Cytokine-stimulated Secretion of Group II Phospholipase A_2 by Rat Mesangial Cells

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

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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 A_2 (PLA_2) 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)E_2 synthesis is observed. We report here that co-incubation with a monoclonal antibody that specifically binds and neutralizes rat group II PLA_2 attenuates IL-1β and TNFα-stimulated PGE_2 production by 45% and 52%, respectively.

CGP43182, a specific inhibitor of group II PLA_2, potently blocks mesangial cell group II PLA_2 in vitro with a half-maximal inhibitory concentration (IC_{50}) of 1.5 μM, while only slightly affecting mesangial cell high molecular weight PLA_2. CGP 43182 markedly attenuates IL-1β- and TNFα-stimulated PGE_2 synthesis in intact mesangial cells with IC_{50}'s of 1.3 and 1.0 μM, respectively.

PLA_2 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 PGE_2. Moreover, addition of purified PLA_2 to a cloned rat glomerular epithelial cell line and cultured bovine glomerular endothelial cells augmented both arachidonic acid release and PGE_2 synthesis, with the endothelial cells being especially sensitive.

Thus, cytokine-triggered synthesis and secretion of group II PLA_2 by mesangial cells contributes, at least in part, to the observed synthesis of PGE_2 that occurs in parallel to the enzyme secretion. Furthermore, extracellular PLA_2 secreted by mesangial cells is able to stimulate arachidonic acid release and PGE_2 synthesis by the adjacent endothelial and epithelial cells. These data suggest that expression and secretion of group II PLA_2 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 A_2 • interleukin 1 • tumor necrosis factor • prostaglandin • mesangial cells

Introduction

Phospholipase A_2 (PLA_2)[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 PLA_2s are a heterogenous family of enzymes that can be classified into two classes, based on their molecular weight. A class of low molecular weight PLA_2s (14 kD) referred to as secretory PLA_2s or $sPLA_2$s and the more recently discovered higher molecular mass enzymes (60–110 kD), also referred to as cytosolic PLA_2s or $cPLA_2$s. Members of both classes from human and rat sources have recently been cloned (3–8). The $sPLA_2$s are further divided into two groups, based on their aminoacid sequence (9). Mammalian group I PLA_2 comprises the pancreatic type of PLA_2 and is characterized by the presence of Cys11. Several nonpancreatic tissues contain group II PLA_2, which is characterized by the lack of Cys11. Mammalian group II PLA_2 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 PLA_2 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 PLA_2 exacerbated the paw edema in rats with adjuvant arthritis (17).

Moreover, two potent proinflammatory cytokines, IL-1 and TNFα, stimulate PLA_2 activity and PG production in several cell systems. IL-1 and TNFα activate membrane-bound PLA_2, but also induce the synthesis and secretion of $sPLA_2$ 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 TNFα increase the level of mRNA for group II PLA_2 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 PLA_2s ($cPLA_2$s) has been purified from a variety of cells and tissues,

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1. *Abbreviations used in this paper:* $cPLA_2$, cytosolic phospholipase A_2; IC_{50}, half-maximal inhibitory concentration; PLA_2, phospholipase A_2; $sPLA_2$, secretory phospholipase A_2.

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 $sPLA_2$, 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 $cPLA_2$ 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-1β, TNFα, or transforming growth factor β_2 also enhance $cPLA_2$ activity (40, 41). It remains difficult to discriminate between contributions of $sPLA_2$ and $cPLA_2$ activities in the liberation of arachidonic acid. Therefore the precise functions of $sPLA_2$ and $cPLA_2$, 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 $sPLA_2$ to IL-1β- and TNFα-stimulated PGE_2 synthesis in rat mesangial cells. By using a neutralizing monoclonal antibody specific for $sPLA_2$ and a new, potent low molecular weight inhibitor specific for $sPLA_2$ we provide evidence for a substantial contribution of $sPLA_2$ to cytokine-induced PGE_2 synthesis. Reconstitution experiments with purified mesangial cell $sPLA_2$ 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-^{14}C]Oleic acid, [5, 6, 8, 9, 11, 13, 14, 15-^{3}H(N)] arachidonic acid and 1-stearoyl-2-[1-^{14}C]arachidonoyl-glycerophosphocholine were purchased from Du Pont de Nemours International (Regensdorf, Switzerland); recombinant human IL-1β 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 TNFα 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).

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 μg/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 trisphosphate upon activation of angiotensin II AT_1 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 cytokeratin; 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 μm stainless steel sieve, and suspended in HBSS. This suspension was then poured through a 180 μm stainless sieve followed by a 100 μm mesh. The glomeruli retained by the 100 μm 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 μg/ml), 50 μg/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. $sPLA_2$ activity was determined using [1-^{14}C]oleate-labeled *Escherichia coli* as substrate as described (48). The substrate was prepared by growing *E. coli* in the presence of [1-^{14}C]-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-^{14}C]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 $CaCl_2$, 2.5×10^8 [1-^{14}C]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 H_2SO_4 (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 × 0.5 cm). Free [1-^{14}C]oleic acid was quantitatively eluted with 1 ml ethyl acetate. Radioactivity was determined in a scintillation counter. PLA_2 activity is expressed as [1-^{14}C]oleate radioactivity released by 100 μl of cell culture supernatant. $cPLA_2$ activity was measured in mesangial cell cytosolic fractions in the presence or absence of CGP 43182 as previously described (40). 1-Stearoyl-2-[1-^{14}C]-arachidonoylglycerophosphocholine was used as substrate. Mesangial cell $cPLA_2$ activity was determined by incubating sonicated substrate vesicles (2 μM 1-stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine [5 nCi], 1 μM *sn*-1,2-dioleoylglycerol) with 50 mM Hepes, pH 7.4, 1 mg/ml fatty acid-free BSA, 2 mM mercaptoethanol, 1 mM $CaCl_2$, 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-^{14}C]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. IC_{50} values were determined graphically.

Monoclonal antibodies. Monoclonal antibodies against rat liver mitochondrial phospholipase A_2 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 A_2. Purification of PLA_2 from the culture supernatant of stimulated mesangial cells was done in a single immunoaffinity chromatography stop as previously described (52). An immunoaffinity matrix was prepared by coupling purified monoclonal anti-PLA_2 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 PLA_2 activity was measured in 25 μl aliquots of the fractions. The eluted PLA_2 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 [^{3}H]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 [^{3}H]arachidonic acid. Approximately 80–90% of the added [^{3}H]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 [^{3}H]arachidonic acid. The cells were then stimulated with purified PLA_2 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 [^{3}H]-arachidonic acid released from total incorporated radioactivity was calculated.

Determination of PGE_2 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 PLA_2 or vehicle for 30 min. At the end of an experiment, the medium was withdrawn and centrifuged. The supernatant was assayed for PGE_2 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 $sPLA_2$ attenuate cytokine-stimulated PGE_2 synthesis. Stimulation of mesangial cells by either IL-1α, IL-1β, or TNFα caused a release of PLA_2 activity in the medium, that was closely paralleled by an increased synthesis of PGE_2 (21). The enzyme secreted by mesangial cells has previously been identified as a group II PLA_2 based on its recognition by monoclonal antibodies raised against rat liver mitochondrial group II PLA_2 (22) and the fact that these antibodies did not recognize rat pancreatic group I PLA_2 (49). Binding experiments using this monoclonal antibody indicated that nearly all (> 93%) of the secreted PLA_2 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 PLA_2 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 PLA_2 activity as shown in Fig. 1. All three types of antibody preparations gave similar inhibitions of secreted $sPLA_2$ 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 PLA_2 activity. Antibody incubation attenuated IL-1β- (Fig. 2) and TNFα- (Fig. 3) stimulated PGE_2 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 PGE_2 synthesis is mediated by the expression and secretion of $sPLA_2$ 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 $sPLA_2$ secretion by mesangial cells presumably via their F_c-part (data not shown).

A specific $sPLA_2$ inhibitor attenuates cytokine-stimulated PGE_2 synthesis. To further substantiate the role of $sPLA_2$ in cytokine-induced PGE_2 formation, we made use of a recently described specific inhibitor of $sPLA_2$, i.e., CGP 43182. CGP 43182 was found to potently inhibit isolated group II PLA_2

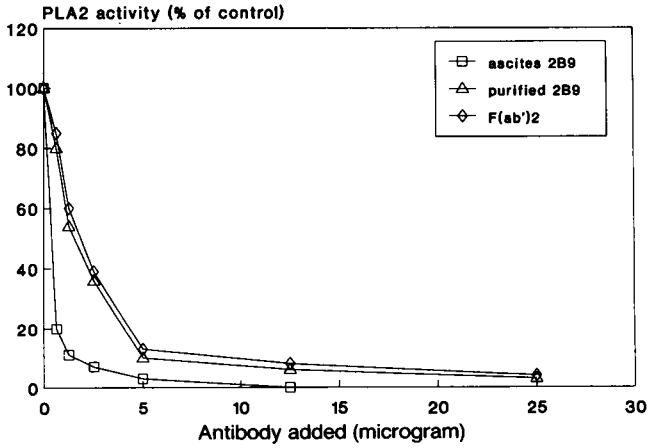

Figure 1. Antibody titration of $sPLA_2$ secreted by mesangial cells. Mesangial cells were stimulated with IL-1β (1 nM) for 24 h and 50 μl of the cell culture supernatant was incubated with the indicated amount of monoclonal $sPLA_2$ antibody (□) ascites fluid; (△) affinity purified $sPLA_2$ antibody; (◇) $F(ab')_2$ fractions of affinity-purified antibody for 15 min at 37°C. The residual PLA_2 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.

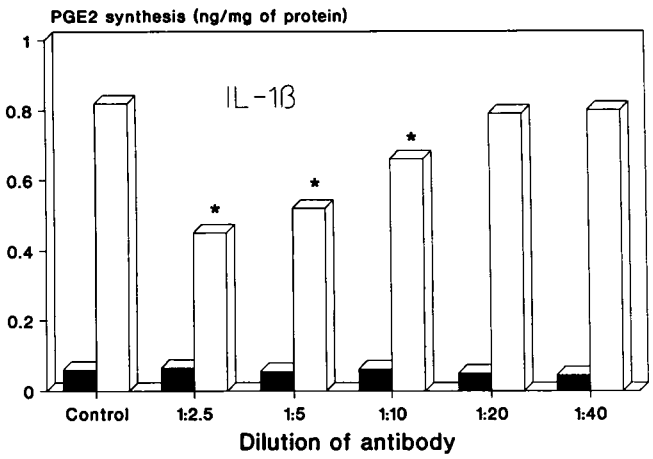

Figure 2. Inhibition of IL-1β-stimulated PGE_2 synthesis by $sPLA_2$ antibodies. Mesangial cells were stimulated with (*open bars*) or without (*closed bars*) IL-1β (1 nM) for 24 h in the presence of the indicated dilutions of $F(ab')_2$ fractions of affinity-purified $sPLA_2$ antibodies, in a total volume of 500 μl of medium. PGE_2 was determined in the culture supernatant and is expressed as ng PGE_2/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.

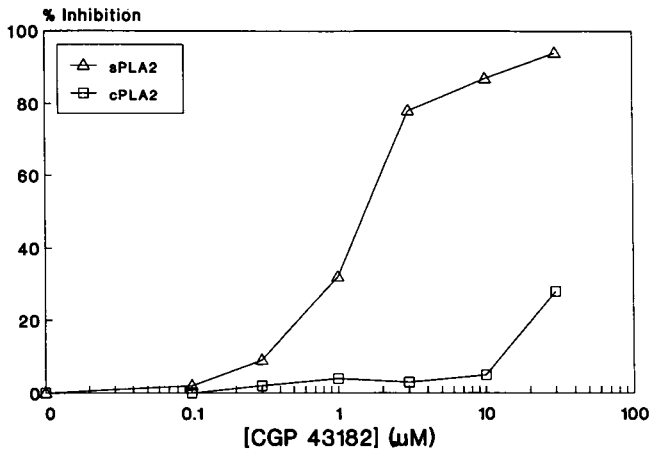

Figure 4. Inhibition of mesangial cell $sPLA_2$ and $cPLA_2$ activities by CGP43182. Culture supernatant of mesangial cells stimulated with IL-1β (1 nM) was incubated with the indicated concentrations of CGP43182 and assayed for $sPLA_2$ activity ($\triangle$) as described in Materials. Cytosolic preparations of mesangial cells were incubated with CGP43182 and assayed for $cPLA_2$ activity ($\square$). Results are expressed as % inhibition of control activity measured in the absence of CGP43182.

from human neutrophils with an IC_{50} of 1.2 μM without having any effect on $cPLA_2$ activity isolated from the human monoblast U937 cell line in concentrations up to 30 μM. The compound is thought to directly inhibit $sPLA_2$ 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 $sPLA_2$ activity secreted by mesangial cell with an IC_{50} of 1.5 μM. In contrast, $cPLA_2$ activity in mesangial cell cytosolic preparations was only slightly attenuated by 28% at the highest concentration tested (30 μM). Thus, CGP 43182 displays a high selectivity also for mesangial cell $sPLA_2$ as compared to $cPLA_2$. When added to intact mesangial cells, CGP 43182 potently inhibited PGE_2 production observed after a 24-h stimulation with IL-1β (1 nM) or TNFα (1 nM) with IC_{50} values of 1.3 μM and 1.0 μM, respectively (Fig. 5). At 10 μM, CGP 43182 suppressed approximately 70% of IL-1β- or TNFα-evoked PGE_2 formation (Fig. 5), thus providing further evidence for a crucial role of $sPLA_2$ in cytokine-induced eicosanoid synthesis in mesangial cells.

Purified mesangial cell $sPLA_2$ induces arachidonic acid release and E_2 synthesis in glomerular cells. In a third experimental approach, we have purified $sPLA_2$ secreted by cytokine-stimulated mesangial cells to homogeneity in a single immunoaf-

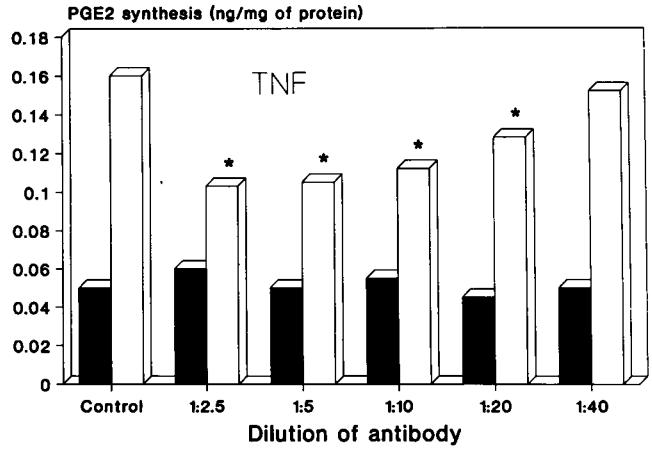

Figure 3. Inhibition of TNFα-stimulated PGE_2 synthesis by $sPLA_2$ antibodies. Mesangial cells were stimulated with (*open bars*) or without (*closed bars*) TNFα (1 nM) for 24 h in the presence of the indicated dilutions of $F(ab')_2$ fractions of affinity-purified $sPLA_2$ antibodies, in a total volume of 500 μl of medium. PGE_2 was determined in the culture supernatant and is expressed as ng PGE_2/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.

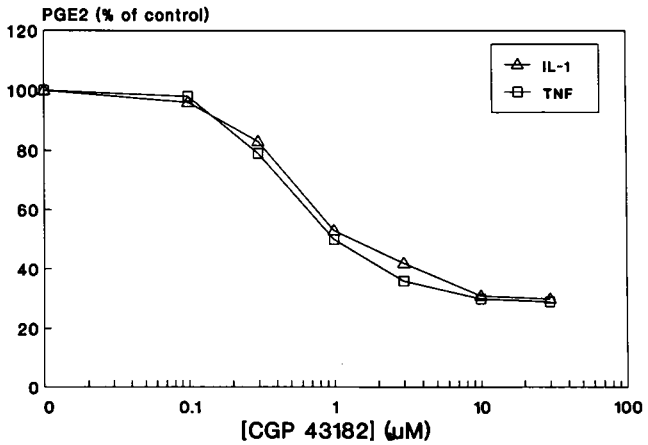

Figure 5. Inhibition of IL-1β- and TNFα-stimulated PGE_2 synthesis by CGP43182. Mesangial cells were stimulated with IL-1β (1 nM, $\triangle$) or TNFα (1 nM: $\square$) in the presence of the indicated concentrations of CGP43182. PGE_2 was determined in the culture supernatants and is expressed as percent 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 $sPLA_2$ (52). We subsequently used this purified mesangial cell $sPLA_2$ 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 $sPLA_2$ induced a marked increase in arachidonic acid release (Fig. 6) and PGE_2 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 $sPLA_2$ with a 88% increase in arachidonic acid release and an approximately fivefold increase in PGE_2 synthesis. Addition of $sPLA_2$ 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 $sPLA_2$ addition as shown in Fig. 8, well in accordance with previously published data (53). Co-incubation of mesangial cells with A23187 and $sPLA_2$ resulted in an additive response of arachidonic acid release, which was increased by 258±12%. The stimulatory action of purified $sPLA_2$, but not of A23187, on arachidonic acid release and PGE_2 synthesis was abolished by pretreatment of the enzyme with 10 μM of the specific group II PLA_2 inhibitor CGP 43182 (Fig. 8).

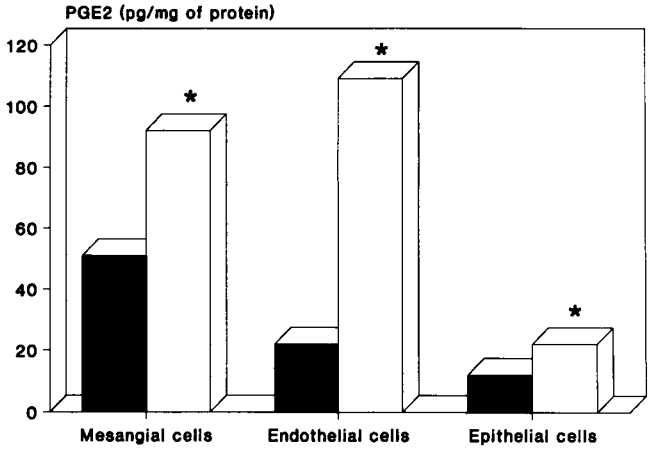

Figure 7. PGE_2 synthesis in glomerular cells stimulated by purified mesangial cell $sPLA_2$. Glomerular mesangial cells, endothelial cells, and epithelial cells were incubated with purified mesangial cell $sPLA_2$ (1.2 μg/ml, *open bars*) or vehicle (*closed bars*) for 30 min. The formation of PGE_2 was determined in the culture supernatant. Results are expressed as pg PGE_2/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.

Discussion

PLA_2 is the primary enzyme regulating arachidonic acid release and subsequent PG synthesis in many cell types. Two main classes of PLA_2 have been described, the secretory low molecular weight PLA_2s and intracellular cytosolic forms of PLA_2. Both types of PLA_2's have been reported to occur in mesangial cells. We were the first to describe the induction and secretion of $sPLA_2$ from mesangial cells in response to IL-1α, IL-1β, and TNFα. The release of $sPLA_2$ was paralleled by a drastic increase in PGE_2 synthesis (21). Furthermore, IL-1β and TNFα exerted a priming effect on PGE_2 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 $sPLA_2$ 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 $sPLA_2$ that cytokines induce a group II PLA_2 in mesan-

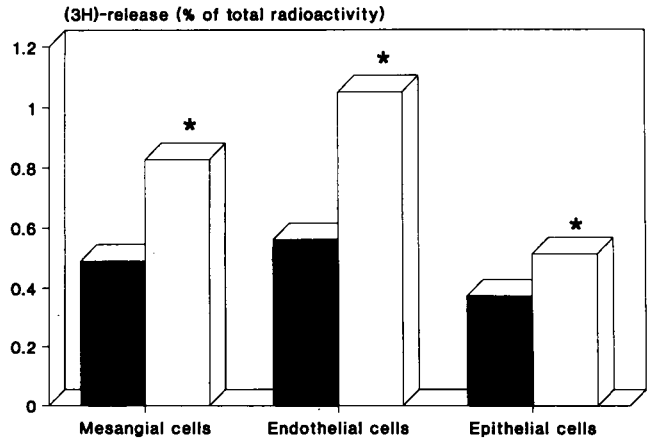

Figure 6. Arachidonic acid release from glomerular cells stimulated by purified mesangial cell $sPLA_2$. Glomerular mesangial cells, endothelial cells, and epithelial cells were labeled with [^{3}H]arachidonic acid and then stimulated with purified mesangial cell $sPLA_2$ (1.2 μg/ml, open bars) or vehicle (closed bars) for 30 min. The release of [^{3}H]arachidonic acid was determined as outlined in Materials. Results are expressed as percentage [^{3}H]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.

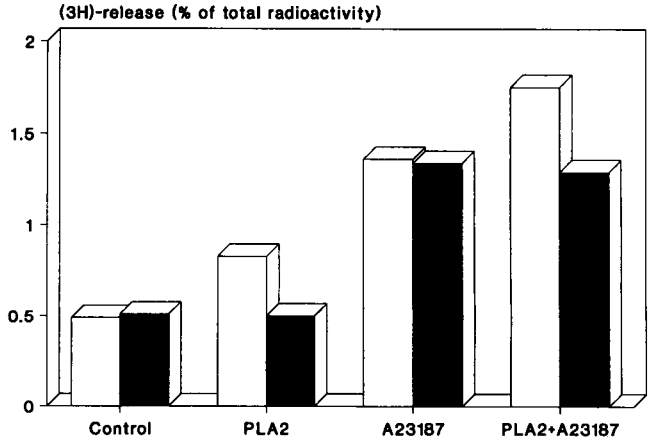

Figure 8. Arachidonic acid release from mesangial cells by purified $sPLA_2$ and Ca^{2+} ionophore. Glomerular mesangial cells were labeled with [^{3}H]arachidonic acid and then stimulated with purified mesangial cell $sPLA_2$ (1.2 μg/ml, *open bars*) or $sPLA_2$ in the presence of CGP 43182 (10 μM, *closed bars*). The release of [^{3}H]arachidonic acid was measured after 30 min incubation as described in the Materials section. Results are expressed as percentage [^{3}H]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 PLA_2 mRNA expression in IL-1α and IL-1β-stimulated mesangial cells (28, 29) that was sustained for at least 60 h (56).

A new family of high molecular weight PLA_2s ($cPLA_2$) 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 PLA_2 (31). A role for protein kinase C in the regulation of mesangial cell $cPLA_2$ 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 $cPLA_2$ activity in mesangial cells. Whereas activation of $cPLA_2$ 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 $cPLA_2$ 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 $cPLA_2$ activation in mesangial cells.

The constitutive expression of $cPLA_2$ 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 $sPLA_2$ 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 PLA_2 that may be responsible for the excessive formation of prostaglandins observed in cells chronically exposed to cytokines, such as IL-1 or TNFα. However, this concept has to be modified since we and others (40, 41, 61, 62) recently demonstrated that chronic treatment with IL-1β, TNFα, TGFβ_2 enhanced $cPLA_2$ activity in mesangial cells, rheumatoid synovial fibroblasts, lung fibroblasts, and the epithelial carcinoma cell line HEp-2. The IL-1-induced increase in $cPLA_2$ activity is due to increased levels of $cPLA_2$, mRNA, and protein, as well as to posttranslational modifications, i.e., phosphorylation of $cPLA_2$ by a yet unidentified kinase (62). Thus, IL-1 induces both synthesis and secretion of $sPLA_2$ and enhanced $cPLA_2$ activity in mesangial cells, and the contribution of both enzymes to arachidonic acid release and subsequent PGE_2 synthesis was not clear.

The contribution of $sPLA_2$ to PGE_2 synthesis in mesangial cells is supported by the suppression of IL-1β-stimulated $sPLA_2$ and PGE_2 synthesis by glucocorticoids (56, 63) and by TGFβ_2 when the cells are pretreated by TGFβ_2 prior to stimulation (52). However, dexamethasone has been shown to inhibit TNFα-triggered increase in $cPLA_2$ activity in the epithelial carcinoma cell line HEp-2 cells (41) and also inhibits $cPLA_2$ activity in mesangial cells (Schalkwijk, C., unpublished observations). The fact that $sPLA_2$ 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 $sPLA_2$, strongly suggests that $sPLA_2$ contributes to cytokine-induced PGE_2 production. The data provided in this report are in line with this view and indicate a marked contribution of $sPLA_2$ to IL-1β- and TNFα-stimulated arachidonic acid release, and to PGE_2 synthesis in mesangial cells. From our antibody neutralization experiments we conclude that at least 50% of the cytokine-induced PGE_2 synthesis is due to extracellular $sPLA_2$ activity. Moreover, the complete inactivation of extracellular $sPLA_2$ and the small portion of $sPLA_2$ remaining in the cells by the specific inhibitor CGP 43182 causes an $\sim$ 70% inhibition of PGE_2 synthesis. Thus, some 50–70% of IL-1β or TNFα-evoked eicosanoid synthesis is due to $sPLA_2$ activity, at least in mesangial cells. Classically, eicosanoid synthesis is regulated by the availability of arachidonic acid released by PLA_2. 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 $sPLA_2$ to unstimulated mesangial cells only increased PGE_2 production twofold (Fig. 7), whereas IL-1 triggered a 15-fold stimulation of PGE_2 formation in the cells (Fig. 2). Co-induction of $sPLA_2$ and cyclooxygenase may result in a synergistically increased synthesis of PGE_2 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 $sPLA_2$ 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, $sPLA_2$ 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 $sPLA_2$ (Figs. 6 and 7).

The participation of $sPLA_2$ in arachidonic acid release has also been reported for other cellular systems. Rat $sPLA_2$ added exogenously to calcium ionophore-activated HL-60 granulocytes augmented their production of PGE_2 (71). Murakami et al. (72) reported that rat mast cells, sensitized by IgE and exposed to antigen and $sPLA_2$, markedly increase PGD_2 production. Both the generation of PGD_2 and the release of arachidonic acid were abolished by inhibitors of $sPLA_2$. Mouse fibroblasts stably transfected with a human $sPLA_2$-cDNA and overexpressing $sPLA_2$ displayed an enhanced stimulated arachidonic acid release (73). Moreover, injection of pure recombinant human $sPLA_2$ into the joint space of healthy rabbits elicited a dramatic increase in PGE_2 production and an inflammatory, arthritogenic response (74). Exogenously applied $sPLA_2$ is thought to liberate fatty acids from phospholipids, which are probably located in the outer leaflet of the plasma membrane. Whether $sPLA_2$ 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-1β and TNFα-stimulated PGE_2 synthesis (Figs. 2 and 3), suggesting that secreted $sPLA_2$ is able to hydrolyze plasma membrane phospholipids. Furthermore, the released arachidonic acid can be used by the cells for prostaglandin synthesis. Alternatively, exogenous $sPLA_2$ may activate cellular phospholipases, e.g., $cPLA_2$, which subsequently liberate arachidonic acid for eicosanoid synthesis. In a simple mechanistic model, $sPLA_2$-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 $sPLA_2$-mediated activation of protein kinase C (76). *Cis*-unsaturated fatty acids, which are produced from phospholipids by the action of a nonselective-type of PLA_2, such as $sPLA_2$, 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-diacyglycerol-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 $cPLA_2$ activity in mesangial cells (31), there may be a cross-communication between $sPLA_2$ and $cPLA_2$ pathways. Further work is required to elucidate the complex network of interaction between $sPLA_2$ and $cPLA_2$-triggered mediator release in mesangial cells to evaluate and establish new therapeutic concepts for the treatment of glomerular inflammatory reactions.

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