

Cytokine-stimulated Secretion of Group II Phospholipase A₂ by Rat Mesangial Cells

Its Contribution to Arachidonic Acid Release and Prostaglandin Synthesis by Cultured Rat Glomerular Cells

Josef Pfeilschifter,* Casper Schalkwijk,† Vreny A. Briner,‡ and Henk van den Bosch†

*Department of Pharmacology, Biocenter, University of Basel, 4056 Basel, Switzerland;

†Centre for Biomembranes and Lipid Enzymology, University of Utrecht, Utrecht, The Netherlands;

and ‡Department of Medicine, University Hospitals, Inselspital, 3010 Bern, Switzerland

Abstract

Potent pro-inflammatory cytokines, such as interleukin 1 (IL-1) or tumor necrosis factor (TNF) α have been found to increase group II phospholipase A₂ (PLA₂) synthesis and secretion by mesangial cells. In all cases 85–90% of the enzyme is secreted from the cells and a parallel increase in prostaglandin (PG)E₂ synthesis is observed. We report here that co-incubation with a monoclonal antibody that specifically binds and neutralizes rat group II PLA₂ attenuates IL-1 β and TNF α -stimulated PGE₂ production by 45% and 52%, respectively.

CGP43182, a specific inhibitor of group II PLA₂, potently blocks mesangial cell group II PLA₂ in vitro with a half-maximal inhibitory concentration (IC₅₀) of 1.5 μ M, while only slightly affecting mesangial cell high molecular weight PLA₂. CGP 43182 markedly attenuates IL-1 β - and TNF α -stimulated PGE₂ synthesis in intact mesangial cells with IC₅₀'s of 1.3 and 1.0 μ M, respectively.

PLA₂ secreted from cytokine-stimulated mesangial cells was purified to homogeneity. Addition of the purified enzyme to unstimulated mesangial cells causes a marked release of arachidonic acid and a subsequent increased synthesis of PGE₂. Moreover, addition of purified PLA₂ to a cloned rat glomerular epithelial cell line and cultured bovine glomerular endothelial cells augmented both arachidonic acid release and PGE₂ synthesis, with the endothelial cells being especially sensitive.

Thus, cytokine-triggered synthesis and secretion of group II PLA₂ by mesangial cells contributes, at least in part, to the observed synthesis of PGE₂ that occurs in parallel to the enzyme secretion. Furthermore, extracellular PLA₂ secreted by mesangial cells is able to stimulate arachidonic acid release and PGE₂ synthesis by the adjacent endothelial and epithelial cells. These data suggest that expression and secretion of group II PLA₂ triggered by pro-inflammatory cytokines may crucially participate in the pathogenesis of inflammatory processes within the glomerulus. (*J. Clin. Invest.* 1993. 92:2516–2523.)

Key words: phospholipase A₂ • interleukin 1 • tumor necrosis factor • prostaglandin • mesangial cells

Address correspondence to Prof. J. M. Pfeilschifter, Department of Pharmacology, Biocenter, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland.

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Introduction

Phospholipase A₂ (PLA₂)¹ comprises a group of lipolytic enzymes that specifically release fatty acids, often arachidonic acid, from the *sn*-2 position of membrane phospholipids for production of important lipid mediators such as eicosanoids and platelet activating factor (1, 2). It has become evident that PLA₂s are a heterogeneous family of enzymes that can be classified into two classes, based on their molecular weight. A class of low molecular weight PLA₂s (14 kD) referred to as secretory PLA₂s or sPLA₂s and the more recently discovered higher molecular mass enzymes (60–110 kD), also referred to as cytosolic PLA₂s or cPLA₂s. Members of both classes from human and rat sources have recently been cloned (3–8). The sPLA₂s are further divided into two groups, based on their amino acid sequence (9). Mammalian group I PLA₂ comprises the pancreatic type of PLA₂ and is characterized by the presence of Cys11. Several nonpancreatic tissues contain group II PLA₂, which is characterized by the lack of Cys11. Mammalian group II PLA₂ is found in soluble form at inflammatory sites such as peritoneal exudates (10, 11) or rheumatoid arthritis (3, 4, 12), is synthesized and secreted from many cell types, and is believed to play a role in the initiation and propagation of inflammatory processes (13, 14). In line with this hypothesis, the PLA₂ purified from human synovial fluid is proinflammatory and vasoactive, and mediates hyperaemia and a marked inflammatory reaction when injected intracutaneously into rabbits (15) or intraarticularly into rats (16). Furthermore, injection of purified rat platelet PLA₂ exacerbated the paw edema in rats with adjuvant arthritis (17).

Moreover, two potent proinflammatory cytokines, IL-1 and TNF α , stimulate PLA₂ activity and PG production in several cell systems. IL-1 and TNF α activate membrane-bound PLA₂, but also induce the synthesis and secretion of sPLA₂ in rabbit and rat chondrocytes (18, 19), human synovial cells (20), rat mesangial cells (21, 22), and MDCK cells (23). The cytokine effect is blocked by actinomycin D and cycloheximide, thus demonstrating a requirement for both transcription and de novo protein synthesis. This is confirmed by the observation that IL-1 and TNF α increase the level of mRNA for group II PLA₂ in rabbit articular chondrocytes (24, 25), rat vascular smooth muscle cells (26), rat astrocytes (27), rat mesangial cells (28, 29), and human hepatoma cells (30).

Recently, a new family of high molecular weight PLA₂s (cPLA₂s) has been purified from a variety of cells and tissues,

1. *Abbreviations used in this paper:* cPLA₂, cytosolic phospholipase A₂; IC₅₀, half-maximal inhibitory concentration; PLA₂, phospholipase A₂; sPLA₂, secretory phospholipase A₂.

including rat mesangial cells (31, 32), rat kidney (33), human monoblast cell line U937 (7, 8, 34, 35), mouse macrophage cell line RAW 264.7 (36), and platelets (37–39). Unlike the sPLA₂, this enzyme displays a preference for substrates containing arachidonic acid esterified at the *sn*-2 position of glycerophospholipids and is activated at physiologic, submicromolar concentrations of calcium. Hormone-stimulated increases in intracellular free calcium are thought to regulate cPLA₂ activity by triggering the translocation of the enzyme from the cytosol to the membrane, thus mediating transient physiological eicosanoid synthesis. Very recently it was shown that cytokines, such as IL-1 β , TNF α , or transforming growth factor β ₂ also enhance cPLA₂ activity (40, 41). It remains difficult to discriminate between contributions of sPLA₂ and cPLA₂ activities in the liberation of arachidonic acid. Therefore the precise functions of sPLA₂ and cPLA₂, and especially the contributions of both enzymes to eicosanoid synthesis under physiological and pathological conditions, are not known. In this report we have addressed the relative contribution of sPLA₂ to IL-1 β - and TNF α -stimulated PGE₂ synthesis in rat mesangial cells. By using a neutralizing monoclonal antibody specific for sPLA₂ and a new, potent low molecular weight inhibitor specific for sPLA₂ we provide evidence for a substantial contribution of sPLA₂ to cytokine-induced PGE₂ synthesis. Reconstitution experiments with purified mesangial cell sPLA₂ also demonstrate that the enzyme secreted by mesangial cells is able to trigger eicosanoid synthesis in a paracrine manner in glomerular endothelial and epithelial cells, as well as in an autocrine manner in mesangial cells themselves.

Methods

Materials. [1-¹⁴C]Oleic acid, [5, 6, 8, 9, 11, 13, 14, 15-³H(N)] arachidonic acid and 1-stearoyl-2-[1-¹⁴C]arachidonoyl-glycerophosphocholine were purchased from Du Pont de Nemours International (Regensdorf, Switzerland); recombinant human IL-1 β was kindly donated by Dr. K. Vosbeck and CGP43182 (2-Hydroxy-4-oxo-1,5-dioxaspiro[5,5]undec-2-ene-3-N-(2,4-dichlorophenyl)-carboxamide) by Dr. H. Wehrli, Ciba-Geigy Ltd. (Basel, Switzerland); recombinant human TNF α was from Boehringer-Mannheim (Germany); all cell culture media and nutrients were from Gibco BRL (Basel, Switzerland), and all other chemicals used were from either Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland).¹

Cell culture and incubation. Renal glomeruli from male Sprague-Dawley rats or from female Wistar rats (80–100 g body wt) were isolated under sterile conditions by a sieving technique, and glomerular cells were cultured as described previously (28, 42). In a second step, single cells were cloned by limited dilution in 96-microwell plates to obtain pure cultures of epithelial cells and mesangial cells (28, 43). Suspensions of cells were diluted with a sufficient volume of medium to a concentration of 10 cells/ml and 0.1 ml was added per well. After 2–3 wk clones with apparent epithelial cells or mesangial cell morphology were used for further processing and cultured in RPMI 1640 supplemented with 20% fetal calf serum and 5 μ g/ml of insulin. Cells were identified as mesangial cells by (a) their typical stellate morphology; (b) positive staining for the intermediate filaments desmin and vimentin, which is considered to be specific for myogenic cells (44); (c) positive staining for Thy 1.1 antigen; (d) negative staining for Factor VIII-related antigen and cytokeratin, excluding endothelial and epithelial contaminations, respectively; and (e) generation of inositol triphosphate upon activation of angiotensin II AT₁ receptors (45) was used as a functional criterion for characterizing the cloned cell line. Cells were identified as parietal epithelial cells by the following criteria (46): (a) typical cobblestone morphology, (b) positive staining for cy-

tokertatin; and (c) negative staining with markers for mesangial cells (Thy 1.1 antigen, smooth muscle actin, desmin), endothelial cells (Factor VIII-related antigen), podocytes (podocyte antigen pp44, antibodies kindly provided by Prof. W. Kriz and Dr. P. Mundel, University of Heidelberg, Germany), tubular epithelial cells (uvomorulin) and macrophages (ED1). For the experiments passages 9–28 of mesangial cells and 18–20 of epithelial cells were used. Calf kidneys were obtained from a local slaughterhouse and were used to obtain glomerular endothelial cell cultures by a method previously described by Ballermann (47) and modified by Briner (47a). Approximately 10 g of renal cortex tissue were minced, passed through a sterile 240 μ m stainless steel sieve, and suspended in HBSS. This suspension was then poured through a 180 μ m stainless sieve followed by a 100 μ m mesh. The glomeruli retained by the 100 μ m sieve were washed three times in HBSS and were then incubated for 10 to 15 min at 37°C in HBSS containing 1 mg/ml of collagenase (type V; Sigma Chemical Co.). After digestion, glomerular remnants were sedimented at 500 g for 5 min. The supernatant was centrifuged at 1,000 g for 5 min, and the pellet was suspended in RPMI 1640 medium containing 20% FCS, penicillin (100 U/ml), streptomycin (100 μ g/ml), 50 μ g/ml heparin sodium, and 5 ng/ml of acidic fibroblast growth factor. Cells were plated on 0.2% gelatin-coated 100 mm-diameter tissue culture plates. Primary cultures of endothelial cell clones were isolated with cloning cylinders, detached with trypsin-EDTA, and passaged at cloning density onto gelatin-coated 35-mm-diameter plates. Individual clones of endothelial cells were characterized by positive staining for Factor VIII-related antigen and uniform uptake of fluorescent acetylated low-density lipoproteins (47). Negative staining for smooth muscle actin and cytokeratin excluded mesangial cell and epithelial cell contaminations. Cells were utilized at passages 5–7.

Phospholipase A₂ assays. sPLA₂ activity was determined using [1-¹⁴C]oleate-labeled *Escherichia coli* as substrate as described (48). The substrate was prepared by growing *E. coli* in the presence of [1-¹⁴C]-oleate, followed by autoclaving to inactivate endogenous phospholipases. More than 95% of the label incorporated by *E. coli* was in phospholipid and, as demonstrated by snake venom hydrolysis (*Crotalus adamanteus*), more than 95% of the [1-¹⁴C]oleate was in the *sn*-2 position of the phospholipids (48).

Assay mixtures (1.0 ml) contained 100 mM Tris/HCl buffer, pH 7.0, 1.0 mM CaCl₂, 2.5 \times 10⁸ [1-¹⁴C]oleate-labeled *E. coli* (5 nmol phospholipid, 5–8,000 cpm), and the enzyme to be tested at a dilution producing ~ 5% substrate hydrolysis. CGP 43182 was added when indicated and reaction mixtures were incubated for 1 h at 37°C in a shaking water bath. The reaction was stopped by the addition of 5 ml propan-2-ol/n-heptane/1M H₂SO₄ (40:10:1, by vol) followed by 2 ml heptane and 3 ml water. After vigorous shaking and phase separation, an aliquot (2.5 ml) of the upper phase was passed over a column of silicic acid (3.5 \times 0.5 cm). Free [1-¹⁴C]oleic acid was quantitatively eluted with 1 ml ethyl acetate. Radioactivity was determined in a scintillation counter. PLA₂ activity is expressed as [1-¹⁴C]oleate radioactivity released by 100 μ l of cell culture supernatant. cPLA₂ activity was measured in mesangial cell cytosolic fractions in the presence or absence of CGP 43182 as previously described (40). 1-Stearoyl-2-[1-¹⁴C]-arachidonoylglycerophosphocholine was used as substrate. Mesangial cell cPLA₂ activity was determined by incubating sonicated substrate vesicles (2 μ M 1-stearoyl-2-[1-¹⁴C]arachidonoyl-*sn*-glycero-3-phosphocholine [5 nCi], 1 μ M *sn*-1,2-dioleoylglycerol) with 50 mM Hepes, pH 7.4, 1 mg/ml fatty acid-free BSA, 2 mM mercaptoethanol, 1 mM CaCl₂, 150 mM NaCl, and test enzyme (diluted in lysis buffer; substrate hydrolysis up to 6%). Stock solution of CGP 43182 was prepared in DMSO and kept at –20°C. Working dilutions (concentration 10 times the assay concentration) were prepared in DMSO/water (1:10, by vol) immediately before use and added to the assay mixture in a volume of 1/10 the assay volume. The free calcium concentration was 1 mM in excess of EGTA and EDTA. After incubation for 30 min at 37°C, the reaction was stopped and the released [1-¹⁴C]arachidonic acid was extracted by a modified Dole extraction as described above. Percent inhibition relative to controls (which contained solvent instead

of solution) was determined at various concentrations of CGP 43182. IC₅₀ values were determined graphically.

Monoclonal antibodies. Monoclonal antibodies against rat liver mitochondrial phospholipase A₂ were prepared as described before (49). Large amounts of these antibodies were obtained by intraperitoneal injection of hybridomas into Pristane-primed mice, and ascites fluids were harvested after appropriate periods. Monoclonal antibodies were purified on protein A-Sepharose CL-4B (Pharmacia LKB, Uppsala, Sweden) as described by the supplier of this affinity material. Column fractions were tested for antibodies by the double immunodiffusion technique of Ouchterlony as described (50) using goat anti-mouse IgG. Purified monoclonal antibodies were digested with pepsin to isolate F(ab')₂ fragments as described (51). The F(ab')₂ fragments were purified by chromatography on HiTrap Protein A pre-packed columns (Pharmacia, Dübendorf, Switzerland) according to the instructions of the supplier. Antibody preparations were dialyzed against DME containing 0.1 mg/ml of fatty acid-free BSA before being added to cell cultures.

Purification of secreted phospholipase A₂ Purification of PLA₂ from the culture supernatant of stimulated mesangial cells was done in a single immunoaffinity chromatography step as previously described (52). An immunoaffinity matrix was prepared by coupling purified monoclonal anti-PLA₂ antibodies to cyanogen bromide-activated Sepharose 4B (Pharmacia LKB) following the instructions of the suppliers. The culture supernatant of mesangial cells stimulated for 48 h with IL-1β (1 nM) plus forskolin (10 μM) was collected and dialyzed against 20 mM Tris/HCl, pH 7.4, 1 M KCl, 2 mM EDTA, and 20% (vol/vol) glycerol (buffer A). The dialysate (100 ml) was applied to the monoclonal antibody-Sepharose column (bed vol 1.5 ml). After washing with buffer A the column was eluted with 0.1 M glycine, pH 2.5, containing 0.5 M KCl. Flow rate was 15 ml/h, and fractions of 4 ml were collected. Protein was measured by A_{280nm} and PLA₂ activity was measured in 25 μl aliquots of the fractions. The eluted PLA₂ activity peak was supplemented with BSA and was dialyzed against DME containing 0.1 mg/ml of fatty acid-free BSA.

Determination of arachidonic acid release. Confluent cells in 16-mm-diameter wells were labeled for 24 h with [³H]arachidonic acid (1 μCi/ml; specific radioactivity 240 Ci/mmol) in DME, containing 0.1 mg/ml of fatty acid-free BSA. Thereafter, the medium was sampled for remaining radioactivity and the cells were washed three times to remove all unincorporated [³H]arachidonic acid. Approximately 80–90% of the added [³H]arachidonic acid was incorporated by this procedure (53). The labeled cells were incubated in DME containing 1 mg/ml of BSA as a trap for the released [³H]arachidonic acid. The cells were then stimulated with purified PLA₂ or vehicle for 30 min. Thereafter the medium was removed and centrifuged. Cells were dissolved in 0.5 M-NaOH, and radioactivity was counted in the supernatants and cell extracts in a scintillation counter. The percentage of [³H]-arachidonic acid released from total incorporated radioactivity was calculated.

Determination of PGE₂ synthesis. Confluent cells in 16-mm-diameter wells were washed, incubated in DME, containing fatty-acid-free BSA (0.1 mg/ml) and stimulated with purified PLA₂ or vehicle for 30 min. At the end of an experiment, the medium was withdrawn and centrifuged. The supernatant was assayed for PGE₂ by RIA (New England Nuclear Boston, MA). Cells were dissolved in 0.5 M-NaOH, and protein was determined by the method of Lowry et al. (54) with BSA as a standard.

Results

Neutralizing antibodies to sPLA₂ attenuate cytokine-stimulated PGE₂ synthesis. Stimulation of mesangial cells by either IL-1α, IL-1β, or TNFα caused a release of PLA₂ activity in the medium, that was closely paralleled by an increased synthesis of PGE₂ (21). The enzyme secreted by mesangial cells has previously been identified as a group II PLA₂ based on its recog-

nition by monoclonal antibodies raised against rat liver mitochondrial group II PLA₂ (22) and the fact that these antibodies did not recognize rat pancreatic group I PLA₂ (49). Binding experiments using this monoclonal antibody indicated that nearly all (> 93%) of the secreted PLA₂ activity was recognized and precipitated by the monoclonal antibodies coupled to Sepharose, irrespective of the stimulator used (22). Fig. 1 shows antibody titration experiments of the PLA₂ secreted by mesangial cells after exposure to IL-1β (1 nM). The activity of this supernatant was 0.6 nmol/min/ml and was comparable to the activities found earlier (21, 22). 50 μl of this supernatant was incubated for 15 min at 37°C with various amounts of ascites fluid, affinity-purified monoclonal antibodies, or F(ab')₂ fractions of affinity-purified monoclonal antibodies and then assayed for residual PLA₂ activity as shown in Fig. 1. All three types of antibody preparations gave similar inhibitions of secreted sPLA₂ activity. As a next step, we performed co-incubation experiments, exposing mesangial cells for 24 h to IL-1β (1 nM) or TNFα (1 nM), in the presence of high amounts of F(ab')₂ fragment, sufficient to block 85–90% of the secreted PLA₂ activity. Antibody incubation attenuated IL-1β- (Fig. 2) and TNFα- (Fig. 3) stimulated PGE₂ synthesis by 45% and 52%, respectively. The inhibitory effect of F(ab')₂ fractions was concentration-dependent (Figs. 2 and 3). These data suggest that at least 50% of the cytokine-triggered PGE₂ synthesis is mediated by the expression and secretion of sPLA₂ in mesangial cells. Ascites fluid and affinity-purified monoclonal antibodies could not be used for this type of experiment as they displayed some intrinsic stimulatory effects on sPLA₂ secretion by mesangial cells presumably via their F_c-part (data not shown).

A specific sPLA₂ inhibitor attenuates cytokine-stimulated PGE₂ synthesis. To further substantiate the role of sPLA₂ in cytokine-induced PGE₂ formation, we made use of a recently described specific inhibitor of sPLA₂, i.e., CGP 43182. CGP 43182 was found to potently inhibit isolated group II PLA₂

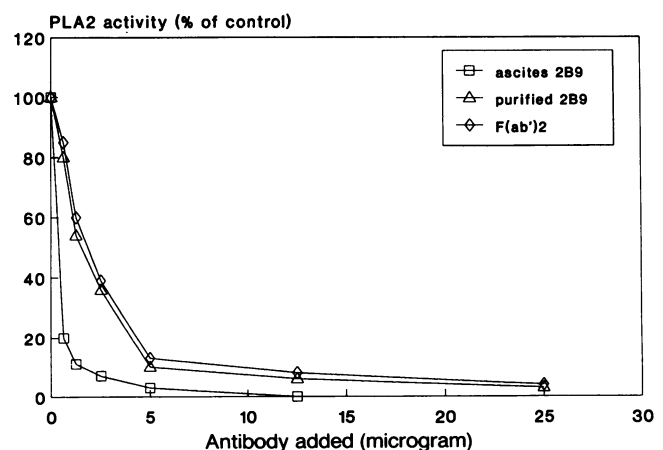


Figure 1. Antibody titration of sPLA₂ secreted by mesangial cells. Mesangial cells were stimulated with IL-1β (1 nM) for 24 h and 50 μl of the cell culture supernatant was incubated with the indicated amount of monoclonal sPLA₂ antibody (□) ascites fluid; (Δ) affinity purified sPLA₂ antibody; (◇) F(ab')₂ fractions of affinity-purified antibody for 15 min at 37°C. The residual PLA₂ activity was assayed by addition of substrate and calcium and is expressed as percent of control measured in cell culture supernatant not treated with monoclonal antibody.

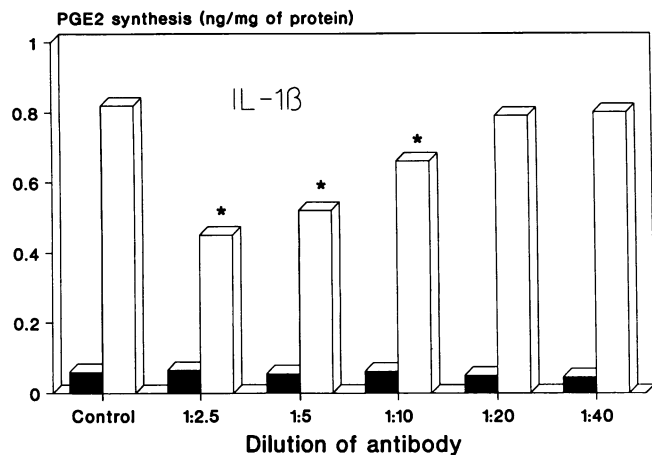


Figure 2. Inhibition of IL-1 β -stimulated PGE₂ synthesis by sPLA₂ antibodies. Mesangial cells were stimulated with (open bars) or without (closed bars) IL-1 β (1 nM) for 24 h in the presence of the indicated dilutions of F(ab')₂ fractions of affinity-purified sPLA₂ antibodies, in a total volume of 500 μ l of medium. PGE₂ was determined in the culture supernatant and is expressed as ng PGE₂/mg of cell protein. Results are means of four experiments and SEM ranges from 3 to 11%. **P* < 0.05 vs. control, by Student's *t* test.

from human neutrophils with an IC₅₀ of 1.2 μ M without having any effect on cPLA₂ activity isolated from the human monoblast U937 cell line in concentrations up to 30 μ M. The compound is thought to directly inhibit sPLA₂ by binding to the active site of enzyme (Märki, F., S. Roggo, H. Wehrli, E. Altmann, W. Breitenstein, I. Wiesenberg, and J. Pfeilschifter, manuscript in preparation). The data in Fig. 4 demonstrate that CGP 43182 also inhibited sPLA₂ activity secreted by mesangial cell with an IC₅₀ of 1.5 μ M. In contrast, cPLA₂ activity in mesangial cell cytosolic preparations was only slightly attenuated by 28% at the highest concentration tested (30 μ M). Thus, CGP 43182 displays a high selectivity also for mesangial

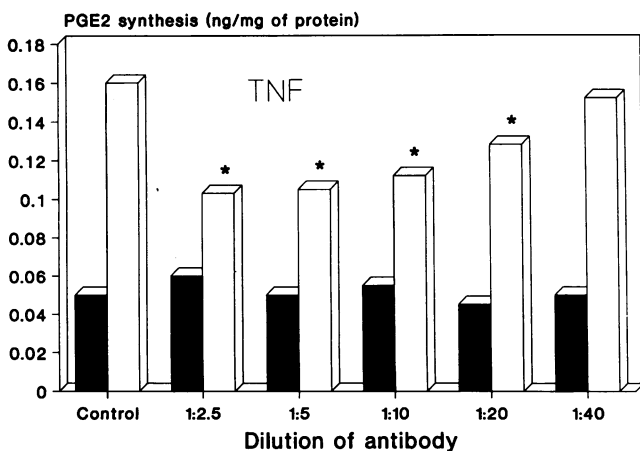


Figure 3. Inhibition of TNF α -stimulated PGE₂ synthesis by sPLA₂ antibodies. Mesangial cells were stimulated with (open bars) or without (closed bars) TNF α (1 nM) for 24 h in the presence of the indicated dilutions of F(ab')₂ fractions of affinity-purified sPLA₂ antibodies, in a total volume of 500 μ l of medium. PGE₂ was determined in the culture supernatant and is expressed as ng PGE₂/mg of cell protein. Results are means of four experiments and SEM ranges from 5 to 13%. **P* < 0.05 vs. control, by Student's *t* test.

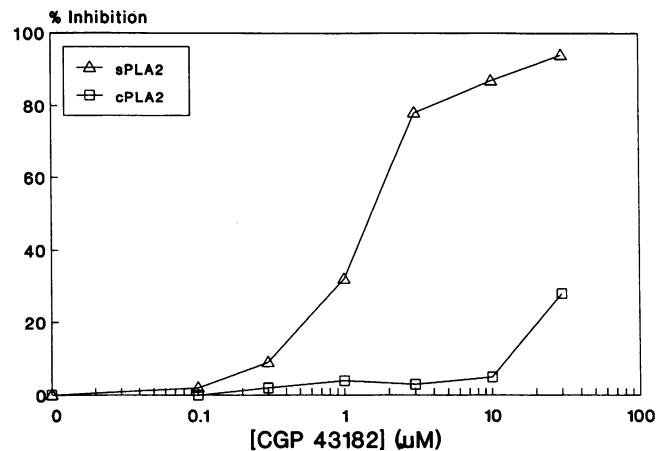


Figure 4. Inhibition of mesangial cell sPLA₂ and cPLA₂ activities by CGP43182. Culture supernatant of mesangial cells stimulated with IL-1 β (1 nM) was incubated with the indicated concentrations of CGP43182 and assayed for sPLA₂ activity (Δ) as described in Materials. Cytosolic preparations of mesangial cells were incubated with CGP43182 and assayed for cPLA₂ activity (\square). Results are expressed as % inhibition of control activity measured in the absence of CGP43182.

cell sPLA₂ as compared to cPLA₂. When added to intact mesangial cells, CGP 43182 potently inhibited PGE₂ production observed after a 24-h stimulation with IL-1 β (1 nM) or TNF α (1 nM) with IC₅₀ values of 1.3 μ M and 1.0 μ M, respectively (Fig. 5). At 10 μ M, CGP 43182 suppressed approximately 70% of IL-1 β - or TNF α -evoked PGE₂ formation (Fig. 5), thus providing further evidence for a crucial role of sPLA₂ in cytokine-induced eicosanoid synthesis in mesangial cells.

Purified mesangial cell sPLA₂ induces arachidonic acid release and E₂ synthesis in glomerular cells. In a third experimental approach, we have purified sPLA₂ secreted by cytokine-stimulated mesangial cells to homogeneity in a single immunoaf-

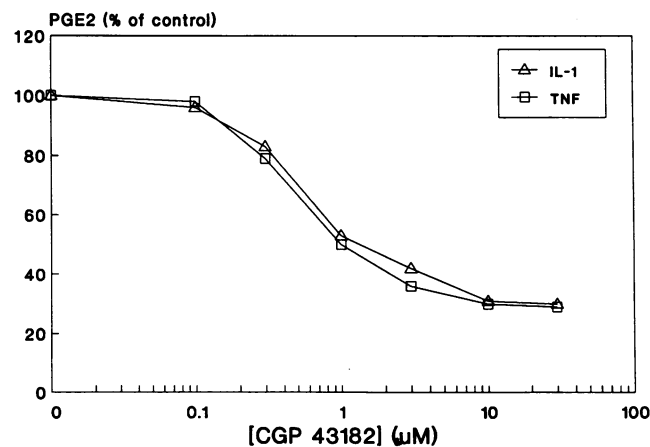


Figure 5. Inhibition of IL-1 β - and TNF α -stimulated PGE₂ synthesis by CGP43182. Mesangial cells were stimulated with IL-1 β (1 nM, Δ) or TNF α (1 nM; \square) in the presence of the indicated concentrations of CGP43182. PGE₂ was determined in the culture supernatants and is expressed as percent of control measured in cytokine-stimulated cells in the absence of CGP43182. Results are means of four experiments and SEM ranges from 2 to 9%.

finity chromatography step as described previously for sequence analysis of mesangial cell sPLA₂ (52). We subsequently used this purified mesangial cell sPLA₂ to examine whether exogenous addition of the enzyme is capable of triggering arachidonic acid release and prostaglandin synthesis in cultures of glomerular cells. When incubated with pure cultures of glomerular endothelial cells, parietal epithelial cells, or mesangial cells, purified sPLA₂ induced a marked increase in arachidonic acid release (Fig. 6) and PGE₂ synthesis (Fig. 7) from all three cell types examined. It is worth noting that the glomerular endothelial cells are especially sensitive to the addition of sPLA₂ with a 88% increase in arachidonic acid release and an approximately fivefold increase in PGE₂ synthesis. Addition of sPLA₂ did not affect cell viability as assessed by examination of cell morphology and by a sensitive colorimetric assay, the MTT test (55).

The calcium ionophore A23187 (1 μM) increased arachidonic acid release from mesangial cells by 178±7% (mean±SEM, n = 4) as compared to 69±3% observed after sPLA₂ addition as shown in Fig. 8, well in accordance with previously published data (53). Co-incubation of mesangial cells with A23187 and sPLA₂ resulted in an additive response of arachidonic acid release, which was increased by 258±12%. The stimulatory action of purified sPLA₂, but not of A23187, on arachidonic acid release and PGE₂ synthesis was abolished by pretreatment of the enzyme with 10 μM of the specific group II PLA₂ inhibitor CGP 43182 (Fig. 8).

Discussion

PLA₂ is the primary enzyme regulating arachidonic acid release and subsequent PG synthesis in many cell types. Two main classes of PLA₂ have been described, the secretory low molecular weight PLA₂s and intracellular cytosolic forms of PLA₂. Both types of PLA₂'s have been reported to occur in mesangial cells. We were the first to describe the induction and

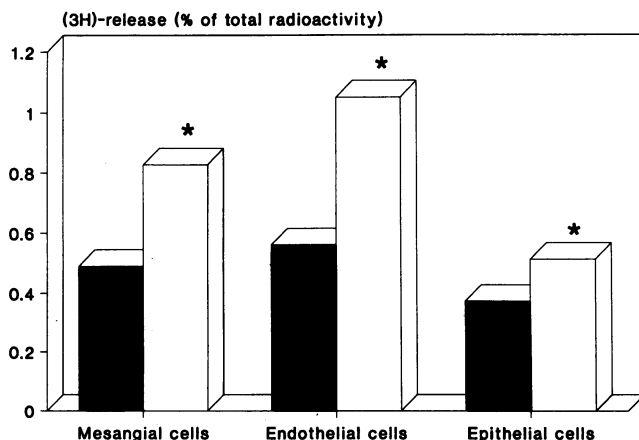


Figure 6. Arachidonic acid release from glomerular cells stimulated by purified mesangial cell sPLA₂. Glomerular mesangial cells, endothelial cells, and epithelial cells were labeled with [³H]arachidonic acid and then stimulated with purified mesangial cell sPLA₂ (1.2 μg/ml, open bars) or vehicle (closed bars) for 30 min. The release of [³H]arachidonic acid was determined as outlined in Materials. Results are expressed as percentage [³H]arachidonic acid released from total incorporated radioactivity and are means of four experiments; SEM ranges from 3 to 14%. *P < 0.05 vs. control, by Student's *t* test.

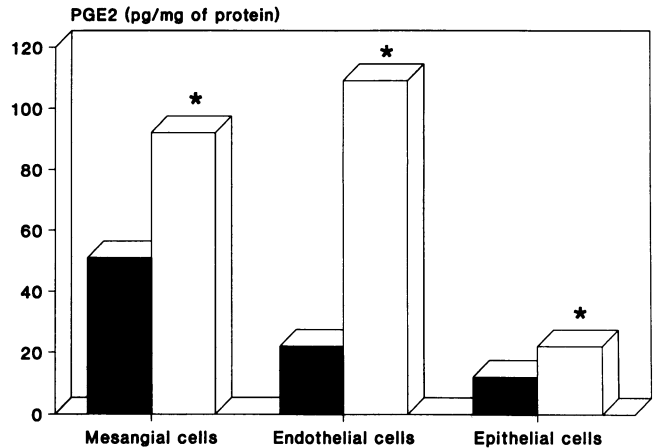


Figure 7. PGE₂ synthesis in glomerular cells stimulated by purified mesangial cell sPLA₂. Glomerular mesangial cells, endothelial cells, and epithelial cells were incubated with purified mesangial cell sPLA₂ (1.2 μg/ml, open bars) or vehicle (closed bars) for 30 min. The formation of PGE₂ was determined in the culture supernatant. Results are expressed as pg PGE₂/mg of cell protein and are means of four experiments; SEM ranges from 4 to 17%. *P < 0.05 vs. control, by Student's *t* test.

secretion of sPLA₂ from mesangial cells in response to IL-1α, IL-1β, and TNFα. The release of sPLA₂ was paralleled by a drastic increase in PGE₂ synthesis (21). Furthermore, IL-1β and TNFα exerted a priming effect on PGE₂ production with an amplified response to subsequent angiotensin II and calcium ionophore stimulation (53). The long lag phase of several hours before the onset of sPLA₂ secretion and the inhibition by actinomycin D and cycloheximide indicated that RNA and protein synthesis are involved in these processes. We also demonstrated by immunoblotting and partial sequence analysis of purified sPLA₂ that cytokines induce a group II PLA₂ in mesan-

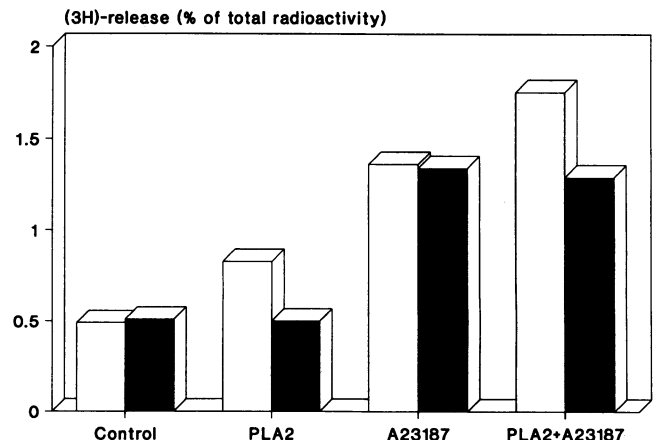


Figure 8. Arachidonic acid release from mesangial cells by purified sPLA₂ and Ca²⁺ ionophore. Glomerular mesangial cells were labeled with [³H]arachidonic acid and then stimulated with purified mesangial cell sPLA₂ (1.2 μg/ml, open bars) or sPLA₂ in the presence of CGP 43182 (10 μM, closed bars). The release of [³H]arachidonic acid was measured after 30 min incubation as described in the Materials section. Results are expressed as percentage [³H]arachidonic acid released from total incorporated radioactivity and are means of four experiments; SEM ranges from 5 to 12%.

gial cells (22, 52). Moreover, Northern blot analysis demonstrated a time-dependent induction of group II PLA₂ mRNA expression in IL-1 α and IL-1 β -stimulated mesangial cells (28, 29) that was sustained for at least 60 h (56).

A new family of high molecular weight PLA₂s (cPLA₂) has been discovered in the cytosol of various cells and tissues, including the kidney and mesangial cells (31–33). Actually, mesangial cells are one of the first cell types that were shown to possess a hormonally regulated high molecular weight PLA₂ (31). A role for protein kinase C in the regulation of mesangial cell cPLA₂ has been proposed several years before. Phorbol esters that specifically activate protein kinase C stimulate arachidonic acid release and prostaglandin synthesis in mesangial cells (57, 58) and inhibitors of protein kinase C were shown to block this response (59). Bonventre and colleagues (31, 32) were the first to demonstrate that the peptide hormone vasopressin, which triggers phosphoinositide turnover in mesangial cells (42), phorbol 12-myristate 13-acetate, and epidermal growth factor stimulated cPLA₂ activity in mesangial cells. Whereas activation of cPLA₂ by vasopressin and phorbol esters depends on the presence of protein kinase C and is completely abolished after down-regulation of protein kinase C, the epidermal growth factor-induced cPLA₂ activation is not mediated via protein kinase C (32). Recently, Huwiler et al. (60) have proposed that the ϵ -isoenzyme of protein kinase C triggers cPLA₂ activation in mesangial cells.

The constitutive expression of cPLA₂ in unstimulated cells, its exclusive intracellular localization, its high sensitivity to calcium, and its regulation by protein kinase C make this enzyme an obvious candidate for the physiological regulation of eicosanoid synthesis in mesangial cells. On the other hand, chronic exposure to proinflammatory cytokines induces the expression of a sPLA₂ that is not present in control cells, is mainly secreted, is less sensitive to calcium, and is regulated on a transcriptional level. It is tempting to designate the latter enzyme an inflammatory PLA₂ that may be responsible for the excessive formation of prostaglandins observed in cells chronically exposed to cytokines, such as IL-1 or TNF α . However, this concept has to be modified since we and others (40, 41, 61, 62) recently demonstrated that chronic treatment with IL-1 β , TNF α , TGF β ₂ enhanced cPLA₂ activity in mesangial cells, rheumatoid synovial fibroblasts, lung fibroblasts, and the epithelial carcinoma cell line HEP-2. The IL-1-induced increase in cPLA₂ activity is due to increased levels of cPLA₂, mRNA, and protein, as well as to posttranslational modifications, i.e., phosphorylation of cPLA₂ by a yet unidentified kinase (62). Thus, IL-1 induces both synthesis and secretion of sPLA₂ and enhanced cPLA₂ activity in mesangial cells, and the contribution of both enzymes to arachidonic acid release and subsequent PGE₂ synthesis was not clear.

The contribution of sPLA₂ to PGE₂ synthesis in mesangial cells is supported by the suppression of IL-1 β -stimulated sPLA₂ and PGE₂ synthesis by glucocorticoids (56, 63) and by TGF β ₂ when the cells are pretreated by TGF β ₂ prior to stimulation (52). However, dexamethasone has been shown to inhibit TNF α -triggered increase in cPLA₂ activity in the epithelial carcinoma cell line HEP-2 cells (41) and also inhibits cPLA₂ activity in mesangial cells (Schalkwijk, C., unpublished observations). The fact that sPLA₂ is predominantly secreted from the cells into the extracellular space, an environment that provides millimolar concentrations of calcium, and these are quantities sufficient to fully activate sPLA₂, strongly suggests that sPLA₂

contributes to cytokine-induced PGE₂ production. The data provided in this report are in line with this view and indicate a marked contribution of sPLA₂ to IL-1 β - and TNF α -stimulated arachidonic acid release, and to PGE₂ synthesis in mesangial cells. From our antibody neutralization experiments we conclude that at least 50% of the cytokine-induced PGE₂ synthesis is due to extracellular sPLA₂ activity. Moreover, the complete inactivation of extracellular sPLA₂ and the small portion of sPLA₂ remaining in the cells by the specific inhibitor CGP 43182 causes an \sim 70% inhibition of PGE₂ synthesis. Thus, some 50–70% of IL-1 β or TNF α -evoked eicosanoid synthesis is due to sPLA₂ activity, at least in mesangial cells. Classically, eicosanoid synthesis is regulated by the availability of arachidonic acid released by PLA₂. However, in certain cell types prostaglandin formation also depends on increased activity of another key enzyme, i.e., cyclooxygenase. In synovial cells and fibroblasts IL-1 was first shown to markedly enhance the expression of cyclooxygenase (64, 65). Two forms of cyclooxygenase are known to be present in eukaryotic cells: a cyclooxygenase 1 that was first purified from ram seminal vesicles and a cytokine- and mitogen-inducible cyclooxygenase 2 (66). In mesangial cells IL-1, TNF, serum, and phorbol ester have been demonstrated to induce the expression of cyclooxygenase (67, 68). In this context it is noteworthy that addition of purified sPLA₂ to unstimulated mesangial cells only increased PGE₂ production twofold (Fig. 7), whereas IL-1 triggered a 15-fold stimulation of PGE₂ formation in the cells (Fig. 2). Co-induction of sPLA₂ and cyclooxygenase may result in a synergistically increased synthesis of PGE₂ in mesangial cells.

It has been demonstrated that arachidonic acid and its metabolites produced by one cell type can be further metabolized by other cell types in the vicinity (69). This is the first report to demonstrate that sPLA₂ secreted by one cell type can also act on adjacent cells to release arachidonic acid, which can be used by the cells to generate eicosanoids. Hence, sPLA₂ secreted by mesangial cells may act on glomerular endothelial cells and epithelial cells. In this context it is noteworthy that the glomerular endothelium is in close apposition to the mesangium without any interfering basement membrane. This is a unique feature and distinguishes the mesangial interstitial space from other interstitial regions of the body (70) and is especially intriguing, considering the high sensitivity of glomerular endothelial cells to mesangial cell sPLA₂ (Figs. 6 and 7).

The participation of sPLA₂ in arachidonic acid release has also been reported for other cellular systems. Rat sPLA₂ added exogenously to calcium ionophore-activated HL-60 granulocytes augmented their production of PGE₂ (71). Murakami et al. (72) reported that rat mast cells, sensitized by IgE and exposed to antigen and sPLA₂, markedly increase PGD₂ production. Both the generation of PGD₂ and the release of arachidonic acid were abolished by inhibitors of sPLA₂. Mouse fibroblasts stably transfected with a human sPLA₂-cDNA and overexpressing sPLA₂ displayed an enhanced stimulated arachidonic acid release (73). Moreover, injection of pure recombinant human sPLA₂ into the joint space of healthy rabbits elicited a dramatic increase in PGE₂ production and an inflammatory, arthritogenic response (74). Exogenously applied sPLA₂ is thought to liberate fatty acids from phospholipids, which are probably located in the outer leaflet of the plasma membrane. Whether sPLA₂ binds to heparan sulfate proteoglycans on mesangial cell surfaces and thus may act as an ectoenzyme as it has been suggested for mast cells (75) remains to be elucidated.

Despite the fact that the monoclonal antibody used in our studies did not pass the plasma membrane of mesangial cells, it blocked ~ 50% of the IL-1 β and TNF α -stimulated PGE₂ synthesis (Figs. 2 and 3), suggesting that secreted sPLA₂ is able to hydrolyze plasma membrane phospholipids. Furthermore, the released arachidonic acid can be used by the cells for prostaglandin synthesis. Alternatively, exogenous sPLA₂ may activate cellular phospholipases, e.g., cPLA₂, which subsequently liberate arachidonic acid for eicosanoid synthesis. In a simple mechanistic model, sPLA₂-triggered inflammatory reaction comprises the generation of arachidonic acid and lysophospholipids, which are subsequently metabolized into eicosanoids and platelet activating factor. Moreover, arachidonic acid and lysophospholipids may act themselves as signaling molecules. An especially intriguing possibility is a sPLA₂-mediated activation of protein kinase C (76). *Cis*-unsaturated fatty acids, which are produced from phospholipids by the action of a non-selective-type of PLA₂, such as sPLA₂, augment the 1,2-diacylglycerol-induced activation of protein kinase C. In addition, lysophospholipids interact with the protein kinase C pathway and potentiate the 1,2-diacylglycerol-dependent activation of the enzyme. This stimulatory effect varied with the protein kinase C isoform examined (76). As protein kinase C is able to activate cPLA₂ activity in mesangial cells (31), there may be a cross-communication between sPLA₂ and cPLA₂ pathways. Further work is required to elucidate the complex network of interaction between sPLA₂ and cPLA₂-triggered mediator release in mesangial cells to evaluate and establish new therapeutic concepts for the treatment of glomerular inflammatory reactions.

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