Liver Cirrhosis Induces Renal and Liver Phospholipase A_2 Activity in Rats

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Abstract

Maintenance of renal function in liver cirrhosis requires increased synthesis of arachidonic acid derived prostaglandin metabolites. Arachidonate metabolites have been reported to be involved in modulation of liver damage. The purpose of the present study was to establish whether the first enzyme of the prostaglandin cascade synthesis, the phospholipase A_2(PLA_2) is altered in liver cirrhosis induced by bile duct excision. the mRNA of PLA_2(group I and II) and annexin-I a presumptive inhibitor of PLA_2 enzyme was measured by PCR using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal standard. The mean mRNA ratio of group II PLA_2/GAPDH was increased in liver tissue by 126% ($P < 0.001$) and in kidney tissue by 263% ($P < 0.006$) following induction of liver cirrhosis. The increase in group II PLA_2 mRNA in cirrhotic animals was reflected by an increase in PLA_2 protein and enzyme activity in both liver and kidney tissues. Since the mRNA of group I PLA_2 was not detectable and Group IV PLA_2 activity measured in liver and kidney tissue samples was very low and not changed following induction of cirrhosis, it is likely that the major PLA_2 activity measured in liver and kidney corresponds to group II PLA_2 enzyme. The mean mRNA ratio of annexin-I/GAPDH was increased in liver tissue by 115% ($P < 0.05$) but unchanged in kidney tissue following induction of cirrhosis. The protein content of annexin-I and -V were not affected by bile duct excision in liver and kidney tissue indicating that upregulation of group II PLA_2 activity was not due to downregulation of annexin-I or -V. Group II PLA_2 activity of glomerular mesangial cells stimulated by interleukin-1β was enhanced by bile juice and various bile salts. In conclusion, activity of group II PLA_2 is upregulated partly due to enhanced transcription and translation in cirrhosis and is furthermore augmented by elevated levels of bile salts. (*J. Clin. Invest.* 1996. 98:365–371.) Key words: phospholipase A_2 • annexins • cirrhosis • mesangial cells • bile salts

Introduction

Cirrhotic patients have a cardiovascular disturbance characterized by low arterial pressure, high cardiac output, and increased plasma volume (1–4). Besides arteriolar vasodilation and arteriovenous shunting, these patients exhibit a diminished sensitivity to pressor hormones and impaired hypoxic vasoconstriction (5–7). Eventually liver failure causes extrahepatic organ dysfuntion such as hepatorenal or hepatopulmonary syndrome (8–10). These changes resemble those seen in patients or animals with septicemia (11–14).

Changes in arachidonic acid derived prostaglandins have been linked to hemodynamic alterations in cirrhotic disease states (11, 15–19). Renal perfusion and glomerular filtration are only maintained through the vasodilatory effect of prostaglandins. Inhibition of the cyclooxygenase enzyme results in a reduction of the effective renal plasma flow and sodium retention (20, 21) and administration of exogenous prostaglandin E derivatives enhance renal function of patients with cirrhosis (22). One of the rate limiting steps in the synthesis of prostaglandins is the release of arachidonic acid from membrane phospholipids by the enzyme phospholipase A_2 (PLA_2).[1] In contrast to the abundant literature about prostaglandins in liver cirrhosis, the relevance of PLA_2 has never been addressed. Therefore, in the present investigation we report the effect of biliary cirrhosis induced by bile duct ligation in rats on mRNA, protein levels and enzyme activity of group II PLA_2. The results revealed an upregulation of group II PLA_2 in cirrhosis.

Materials and Methods

Animals and bile duct excision. Sprague-Dawley male rats (Deutsche Versuchstierfarm Hartmutt-Voss, Tuttlingen, Germany) were maintained on standard laboratory chow (Kliba-Futter, Basel, Switzerland) and tap water with a 12-h light–dark cycle at constant temperature and humidity. At the time of surgery, rats weighed 300 to 400 g. Animals were allotted to bile duct excision ($n = 7$) or sham surgery ($n = 5$). The experimental protocol had been approved by the Animal Ethics Committee of the state of Berne.

We used bile duct excision to produce cirrhosis (23). A 2-cm upper abdominal midline incision was made under ether anesthesia. In the experimental group, the bile duct was isolated and a 1-cm section excised after double ligature. In the sham group, the bowel and mesentery were manipulated and replaced. 8 wk after surgery the animals were anesthetized using pentobarbital sodium (50 mg/kg i.p.). In situ perfusion with ice-cold saline was performed. Liver and kidneys were removed and immediately frozen in liquid nitrogen, and stored at −80°C until further use.

Bile juice collection. Sprague-Dawley rats were anesthetized with pentobarbital sodium (50 mg/kg i.p.). The body temperature, mea-

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1. *Abbreviations used in this paper:* CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GLCA, glycolithocholic acid; IL-1β, interleukin-1β; LCA, lithocholic acid; PLA_2, phospholipase A_2, RAS, Ribi Adjuvant system; RT, reverse transcription; TBS, Tris-buffered saline; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TS, Tris-sucrose buffer, TUDCA, tauroursodeoxycholic acid.

sured by a rectal thermometer, was maintained at 37–37.5°C with a heating lamp. Bile juice was collected by cannulating the common bile duct with PE-50 tubing (0.51 × 0.94 mm).

Extraction of total RNA. Kidney and liver tissues frozen at −80°C were powdered using pestle and mortar kept in a mixture of dry ice and acetone. Total RNA was extracted from a known amount of tissue according to the method of Chomczynski and Sacchi (24). The RNA concentration was determined by absorbance at 260 nm. The quality of RNA was controlled by running an aliquot on a 1% agarose formaldehyde gel.

Reverse transcription of mRNA. The reverse transcripton (RT) reaction mixture of 20 μl contained 50 mM Tris-HCl (pH 8.2), 6 mM $MgCl_2$, 10 mM DTT, 100 mM NaCl, 200 μM dNTPs, 11 U RNAse inhibitor, 10 pmol primer (3′ primer of the corresponding cDNA: positions 695–719 for group II PLA_2 cDNA [25], 335–359 for group I PLA_2 [26], 978–1007 for annexin-I [27], and 980–1004 for GAPDH [28] from Microsynth GmbH, Balgach, Switzerland), 2–5 μg of total RNA and 1 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals, Rotkreuz, Switzerland). For competitive RT-PCR 18 fg of modified group II PLA_2 transcript was added to the reverse transcription reaction (29). Initially, the 3' primer and total RNA with and without modified group II PLA_2 transcript, were incubated together for 5 min at 65°C, and then cooled at room temperature for 15 min. The remaining reaction components, including avian myeloblastosis virus reverse transcriptase, were then added and incubated at 42°C for 60 min.

PCR. PCR was performed in a total volume of 30 μl with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 10 pmol of the appropriate 3′ and 5′ cDNA primers (5′ primers of corresponding cDNA: positions 58–82 for group II PLA_2, 38–62 for group I PLA_2, 1–29 for annexin-I, and 66–90 for GAPDH), 6.0 μg BSA, 1 μCi [α-^{32}P]dCTP, 2–10 μl reverse transcribed cDNA, 1 U *Thermus aquaticus* DNA polymerase (Perkin Elmer Cetus Corp., Norwalk, CT). Before adding the enzyme and cDNA the samples were irradiated under ultraviolet light for 20 min to eliminate any contaminating DNA (30). The mixture was overlaid with mineral oil, and cDNA was amplified with a DNA thermal cycler (Perkin Elmer Cetus Instruments) for 35 cycles. The amplification profile involved denaturation at 94°C for 1 min and 15 s, primer annealing at 72°C for 2 min (group II PLA_2) or 62°C (group I PLA_2, annexin-I and GAPDH) and elongation of annealed primers at 72°C for 2 min (group II PLA_2) or for 3 min (group I PLA_2, annexin-I and GAPDH). 10 μl of each PCR reaction mixture were mixed with 2 μl of sixfold concentrated loading buffer and applied on either 1.5 or 0.9% agarose gels containing ethidium bromide. Electrophoresis was carried out in Tris-borate-EDTA buffer (pH 8.0) with a constant voltage of 8 V/cm for 40 min. Bands were visualized under ultraviolet light and excised from the gel. The radioactivity of the bands was measured in a scintillation counter using a Cerenkov program. Using the above mentioned primers the sizes of the fragment for different mRNA were 662 bp for group II PLA_2, 254 bp for modified group II PLA_2 transcript, 222 bp for group I PLA_2, 1,007 bp for annexin-I, and 939 for GAPDH.

Protein extraction. Small amounts of powdered tissue were extracted with acid according to the method of Märki and Franson (31). The prepared material designated as tissue acid extract was used to determine PLA_2 (group II) protein by Western blot analysis and enzyme activity. To determine specifically group IV PLA_2 protein by Western blot and its enzymatic activity, powdered tissue was extracted with Tris-sucrose (TS) buffer (10.0 mM Tris-HCl, pH 7.0; 25.0 mM sucrose). For the determination of annexin-I and -V by Western blot analysis powdered tissue was extracted with 10 mM Tris-HCl buffer, pH 9.0, containing 1% Triton X-100, 2 mM phenylmethylsulfonylfluoride and 5 mM EDTA using a Dounce homogenizer, stored on ice for 60 min. The extracts were centrifuged at 13,000 rpm at 4°C for 30 min to sediment cell debris. The supernatants from TS buffer and from Triton buffer were designated as TS extract and Triton extract, respectively.

Determination of protein concentrations. Protein concentration in tissue acid extract, TS extract and in Triton extract was determined using the bicinchonic acid protein assay reagent (Pierce Chemical Co., Rockford, IL).

Monoclonal antibodies. Rat lung tissues were extracted with acid according to the method of Märki and Franson (31). PLA_2 enzyme from lung acid extract was partially purified on a Sephadex G-50 column (dimensions 0.75 × 60 cm; elution buffer 50 mM sodium acetate buffer, pH 4.5, containing 1.5 M NaCl). The major PLA_2 fraction eluted in the 14-kD protein region were pooled and used to raise monoclonal antibody in mice.

Partially purified rat lung PLA_2 in a concentration of 1 mg/ml of saline was mixed with an equal volume of Ribi adjuvant system (RAS; RIBI Immunochem Research Inc., Hamilton, MT) and injected into the peritoneal cavity of 6-wk-old mice. Mice were boosted twice with 100 μg of PLA_2 in RAS and sacrificed 3 d after the last booster injection. The spleen was excised and somatic–cell fusion was performed by the method initially described by Koehler and Milstein (32). Suspensions of fused cells in selective medium containing hypoxanthine, aminopterine, and thymidine were divided into 10 tissue culture plates (Falcon 3072; 96 wells/plate from Becton Dickinson Labware, Lincoln Park, NJ) at the rate of 200 μl/well. Samples of medium from each well were screened for anti- PLA_2 antibody activity using the dot blot procedure and Western blots. Cells from positive wells were subcloned by limiting dilution in 96-well tissue-culture plates. The selected clone (7G7) was expanded in vitro using Iscove's modified Dulbecco's medium enriched with 10% fetal calf serum. The monoclonal antibody was isolated from the hybridoma supernatants by protein G–Sepharose (Pharmacia Biotech AG, Duebendorf, Switzerland) affinity purification using buffers recommended by the manufacturer.

Monoclonal antibody (6G6) was prepared similarly using recombinant human annexin-I (33). Monoclonal antibodies against annexin-V and group IV PLA_2 were obtained from Alexis Corporation, (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Western blot analysis. For Western blots indicated amounts of proteins from tissue acid extract (to detect group II PLA_2), Triton extract (to detect annexin-I and -V) and 50.0 μg of TS extract (to detect group IV PLA_2) were reduced (100 mM DTT) and denatured (100°C) before being resolved by 12.5 or 8.0% SDS–polyacrylamide gel electrophoresis (34). The proteins were transferred to PVDF Immobilon membrane (Millipore AG, Volketswil, Switzerland) by electroblot using Tris-glycine buffer containing 20% methanol. The transfer was performed at a constant voltage of 65 V for 60 min. Protein transfer was monitored by staining the Immobilon membrane with Ponceau S (Sigma Chemie, Buchs, Switzerland) and the gel with Coomassie blue. Purified recombinant annexin-I (0.94-7.5 ng) was used in each set of experiments as a standard to measure the annexin-I concentration in the protein samples. The blots were agitated with Tris-buffered saline (TBS), pH 7.5, containing 5% BSA for 60 min at room temperature, washed three times with TBS, and incubated with primary antibodies as follows.

To detect group II PLA_2 protein membranes were incubated for 120 min at 37°C with the monoclonal antibody 7G7 in TBS containing 1.0% Tween-20 and 0.05% BSA. To detect group IV PLA_2, annexin-I and -V membranes were incubated for 120 min at 37°C with their corresponding monoclonal antibodies in TBS containing 0.5% BSA. Membranes were washed three times with TBS and blocked again with TBS containing 5% BSA for 60 min at room temperature. Membranes were washed three times with TBS and incubated with peroxidase conjugated goat anti–mouse IgG (H + L) (Bio-Rad, Glattbrugg, Switzerland) for 60 min at room temperature in TBS containing 1% Tween-20 and 0.05% BSA for group II PLA_2 or in TBS containing 0.5% BSA for group IV PLA_2 and annexins. Membranes were washed three times with TBS and the peroxidase activity was determined using the enhanced chemiluminescence Western blotting detection system (Amersham International, Buckinghamshire, UK) according to the manufacturerís instructions. The bands on the X-ray films were scanned with a transmittance scanning densitometer (Scananalytics CSPI, MA).

Cell culture. Mesangial cells were cultured from isolated rat (Sprague-Dawley) glomeruli (35). Briefly, the cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) transferrin (5 μg/ml), insulin (5 μg/ml), and sodium selenite (5 ng/ml). For the experiments passages 27–30 of mesangial cells were used. Confluent mesangial cells cultured in 15-mm-diam wells (24 wells tissue culture plate; Falcon 3047) were incubated with 500 μl of RPMI 1640 medium containing 10% FCS and 1 nM of interleukin-1β (IL-1β; Pharma Biotechnologie, Hannover, Germany) with and without bile juice (5–50 μl/ml) or bile salts (100 μM) for 24 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The following bile salts were tested: sodium salts of cholic acid (CA); chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycolithocholic acid (GLCA), lithocholic acid (LCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), and tauroursodeoxycholic acid (TUDCA) (Calbiochem AG, Lucerne, Switzerland). After 24 h incubation the medium was removed and centrifuged for 5 min at 4,000 rpm. The supernatant was removed and used for PLA_2 activity measurements.

Assay of PLA_2 activity. [^{3}H]oleic acid (specific activity 10 Ci/mmol; Amersham International, Buckinghamshire, UK) labeled *Escherichia coli* and [^{3}H]arachidonic acid (specific activity 205 Ci/mmol; Amersham International) labeled *E. coli* were prepared according to the procedure of Patriarca et al. (36). Group II PLA_2 was assayed using [^{3}H]oleate–labeled, autoclaved *E. coli* as the substrate (37). The reaction mixture of 350 μl contained 100 mM Tris-HCl, pH 8.0, 5 mM Ca^{2+}, 2.85×10^8 cells of autoclaved *E. coli* (corresponding to 10,000 cpm and 5.5 nmol lipid phosphorus), and tissue acid extracts or supernatants of mesangial cells were incubated at 37°C for 120 min. Activity of group IV PLA_2 was assayed using [^{3}H]arachidonate-labeled, autoclaved E. coli as the substrate for TS-extracted samples (38). The reaction mixture of 350 μl contained 100 mM Tris-HCl, pH 7.0, 1 mM Ca^{2+}, 5.0 mM DTT, and TS-extract. This mixture was incubated for 60 min at 37°C. The reaction was started by adding autoclaved *E. coli* (2.1×10^8 cells corresponding to 8,000 cpm and 4.0 nmol lipid phosphorus) and the incubation was continued for another 120 min at 37°C. The amount of protein was chosen such that hydrolysis of the substrate was 6–15%. The reaction was terminated by adding 100 μl of 2 N hydrochloric acid. Fatty acid–free BSA, (100 μl, 100 mg/ml) was added, and the tubes were vortexed and centrifuged at 13,000 *g* for 5 min. An aliquot (140 μl) of the supernatant containing released [^{3}H]oleic acid or [^{3}H]arachidonic acid was mixed with scintillation cocktail (Dynagel, J.T. Baker B.V., Deventer, Holland) and counted in a liquid scintillation counter (model SL 4000; Intertechnique, Nucleotron, Lausanne, Switzerland).

Statistics. All results in the text and in the figures are mean (+SD). Differences between two groups were analyzed by Student's *t*-test.

Results

All animals with bile duct ligation had hepatomegaly; the liver was nodular in appearance and all had portal hypertension. The animal characteristics are given in Table I.

The mean amount of total RNA isolated from liver and kidney (μg/g tissue) was significantly lower in cirrhotic liver (−27%) and kidney (−35%) tissue samples compared to their corresponding sham-operated controls. The mRNA of GAPDH and its activity have been shown to be unchanged during cirrhosis (39). Therefore the mRNA of GAPDH was used as an internal standard. In control and cirrhotic samples the mRNA of group I PLA_2, group II PLA_2, annexin-I and GAPDH were measured by RT-PCR. The mRNA of group II PLA_2 was additionally quantified by RT-PCR using a constant amount of a modified group II PLA_2 cDNA transcript. Both specific target mRNA and the modified transcript were coamplified in one reaction using the same primers (29). Visual inspection of the bands on the agarose gels revealed qualitatively that mRNA of group II PLA_2 was increased in cirrhotic liver and kidney tissue when compared with their corresponding controls (data not shown). The inclusion and coamplification of the modified group II PLA_2 transcript using the same primers (29) excluded the possibility that increased concentrations of group II PLA_2 in cirrhotic rats were caused by an artifact during the PCR amplification procedure (40). An increased signal for annexin-I mRNA was observed in cirrhotic liver but not in kidney tissue (data not shown). In accordance with the previous report (39) no change in the mRNA of GAPDH was observed in cirrhotic liver and kidney tissue when compared with the corresponding controls in the present investigation. No signal for mRNA of group I PLA_2 was found in liver or in kidney tissue.

Table I. Characteristics of the Animals at the Time of the Experiments

	Controls ($n = 5$)	Bile duct ligation ($n = 7$)
Body weight (g)	500±20	404±38*
Liver weight (g)	18.8±1.4	32.4±2.5*
Kidney weight (g)	3.3±0.3	3.3±0.3*
Portal pressure (cm H_2O)	12.4±0.7	20.2±2.1*
Bilirubin (μmoles/l)	2±0	130±26*
Serum bile acids (μmoles/l)	3±2	91±51*
Alkaline phosphatase (IU/l)	155±41	605±288*
Alanine aminotransferase (IU/l)	47±4	100±37*

Mean±1 standard deviation are given. *Denotes a statistically significant difference ($P < 0.05$).

To assess quantitatively the change in mRNA, [α-^{32}P]dCTP was included during PCR and the incorporation of radioactivity into group II PLA_2, annexin-I and GAPDH was measured. The ratios between group II PLA_2 and GAPDH and the ratios between annexin-I and GAPDH were calculated. In sham-operated animals the liver contained more group II PLA_2 mRNA (difference between the mean values 213%, $P < 0.006$) than the kidney (Fig. 1). Cirrhosis induced transcription of group II PLA_2 mRNA in liver by 126% ($P < 0.001$) and in kidney tissue by 263% ($P < 0.006$; Fig. 1). Similar results were obtained when the ratio was calculated between group II PLA_2 and the modified transcript (128% increase in liver $P < 0.05$ and 381% increase in kidney $P < 0.02$). Compared to group II PLA_2 mRNA, cirrhosis exhibited a different effect on the mRNA of annexin-I. After the induction of cirrhosis annexin-I mRNA was increased in liver by 115% (annexin-I mRNA/GAPDH mRNA: control 0.86±0.37 vs cirrhotic 1.84±0.87, $P < 0.05$) tissue but unchanged in kidney tissue (annexin-I mRNA/GAPDH mRNA: control 1.98±0.5 and cirrhotic 1.83±0.72).

To establish whether the changes in group II PLA_2 and annexin-I mRNA due to cirrhosis were associated with corresponding changes of group II PLA_2 and annexin-I proteins, these proteins were analyzed together with annexin-V proteins in liver and kidney samples by Western blot using specific monoclonal antibodies (Fig. 2). In cirrhotic liver and kidney tissue increased amounts of group II PLA_2 but not annexin-I or -V proteins were found when compared with the corresponding control tissues (Fig. 2). In cirrhotic tissue samples

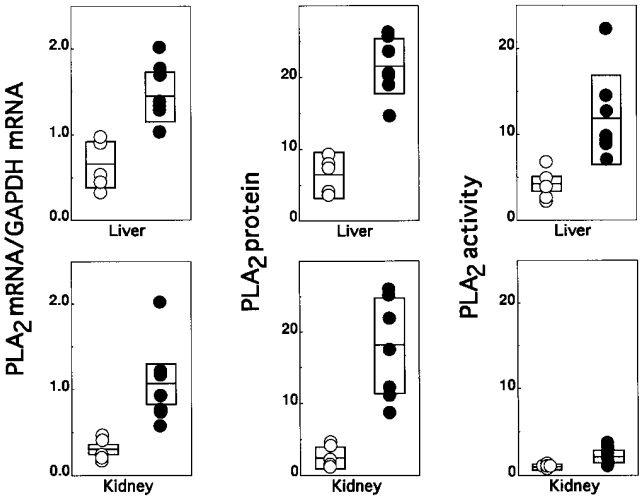

Figure 1. Measurements of group II PLA_2 mRNA, protein and enzyme activity in kidney and liver tissue samples. Messenger RNA was quantified from the ratio of group II PLA_2 cpm / GAPDH cpm. Increased PLA_2 mRNA was observed in cirrhotic liver ($P < 0.001$) and kidney tissues ($P < 0.006$). Group II PLA_2 protein was analyzed by Western blot analysis and the signals were determined by a transmission scanning densitometer. The relative transmission is plotted. PLA_2 protein was higher in liver ($P < 0.001$) and kidney tissues ($P < 0.001$) from cirrhotic than control animals. PLA_2 activity measured in liver and kidney acid extracts is expressed as nmol fatty acid/mg protein/min. The PLA_2 activity was increased following induction of cirrhosis in both liver ($P < 0.002$) and kidney tissue ($P < 0.02$). Open circles represent control ($n = 5$) and closed circles represent cirrhotic ($n = 7$) samples. The values are mean±SD.

group II PLA_2 protein was increased in liver by 230% ($P < 0.001$) and in kidney tissue by 690% ($P < 0.001$, Fig. 1). In these tissue samples group IV PLA_2 protein was also measured using a specific monoclonal antibody. Kidney tissue exhibited a threefold higher amount of group IV PLA_2 protein than liver tissue ($P < 0.04$), however, cirrhosis did not influence the mean amount of group IV PLA_2 in these tissues (data not shown).

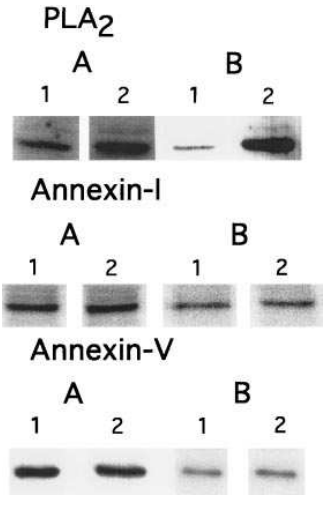

Figure 2. Western blot analysis of group II PLA_2 protein, annexin-I and -V from liver (*A*) and kidney (*B*) tissues from control (lane *1*) and cirrhotic (lane *2*) animals. Group II PLA_2 (0.5 μg of tissue acid extract) was analyzed using 7G7 monoclonal antibody. Annexin-I (25 μg of Triton-extract) was analyzed using 6G6 monoclonal antibody. Annexin-V (20 μg of Triton extract for liver and 8 μg for kidney) was analyzed using anti–annexin-V monoclonal antibody. In cirrhotic animals an increased amount of group II PLA_2 protein was found in both kidney and liver tissues (see Fig. 1) but not of annexin-I (control vs cirrhotic: 109.0±8.0 vs 118.0±22.0 in liver and 78.0±4.4 vs 79.0±4.6 ng/mg protein in kidney tissue) or annexin-V protein (control vs cirrhotic: 27.7±2.6 vs 27.2±5.5 in liver and 8.96±2.6 vs 11.0±3.5 in kidney; for annexin-V protein the values are given as relative transmission).

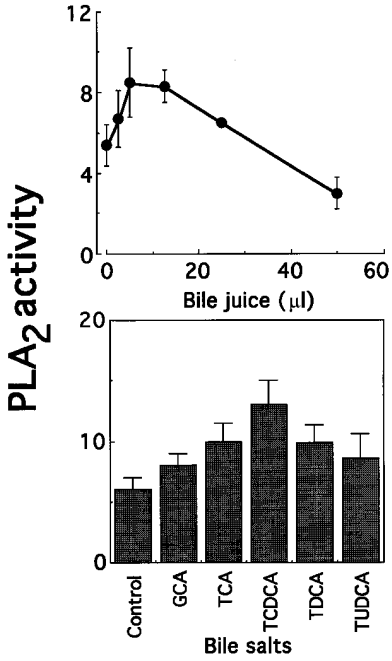

Figure 3. Effect of bile juice and bile salts on IL-1β stimulated PLA_2 activity in the supernatants of mesangial cells. Mesangial cells were incubated with 1 nM of IL-1β in the presence or absence of indicated amounts of bile juice per 500 μl or of 100 μM of different bile salts for 24 h. Cell supernatants were adjusted to get 6–15% hydrolysis as described in the method section. Each value represents the mean of three determinations. PLA_2 activity is expressed as nmole fatty acid/ml of cell supernatant/min. *GCA,* glycocholic acid; *TCA*, taurocholic acid; *TCDCA*, taurochenodeoxycholic acid; *TDCA*, taurodeoxycholic acid; *TUDCA*, tauroursodeoxycholic acid.

For the assessment of group II PLA_2 activity in liver and kidney tissue the PLA_2 enzyme from these tissues was solubilized by acid extraction. The extracted PLA_2 enzyme showed an optimum pH of 7.5–9.0 and required 5 mM Ca^{2+} for optimal activity (data not shown). The results from PLA_2 activity measurements are given in Fig. 1. As previously shown, liver exhibited a higher PLA_2 activity (487%, $P < 0.001$) than kidney tissue in control animals (29). PLA_2 activity was increased by 138% in liver ($P < 0.002$) and by 135% in kidney tissue ($P < 0.02$) when samples from cirrhotic animals were compared with those from control animals (Fig. 1). In the TS extract of these tissues group IV PLA_2 activity was measured using [^{3}H]arachidonate–labeled *E. coli* in the presence of DTT (38). Control liver and kidney exhibited a 47- and 20-fold lower group IV PLA_2 activity when compared with group II PLA_2 activity (0.0133±0.006 vs 0.63±0.27 nmol fatty acid/mg protein/min in liver and 0.0148±0.007 vs 0.29±0.07 nmol fatty acid/mg protein/min in kidney tissues). Cirrhosis did not influence the group IV PLA_2 activity in these tissue samples (control vs cirrhosis: 0.0133±0.006 vs 0.0162±0.009 nmol fatty acid/mg protein/min in liver and 0.0148±0.007 vs 0.0146±0.005 nmol fatty acid/mg protein/min in kidney tissue).

When the ratio of group II PLA_2 mRNA/GAPDH mRNA was plotted versus the corresponding group II PLA_2 protein or activity for each animal separately the following correlation coefficients were found: mRNA vs protein 0.66 ($P < 0.02$) for liver and 0.65 ($P < 0.05$) for kidney; mRNA vs activity 0.59 ($P < 0.05$) for liver and 0.72 ($P < 0.01$) for kidney. Since there was no change in the activity of group IV PLA_2 and its protein content during cirrhosis the increased PLA_2 activity was most likely due to an increase in group II PLA_2 enzyme activity. The

direct relationships between mRNA, protein and activity supports the hypothesis that a defined enzyme was upregulated by biliary obstruction.

During biliary duct ligation the concentrations of bile salts increased (Table I). Therefore, the effect of bile juice and bile salts on the secreted group II PLA_2 enzyme from cultured mesangial cells stimulated by IL-1β was studied. Bile juice or bile salts by themselves did not influence the transcription or secretion of mesangial cell PLA_2 enzyme. However, the activity of secreted PLA_2 by IL-1β was enhanced by bile juice and various bile salts (Fig. 3). Bile juice increased PLA_2 activity in a dose-dependent manner. Maximum increased activity was observed at 10–25 μl/ml of bile juice. At higher concentrations of bile juice, inhibition of PLA_2 activity was observed. Among various bile salts tested only GCA, TCA, TCDCA, TDCA, and TUDCA activated the IL-1β–stimulated PLA_2 enzyme (Fig. 3). Other bile salts such as CA, CDCA, DCA GCDCA, GLCA, and LCA did not influence the IL-1β–stimulated PLA_2 activity (data not shown).

Discussion

PLA_2 enzymes characterized so far are classified into group I, II, III, and IV based on their primary and secondary structure (41,42). PLA_2 enzymes purified from human platelets and from human synovial fluid are classified under group II and are considered to account for inflammatory reactions, whereas the group I from pancreas appears not to be involved in inflammation (29, 43, 44). Group III enzyme has not been detected in mammalians and the function of cytosolic PLA_2 (group IV) is not very well defined yet (42). Inflammatory human synovial fluid PLA_2 (group II) is resistant to low pH , optimally active in the presence of mM concentrations of calcium and at basic pH (38, 45). In the present study PLA_2 activity measured in the acid extracts of kidney and liver exhibited similar biochemical/biophysical properties as those known for PLA_2 derived from inflammatory synovial fluid PLA_2. Group IV PLA_2 selectively hydrolyzes *sn*-2-arachidonoyl–containing phospholipids and is resistant to reducing agents like DTT (38). Group IV PLA_2 activity measured in kidney and liver was 20–47-fold lower compared to the activity of group II PLA_2. Since no mRNA for group I PLA_2 was detected its contribution for total PLA_2 activity is very unlikely. Therefore the PLA_2 activity measured in these tissues corresponds mainly to group II PLA_2 enzyme.

The present investigation revealed an upregulation of group II PLA_2 enzyme in obstructive jaundice but not of group I or of group IV PLA_2. For such an enhanced PLA_2 activity three mechanisms have to be considered. First, an enhanced transcription and translation of the group II PLA_2 enzyme, second an increased concentration of PLA_2 activity enhancing agents and third, a decline of an endogenous PLA_2 inhibitor. The quantitative RT-PCR study revealed that group II PLA_2 mRNA levels were elevated after bile duct ligation in renal and liver tissue. The upregulation of PLA_2 was not a nonspecific phenomenon, since mRNA of GAPDH was unaffected and was used as internal standard. The increase of group II PLA_2 mRNA was reflected by an increase of group II PLA_2 protein and group II PLA_2 enzyme activity. Thus, an enhanced transcription and translation of group II PLA_2 enzyme accounted at least partly for the augmented enzyme activity in cirrhotic rats.

With respect to the second mechanism involving the PLA_2 activating agents during cirrhosis we analyzed the impact of bile juice and different bile salts known to be increased during cirrhotic conditions on the secretion and on the activation of group II PLA_2 enzyme activity. Bile juice and bile salts did not activate PLA_2 enzyme activity by inducing the transcription and translation of PLA_2 protein (data not shown). However, bile juice by itself and several bile salts like GCA, TCA, TCDCA, TDCA, and TUDCA individually activated IL-1β released group II PLA_2 enzyme activity indicating the synergistic action of these salts. The present finding, that some bile acids enhance PLA_2 activity is novel, and in line with the description of other endobiotics such as PLA_2 activating protein enhancing PLA_2 activity (46).

For the third mechanism, a decline of an endogenous inhibitor of PLA_2 enzyme during cirrhosis, the role of the presumptive PLA_2 inhibitor annexin-I has been studied together with annexin-V. The annexin family presently consists of some 13 members capable of binding phospholipids in the presence of calcium (27, 47–49). It had been suggested that annexin-I (sive lipocortin-I or calpactin-II) is a mediator of glucocorticoid action and acts as a specific inhibitor of PLA_2 (50, 51), a concept which remains controversial, however (33, 52). In the present investigation annexin-I and -V have been quantitatively assessed. Bile duct ligation did not affect the concentrations of these proteins. Thus, enhanced transcription of PLA_2 and subsequent activation by bile salts is the most likely factor accounting for the enhanced PLA_2 activity following bile duct ligation.

The mechanism accounting for the enhanced release of PLA_2 in obstructive cirrhosis is unknown; however, the following factors have to be considered. First, PLA_2 enzyme is activated by endotoxins and cytokines like IL-1β and tumor necrosis factor-α (53–55). Chronic endotoxemia due to increased intestinal absorption and/or decreased hepatic clearance of lipopolysacharide has been documented in patients with cirrhosis (56, 57) and may thus account for the activation of PLA_2. Second, glucocorticoids which are known to regulate the activity of group II PLA_2 enzyme could be involved in the observed activation of PLA_2. Glucocorticoid deficiency enhances and pharmacological doses reduce PLA_2 activity by means of inhibiting cAMP-dependent, but not cAMP-independent expression of group II PLA_2 mRNA (43, 58). The increased PLA_2 activity under glucocorticoid deficiency was positively correlated to the decreased expression of the presumptive inhibitor annexin-I (29). Since there was no change in the level of annexin-I during cirrhosis, the enhanced PLA_2 activity in biliary obstruction is probably not related to deranged glucocorticoid effects. Third, activation of PLA_2 by catecholamines and angiotensin II has to be considered (59). Numerous studies have demonstrated an increased release of both pressor agents in humans and various animal models with cirrhosis (60, 61), suggesting a role of these endobiotics for PLA_2 activation in liver disease.

Arachidonate metabolites have been reported to be involved in liver damage. For instance there is evidence for 16,16, dimethyl PGE_2 to attenuate liver cell damage induced by carbon tetrachloride and aflatoxin (62-64). Similarly, a PGI_2 analogue protects against carbon tetrachloride or acetaminophen-induced liver injury (65, 66) and 5-lipoxygenase inhibitors or LT receptor antagonists reduce liver injury from D-galactosamine (67–71). On the other hand, there is evidence that

leukotrienes and thromboxane could play a major role in the pathogenesis of cirrhosis and portal hypertension. Thus, the demonstration of increased leukotriene excretion by patients with liver cirrhosis is compatible with PLA_2 activation (72). This contention is further supported by the presence of elevated prostacyclin levels in patients with cirrhosis which correlate with portal hypertension (73).

While the role of prostanoids in the liver is ambiguous, at least exogeneous prostaglandins being protective but leukotrienes, prostacyclin, and thromboxane being pathogenetically involved in cirrhogenesis and maintenance of portal hypertension and hyperdynamic circulation, the situation appears much clearer in the kidney. Through their vasodilator effect, prostaglandins modify intrarenal vascular resistance and thereby maintain glomerular filtration rate (74). Furthermore, they promote natriuresis and free water clearance (20, 22). This explains the deleterious effect of cyclooxygenase inhibitors on renal function in cirrhosis (75).

In conclusion, our studies demonstrate an upregulation of group II PLA_2 at the enzyme and message level in biliary cirrhosis in rat kidney and liver. While this could account for the renal protective effects of prostaglandins in the kidney, its role in cirrhogenesis in the liver remains to be established.

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