Heparin-enhanced Plasma Phospholipase A_2 Activity and Prostacyclin Synthesis in Patients Undergoing Cardiac Surgery

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Abstract

Although eicosanoid production contributes to physiological and pathophysiological consequences of cardiopulmonary bypass (CPB), the mechanisms accounting for the enhanced eicosanoid production have not been defined. Plasma phospholipase A_2 (PLA_2) activity, 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), and thromboxane B_2 (TXB_2) levels were measured at various times during cardiac surgery. Plasma PLA_2 activity increased after systemic heparinization, before CPB. This was highly correlated with concurrent increases in plasma 6-keto-$PGF_{1\alpha}$. TXB_2 concentrations did not increase with heparin administration but did increase significantly after initiation of CPB. High plasma PLA_2 activity, 6-keto-$PGF_{1\alpha}$, and TXB_2 concentrations were measured throughout the CPB period. Protamine, administered to neutralize the heparin, caused an acute reduction of both plasma PLA_2 activity and plasma 6-keto-$PGF_{1\alpha}$, but no change in plasma TXB_2 concentrations. Thus the ratio of TXB_2 to 6-keto-$PGF_{1\alpha}$ increased significantly after protamine administration. Enhanced plasma PLA_2 activity was also measured in patients with lower doses of heparin used clinically for nonsurgical applications. Human plasma PLA_2 was identified as group II PLA_2 by its sensitivity to deoxycholate and dithiothreitol, its substrate specificity, and its elution characteristics on heparin affinity chromatography. Heparin addition to PMNs in vitro resulted in dose-dependent increases in cellular PLA_2 activity and release of PLA_2. The PLA_2 released from the PMN had characteristics similar to those of post-heparin plasma PLA_2.

In conclusion, plasma PLA_2 activity and 6-keto-$PGF_{1\alpha}$ concentrations are markedly enhanced with systemic heparinization. Part of the anticoagulant and vasodilating effects of heparin may be due to increased plasma prostacyclin (PGI_2) levels. In addition the pulmonary vasoconstriction sometimes associated with protamine infusion during cardiac surgery might be due to decreased plasma PLA_2 activity, with an associated increased TXB_2/6-keto-$PGF_{1\alpha}$ ratio. (*J. Clin. Invest.* 1995. 95:1062–1070.) Key words: phospholipids • polymorphonuclear leukocytes, signal transduction • cardiopulmonary bypass • thromboxane • protamine

Introduction

Cardiopulmonary bypass (CPB)[1] has been associated with increased plasma concentrations of prostacyclin (PGI_2) and thromboxane A_2 (TXA_2), measured as the stable metabolites, 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and thromboxane B_2 (TXB_2) (1–5). Another eicosanoid, leukotriene B_4, also has been found in pulmonary edema fluid after CPB (6). PGI_2, the major arachidonate metabolite of endothelial cells, is a potent vasodilator and inhibits platelet aggregation. TXA_2, the predominant cyclooxygenase product of arachidonic acid in platelets, induces vasoconstriction and platelet aggregation and has been implicated in the etiology of pulmonary hypertension sometimes seen with cardiac surgery after protamine infusion for neutralization of heparin (7–11). It has been suggested that increased synthesis or imbalance in the relative synthesis of these potent vasoactive eicosanoids may contribute to organ dysfunction in various pathological states (12) and result in depressed renal, pulmonary, and cardiac function as well as thrombocytopenia, significant problems after cardiac surgery. It is not known, however, why eicosanoid production is enhanced with CPB.

Phospholipases A_2 (PLA_2) comprise a family of enzymes that hydrolyze membrane phospholipids at the *sn*-2 position to release fatty acids and lysophospholipids (13). Different forms of PLA_2 are involved in digestion, inflammation, and intracellular and intercellular signaling. Some forms are secreted, while others act intracellularly. When PLA_2 acts upon lipids containing arachidonic acid in the *sn*-2 position the resulting free arachidonic acid can serve as a substrate for cyclooxygenases and lipoxygenases. Platelet damage or excess secretion of humoral factors such as catecholamines or angiotensin II during cardiac surgery (14–16) has been implicated in the elevation of cellular PLA_2 activity. Complement activation during CPB (17) causes chemotaxis and leukosequestration, together with the production of oxygen free-radicals by neutrophils, which is thought to affect PLA_2 activity (18).

We report that high-dose heparin administration during surgery, before CPB, increases plasma PLA_2 activity. Furthermore, this increase in plasma PLA_2 activity is directly correlated with

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1. *Abbreviations used in this paper:* 2-[1-^{14}C]AA-GPC, 1-stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycerol-3-phosphocholine; 2-[1-^{14}C]AA-GPE, 1-acyl-2-[1-^{14}C]arachidonoyl-*sn*-glycerol-3-phosphoethanolamine; BPB, *p*-bromophenacyl bromide; CPB, cardiopulmonary bypass; $cPLA_2$, cytosolic phospholipase A_2; PLA_2, phospholipase A_2.

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plasma concentrations of 6-keto-$PGF_{1\alpha}$. The PLA_2 activity is identified as a group II form of the enzyme and may derive, at least in part, from polymorphonuclear leukocytes (PMNs) since the administration in vitro of heparin to these cells results in enhancement of cellular and released PLA_2 activities, which have identical characteristics to those of post-heparin plasma PLA_2. Our study suggests that heparin-stimulated PLA_2 activity may be partially responsible for the anticoagulant action of heparin in vivo due to conversion of arachidonic acid, liberated by PLA_2, to PGI_2 in the endothelial cell.

Methods

Materials

Radioactively labeled phospholipid substrates, 1-acyl-2-[1-^{14}C]arachidonoyl-*sn*-glycerol-3-phosphoethanolamine (2-[1-^{14}C]AA-GPE) and 1-stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycerol-3-phosphocholine (2-[1-^{14}C]AA-GPC) were obtained from Amersham Corp. (Arlington Heights, IL). Arachidonic acid (AA), porcine pancreatic PLA_2, and molecular weight standards for gel filtration (vitamin B_{12}, cytochrome *c*, blue dextran, ovalbumin, and human serum albumin) were obtained from Sigma Chemical Co. (St. Louis, MO), and Silica gel LK5D plates were from Whatman Inc. (Clifton, NJ). Scintillation fluid (Ecoscint®) was purchased from National Diagnostics, Inc. (Atlanta, GA) and lymphocyte separation medium® from Organon Teknica (Rockville, MD). The heparin-5PW HPLC column was purchased from TosoHAAS (Montgomeryville, PA). Heparin sodium, derived from porcine intestines, was obtained from Elkins-Sinn, Inc. (Cherryville, NJ). Protamine sulfate was obtained from Eli Lilly & Co. (Indianapolis, IN).

Patients

12 patients scheduled for elective aorto-coronary artery bypass grafting were selected for study with institutional approval and informed consent. Each patient was anesthetized with fentanyl (100 μg/kg) and muscle relaxants. All received identical preoperative and perioperative medications, except one patient who received low dose (500 U/h) heparin infusion preoperatively for anticoagulation therapy. For CPB, a bubble oxygenator and nonpulsatile flow pump were used and systemic hypothermia (24°C) was maintained. 10 serial measurements were performed as follows: period 1, Control: after placement of monitoring catheters before induction of anesthesia; period 2, Anesthesia: 10 min after induction of anesthesia before incision; period 3, Before heparin: after incision, before heparin administration; period 4: 5 min after heparin administration (300 U/kg intravenously); periods 5–7: at 15, 30, and 60 min on CPB, respectively; period 8: Before protamine administration; period 9: 5 min after protamine administration (3 mg/kg); and period 10: at the end of the operation. At each point, hemodynamic determinations and arterial blood gas measurements were performed. Arterial blood samples were transferred immediately into glass tubes containing EDTA for measurement of PLA_2 activity. Indomethacin was added to samples to be analyzed for plasma 6-keto-$PGF_{1\alpha}$ and TXB_2. Samples were centrifuged at 1,700 *g* for 20 min at 4°C, and plasma was stored at −70°C until assayed. Plasma heparin levels were determined by the heparin anti-10A functional assay (chromostrate assay; Organon Teknika).

Experiments were performed to determine if lower doses of heparin also resulted in elevation in plasma PLA_2 activity. In three patients blood was taken before and 5 min after a bolus of 5,000–6,000 U of heparin and in two patients blood was taken while they were receiving 1,400–1,500 U/h of heparin by continuous infusion.

Plasma prostaglandin assays

Plasma stable metabolites of TXA_2 and PGI_2, TXB_2 and 6-keto-$PGF_{1\alpha}$, respectively, were measured by double antibody radioimmunoassay (19).

Isolation of polymorphonuclear leukocytes

Polymorphonuclear leukocytes (PMNs) were prepared from fresh human blood using a method described previously (20) with minor modifications. After sedimentation of erythrocytes in 3% (wt/vol) dextran at room temperature for 45 min, lymphocyte separation medium was added to the leukocyte-rich supernatant which was then centrifuged at 500 *g* for 35 min. The supernatant was discarded and residual erythrocytes were destroyed by hypotonic (0.2% NaCl) lysis. Hypertonic NaCl (1.6%) was then added to return the solution to isotonicity. Lysis was repeated, and PMNs were suspended in buffer (2.5×10^6 cells/ml) consisting of 250 mM sucrose and 50 mM Hepes, pH 7.5, that contained the protease inhibitors pepstatin 20 μM, leupeptin 20 μM, Trasylol 1,000 kallikrein inactivating unit/ml, and phenylmethyl sulfonyl chloride 0.1 mM. Cell preparations were examined after Wright staining and found to contain > 95% PMNs.

Plasma PLA_2 assay

Plasma PLA_2 activity was measured using methods described previously (21) with some modifications. 2-[1-^{14}C]AA-GPE was the most commonly used substrate. Preliminary experiments, using 2-[1-^{14}C]AA-GPC and 2-[1-^{14}C]AA-GPE as substrates, indicated that plasma PLA_2 activity was greatest when 2-[1-^{14}C]AA-GPE was used. Sample protein concentrations were matched using a Bio-Rad Labs (Hercules, CA) protein kit with bovine serum albumin as a standard. 2-[1-^{14}C]AA-GPE was dried under a stream of N_2 gas and resuspended in dimethylsulfoxide. 2-[1-^{14}C]AA-GPE (final concentration, 15 mM) was added to microcentrifuge tubes. Reactions were initiated by the addition of plasma, diluted 100-fold in 250 mM Tris-HCl, pH 8.5, buffer containing 3 mM Ca^{2+}. The mixture was incubated for 60 min at 37°C, and the reaction was terminated by the addition of ethanol containing 2% (vol/vol) acetic acid and 10% (vol/vol) AA. Aliquots of the reaction mixture were spotted onto a silica gel LK50DF thin-layer chromatography plate, and the plates were developed in the organic phase of ethyl acetate/isooctane/H_2O/acetic acid (55:75:100:8). The lipids were visualized by I_2 staining. The phospholipid and arachidonate bands were scraped, and radioactivity was counted with a liquid scintillation counter (Hewlett-Packard Co., Palo Alto, CA) in 3 ml of Ecoscint®. PLA_2 activity was defined as picomoles of radiolabeled AA released from 2-[1-^{14}C]AA-GPE per minute per milligram of protein at 37°C. In some experiments, PLA_2 activity was determined using a different method (22). Substrate 2-[1-^{14}C]AA-GPE was dried down with an N_2 gas stream and resuspended in ethanol by vigorous vortexing. The PLA_2 assay buffer (100 μl) contained 75 mM Tris-HCl, 5 mM $CaCl_2$, and 0.5 nmol of the phospholipid (~ 65,000 cpm) at pH 8.5. The reaction was carried out at 37°C for 30 min and was stopped by adding 0.56 ml of Dole's reagent: 48.75% isopropyl alcohol, 50% *n*-heptane, 1.25% 1 N H_2SO_4 in water (23). Free fatty acid was extracted in the following manner: 0.11 ml of water was added and the sample was vortexed and centrifuged for 3 min. 0.15 ml of the upper phase was transferred to a new tube to which 25 mg silica gel and 0.8 ml of *n*-heptane were added. The samples were vortexed and centrifuged again for 3 min each. 0.8 ml of supernatant was then counted in a liquid scintillation counter.

PLA_2 activity intrinsic to PMNs and released from PMNs and endothelial cells

After incubation of PMNs for 20 min with various doses of heparin (0, 1, 10, and 100 U/ml) at 37°C, samples were centrifuged at 3,000 *g* for 30 min, and PLA_2 activity was measured in the supernatant to determine the release of enzymatic activity by PMNs into the medium. PMNs were washed twice with the same buffer not containing heparin, homogenized with 25 strokes of a tight Dounce homogenizer, and then centrifuged at 100,000 *g* at 4°C for 60 min. PLA_2 activity in the 100,000 *g* supernatant was measured as described above and taken to be the intrinsic soluble PMN PLA_2 activity. Human umbilical endothelial cells were grown in M199 medium with 20% fetal bovine serum. Endothelial cell medium PLA_2 activity was measured under control conditions and after exposure of the cells to heparin (15 U/ml) for periods up to 24 h.

Chromatographic characterization of PLA_2 activity

Superose 12 gel filtration FPLC column chromatography. Samples of plasma, PMN cellular extracts, or supernatants (400 μl) of intact PMNs

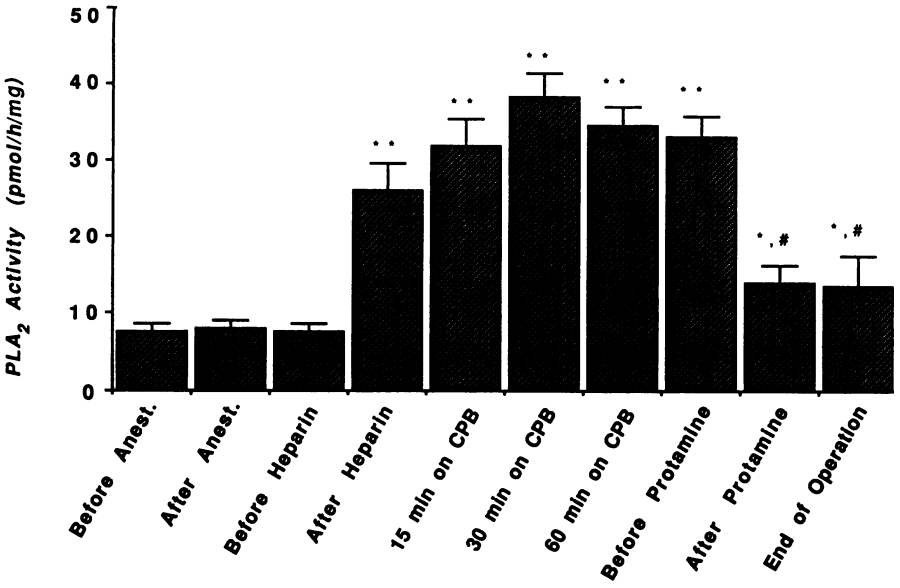

Figure 1. Plasma PLA_2 activity changes during CPB surgery. Plasma samples were collected from 12 patients: just before and 10 min after induction of anesthesia (*Anest.*); just before and 5 min after heparin administration; 15, 30, and 60 min on CPB; just before and 5 min after protamine administration; and at the end of the operation at skin closure. There was a significant increase in PLA_2 activity after heparin administration. PLA_2 activity remained elevated during CPB. Protamine infusion resulted in a marked decrease of plasma PLA_2 activity, although PLA_2 activity remained higher than values before heparin administration. $^*P < 0.05$, $^{**}P < 0.001$, compared with control values before anesthesia, and $^{\#}P < 0.05$, compared with the values before protamine.

were loaded onto a Superose 12 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) 24-ml gel filtration column, previously equilibrated with buffer consisting of 250 mM Tris-HCl, pH 8.5, 0.15 M NaCl, 1 mM EDTA, and 1 mM EGTA buffer at 4°C. Proteins were eluted with this buffer at a flow rate of 0.5 ml/min. The column was calibrated using blue dextran (2,000 M_r), bovine serum albumin (66 kD), ovalbumin (45 kD), porcine pancreatic PLA_2 (13.5 kD), cytochrome *c* oxidase (12.8 kD), and vitamin B_{12} (1.35 kD). 1-ml fractions were collected and assayed for PLA_2 activity. The protein concentration of each fraction was monitored by its absorbance at 280 nm.

Heparin-5PW HPLC column chromatography. Human plasma samples were diluted 1:1 with buffer A (50 mM Tris-HCl, 1 mM EDTA, pH 7.4) and loaded onto a heparin-5PW HPLC column (0.75 × 7.5 cm) preequilibrated with buffer A. Activity was eluted with buffer A (nonbinding fraction) at 1 ml/min for 20 min. Additional activity was then eluted with a linearly increasing gradient from 0 to 2 M of NaCl concentration in buffer A.

Ca^{2+} dependency of plasma PLA_2 activity

Aliquots of plasma samples were diluted 1:8 with buffers containing 140 mM NaCl, 25 mM Hepes, and varying amounts of $CaCl_2$ with or without 1 mM EGTA, at pH 8.5. Enzymatic activity was determined after an aliquot of the mixture was taken to measure the free Ca^{2+} concentration. Enzymatic activity and free Ca^{2+} concentration were measured at 37°C. Ca^{2+} concentrations below 1 μM were determined using the dual wavelength fluorescence characteristics of Fura-2 free acid with a dual wavelength spectrofluorometer (Deltascan; Photon Technology Inc., Princeton, NJ). For concentrations > 1 μM, the free Ca^{2+} concentration of the assay buffer was determined with a Ca^{2+} selective electrode, which we constructed and calibrated as described previously (24).

pH dependency of plasma PLA_2 activity

Plasma samples were diluted with buffers of different pH (pH 5.0–11.0). Tris-HCl buffers were used to make up solutions at pH ≤ 7.5, and glycine-NaOH buffers were used for pH 8.0 and above. Actual pH and PLA_2 activity of each sample were determined in the presence of 3 mM Ca^{2+} (1 mM greater than the sum of the EGTA and EDTA concentrations in the assay buffer).

Effect of patients' plasma on porcine pancreatic PLA_2 activity

Porcine pancreatic PLA_2 (50 ng) was incubated in 250 mM Tris-HCl, pH 8.5, at 37°C, for 15 min with or without plasma (25 μg protein) obtained intraoperatively. PLA_2 activity was then measured as described above.

Statistics

All values are presented as the mean ± 1 standard error of the mean. Statistical significance was evaluated using one-way analysis of variance and the Student's *t* test for paired comparisons. $P < 0.05$ was regarded as significant.

Results

Plasma PLA_2 activity during CPB surgery. As shown in Fig. 1, plasma PLA_2 activity before anesthesia was 7.5±1.1 pmol/h/mg protein and did not change significantly either after induction of anesthesia or after skin incision before heparin. A marked increase in plasma PLA_2 activity was seen before CPB after administration of heparin, from 7.4±1.0 to 25.5±3.5 pmol/h/mg, $P < 0.001$. PLA_2 activity remained elevated throughout the CPB period. Protamine, administered to neutralize the heparin, resulted in a marked decrease of PLA_2 activity (to 13.8±2.1 pmol/h/mg, $P < 0.01$ compared with values before protamine infusion), although PLA_2 activity remained significantly higher than values before heparin administration. Neither heparin nor protamine affected enzyme activity when added to plasma in vitro (data not shown). Plasma heparin concentrations measured 5 min after the administration of heparin were 6.0, 6.7, and 7.6 U/ml in three patients. The heparin concentration was slightly higher (8.5 U/ml) in one patient in which it was measured 25 min after heparin administration and lower (3.4 and 3.1 U/ml) in two patients in which it was measured 5 min before protamine administration.

Plasma 6-keto-$PGF_{1\alpha}$ and TXB_2 concentration. No significant changes in plasma 6-keto-$PGF_{1\alpha}$ and TXB_2 concentrations were observed after induction of anesthesia or after the initial surgical incision before heparin administration (Fig. 2). Marked increases of plasma 6-keto-$PGF_{1\alpha}$ concentrations were measured after heparin administration (from 96±28 to 454±92 pg/ml, $P < 0.001$), but heparin did not alter plasma TXB_2. TXB_2 concentrations increased significantly after initiation of CPB (from 124±20 to 197±36 pg/ml at 15 min on CPB, $P < 0.05$).

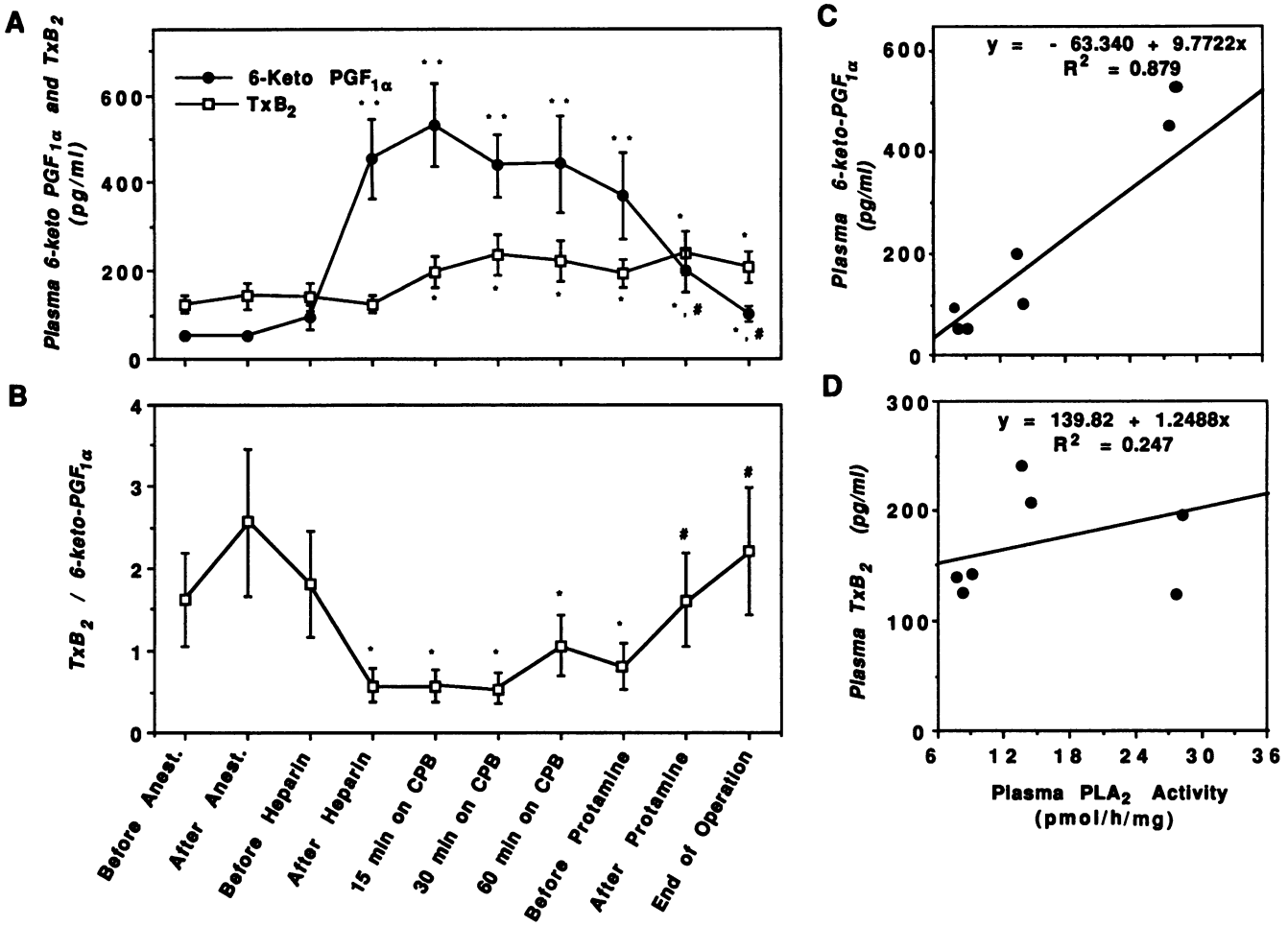

Figure 2. Plasma 6-keto-$PGF_{1\alpha}$, TXB_2 levels (*A*), and TXB_2/6-keto-$PGF_{1\alpha}$ ratio (*B*) during CPB surgery. Marked increases in plasma 6-keto-$PGF_{1\alpha}$ levels were seen after heparin administration, while plasma TXB_2 increased only after initiation of CPB (*A*). Protamine acutely reduced plasma levels of 6-keto-$PGF_{1\alpha}$ but did not change TXB_2 levels, thus the ratio of TXB_2/6-keto-$PGF_{1\alpha}$ decreased after heparin and increased after protamine infusion (*B*). $^*P < 0.05$, $^{**}P < 0.01$, compared with controls, and $^{\#}P < 0.05$, compared with the values before protamine. $n = 12$ patients. Plasma 6-keto-$PGF_{1\alpha}$ (*C*) and TXB_2 levels (*D*) plotted against PLA_2 activity of plasma taken during CPB surgery. There was a close correlation between mean plasma 6-keto-$PGF_{1\alpha}$ levels and PLA_2 activity (*C*), whereas no correlation was found between PLA_2 activity and TXB_2 levels (*D*).

Protamine infusion acutely reduced plasma 6-keto-$PGF_{1\alpha}$, from 370±100 to 200±47 pg/ml (Fig. 2 *A*, $P < 0.001$). Protamine did not change plasma TXB_2 concentrations. Thus, the ratio of TXB_2 to 6-keto-$PGF_{1\alpha}$ decreased after heparin from 2.22±0.64 to 0.49±0.21 and increased significantly after protamine infusion (to 1.85±0.57, Fig. 2 *B*, $P < 0.05$).

Correlation of plasma PLA_2 activity and plasma 6-keto-$PGF_{1\alpha}$ and TXB_2 levels during CPB surgery. To determine interrelationships between plasma PLA_2 activity and plasma 6-keto-$PGF_{1\alpha}$, mean 6-keto-$PGF_{1\alpha}$ concentration at each operative period was plotted as a function of plasma PLA_2 activity. As demonstrated in Fig. 2 *C*, there was a close correlation between plasma 6-keto-$PGF_{1\alpha}$ levels and PLA_2 activity during surgery ($y = 9.772x - 63.34$, $r^2 = 0.879$). No correlation was found between plasma TXB_2 levels and PLA_2 activity (Fig. 2 *D*).

Effects of lower clinical doses of heparin on plasma PLA_2 activity. PLA_2 activity was measured in another group of patients who received a bolus of only 5,000 or 6,000 U of heparin. This dose of heparin had a significant effect on plasma PLA_2 activity (12.3±1.1 pmol/h/mg in post-heparin plasma vs 1.2±0.2 pmol/h/mg in pre-heparin plasma, $P < 0.005$, $n = 3$). Thus, plasma PLA_2 activity is increased in patients receiving amounts of heparin that are routinely used for therapy in the nonsurgical setting.

Characterization of plasma PLA_2 activity. To further characterize post-heparin plasma PLA_2 activity, samples were fractionated by Superose 12 gel filtration chromatography (Fig. 3). Recovery of activity from the column was between 90 and 110%. Plasma PLA_2 activity, measured using 2-[1-^{14}C]AA-GPE as substrate, both before and after heparin, migrated as a single peak of activity eluting in the same fraction as porcine pancreatic PLA_2 with mobility characteristics corresponding to an approximate molecular mass of 14 kD. Greater peak and integrated activities were observed consistently in post-heparin plasma than in pre-heparin plasma.

There were additional features of the post-heparin plasma PLA_2 which clearly distinguished it as group II PLA_2. No increase in plasma PLA_2 activity was measured during surgery when 2-[1-^{14}C]AA-GPC was used as substrate in the assay, indicating that post-heparin PLA_2 preferentially hydrolyzed 2-[1-^{14}C]AA-GPE when compared with 2-[1-^{14}C]AA-GPC. The phospholipid subclass substrate preference for 2-[1-^{14}C]AA-

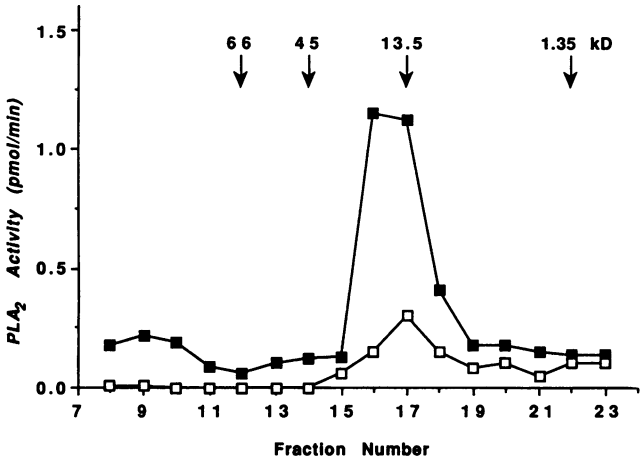

Figure 3. Fractionation of plasma by FPLC Superose 12 gel filtration chromatography. Pre- (*open boxes*) and post-heparin (*filled boxes*) plasma samples were applied to a 24-ml column and 1-ml fractions were collected and assayed for PLA_2 activity. A representative elution profile is presented. PLA_2 activity directed against 2-[1-^{14}C]AA-GPE migrated as a single peak with mobility characteristics corresponding to an apparent molecular mass of 14 kD. Greater activity was seen in the peak from the post-heparin plasma. The column was calibrated using bovine serum albumin (66 kD), ovalbumin (45 kD), porcine pancreatic PLA_2 (13.5 kD), and vitamin B_{12} (1.35 kD).

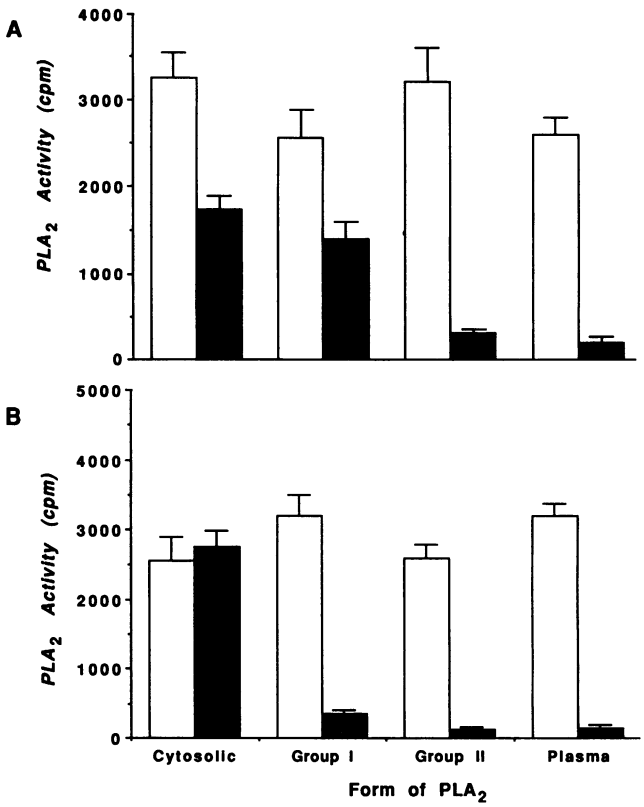

Figure 4. (*A*) Phospholipid head group specificity of human plasma PLA_2 activity. Cytosolic (porcine spleen), group I (porcine pancreas), and group II (rat platelet) PLA_2 and post-heparin human plasma were tested for activity using 2-[1-^{14}C]AA-GPE (*open bars*) and 2-[1-^{14}C]AA-GPC (*hatched bars*) as substrates.(*B*) Effect of DTT on human plasma PLA_2 activity. Partially purified porcine spleen (*Cytosolic*), purified porcine pancreatic (*Group I*), partially purified rat platelet (*Group II*), and human post-heparin plasma samples were preincubated in 50 mM Tris-HCl, 5 mM $CaCl_2$, pH 8.5, without (*open bars*) or with 5 mM DTT (*hatched bars*) at 37°C for 30 min. The reactions were initiated by adding substrate, 2-[1-^{14}C]AA-GPE, and assayed for PLA_2 activity at 37°C for 30 min by the Dole's extraction method as described in Methods. Data shown are reflective of three independent experiments. Each bar reflects the mean and standard error of triplicate determinations and is representative of similar results from three independent experiments.

GPE over 2-[1-^{14}C]AA-GPC of plasma PLA_2 activity is characteristic of group II PLA_2 as compared with group I PLA_2 which is also ~ 14 kD in molecular mass but has greater hydrolytic activity against 2-[1-^{14}C]AA-GPC than do group II forms of PLA_2 in our assay (Fig. 4 *A*).

The identity of the post-heparin plasma PLA_2 as a group I or II enzyme was further confirmed by its inhibition by *p*-bromophenacyl bromide (BPB) and dithiothreitol (DTT). BPB inactivates group I and group II PLA_2s by reacting specifically with ^{48}His at the catalytic site (25) which is conserved among group I and group II forms (26). BPB has no activity against cytosolic PLA_2 ($cPLA_2$) (22). BPB inhibited plasma PLA_2 activity by ~ 80% (data not shown). DTT markedly inhibits human plasma PLA_2 in a manner similar to its inhibition of group I and group II PLA_2s (Fig. 4 *B*). DTT has no effect on the large molecular mass $cPLA_2$.

The calcium and pH dependencies of post-heparin PLA_2 activities were also determined. The enzyme was Ca^{2+} dependent, was maximally active at Ca^{2+} concentrations $\geq$ 50 μM, and had a pH optimum of ~ 8.5. Approximately one-half maximal activity was present at physiological pH of 7.4–7.5. These are properties typical for a group II PLA_2 (data not shown).

We further characterized the PLA_2 activity by heparin-5PW HPLC chromatography (Fig. 5). On this column, group I is clearly distinguished from group II PLA_2 by elution at different NaCl concentrations. As shown in Fig. 5, the elution pattern of plasma enzymatic activity is clearly that of group II.

The concentration dependency of inhibition with sodium deoxycholate is typical for group II PLA_2 (Fig. 6) and is distinct from the pattern seen with group I or $cPLA_2$. No PLC activity was identified since no radioactivity was found in the mono- and diglyceride regions when PLA_2 assay samples were separated by TLC.

Effects of patients' plasma on porcine PLA_2 activity. When plasma (containing 25 μg protein), taken at various times during surgery, was added to 50 ng of purified porcine pancreatic PLA_2, there was no modulation of PLA_2 activity regardless of when, during the course of the operation, the plasma was collected. In addition, actual and predicted activity of PLA_2 was the same when plasma samples, collected before and after heparin administration, were mixed at various ratios. Thus, the patients' plasma did not contain any PLA_2 activating or inhibiting factor detectable by this assay with pancreatic PLA_2 even in the presence of heparin.

PLA_2 activity in PMNs and release of PLA_2 activity from PMNs into the medium by heparin. As demonstrated in Fig. 7 *A*, there was a dose-dependent increase in soluble PLA_2 activity in PMNs when cells were treated with heparin. There was also a dose-dependent increase in PLA_2 activity released into the medium when PMNs were incubated with heparin (Fig. 7 *B*). In additional experiments (not shown) we found that PMNs isolated from patients on CPB receiving high dose heparin also

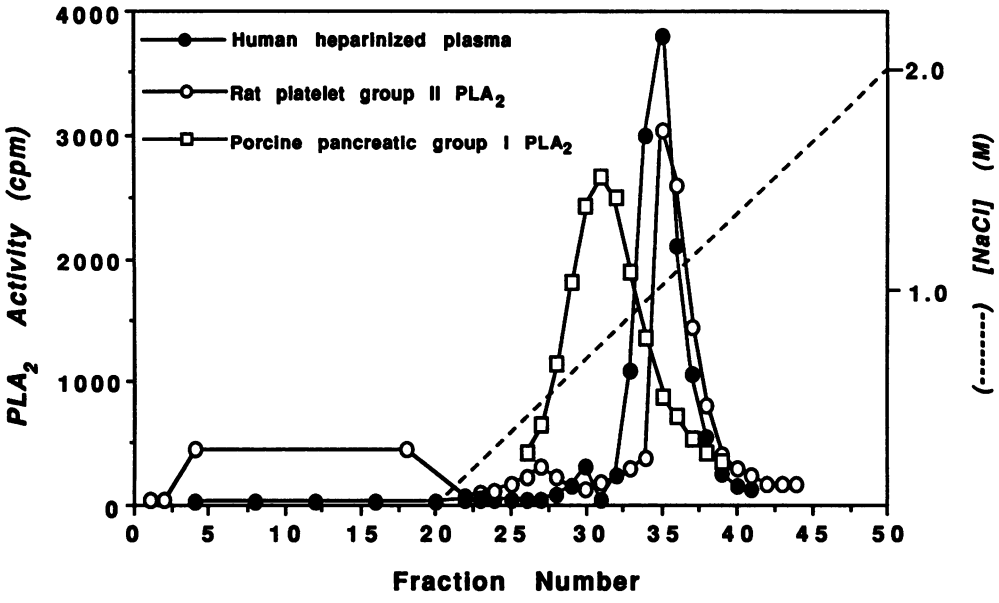

Figure 5. Heparin-5PW HPLC profile of post-heparin human plasma PLA_2 activity. Plasma PLA_2 was loaded onto a heparin affinity column. For comparison, rat platelet 100,000 *g* supernatant was used as a standard for group II PLA_2 and purified porcine pancreatic PLA_2 diluted with buffer A (see Methods) containing 1 mg/ml bovine serum albumin as a group I standard. Each sample was applied to the same column under the same conditions.

had higher levels of cellular PLA_2 activity when compared with PMNs isolated before heparin therapy. PLA_2 activity was not increased in the culture medium of human umbilical endothelial cells after exposure to heparin for periods up to 24 h (data not shown). Exposure of PMNs to protamine had no effect on cellular (Fig. 8 *A*) or secreted (Fig. 8 *B*) PLA_2 activity, but pretreatment with protamine completely prevented the increases in cellular and medium activities observed after heparin treatment.

Gel filtration chromatography of PMN soluble extracts and medium after heparin exposure. To partially characterize the heparin-induced PLA_2 activation of PMNs, soluble cellular extracts and supernatants of intact PMNs exposed to heparin were fractionated by Superose 12 gel filtration chromatography. The

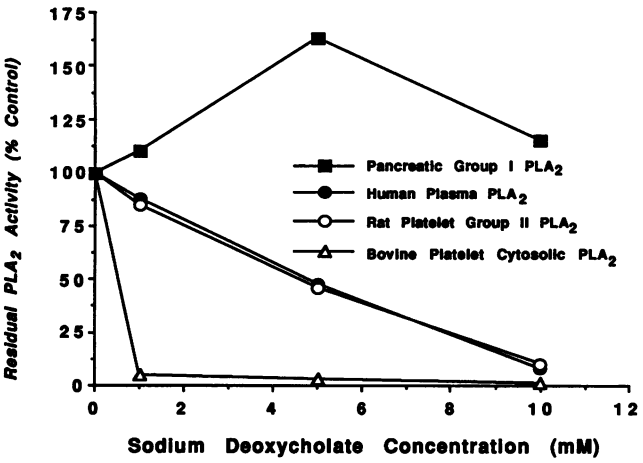

Figure 6. Effect of sodium deoxycholate on human plasma PLA_2 activity. The indicated concentrations of sodium deoxycholate were added to buffer containing 75 mM Tris-HCl (pH 9.0), 5 mM $CaCl_2$, and 20% glycerol. The mixture was vigorously vortexed for 15 s. Human post-heparin plasma or PLA_2 enzymes were then added and incubated at 37°C for 30 min. Released free fatty acid was extracted using Dole's extraction method as described in Methods. 100-kD cytosolic PLA_2 was partially purified from bovine platelets with sequential uses of DEAE-Sephacel, Butyl-Toyopearl, Sephacryl S-300, and DEAE-5PW HPLC as described previously (51). In sodium deoxycholate–free assay, porcine pancreatic, rat platelet, bovine platelets, and human plasma PLA_2 released 3,623, 2,810, 3,250, and 2,200 cpm, respectively. Data are presented as means of three experiments.

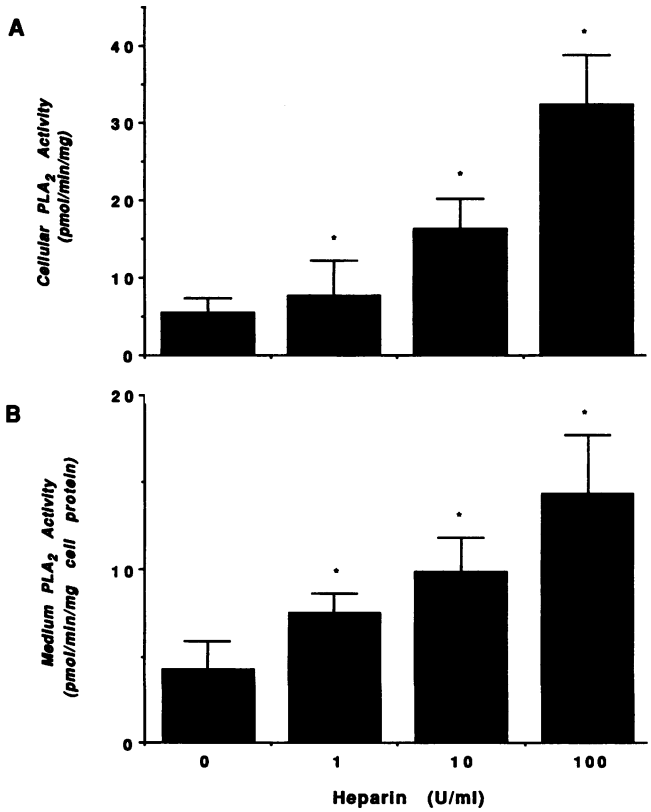

Figure 7. PLA_2 activity in soluble extracts of PMNs (*A*) and in media from heparin-activated PMNs (*B*). In PMNs exposed to heparin there was a dose-dependent enhancement of soluble PLA_2 activity (*A*) as well as a dose-dependent release of PLA_2 activity from cells (*B*). $n = 7$, $^*P < 0.05$ compared with control values.

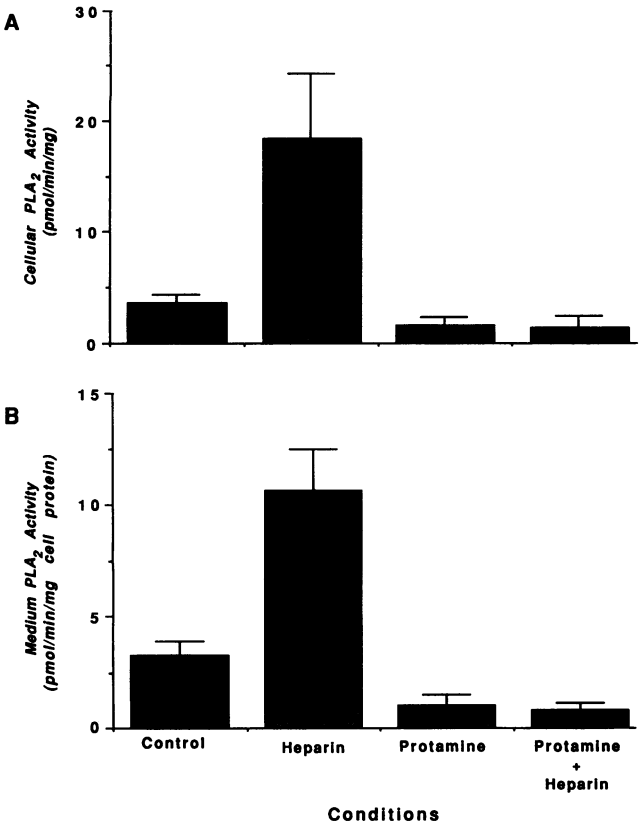

Figure 8. Effect of protamine on PLA_2 activity in soluble extracts (*A*) and medium (*B*) of PMNs. PMNs were treated with heparin (100 U/ml), protamine sulfate (0.87 mg/ml), which will neutralize 100 U/ml of porcine intestine heparin, or protamine sulfate followed by heparin. Control cells were treated with vehicle: 0.9% sodium chloride, 0.01% benzyl alcohol (final vol/vol). PLA_2 activities in media of intact PMNs and 100,000 *g* supernatants of PMN homogenates were measured by Dole's extraction method as described in Methods. Values shown reflect the results of three experiments.

major peak of PLA_2 activity in both soluble cellular fractions and medium migrated at the same position as the peak of activity which was seen in post-heparin plasma (Fig. 3) (data not shown). The specificity for 2-[1-^{14}C]AA-GPE over 2-[1-^{14}C]AA-GPC was also observed in these fractions.

Discussion

Enhanced eicosanoid production during cardiac surgery has been shown to contribute to pathophysiological responses associated with extracorporeal circulation. Platelets have been proposed to be the source of enhanced thromboxane generation (2). It has been suggested that PGI_2 production is increased secondary to elevated levels of thromboxane or activation of the endothelial cell by CPB (4). Our data demonstrate that these explanations are inadequate since we have observed elevated plasma concentrations of 6-keto-$PGF_{1\alpha}$ before increases in plasma thromboxane concentrations and before initiation of CPB (4, 5, 27). It has also been suggested previously that increases in PGI_2 were due to manipulation of vascular endothelial cells during surgery rather than to the direct effects of CPB (19). In our study, although plasma levels of TXB_2 were not increased until 15 min after initiation of CPB, increases in plasma 6-keto-$PGF_{1\alpha}$ levels were seen before CPB, before cannulation of large vessels. Therefore, another explanation must be invoked to explain increased plasma PGI_2 concentrations during CPB surgery.

Heparin is known to increase plasma lipolytic activities, including lipoprotein lipase (28, 29) and PLA_1 (30) activities. Although release of lipolytic enzyme activity after heparin and CPB has been reported (31, 32), the enzymatic activity identified in these reports did not have PLA_2 specificity. Previous studies have not identified heparin-stimulated PLA_2 activity distinct from other lipase activity, nor have they characterized a possible cell source of the elevated plasma PLA_2 activity. Furthermore, a relationship between changes in plasma PLA_2 activity and changes in prostaglandin levels has not been previously delineated.

Our study demonstrates directly that heparin administration to humans results in a marked enhancement of plasma PLA_2 activity. Activation of plasma PLA_2 by heparin is not dependent upon an interaction between heparin and anesthetic agents, since plasma PLA_2 activity was elevated in heparin-treated patients who received relatively low doses of heparin used for anticoagulation in the nonsurgical setting (5,000 U). PLA_2 enzymatic activity was distinguished from sequential action of PLA_1 and lysophospholipase by: Ca^{2+} dependency (33), inhibition by BPB, the alkaline pH optimum (34, 35), and migration as a single enzymatic activity on gel filtration chromatography, with an apparent M_r similar to that of previously defined plasma or serum PLA_2s (36, 37). Although AA could be released from phospholipids by the sequential hydrolysis of substrate via PLC and diacylglycerol lipase (13), this pathway is unlikely to be important because no radioactivity was found in mono- or diglyceride regions on TLC separations. The phospholipase activity we have measured is not due to lipoprotein lipase or hepatic triglyceride lipase, which have considerably larger molecular masses (67 and 65.5 kD, respectively [38]) than the PLA_2 activity we have characterized (14 kD).

The increase of plasma PLA_2 activity associated with heparin administration was highly correlated with concurrent increases in plasma 6-keto-$PGF_{1\alpha}$ levels during surgery. In patients with coronary disease, heparin increases the PGI_2 concentration in coronary sinus blood (39). Itoh and colleagues (40) demonstrated that heparin enhances thrombin and Ca^{2+} ionophore–stimulated PGI_2 production by cultured endothelial cells, perhaps related to enhanced intracellular PLA_2 activity associated with heparin-induced physical changes in cell membranes. Our study suggests that heparin-stimulated PLA_2 activity may be partially responsible for the anticoagulant action of heparin in vivo due to conversion of AA to PGI_2 in the endothelial cell. AA may be liberated by PLA_2 from serum lipoproteins or by direct interaction between PLA_2 and cell membranes.

The source of the increased plasma PLA_2 activity is not established by our study. The PMN likely contributes, but our studies do not rule out other cells as potential sources of PLA_2. The post-heparin plasma PLA_2 is unlikely to originate from the pancreas. Our characterization of PLA_2 as group II distinguishes it from pancreatic group I PLA_2. Our data indicate enhanced soluble PLA_2 activity in PMNs and enhanced release of PLA_2 from PMNs after exposure to heparin. The gel filtration elution pattern of PMN-derived PLA_2 activity was identical to that of post-heparin plasma. Another group has also demonstrated enhanced PLA_2 activity in neutrophils exposed to heparin (42).

PLA_2 may be released from PMNs under other conditions. Plasma PLA_2 activity is enhanced in experimental endotoxemia (43). Patients with sepsis have elevated plasma 14-kD PLA_2 activity (36). Our experiments demonstrate no release of PLA_2 activity from endothelial cells in response to heparin. It is possible, however, that a plasma factor, not present in these experiments in vitro, might potentiate the effect of heparin to release PLA_2 from endothelial cells in vivo. Murakami et al. (44) have recently demonstrated that heparin addition after preexposure of cells to tumor necrosis factor for 6 h resulted in release of group II PLA_2 from human umbilical vein endothelial cells. Platelets are another potential source of group II PLA_2 (26).

While our studies do not prove a direct cause and effect relationship between increases in plasma PLA_2 activity and prostacyclin synthesis, the two are highly correlated, and we propose that the elevated plasma concentrations of group II PLA_2 act directly on the endothelial cells to induce prostacyclin synthesis. When group II PLA_2 is added to human umbilical vein endothelial cells there is a stimulation of prostacyclin release into the medium (44).

Protamine sulfate administration to patients can result in severe pulmonary vasoconstriction (45). Eicosanoids, in particular TXA_2, have been implicated in this pathophysiological response to protamine (7–9, 11, 46–48), and hemodynamic side effects of protamine have been prevented by pretreatment with a cyclooxygenase inhibitor (47). In other studies, however, no significant changes were observed in eicosanoid levels after protamine administration (19, 49, 50). In our study, protamine administration reverses heparin-enhanced plasma PLA_2 activity and reduces plasma 6-keto-$PGF_{1\alpha}$ levels. Protamine also prevented the release of PLA_2 from PMNs. Because TXB_2 did not change after protamine, the ratio of TXB_2/6-keto-$PGF_{1\alpha}$ increased markedly. Thus, protamine-induced changes in the balance between the two prostanoids with opposing vasoactivities may account for some of the adverse effects of protamine.

In conclusion, we have demonstrated that plasma group II PLA_2 activity is enhanced markedly during cardiac surgery and that this is due to heparin administration. This enhanced PLA_2 activity in post-heparin plasma correlates highly with plasma concentrations of 6-keto-$PGF_{1\alpha}$ but not TXB_2. Post-heparin PLA_2 activity may derive in part from PMNs, but our data do not exclude other cells such as platelets and/or endothelial cells as potential sources for the plasma PLA_2 activity. The anticoagulant, and possibly vasodilatory, effects of heparin might be due in part to enhanced plasma PLA_2 activity with subsequent increases in plasma levels of PGI_2. After protamine administration, plasma PLA_2 activity and PGI_2 concentrations are reduced. An increased ratio of TXB_2/6-keto-PGF1a may explain, at least in part, the pathophysiological consequences sometimes observed with protamine administration to patients.

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