

## PDGF suppresses the activation of group II phospholipase A<sub>2</sub> gene expression by interleukin 1 and forskolin in mesangial cells

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Treatment of rat mesangial cells with interleukin 1 $\beta$  (IL-1 $\beta$ ) and forskolin greatly enhanced the expression of group II phospholipase A<sub>2</sub> (PLA<sub>2</sub>) mRNA, with subsequent increased synthesis and secretion of PLA<sub>2</sub>, as detected by PLA<sub>2</sub> activity measurements and immunoprecipitation of culture media of [<sup>35</sup>S]methionine-labelled mesangial cells. PDGF-BB dose-dependently suppressed the IL-1 $\beta$ - and forskolin-induced elevation of PLA<sub>2</sub> mRNA, as well as PLA<sub>2</sub> synthesis and secretion. In contrast, PDGF-AA had no inhibitory effect. The tyrosine kinase inhibitor genistein dose-dependently antagonized the inhibitory effect of PDGF-BB on IL-1 $\beta$ -stimulated PLA<sub>2</sub> secretion, thus suggesting that tyrosine phosphorylation may be required for PDGF-BB inhibition of PLA<sub>2</sub> gene expression in mesangial cells.

Phospholipase A<sub>2</sub>, Interleukin 1, Phospholipase A<sub>2</sub> mRNA, PDGF, Genistein, Mesangial cell

### 1 INTRODUCTION

Arachidonic acid release from the sn-2 position of phospholipids by PLA<sub>2</sub> activation is thought to be the rate-limiting step of eicosanoid synthesis in many cells [1,2]. But PLA<sub>2</sub> not only plays an important intracellular role, it is also secreted from a variety of cells during inflammatory processes and may thereby contribute to the pathological alterations observed [3]. PLA<sub>2</sub>s comprise a diverse group of enzymes that can be divided into a class of high mol. wt. PLA<sub>2</sub>s of 60–110 kDa and a class of low mol. wt. PLA<sub>2</sub>s of 14 kDa. Members of both classes from human and rat sources have recently been cloned [4–8]. The low mol. wt. PLA<sub>2</sub>s are further divided into group I (the pancreatic type of PLA<sub>2</sub>) and group II PLA<sub>2</sub> [9]. Group II PLA<sub>2</sub> is synthesized and secreted from many tissues and cell types and is believed to play a role in the initiation and propagation of inflammatory processes [3]. In addition, potent pro-inflammatory cytokines, such as IL-1 or tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) have been found to increase group II PLA<sub>2</sub> gene expression in rabbit chondrocytes [10,11], rat vascular smooth muscle cells [12] and HepG2 cells [13].

Glomerular mesangial cells are being increasingly recognized as important target and effector cells in the pathogenesis of acute and chronic forms of renal dis-

eases [14]. Recently we have shown that stimulation of mesangial cells with IL-1 $\alpha$ , IL-1 $\beta$ , TNF $\alpha$  or forskolin increases the synthesis and secretion of prostaglandin E<sub>2</sub> and group II PLA<sub>2</sub> [15–17]. Extracellular PLA<sub>2</sub> secreted by mesangial cells may participate in inducing and maintaining inflammatory reaction within the glomerulus. The latter suggestion gains support from our recent observations, demonstrating that anti-inflammatory steroids and transforming growth factor  $\beta$ <sub>2</sub> potentially antagonize IL-1 $\beta$ - and TNF $\alpha$ -induced PLA<sub>2</sub> synthesis [18–20].

Platelet-derived growth factor (PDGF) is a dimeric protein composed of two closely related but not identical chains, denoted A and B, which are linked by disulphide bonds. All three possible dimeric molecules of PDGF have been isolated from natural sources. We report here that PDGF-BB is another potent inhibitor of IL-1 $\beta$  and forskolin-induced PLA<sub>2</sub> synthesis acting at the level of PLA<sub>2</sub> gene expression.

### 2. MATERIALS AND METHODS

#### 2.1 Cell cultures and incubation

Rat glomerular mesangial cells were cultivated as described previously [21]. In a second step, single cells were cloned by limiting dilution using 96-microwell plates. Clones with apparent mesangial cell morphology were characterized [22] and used for further processing. The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml) and bovine insulin at 0.66 U/ml. For the experiments passages 12–41 of mesangial cells were used. Confluent mesangial cells cultured in 16 mm diameter wells or 60 mm diameter dishes (for PLA<sub>2</sub> immunoprecipitation), were washed twice with PBS and incubated with 1 ml of RPMI 1640, containing 0.1 mg/ml of fatty acid-free bovine serum albumin (Sigma)

**Abbreviations:** PLA<sub>2</sub>, phospholipase A<sub>2</sub>; IL-1 $\beta$ , interleukin 1 $\beta$ ; PDGF, platelet derived growth factor.

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with or without agents for the indicated time periods. Thereafter, the medium was withdrawn and centrifuged for 10 min at 1000 rpm in an IEC Centra-7R lab centrifuge. The supernatant was removed and frozen in liquid nitrogen and stored until assayed for protein content and PLA<sub>2</sub> activity. Protein was determined by the method of Bradford [23] with bovine serum albumin as the standard. 80  $\mu$ Ci of [<sup>35</sup>S]methionine were added to 2 ml of methionine-free culture medium for the labelling of newly synthesized proteins. The incubation time was 6 h.

## 2.2 Phospholipase A<sub>2</sub> assay

PLA<sub>2</sub> activity was determined using [1-<sup>14</sup>S]oleate-labelled *E. coli* as substrate as described [24]. Assay mixtures (1.0 ml) contained 100 mM Tris-HCl (pH 7.4), 1.0 mM CaCl<sub>2</sub>, 5 mM *E. coli* phospholipid (3000–5000 cpm) and the enzyme to be tested at a dilution producing approximately 5% substrate hydrolysis. Reactions were stopped after 1 h and the liberated [<sup>14</sup>S]oleate was extracted by a modified Dole extraction procedure [24].

## 2.3 Immunoprecipitation

For immunoprecipitation, 1 ml of culture supernatant was added to 6 ml of buffer A (20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride. After addition of 10  $\mu$ l of antibodies [25,26] and incubation at 4°C overnight, the antigen-antibody complexes were bound to 10 mg (dry weight) of a mixture of protein A-Sepharose and protein G-Sepharose, washed four times with buffer A and twice with 50 mM sodium phosphate buffer, pH 7.5. Elution was routinely achieved by incubation with 0.1 M Tris-HCl, pH 6.8, 5% mercaptoethanol, 5% sodium dodecyl sulfate, and 10% glycerol at 95°C for 3 min. The eluted proteins were analyzed by SDS-PAGE using a 10–15% polyacrylamide step gel [27] and subjected to fluorography [28].

## 2.4 Northern blot analysis

Confluent mesangial cells were harvested from 60 mm diameter culture dishes with a rubber policeman. Total RNA was extracted from the cells using the guanidinium thiocyanate/phenol/chloroform method [29]. The RNA was precipitated with isopropyl alcohol, the pellets were washed with 75% ethanol, and samples of 15  $\mu$ g RNA were separated on 1% agarose gels containing 6.6% formaldehyde prior to transfer to Gene Screen membranes (New England Nuclear) [30]. After baking the filters at 80°C for 2 h and prehybridization for 6 h, the filters were hybridized with a 39-mer antisense oligonucleotide for rat group II PLA<sub>2</sub> (nucleotide 58–96, [6]), that had been labelled with T4-polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP [30]. To correct for variations in RNA amount a parallel filter was hybridized with a genomic clone for rat 28 S ribosomal RNA [31], labelled with the random primer technique described by Feinberg and Vogelstein [32]. After hybridization, the membrane was washed 3 times in 4 × SSPE, 0.1% sodium dodecyl sulfate at 50°C for 20 min (20 × SSPE: 3.6 M sodium chloride, 0.2 M sodium phosphate, 0.02 M EDTA, pH 7.4). The signal was detected by autoradiography.

## 2.5 Chemicals

[1-<sup>14</sup>C]oleic acid and nylon membranes (Gene Screen) were purchased from DuPont de Nemours International (Regensdorf, Switzerland), adenosine 5'-[ $\gamma$ -<sup>32</sup>P]triphosphate (specific activity >110 TBq/mM) and [<sup>35</sup>S]methionine (specific activity >37 TBq/mM) were purchased from Amersham International (UK), forskolin was from Calbiochem (Lucerne, Switzerland), recombinant human IL-1 $\beta$  was prepared by the Biotechnology Department of Ciba-Geigy Ltd (Basel, Switzerland), recombinant human PDGF-AA and -BB were kindly donated by Dr M. Pech from F. Hoffmann-La Roche Ltd (Basel, Switzerland), prestained markers were from Bio-Rad Laboratories (Glattbrugg, Switzerland), T4-polynucleotide kinase and all cell culture nutrients were from Boehringer-Mannheim (Germany), and all other chemicals used were from either Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland).

## 3 RESULTS

As already shown previously, mesangial cells secrete low amounts of PLA<sub>2</sub> into the culture medium, and this secretion of PLA<sub>2</sub> is markedly stimulated by IL-1 $\beta$  (Table I). Addition of PDGF-BB (100 ng/ml) significantly inhibited the basal release of PLA<sub>2</sub> from mesangial cells. Furthermore, PDGF-BB dose-dependently attenuated the stimulatory effect of IL-1 $\beta$  on PLA<sub>2</sub> secretion. In contrast, PDGF-AA at 100 ng/ml had no effect on both basal and cytokine-induced PLA<sub>2</sub> release.

To study the biosynthesis of PLA<sub>2</sub>, mesangial cells were stimulated with IL-1 $\beta$  and forskolin (10  $\mu$ M) for 22 h and [<sup>35</sup>S]methionine was added for the last 6 h of the stimulation periods. A combination of IL-1 $\beta$  and forskolin has been shown to synergistically stimulate PLA<sub>2</sub> secretion from mesangial cells [16,17]. PLA<sub>2</sub> was immunoprecipitated from the medium with a monoclonal group II PLA<sub>2</sub> antibody [25,26] and subjected to SDS-PAGE. As shown in Fig. 1, no 14 kDa group II PLA<sub>2</sub> could be detected in the culture supernatants of unstimulated cells. Addition of IL-1 $\beta$  plus forskolin markedly stimulated the synthesis of PLA<sub>2</sub>, an effect that was dose-dependently antagonized by PDGF-BB (Fig. 1).

Northern blot analysis using an oligonucleotide probe for group II PLA<sub>2</sub> revealed that the amount of group II PLA<sub>2</sub> mRNA was massively increased by IL-1 $\beta$  plus forskolin as compared to the levels found in control cells (Fig. 2). Addition of PDGF-BB potentially antagonized IL-1 $\beta$  plus forskolin-induced PLA<sub>2</sub> mRNA accumulation as shown in Fig. 2.

In order to get some hints on the mechanisms of PDGF-BB inhibition of cytokine-induced PLA<sub>2</sub> secre-

Table I

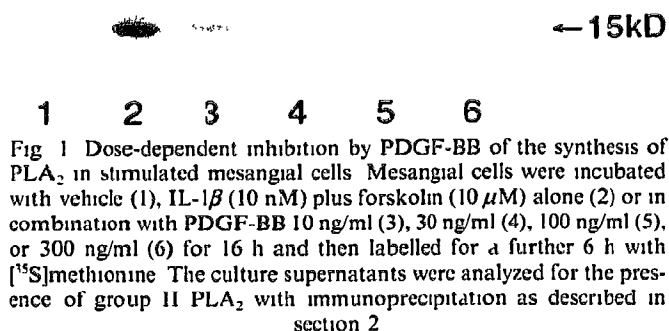
Effects of PDGF-homodimers on IL-1 $\beta$ -stimulated PLA<sub>2</sub> secretion from mesangial cells

Addition	PLA <sub>2</sub> secretion (cpm/100 $\mu$ l)
Control	321 $\pm$ 22
PDGF-BB (100 ng/ml)	201 $\pm$ 19*
PDGF-AA (100 ng/ml)	288 $\pm$ 22
IL-1 $\beta$ (1 nM)	5695 $\pm$ 746*
IL-1 $\beta$ (1 nM) + PDGF-BB (1 ng/ml)	5695 $\pm$ 385
IL-1 $\beta$ (1 nM) + PDGF-BB (10 ng/ml)	4571 $\pm$ 195
IL-1 $\beta$ (1 nM) + PDGF-BB (50 ng/ml)	1382 $\pm$ 95**
IL-1 $\beta$ (1 nM) + PDGF-BB (100 ng/ml)	744 $\pm$ 27**
IL-1 $\beta$ (1 nM) + PDGF-AA (100 ng/ml)	6435 $\pm$ 200

Confluent mesangial cells were incubated with the indicated concentrations of IL-1 $\beta$ , PDGF-AA or PDGF-BB for 24 h. Thereafter, the medium was withdrawn and PLA<sub>2</sub> activity determined as described in section 2. Results are means  $\pm$  SE (n=4).

\*P<0.05 versus control.

\*\*P<0.05 versus IL-1 $\beta$  (Student's *t*-test).



tion, we investigated the effect of genistein, a tyrosine kinase inhibitor, that is known to be active against the PDGF receptor tyrosine kinase [33]. The data in Table II show that genistein completely reversed the inhibitory action of PDGF-BB on IL-1 $\beta$ -stimulated PLA<sub>2</sub> secretion from mesangial cells.

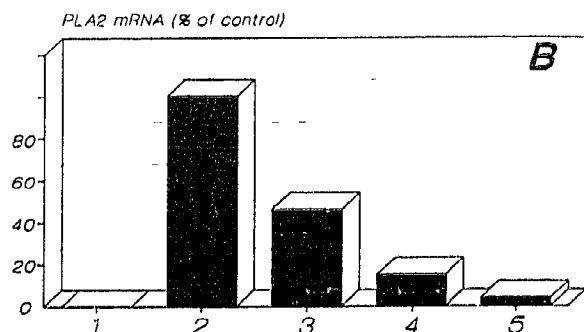
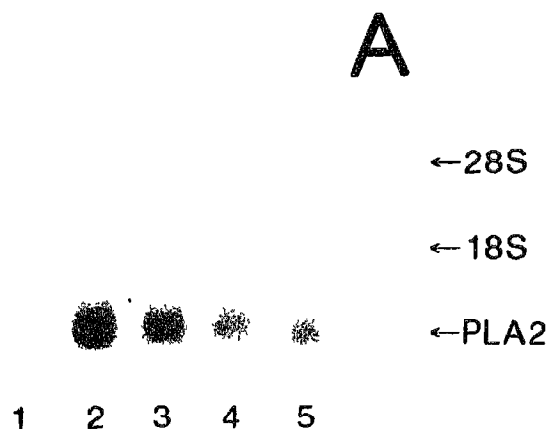


Fig 2 Effect of PDGF-BB on group II PLA<sub>2</sub> mRNA levels in stimulated mesangial cells. (A) Mesangial cells were incubated with vehicle (1), IL-1 $\beta$  (10 nM) plus forskolin (10  $\mu$ M) alone (2) or in combination with PDGF-BB 10 ng/ml (3), 100 ng/ml (4), or 300 ng/ml (5) for 21 h. Cellular RNA (15  $\mu$ g) was analyzed by Northern blotting as described in section 2. (B) For quantitation, autoradiograms of the filters were scanned densitometrically and expressed as a percentage of RNA found in maximally stimulated cells.

#### 4. DISCUSSION

Previously we have shown that pro-inflammatory cytokines, such as IL-1 $\alpha$ , IL-1 $\beta$ , and TNF $\alpha$  as well as cAMP elevating agents, stimulate the synthesis and secretion of group II PLA<sub>2</sub> from mesangial cells [15–19]. Addition of actinomycin D or cycloheximide completely suppressed the basal, as well as the cytokine- and forskolin-stimulated release of PLA<sub>2</sub> [15,16]. These results, together with a lag period of several hours before the onset of PLA<sub>2</sub> secretion, indicated that RNA synthesis and protein synthesis are necessary for the observed enzyme secretion [15–17]. In the present study, we demonstrate that IL-1 $\beta$  and forskolin indeed stimulate group II PLA<sub>2</sub> gene expression in mesangial cells (Fig. 2). Increased levels of group II mRNA could be detected within 4 h after addition of IL-1 $\beta$  plus forskolin, and maximum levels were obtained between 22 h and 40 h after onset of stimulation (H. Muhl, T. Geiger and J. Pfeilschifter, unpublished observations). An increased synthesis and secretion of PLA<sub>2</sub> after stimulation with IL-1, TNF or cAMP have also been reported for vascular smooth muscle cells [12,34] and were found to be due to augmented group II PLA<sub>2</sub> gene expression [12].

The main finding of the present report is the potent inhibition of IL-1 $\beta$ - and forskolin-induced PLA<sub>2</sub> gene expression by PDGF-BB (Fig. 2). This inhibitory action of PDGF-BB was also evident from the marked reduction of PLA<sub>2</sub> protein (Fig. 1) and PLA<sub>2</sub> activity (Table I) in the culture supernatant of stimulated mesangial cells. The tyrosine kinase inhibitor genistein reversed the inhibitory effect of PDGF-BB on IL-1 $\beta$  and forskolin-stimulated PLA<sub>2</sub> secretion, thus suggesting that tyrosine phosphorylation may be required for PDGF-BB inhibition of PLA<sub>2</sub> gene expression in mesangial cells.

Table II

Effects of genistein on PDGF-BB inhibition of IL-1 $\beta$ -stimulated PLA<sub>2</sub> secretion from mesangial cells

Addition	PLA <sub>2</sub> secretion (cpm/100 $\mu$ l)
Control	537 $\pm$ 57
IL-1 $\beta$	9331 $\pm$ 882
IL-1 $\beta$ + PDGF-BB	1439 $\pm$ 87*
IL-1 $\beta$ + PDGF-BB + Genistein (1 $\mu$ M)	1355 $\pm$ 89
IL-1 $\beta$ + PDGF-BB + Genistein (10 $\mu$ M)	2472 $\pm$ 122**
IL-1 $\beta$ + PDGF-BB + Genistein (50 $\mu$ M)	10826 $\pm$ 402**

Confluent mesangial cells were incubated with IL-1 $\beta$  (1 nM), PDGF-BB (100 ng/ml) or vehicle together with the indicated concentrations of genistein for 24 h. Thereafter, the medium was withdrawn and PLA<sub>2</sub> activity determined as described in Section 2. Results are means  $\pm$  SE ( $n=4$ ).

\* $P < 0.05$  versus IL-1 $\beta$ .

\*\* $P < 0.05$  versus IL-1 $\beta$  + PDGF-BB (Student's  $t$ -test).

In contrast, PDGF-AA had no significant effect on PLA<sub>2</sub> induction in mesangial cells. This may be explained by the observation that mesangial cells exclusively bind the BB homodimer of PDGF and respond only to the BB isoform in terms of phosphoinositide turnover, prostaglandin and DNA synthesis [35], thus suggesting that mesangial cells possess only the  $\beta$ -type of PDGF receptor. Abboud and colleagues [36,37] have reported that mesangial cells express both PDGF A-chain and B-chain mRNAs, release a PDGF-like factor and proliferate in response to PDGF, thus suggesting a possible autocrine function for PDGF in glomerular pathophysiology. In addition, an up-regulation of PDGF receptors has been reported for inflamed kidney as compared to normal kidney [38]. Inhibition of cytokine-induced PLA<sub>2</sub> secretion by PDGF may represent an attenuating mechanism for the acute inflammatory process. Extracellular PLA<sub>2</sub> is vasoactive and pro-inflammatory when administered by different routes (for review see [3]). PDGF may act in line with TGF $\beta$  [20] and glucocorticoids [18,19] to protect the kidney from damage resulting from cytokine-stimulated mediator release and subsequent inflammatory reactions. In a manner reminiscent of TGF $\beta$  [20], PDGF-BB inhibits cytokine-induced PLA<sub>2</sub> expression and secretion (this paper), but potentiates cytokine-stimulated prostaglandin synthesis [39]. TGF $\beta$  has been reported to modulate the expression of PDGF receptor  $\alpha$ - and  $\beta$ -subunits in fibroblasts and smooth muscle cells [40,41]. Further experiments are required to investigate whether this also applies to our cell culture model. Thus, mesangial cells may provide a model system to study the cross-talk between both cytokines and polypeptide growth factors.

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