffer cells in rats. Treatment of rats with bacterial lipopolysaccharide (endotoxin) resulted in a dramatic time- and dose-dependent increase in liver phospholipase A_2 activity. (4) It is known that injection of endotoxin into rodents results in elevated serum phospholipase A_2 activity, while a similar phenomenon is seen in the condition of septic shock in man. The source of this serum enzyme was unknown. In this study perfusion of livers from rats pretreated with lipopolysaccharide with physiological saline demonstrated a 6-fold increase in phospholipase A_2 activity in the perfusate compared with sham-treated controls, with only minor release of hepatic lipase. (5) Western-blot analysis confirmed an increased release of this Group-II phospholipase A_2 into the perfusate of lipopolysaccharide-treated rats compared with sham-treated controls. These results suggest that liver Kupffer cells are a major source of the endotoxin-induced serum Group-II phospholipase A_2 activity associated with bacterial infection and trauma.

INTRODUCTION

The enzyme phospholipase A_2 (PLA_2; EC 3.1.1.4) is of considerable interest because of its possible role in a number of biological phenomena. These processes include (a) signal transduction and the generation of eicosanoids and other cellular mediators [1], (b) the remodelling of the fatty acyl chains of fatty acids in phospholipids, including the removal of oxidatively damaged fatty acids [2,3], and (c) the degradation of invading micro-organisms and necrotic tissue [4], in addition to the well-established digestive function of the enzyme from pancreas. The ability of PLA_2 to release arachidonic acid as the controlling step in the biosynthesis of the eicosanoids and platelet-activating factor by most tissues of the body has focused much attention on the potential role of this enzyme in inflammatory processes [5] including the condition of septic shock [6]. The level of Group-II serum PLA_2 is dramatically increased in animals treated with bacterial endotoxin [5]. It is also known that Kupffer cells in liver are a major site for removal of endotoxin [7].

A continuous fluorescence-displacement technique has been developed that allows the rapid assay of PLA_2 and lipases that release long-chain fatty acids. The versatility of this assay has been established with pure enzymes [8–10]. We wished to evaluate the use of this assay for measurement of phospholipases in crude fractions from homogenized tissues and cells in culture. The hepatocyte was chosen as an appropriate system for the measurement of PLA_2 activity using the fluorescence assay because of the relatively high specific activity of the Group-II liver mitochondrial PLA_2 [11].

We now demonstrate that the liver mitochondrial PLA_2 activity [11] is located in Kupffer cells of the liver and not in hepatocytes. Moreover, the activity was not primarily mitochondrial but had a more general membrane distribution. PLA_2 activity was greatly enhanced on lipopolysaccharide (LPS) treatment *in vivo*, supporting a Kupffer cell location of this enzyme. Liver perfusion studies indicated the potential of this enzyme to be released into the serum under physiological or pathological conditions. Thus, Kupffer cells in liver may be a major source of the elevated serum PLA_2 that is seen under conditions producing trauma, particularly septic shock [12].

EXPERIMENTAL

Materials

Dioleoylphosphatidylglycerol (DOPG) and dioleoylphosphatidylcholine were obtained from Sigma and redissolved (2 mg/ml) in methanol. 11-(Dansylamino)undecanoic acid was obtained from Molecular Probes and was dissolved at a concentration of 1.16 μM in methanol. Purified LPS from *Escherichia coli* K-235 was obtained from Sigma. Poly(ethylene glycol) 8000 was obtained from BDH, Toronto, Canada. Monoclonal antibody raised to PLA_2 was a gift from Dr. H. Van den Bosch. All other biochemicals were of analytical grade and obtained from either Sigma Chemical Company, St. Louis, MO, U.S.A. or

Abbreviations used: PLA_2, phospholipase A_2; LPS, lipopolysaccharide; DOPG, dioleoylphosphatidylglycerol.

‡ Present address: Departments of Medicine and Biochemistry, University of Manitoba, Winnipeg, Manitoba, R3E OZ3, Canada.
§ To whom correspondence should be addressed.

Fisher Scientific, Edmonton, Canada. Male Sprague–Dawley rats (100–125 g body wt.) were used throughout the study.

Subcellular fractionation of rat liver, hepatocytes and Kupffer-cell-enriched fractions

A 20% homogenate (w/v) of tissue (or cells) was prepared in buffer A (0.25 M sucrose, 20 mM Tris/HCl, pH 7.4, 0.025% sodium azide, 2 mM dithiothreitol, 2.5 mM EDTA) with 15 strokes (50 strokes for cells) of a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 500 *g* for 5 min to sediment cell debris. The supernatant was centrifuged at 12000 *g* for 10 min and the resulting pellet designated the mitochondrial fraction. The post-mitochondrial supernatant was centrifuged at 105000 *g* for 60 min, the supernatant discarded and the resulting pellet was designated the microsomal fraction.

Preparation of hepatocytes and Kupffer-cell-enriched fractions

Rat liver hepatocytes and Kupffer-cell-enriched fractions were prepared using a modified collagenase-perfusion technique [13]. Subsequent to collagenase perfusion, the liver was removed and cut into small pieces. The tissue was further digested with 15 ml of collagenase solution for 5 min. The collagenase reaction was terminated by the addition of Dulbecco's minimal essential medium containing 17% (v/v) fetal-calf serum to a final volume of 50 ml. The suspension was centrifuged at 25 *g* for 2 min and the resulting supernatant was carefully removed and centrifuged at 300 *g* for 10 min. This pellet, which contains the sinusoidal cells, was washed four times with ice-cold PBS and subsequently designated the Kupffer-cell-enriched fraction. The pellet from the first centrifugation (25 *g*) was washed three times with serum-containing medium and then twice with ice-cold PBS and designated the hepatocyte fraction. More than 95% of hepatocytes prepared by this method excluded Trypan Blue.

Preparation of purified Kupffer cells

Purified Kupffer cells were kindly provided by Dr. P. Winwood from the Department of Medicine, University of Southampton, U.K. Cells were obtained from normal Sprague–Dawley rats following perfusion with collagenase then Pronase. Purification was achieved by arabinogalactan-density-gradient centrifugation and centrifugal elutriation following the procedure of Arthur et al. [14]. The yields varied between 50×10^6 and 150×10^6 cells per liver and purity was greater than 98%.

Preparation of PLA_2 from control, sham-operated, and LPS-injected rats

Liver perfusates from sham- (intraperitoneal injection of 0.5 ml of PBS) and LPS- (intraperitoneal injection of 0.5 ml of PBS containing LPS at a dose of 10 mg/kg) treated rats were prepared as follows. The rats were anaesthetized with ether 4 h after injection and an incision from the lower abdomen up to the thoracic cavity was made. The portal vein was cannulated with a blunt-end needle and immediately the inferior vena cava was cut below the kidney. The vena cava was then tied above the kidney and, after a thoracic incision, the aorta was also quickly cannulated. The perfusion of Hanks balanced salt solution was allowed to flow via gravity to a collecting tube located 30 cm below the operating table. The flow rate (5.5 ml/min per liver) was maintained throughout this period (2–4 min) to provide an initial perfusate of 20 ml after which a 50 ml sample of Hanks perfusate was collected for analysis. These perfusion conditions represent non-digestive modification of the longer and more aggressive conditions employed for the preparation of hepatocytes and Kupffer cells in high yield. Therefore liver cell integrity would be maintained during the short duration of this particular perfusion procedure. Although some anoxia may result it should not affect cell integrity and this was confirmed by measurement of lactate dehydrogenase levels in the perfusate.

The 50 ml fraction was concentrated to 5 ml in a dialysis membrane (Spectra/Por 3, molecular-mass cut-off 3500) layered on a bed of poly(ethylene glycol) 8000 (for 6–8 h). An aliquot of the concentrate was removed for assay of lactate dehydrogenase activity (EC 1.1.1.27) [15]. A unit of lactate dehydrogenase activity is defined as 1 μmol of NADH converted into NAD/ml per min in the standard assay. The concentrate was then applied to a Sephadex G-75 column (dimensions: 100 cm × 1.5 cm, 150 ml bed volume) and PLA_2 eluted with 20 mM Tris/HCl, pH 7.4, 1 M KCl, 1 mM EDTA, 0.025% sodium azide (flow rate 0.82 ml/min). Fractions (10 ml) were collected and PLA_2 activity assayed. The fractions containing the highest PLA_2 activity were pooled and concentrated with poly(ethylene glycol) as described above. An aliquot of concentrated protein was incubated in a boiling water-bath for 2 min with an equivalent volume of sample buffer [8 M urea, 10% (w/v) glycerol, 2% (w/v) SDS, 60 mM Tris/HCl, 6.8). Proteins were electrophoresed (Bio-Rad Mini Gel system) on gels (0.5 mm × 6.5 cm × 8.0 cm) of 10% (w/v) polyacrylamide with a 3% stacking gel at 15 mA (constant current) until the Bromophenol Blue in the samples was within 2 mm of the bottom of the gel (about 45 min) [16]. Proteins were stained with 0.1% Coomassie Blue G-250 in 2% (v/v) phosphoric acid overnight and destained with several changes of distilled water.

Western-blot analysis of PLA_2

The perfusates from LPS-treated rats were concentrated on a Sephadex G-75 column and subjected to SDS/PAGE as described above. Using the Bio-Rad Minisystem Transblot apparatus, proteins were transfered on to nitrocellulose at 200 mA (constant current) for 1.5–2 h in transfer buffer [25 mM Tris/HCl, pH 8.3, 0.2 M glycine, 20% (v/v) methanol]. The blots were blocked overnight with 50 ml of Tris-buffered saline (10 mM Tris/HCl, pH 7.5, 0.154 M NaCl, 0.2% sodium azide) containing 5 g of non-fat dry milk. Blots were washed three times for 10 min each wash with Tris-buffered saline and subsequently incubated in 25 ml of a 1:500 dilution of a monoclonal antibody raised to rat liver mitochondrial PLA_2 ([11], kindly provided by Dr. Henk van den Bosch) for 1–2 h at room temperature. Subsequently, the blot was washed four times (for 10 min each wash) with 30 ml of Tris-buffered saline containing 0.5 ml/l Tween-20 and no sodium azide. The blot was treated for 1–2 h with sheep anti-(mouse IgG) peroxidase (1:5000) in the Tween-20 buffer. The blot was then washed four times for 5 min with the Tween buffer, then once for 15 min. The blots were incubated for 1 min in 20 ml of enhanced chemiluminescent Western-blotting detection reagent and subsequently exposed to X-ray film for 30 s. In some experiments, rat liver perfusate (50 ml) from sham-operated (control) and LPS-treated rats was concentrated to 3 ml as described above and Western-blot analysis performed on aliquots of the crude perfusate that were subjected to SDS/PAGE.

Release of PLA_2 from membrane fractions

Membrane fractions obtained by centrifugation were resuspended in a buffer containing 250 mM sucrose, 20 mM Tris/HCl,

pH 7.4, 1 mM EDTA and 1 M KCl with five strokes of a dounce homogenizer. Typically the liver mitochondrial fraction was suspended to give a total volume of 10 ml. Samples, 2 × 0.5 ml, were placed in 11 mm × 34 mm centrifuge tubes and sonicated for 2 × 15 s using a Heat Systems-Ultrasonics W-385 sonicator fitted with a microtip probe on a power setting of 2. The samples were centrifuged at 350000 *g* for 15 min in a Beckman TL-100 ultracentrifuge and the supernatant (2–50 μl) was assayed.

Assay of PLA_2 activity

The procedure used was essentially that described previously [8]. The assay cocktail (1 ml) contained 1.116 μM 11-(dansylamino)undecanoic acid and 0.01 mg/ml DOPG in 0.1 M Tris/HCl, pH 8.0, containing 0.1 M NaCl and 2.5 mM $CaCl_2$. The 70% satn. $(NH_4)_2SO_4$ supernatant, obtained during purification of recombinant rat liver fatty-acid-binding protein, or pure protein [17] (at a concentration of about 10 μg/ml) were used in these studies. Calibration was performed by adding up to 1 nmol of oleic acid as a 1 mM solution prepared in methanol. EGTA, when used, was added at a final concentration of 5 mM to this assay. Enzyme fractions (2–50 μl) were added to the complete assay and the initial rate of fall in fluorescence was recorded. Fluorescence measurements were made at 25 °C in 4 ml polystyrene fluorimeter cells using an Hitachi F2000 fluorimeter fitted with an external recorder.

Other analyses

Protein was estimated by the Bradford method [18]. Student's *t*-test was used for determination of significance. The level of significance was defined as $P < 0.05$.

Table 1 Distribution of the KCl-releasable PLA_2 activity of rat liver

Liver fractions were prepared as described in the Experimental section and the peripheral PLA_2 was released by sonication in the presence of 1 M KCl. PLA_2 activity was determined in the supernatant after sonication using DOPG as substrate. Values represent the mean ± S.D. Values in parentheses indicate number of separate experiments performed. For PLA_2 activity 1 m-unit is defined as 1 nmol of DOPG hydrolysed/min in the fluorescence assay after subtraction of the rate in the presence of EGTA. In one experiment the distribution of total KCl-releasable activity was determined between hepatocytes, the non-hepatocyte cell fraction and the cell-debris fraction. The percentage of total distribution was hepatocytes (2%), non-hepatocyte cell fraction (30%) and cell-debris fraction (68%).

Liver fraction	PLA_2 specific activity (m-unit/mg)
Whole liver mitochondria	13.6 ± 2.9 (5)
Whole liver microsomes	2.07 ± 0.57 (3)
Hepatocyte mitochondria	0.57 ± 0.28 (4)
Hepatocyte microsomes	< 0.1* (3)
Non-hepatocyte cell fraction mitochondria	22.7 ± 7.7 (3)
Non-hepatocyte cell fraction microsomes	30.1 ± 5.0 (3)
Cell-debris-fraction mitochondria	6.9 ± 1.4 (3)
Cell-debris-fraction microsomes	40.7 ± 17.9 (3)
Purified Kupffer cell membranes†	169 ± 135 (4)

* Activity was below the level of detection by this assay.

† The 500 *g* supernatant was centrifuged at 25000 *g* for 20 min and the membrane pellet was assayed for activity.

RESULTS

Assay of liver PLA_2 activity

Liver mitochondria were prepared and solubilized by sonication in the presence of 1 M KCl following essentially the method of Aarsman et al. [11]. Enzyme activity in the supernatant was measured by the fluorescence assay using DOPG as substrate. We have previously shown that DOPG is a preferred substrate for the liver mitochondrial PLA_2 using the fluorescence assay [19] and in the present study DOPG showed activity approx. 1000 times greater than with dioleoylphosphatidylcholine, when measured under identical assay conditions.

High PLA_2 activity was released from the liver mitochondria after sonication (Table 1) although no significant activity was released by sonication in the absence of 1 M KCl. This released activity could be inhibited by over 90% in the presence of excess EGTA, consistent with a PLA_2 which requires Ca^{2+} as an essential component of the catalytic mechanism. The solubilized activity, when fractionated on a Sephadex G-75 column, was detected in the low-molecular-mass fraction of the column effluent consistent with the 14 kDa mass of mitochondrial PLA_2 [11]. The total liver mitochondrial activity for this enzyme under these assay conditions was about 400 m-unit for 125 g male rats.

The presence or absence of Ca^{2+} in the homogenizing buffer could affect the subcellular distribution of this PLA_2 during cell disruption. Therefore, in one experiment, samples from the same liver were homogenized either in the presence of EDTA (normal protocol) or without EDTA and in the presence of 1 mM Ca^{2+}. The specific activities of the KCl-released mitochondrial associated enzymes were 15.4 and 14.9 nmol/min per mg after homogenization in the absence or presence of Ca^{2+} respectively. Thus, Ca^{2+} appears to have no effect on the subcellular location of this enzyme after cell disruption.

The microsomal fraction was also solubilized and tested for PLA_2 activity (Table 1). Low levels of activity were detected, consistent with a previous report [11]. Phospholipase activity was relatively insensitive to EGTA, indicating the presence in this fraction of other Ca^{2+}-independent lipases. The other relatively abundant phospholipase activity in liver is hepatic lipase. This 53 kDa protein, which does not have an absolute requirement for Ca^{2+}, is readily assayed with the fluorescence method using DOPG as substrate. About 1000 m-unit of this activity was released by heparin in liver perfusion studies (results not shown). Mono-oleoylglycerol is a well-documented substrate for hepatic lipase [20] and showed similar activity to DOPG in the fluorescence assay. The KCl-released liver mitochondrial enzyme was completely inactive with this substrate in the fluorescence assay.

Thus, although the fluorescence assay is non-specific and detects the release of long-chain fatty acids, the properties of the KCl-releasable liver mitochondrial activity confirm that it is the PLA_2 described by Aarsman et al. [11].

Cellular distribution of liver PLA_2

Liver contains hepatocytes and other non-parenchymal cells of which Kupffer cells, the resident macrophage cells of the liver, are the most abundant. Collagenase perfusion of liver allows the preparation of virtually pure samples of hepatocytes and, by further differential centrifugation, a sinusoidal cell fraction that contained the Kupffer cells. Following this procedure relatively large amounts of these two cell types could be prepared rapidly and subjected to homogenization and subcellular fractionation before assay for PLA_2 activity.

Significant liver mitochondrial PLA_2 activity could not be detected in hepatocytes using the fluorescence assay (Table 1).

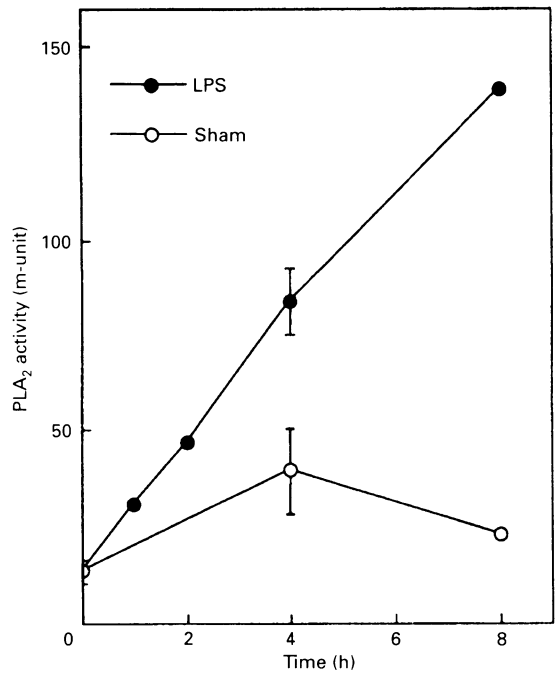

Figure 1 Time-dependent stimulation of the KCl-releasable liver mitochondrial PLA_2 activity by LPS

Rats were injected intraperitoneally with purified LPS in saline (10 mg/kg) (LPS) or saline alone (Sham). After various times the livers were perfused with Hanks solution to remove blood, homogenized and the KCl-releasable liver mitochondrial PLA_2 activity was measured. The zero time value is for normal non-injected rats. Points with error bars represent the mean ± S.D. of three separate experiments performed in duplicate. Points without error bars represent the average of duplicate measurements from individual rats.

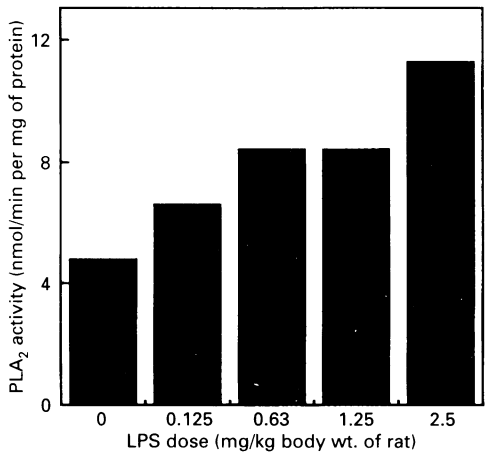

Figure 2 Concentration-dependent stimulation of the KCl-releasable liver mitochondrial PLA_2 by LPS

Rats were injected intraperitoneally with various concentrations of purified LPS or saline and releasable liver mitochondrial PLA_2 activity was measured after 4 h as described in Figure 1. Each bar represents the average of duplicate measurements from two rats.

However, high levels of activity were present in the Kupffer-cell-enriched fractions (Table 1) and this activity was 98–100% inhibited by EGTA. An additional observation was that the majority of enzyme activity and the highest specific activity was found in the microsomal fraction of the Kupffer-cell-enriched fraction.

The Kupffer-cell-enriched fraction accounted for about 30% of the total PLA_2 activity in this non-hepatocyte-containing residue after liver disruption. The majority of the PLA_2 activity was in the membrane fractions derived from the 300 *g* supernatant (cell-free debris fraction). Differential centrifugation of this cell debris fraction also gave a preferential distribution of PLA_2 activity in the microsomal fraction (Table 1).

Assay of PLA_2 activity in pure Kupffer cells

The Kupffer cell fraction described above would contain other small cells from the liver including lipocytes and endothelial cells. When the PLA_2 activity was measured in highly purified Kupffer cells high PLA_2 activity was observed (Table 1) which was completely inhibited with EGTA. This result confirms a Kupffer cell location of this liver PLA_2. After these studies were completed, Inada et al. [21] reported a preferential distribution of PLA_2 activity in Kupffer cells.

Effect of LPS on liver PLA_2 activity

Kupffer cells play a major role in the removal of LPS (bacterial endotoxin) in the rat while endotoxin challenge is associated with elevated levels of non-pancreatic serum PLA_2 in many species, including man [6]. Having identified high levels of liver PLA_2 in the Kupffer cell fraction it was of particular interest to investigate how this enzyme activity responded *in vivo* to administration of LPS.

In Figure 1 we demonstrate the dramatic time-dependent effect of LPS injection on liver PLA_2 activity, showing a 10-fold increase after 8 h compared with saline-injected controls. A significant increase was also seen in saline-injected controls after 4 h, suggesting that even the minor trauma of intraperitoneal injection of a significant volume of saline may stimulate the activity of this enzyme.

In order to determine the effect of lower doses of LPS on PLA_2 activity, rats were injected with saline or various concentrations of LPS and 4 h later the liver was removed and PLA_2 activity assayed. As seen in Figure 2 the effect of LPS on rat liver PLA_2 activity was concentration-dependent.

When livers from LPS-treated rats (4 h exposure) were subjected to collagenase perfusion and cell fractionation, it was impossible to obtain discrete fractions of hepatocytes and non-parenchymal cells due to cell clumping. Therefore, it was not possible to demonstrate enhanced levels of PLA_2 activity in the Kupffer cell fraction. After these studies were completed, Inada et al. [22] reported stimulation of PLA_2 by LPS in both hepatocytes and non-parenchymal cells.

Is liver PLA_2 activity released during perfusion?

As well as the classic example of PLA_2 release from platelets [23] a number of mammalian cells in culture have now been shown to release PLA_2 [24–27]. Therefore, it was important to investigate the possible release of PLA_2 from the liver in view of the similarity to PLA_2 from rat spleen and platelets [27]. Both of these enzymes have a cleavable signal sequence, suggesting export [28,29].

Liver perfusions were performed on 4 h LPS-treated and sham-treated control rats, since this has been observed to be the time at which serum PLA_2 reaches its maximum level after LPS injection in rabbits [5]. After an initial 20 ml perfusion to remove blood components, a 50 ml perfusion (10–12 min) was collected and, after concentration, subjected to fractionation on a

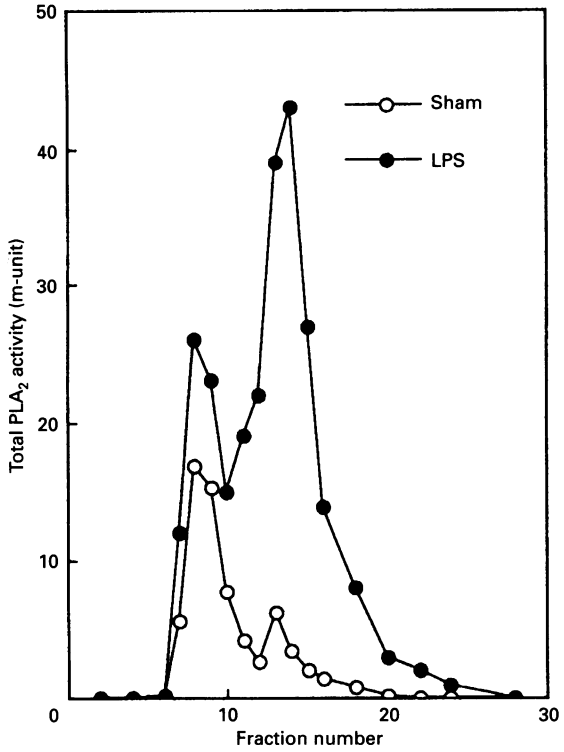

Figure 3 Fractionation of liver perfusate on a Sephadex G-75 column to separate hepatic lipase from PLA_2

Livers from LPS-injected (LPS) or saline-injected (Sham) rats were perfused and the perfusate concentrated as described in the Experimental section. The concentrated suspension was applied to a Sephadex G-75 column and proteins eluted. Traces are shown for both saline- and LPS-injected rats and are the average of two separate liver perfusions. Total releasable PLA_2 activity, collected in fractions 11–18, was 156 m-unit for LPS-injected rats and 27 m-unit for saline-injected controls. The PLA_2 activities from all rats was 100% inhibited by EGTA.

Sephadex G-75 column to separate PLA_2 activity from the heparin-releasable hepatic lipase which is also released to a small extent from liver in the absence of heparin. The results of such fractionations for control and LPS-treated liver perfusates are shown in Figure 3 and clearly demonstrate an enhanced release of PLA_2 activity (fractions 12–15) after LPS treatment. The initial peak (fractions 8–10) is hepatic lipase which was dramatically enhanced (20-fold) by addition of heparin to the perfusion buffer (results not shown). The total PLA_2 activity released from LPS-treated liver in 10–12 min of perfusion (about 150 m-unit) represented about 6% of the total mitochondrial-associated activity (2500 m-unit) in these livers. This remarkable rate of loss would suggest a physiological release process rather than liver damage. This conclusion is supported by lactate dehydrogenase levels which were low in the total perfusate of both sham-operated control (0.21 unit) and LPS-treated (0.26 unit) rats. Of interest was the appearance of a yellow protein(s) in the fractions eluting between hepatic lipase and PLA_2 which showed absorbance at 414 nm. The release of this chromophore paralleled the release of PLA_2. The nature of this protein remains to be established but could be related to the respiratory burst oxidase of macrophages [30].

SDS/PAGE followed by Western-blot analysis was performed on the 50 ml fraction concentrated by Sephadex G-75 chromatography. The results clearly showed cross-reaction of the 14 kDa band with the rat liver PLA_2 antibody (Figure 4, lanes 1 and 2).

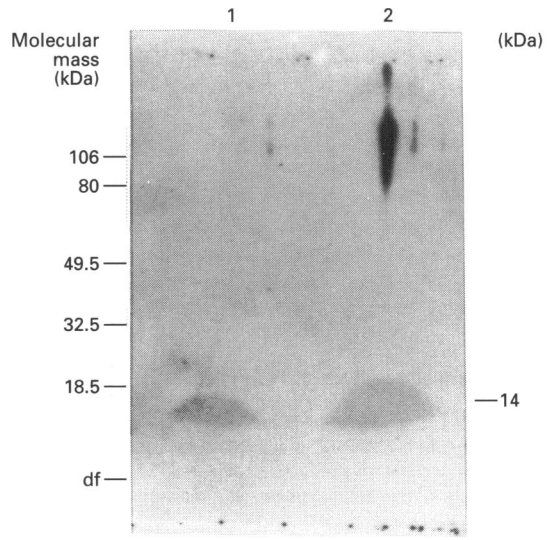

Figure 4 Western-blot analysis of PLA_2 concentrated by Sephadex G-75 chromatography

Rats were treated with LPS (10 mg/kg) and 4 h later their livers were perfused with 50 ml of Hanks balanced salt solution. The perfusate was concentrated to 5 ml as described in the Experimental section and passed down a Sephadex G-75 column. Fractions with the highest levels of activity were pooled and concentrated. Samples, 0.5 μg (lane 1) and 1.0 μg (lane 2) or protein, were subjected to SDS/PAGE followed by Western-blot analysis with antibody raised to rat liver PLA_2. Abbreviation: df, dye front.

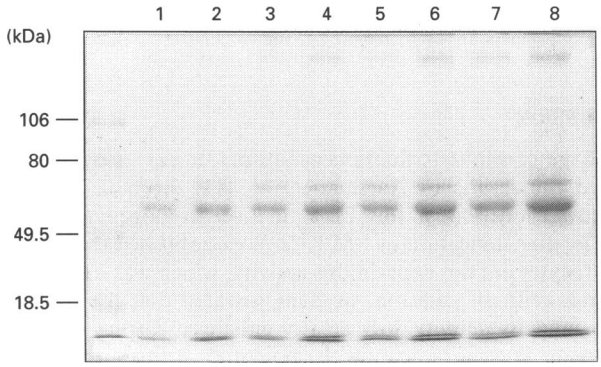

Figure 5 SDS/PAGE analysis of proteins released into the perfusate

Rats were treated with LPS (10 mg/kg) or saline (sham) and 4 h later their livers were perfused with 50 ml of Hanks balanced salt solution. The 50 ml perfusate was concentrated to 3 ml using poly(ethylene glycol). Subsequently, increasing equivalent volumes of perfusate from each group were analysed by SDS/PAGE as described in the Experimental section. Lanes 1, 3, 5 and 7, sham; lanes 2, 4, 6 and 8, LPS-treated. Gel loading: lanes 1 and 2, 5 μl; lanes 3 and 4, 10 μl; lanes 5 and 6, 15 μl; lanes 7 and 8, 20 μl of concentrated perfusate. The protein concentration was 0.6 μg/μl and 1.2 μg/μl of sham-treated control and LPS-treated groups respectively.

The bands appeared quite diffuse in this analysis and this was probably due to the high concentration of KCl present in the Sephadex G-75-concentrated perfusate before electrophoresis. Despite the diffuse nature of the bands the antibody response was clearly concentration-dependent and restricted to the 14 kDa region of the gel.

To determine whether the amount of PLA_2 released into the perfusate was altered in LPS-treated rats compared with sham-

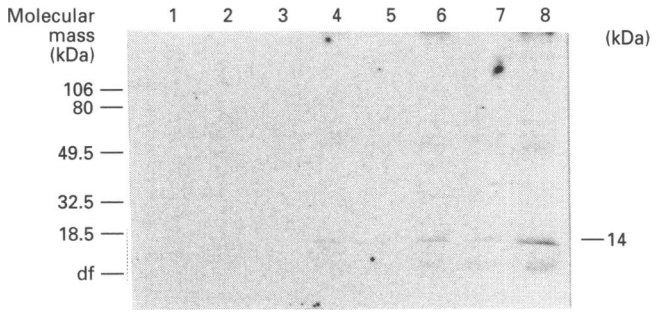

Figure 6 Western-blot analysis of PLA_2 released into the perfusate

Rats were treated with LPS or saline and 4 h later the livers were perfused and the perfusate concentrated and subjected to SDS/PAGE as described in the legend to Figure 4, except that equivalent amounts of protein from sham-treated control or LPS-treated rat perfusates were analysed. Subsequently, Western-blot analysis was performed using antibody raised to rat liver PLA_2 as described in the Experimental section. Lanes 1, 3, 5 and 7, sham; lanes 2, 4, 6 and 8, LPS-treated. Gel loading: lanes 1 and 2, 5 μg; lanes 3 and 4, 10 μg; lanes 5 and 6, 20 μg; lanes 7 and 8, 30 μg of protein. Abbreviation: df, dye front.

treated controls, SDS/PAGE followed by Western-blot analysis was performed on 50 ml of liver perfusate that was concentrated from these rats. SDS/PAGE revealed a significant 2-fold increase in release of protein (Figure 5). This was confirmed by Bradford protein estimation [18]. When equivalent amounts of protein were subjected to SDS/PAGE followed by Western-blot analysis using antibody raised to rat liver PLA_2, a significantly larger amount of PLA_2 was released from the liver into the perfusate of LPS-treated rats compared with sham-treated controls (Figure 6).

DISCUSSION

With the continuous fluorescence-displacement assay, we confirmed the results of Aarsman et al. [11] for both the mitochondrial location of this PLA_2 in liver homogenates and its release after sonication in 1 M KCl. We established that isolated hepatocytes did not contain this activity, which was found in the Kupffer cells. In addition, we demonstrated that the release of PLA_2 and its activity was greatly enhanced during endotoxin treatment *in vivo*, indicating the potential of this enzyme to be released into the serum.

The high specific activity of the liver mitochondrial PLA_2 put the measurement of this enzyme well within the sensitivity range of the fluorescence-assay method. The fluorescence-displacement-assay method has a lower sensitivity of about 0.02 m-unit of enzyme activity per ml of assay mixture under optimal conditions. However, sensitivity is compromised if membrane fractions are measured. This is because the fluorescent fatty-acid probe that is used will partition into the membranes and give a high background fluorescence. Therefore, the fact that the liver mitochondrial enzyme is readily released from membrane fractions by sonication in 1 M KCl greatly facilitates the assay of this enzyme [11].

A surprising feature of the activity, in both the Kupffer-cell-enriched fraction and the debris obtained after collagenase treatment, was that the majority of this activity was not found in the mitochondrial fraction but was located in the post-mitochondrial membrane fractions, which would include smaller granules and microsomes. An identical homogenization procedure was used as for the preparation of the mitochondrial fraction from whole liver. The explanation of the different distribution in these systems probably reflects the cells being homogenized. One possible explanation for the subcellular distribution of PLA_2 activity is that this peripheral cationic enzyme binds to subcellular membranes according to their availability. It would be anticipated that the negatively charged membrane domains would be the preferred site of interaction. The separation of hepatocytes from other cell types would remove a major source of mitochondrial membranes and, hence, binding to other subcellular membranes would be observed. Alternatively there may be other factors present in the homogenate of whole liver that affect enzyme distribution as compared with that seen in hepatocyte-free homogenates. The presence or absence of Ca^{2+} in the buffer does not appear to affect the subcellular location of the enzyme.

An explanation is required for why the majority of the PLA_2 activity was found in the cell-debris fraction during cell fractionation and two possibilities should be considered. First, the collagenase-perfusion procedure is optimized for hepatocyte preparation and may not be so effective in releasing intact Kupffer cells in high yield. Secondly, in view of the potential for the PLA_2 to be released from the liver it is possible that considerable release of the enzyme from whole cells has occurred during the overall trauma of the collagenase-perfusion procedure.

The precise intracellular location of this enzyme in Kupffer cells *in vivo* remains to be established. In view of the anticipated role of this enzyme in phagocytic degradation by these macrophages, a granular location might be expected. Preliminary studies involving an initial centrifugation of the post-mitochondrial supernatant at 25000 *g* for 20 min yielded a pellet of high specific activity of PLA_2 consistent with a granular location.

We have observed previously that DOPG is a preferred substrate for liver PLA_2, and we have observed a similar preference with the recombinant human-platelet-derived enzyme. Using the fluorescence assay, the expressed activity was in the order DOPG > dioleoylphosphatidylethanolamine > dioleoylphosphatidylcholine [19]. This preference for the negatively charged DOPG may have physiological significance that reflects the probable degradative role of the enzyme. This macrophage-derived PLA_2 would preferentially degrade negatively charged phospholipids that are exposed in broken or permeabilized cells of host or bacterial origin. A similar phosphatidylglycerol preference has been observed for PLA_2s from pig intestine [31] and rat spleen [32]. The spleen enzyme is also associated with phagocytic cells [21,22].

The level of serum PLA_2 is dramatically increased *in vivo* by treatment with endotoxin [5]. Moreover Kupffer cells are a major site of removal of this LPS in rat [7], possibly as a result of uptake via the macrophage scavenger system [33]. Therefore, the effect of LPS treatment on the PLA_2 activity in the liver and isolated cell fractions was investigated. A dramatic 6-fold enhancement of liver PLA_2 activity was observed after 4 h. The characteristics of this enhanced enzyme activity were identical to normal liver mitochondrial-associated PLA_2 activity. It was not possible to obtain discrete cell fractions after collagenase perfusion of livers from LPS-treated animals, possibly due to a significant disruption of the cellular integrity of the liver by LPS.

A major question in inflammation research is the origin of the elevated serum PLA_2 levels observed after infection or trauma, particularly septic shock. An obvious candidate is the releasable platelet PLA_2. However, according to the calculations of Aarsman et al. [11] the total mitochondrial-associated PLA_2 activity is similar to that of total platelet activity. The ability of LPS to enhance the liver PLA_2 activity many fold focuses attention on the Kupffer cell enzyme as a major source of serum

PLA_2 in the traumatized rat. The greatly enhanced loss of PLA_2 in the perfusates of livers from animals previously injected with LPS supports this proposal and is quantitatively high enough to produce elevated serum levels of the enzyme. There are now many precedents for the stimulation and release of PLA_2 from cells by various mediators, including cytokines and platelet-activating factor. A role for LPS has been directly implicated in PLA_2 expression in murine macrophage-like P338D1 cells [34]. In addition, it has recently been reported that HepG2 cells respond to various cytokines, and in particular interleukin-6, by expressing and releasing PLA_2 in parallel to the release of acute-phase proteins [26]. The fact that HepG2 cells are derived from human hepatocytes rather than liver macrophages will require clarification.

It should be noted that whereas Kupffer cells represent a major site of endotoxin removal in rats, in some species other macrophage-containing organs such as lung play a more dominant role [7]. The macrophages resident in other tissues must also be considered as additional/alternative sources of serum PLA_2 during trauma, depending on the nature of the trauma and the animal species.

If, as appears to be the case, this release of PLA_2 by the liver is a physiological or pathophysiological phenomenon then the precise function or effect of the serum PLA_2 is obscure. Certainly injected PLA_2 is able to release arachidonic acid in tissues as the first step in eicosanoid synthesis. Moreover, addition of this PLA_2 to cells in culture produces increased synthesis of prostanoids; however, this is only observed in activated or damaged cells [35,36]. Therefore, it is possible that enhanced levels of this Group-II PLA_2 in serum as a result of trauma may provoke an excessive inflammatory response by hydrolysing the membrane phospholipid of suitably modified or damaged tissues to release archidonic acid or other biologically active lipids. However, it is now accepted that the enzyme which normally mediates eicosanoid synthesis is the high-molecular-mass arachidonic-acid-specific cytosolic PLA_2 [37,38].

Alternatively, the elevated serum PLA_2 may play a role in the degradation of invading bacteria or damaged cells in the blood. Functionally this PLA_2 shows a similarity to the pancreatic enzyme in terms of its preference for DOPG [9]. Moreover, the pancreatic enzyme will bind essentially irreversibly to negatively charged phospholipids [39] but has low affinity for zwitterionic phospholipids such as phosphatidylcholine that predominate on the external surface of cell membranes. If the liver enzyme had similar binding characteristics, it would bind preferentially to damaged cells in the blood and not bring about the destruction of normal cells. This destruction may be the result of the uptake of this PLA_2 into phagocytic cells in a 'piggy-back' fashion while still attached to damaged cell membranes and thus facilitates degradation within the macrophage [40].

Further work will be required to clarify the mechanism of PLA_2 stimulation by LPS challenge. However, there are a number of precedents based on studies with cultured cells which show that enhanced PLA_2 activity upon stimulation by LPS and cytokines is associated with increased levels of mRNA for this Group-II enzyme [24–27]. Since hepatocytes are the dominant type of cells in liver, Kupffer cells in culture rather than experiments involving whole liver should provide the best system for this future work. The potential difficulty with studies involving whole liver could explain the report that LPS injection did not apparently affect mRNA levels for this Group-II enzyme in rat liver [41].

In conclusion, we have demonstrated using a rapid fluorescence assay that liver mitochondrial PLA_2 is located in Kupffer cells, the resident macrophages of the liver, and that this enzyme activity is dramatically enhanced *in vivo* by endotoxin. The release of this enzyme in perfusion studies was also demonstrated and suggests that the liver may be a major source of serum PLA_2 after trauma and infection. Thus, this macrophage-derived enzyme may play a pivotal role in a variety of clinical disorders including septic shock, adult respiratory distress system, psoriasis, renal failure, gout and arthritis where serum levels of PLA_2 are elevated [5,42]. The mechanisms and mediators of stimulation and release of enzyme activity from Kupffer cells remain to be established. The isolated perfused liver system together with cultured Kupffer cells should provide a useful model for this purpose. The possible role of the other small cells from the liver, lipocytes and endothelial cells, in PLA_2 stimulation and release must also be investigated.

This work was supported by a grant from the Medical Research Council of Canada. G. M. H. was supported by a Fellowship from the Alberta Heritage Foundation for Medical Research. D.E.V. is a Medical Scientist of the Alberta Heritage Foundation for Medical Research. D.C.W. was the recipient of Visiting Scientist Awards from the Alberta Heritage Foundation for Medical Research and the Medical Research Council of Canada. Support to D.C.W. from the Wellcome Trust is also acknowledged. We are very grateful to Dr. Henk van den Bosch for providing monoclonal antibody to PLA_2 from rat liver.

REFERENCES

1 Dennis, E. A., Rhee, S. G., Billah, M. M. and Hannun, Y. A. (1991) FASEB J **5**, 2068–2076
2 Samborski, R., Ridgway, N. D. and Vance, D. E. (1991) J. Biol. Chem. **265**, 18322–18329
3 van Kuijk, F. J. G. M., Sevanian, A., Handelman, G. J. and Dratz, E. A. (1987) Trends Biochem. Sci. **12**, 31–34
4 Wright, G. C., Ooi, C. E., Weiss, J. and Elsbach, P. (1990) J. Biol. Chem. **265**, 6675–6681
5 Vadas, P. and Pruzanski, W. (1989) in Phospholipase A_2: Role and Function in Inflammation (Wong, P. Y.-K. and Dennis, E. A., eds.), pp. 83–101, Plenum Press, NY
6 Vadas, P., Pruzanski, W. and Stefanski, E. (1988) Agents Actions **24**, 320–325
7 Handley, D. A. (1990) in Platelet-Activating Factor in Endotoxin and Immune Diseases (Hanley, D. A., Saunders, R. N., Houlihan, W. J. and Tomesch, J. C., eds.), pp. 451–495, Marcell Dekker, NY
8 Wilton, D. C. (1990) Biochem. J. **266**, 435–439
9 Wilton, D. C. (1991) Biochem. J. **276**, 129–133
10 Kinkaid, A. and Wilton, D. C. (1991) Biochem. J. **278**, 843–848
11 Aarsman, A. J., de Jong, J. G. N., Arnoldussen, E., Neys, F. W., Van Wassenaar, P. D. and Van den Bosch, H. (1989) J. Biol. Chem. **264**, 10008–10014
12 Vadas, P. and Pruzanski, W. (1986) Lab. Invest. **55**, 391–404
13 Davis, R. E., Engelhorn, S. C., Pangburn, S. H., Weinstein, D. B. and Steinberg, D. (1979) J. Biol. Chem. **254**, 2010–2016
14 Arthur, M. J. P., Friedman, S. L., Roll, F. J. and Bissell, D. M. (1989) J. Clin. Invest. **84**, 1076–1085
15 Saggerson, E. D. and Geenbaum, A. L. (1969) Biochem. J. **115**, 405–417
16 Laemmli, U.K. (1970) Nature (London) **270**, 680–685
17 Worrall, A. F., Evans, C. and Wilton, D. C. (1991) Biochem. J. **278**, 365–368
18 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
19 Kinkaid, A. R. and Wilton, D. C. (1992) Biochem. Soc. Trans. **20**, 287S
20 Belcher, J. D., Sisson, P. J. and Waite, M. (1985) Biochem. J. **229**, 343–351
21 Inada, M., Tojo, H., Kawata, S., Tarui, S. and Okamoto, M. (1991) Eur. J. Biochem. **197**, 323–329
22 Inada, M., Tojo, H., Kawata, S., Tarui, S. and Okamoto, M. (1991) Biochem. Biophys. Res. Commun. **174**, 1077–1083
23 Horigome, K., Hayakawa, M., Inoue, K. and Nojima, S. (1987) J. Biochem. (Tokyo) **101**, 53–61
24 Nakano, T., Ohara, O., Teraoka, H. and Arita, H. (1990) J. Biol. Chem. **265**, 12745–12748
25 Oka, S. and Arita, H. (1991) J. Biol. Chem. **266**, 9956–9960
26 Crowl, R. M., Stoller, T. J., Conroy, R. R. and Stoner, C. R. (1991) J. Biol. Chem. **266**, 2647–2651
27 Schalkwijk, D., Pfeilschifter, J., Märki, F. and van den Bosch, H. (1992) J. Biol. Chem. **267**, 8846–8851
28 Ishizaki, J., Ohara, O., Nakamura, E., Tamaki, M., Ono, T., Kanda, A., Yoshida, N., Teraoka, H., Tojo, H. and Okamoto, M. (1989) Biochem. Biophys. Res. Commun. **162**, 1030–1036

29 Komada, M., Kudo, I., Mizushima, H., Kitamura, N. and Inoue, K. (1989) J. Biochem. (Tokyo) **106**, 545–547
30 Babior, B. M. (1987) Trends Biochem. Sci. **12**, 241–243
31 Verger, R., Ferrato, F., Mansbach, C. M. and Pieroni, G. (1982) Biochemistry **21**, 6883–6889
32 Ono, T., Tojo, H., Kuramitsu, S., Kagamiyama, H. and Okamoto, M. (1988) J. Biol. Chem. **263**, 5732–5738
33 Hampton, R. Y., Golenbock, D. T., Penman, M., Krieger, M. and Raetz, C. R. H. (1991) Nature (London) **352**, 342–344
34 Glaser, K. B., Asmis, R. and Dennis, E. A. (1990) J. Biol. Chem. **265**, 8658–8664
35 Hara, S., Kudo, I. and Inoue, K. (1991) J. Biochem. (Tokyo) **110**, 163–165
36 Murakami, M., Kudo, I. and Inoue, K. (1991) FEBS Lett. **294**, 247–251
37 Clark, J. D., Lin, L.-L., Kriz, R. W., Ramesha, C. L., Sultzman, L. A., Lin, A. Y., Milona, N. and Knopf, J. L. (1991) Cell **65**, 1043–1051
38 Sharp, J. D., White, D. L., Chiou, X. G., Goodson, G., Gamboa, G. C., McClure, D., Burgett, S., Hoskins, J., Skatrud, P. L., Sportsman, J. L., Becker, G. W., Kang, L. H., Roberts, E. F. and Kramer, R. M. (1991) J. Biol. Chem. **266**, 14850–14853
39 Ramirez, F. and Jain, M. K. (1991) Proteins: Struct. Funct. Genet. **9**, 229–239
40 Wright, G. C., Weiss, J., Kim, K. S., Verheij, H. and Elsbach, P. (1990) J. Clin. Invest. 85, 1925–1935
41 Nakano, T. and Arita, H. (1990) FEBS Lett. **273**, 23–26
42 Pruzanski, W. and Vadas, P. (1990) in Biochemistry, Molecular Biology, and Physiology of PLA_2 and its Regulatory Factors (Mukherjee, A. B., ed.), pp. 239–251, Plenum Press, NY

Received 12 October 1992/19 January 1993; accepted 25 January 1993