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# Regulation of platelet-derived growth factor isoform-mediated expression of prostaglandin G/H synthase in mesangial cells

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**Regulation of platelet-derived growth factor isoform-mediated expression of prostaglandin G/H synthase in mesangial cells.** Incubation of rat renal mesangial cells with platelet-derived growth factor (PDGF) -AB or -BB led to a transient increase in prostaglandin G/H synthase-2 (PGHS-2) mRNA expression with a maximum after two hours. Expression of PGHS-1 mRNA remained unchanged during short term incubation, but was enhanced about twofold after 8 to 12 hours incubation with PDGF-AB or -BB. Enhanced PGHS activity was still observed after 24 hours. Nevertheless, $PGE_2$ release from mesangial cells was not enhanced by PDGF, hinting to the availability of arachidonic acid as rate-limiting step. PDGF receptors are coupled to multiple signaling pathways, among them phospholipase $C_\gamma$. PDGF-BB rapidly phoshorylated $PLC_\gamma$, while phosphorylation by PDGF-AB was barely detectable. The differential effect of PDGF-BB and PDGF-AB was also seen with respect to calcium signaling: PDGF-BB but not PDGF-AB induced release of $Ca^{2+}$ from internal stores. Activation of PLC and the resulting transient release of $Ca^{2+}$ were not considered to be essential for PGHS-2 mRNA induction as both PDGF isoforms were equally effective in mRNA induction. Both PDGF isoforms led to a $Ca^{2+}$ influx resulting in a long lasting elevation of $[Ca^{2+}]_i$. Enhanced $[Ca^{2+}]_i$ seemed to be related to PGHS-2 mRNA expression, because PDGF-induced PGHS-2 mRNA was significantly reduced under $Ca^{2+}$ free conditions. Diacylglycerol, liberated by PLC, is an activator of protein kinase C (PKC). Down-regulation of PKC by overnight incubation with phorbol ester (0.1 $\mu$M) attenuated PGHS-2 mRNA induction by PDGF-AB and -BB. Involvement of PKC was substantiated by the PKC inhibitor H7, which interfered with PDGF-mediated PGHS-2 mRNA expression, while HA1004, a considerably specific inhibitor of protein kinases A and G, was without effect. Taken together, signaling pathways other than $PLC_\gamma$ seem to be involved in activation of PKC and elevation of $[Ca^{2+}]_i$, which were shown to be essential elements of PDGF-mediated induction of PGHS-2 mRNA expression in mesangial cells.

Platelet-derived growth factor (PDGF) plays a major role in the pathology of glomerular diseases [1–3]. Main target cells within the glomerulus are mesangial cells which are affected by PDGF in multiple ways. Biological effects of PDGF may include induction of proliferation, stimulation of chemotaxis and contraction, or synthesis and secretion of mediators of inflammation [4].

PDGF consists of two polypeptide chains which form three different isoforms, PDGF-AA, -AB and -BB [5]. Mesangial cells express mainly PDGF-A chains and secrete predominantly the AA isoform; the number of corresponding PDGF$\alpha\alpha$-receptors, however, is very low on mesangial cells [6]. This was taken as an explanation that PDGF-AA does not seem to be able to induce any biological response in mesangial cells [7, 8], whereas PDGF-AB and -BB are highly active.

Prostaglandins, especially $PGE_2$, are lipid mediators that are involved in the regulation of glomerular function under physiological and pathophysiological conditions [9]. As a vasodilator, $PGE_2$ is active as counterpart of vasoconstrictors such as endothelin, serotonin or even PDGF itself [10–13]. In the inflamed glomerulus, $PGE_2$ may act as immunomodulator and local hormone affecting the secretion and action of cytokines.

The biosynthesis of $PGE_2$ is mainly controlled at two levels, the liberation of the precursor fatty acid, arachidonic acid, from cellular membranes by phospholipases and by the abundance and activity of cyclooxygenases (prostaglandin G/H synthases, PGHS), which metabolize arachidonic acid yielding the immediate prostanoid precursor $PGH_2$ [14–16]. Two types of PGHS have been characterized in mesangial cells, PGHS-1, which is expressed constitutively in these cells, and PGHS-2, which is expressed at very low levels in resting mesangial cells but can be induced by a variety of stimuli including IL-1, serotonin or endothelin [17–19]. PGHS-2 was thus related to the enhanced $PGE_2$ synthesis observed in mesangial cells after prolonged incubation with these stimuli. Thus far, little information is available regarding the effect of PDGF on PGHS-2 expression. In fibroblasts [20] and megacaryocytic cells [21], PGHS was described to be induced by PDGF at a time, when specific probes for PGHS-2 were not yet available. Induction of a second isozyme was suggested and later shown in smooth muscle cells [22]. Induction of either PGHS isoform in mesangial cells by PDGF has not yet been described, although PDGF is one of the most important cytokines involved in mesangial cell activation in glomerular pathology [3].

Therefore, we investigated the effect of PDGF isoforms (PDGF-AA, -AB and -BB) on PGHS mRNA and protein expression in mesangial cells. The PDGF signaling pathway was studied with special reference to the involvement of phospholipase $C_\gamma$, intracellular calcium and protein kinase C in PGHS mRNA induction.

## Methods

### *Materials*

Recombinant PDGF isoforms were prepared as described before [23, 24]. Serotonin, calcium ionophore A23187, 12-O-tetradecanoylphorbol-13-acetate (TPA), Fura-2/AM, BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid)/AM

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and the anti-phosphotyrosine antibody were from Sigma. Antibodies directed against bovine phospholipase $C_{\gamma 1}$ were from UBI. Protein G Plus-agarose was obtained from Santa Cruz. Peroxidase-conjugated anti-mouse conjugate, biotinylated goat anti-mouse antibody, and the ECL Western blotting analysis system and detection reagent were from Amersham. ABTS (2,2′-azino-di-[3-ethylbenzthiazoline-6-sulfonate] was from Boehringer.

### *Cell culture*

Rat mesangial cells were isolated and cultured as described previously [25]. Cells were grown in DMEM supplemented with 2 mM L-glutamine, 5 μg/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS. For the experiments cells were used between passages 10 and 25. Mesangial cells ($0.5 \times 10^6$/10 ml) were plated in 100 mm Petri dishes in medium with 10% FCS. At subconfluency (after 3 to 5 days), cells were growth-arrested by serum deprivation in DMEM containing 0.5% FCS for three days and then stimulated as indicated.

### *Northern blot analysis*

Northern blot analysis was performed similarly as previously described [26]. After stimulation for the times indicated cells were scraped with a rubber policeman into chilled 50 ml polypropylene centrifuge tubes and spun down at 225 × g at 4°C. Each pellet was lysed by adding 300 μl guanidinium isothiocyanate-containing lysis solution and total RNA was extracted according to the protocol of Chomczynski and Sacchi [27] with minor alterations. RNA yield usually was 30 to 40 μg/Petri dish.

Separation of total RNA (10 μg/lane) was achieved by use of 1.2% agarose gels containing 2% (vol/vol) formaldehyde with 1xMOPS as gel/running buffer. Separated RNA was transferred to nylon membranes by capillary blotting and fixed by baking at 80°C for two hours.

Hybridization was performed with cDNA probes labeled with $^{32}$P-dCTP using the Megaprime random prime kit (Amersham) as described [26]. Specific PGHS-1 and PGHS-2 probes were 2.767 kb and 1.156 kb *Eco*RI fragments from the 5′-end of mouse cDNA, respectively [28]. Both PGHS probes were added simultaneously, since no cross hybridization was observed. The length of the mRNAs recognized was 3.3 kb (PGHS-1) and 5.0 kb (PGHS-2).

The GAPDH probe was obtained with a 500 bp reverse transcribed fragment with the following primers: 5′-AATGCATCCTGCACCACCAA (sense), 5′-GTCATTGAGAGCAATGCCAGC (antisense). DNA/RNA-hybrids were detected by autoradiography using Kodak X-OMAT AR film; exposure time ranged between one and seven days.

Quantitative analysis was performed by densitometric scanning of the autoradiographs (Bioprofil, Fröbel, Germany). All values were corrected for differences of RNA loading by calculating the ratio PGHS to GAPDH expression or ethidium bromide staining of 18S rRNA.

### *Determination of intracellular $Ca^{2+}$ levels*

Serum-deprived cells were washed twice in PBS and trypsinized with 0.05% trypsin/0.02% EDTA for 10 minutes. Trypsinization was stopped by addition of an equal volume of medium containing 10% FCS. After centrifugation cells were resuspended in medium containing 10% FCS ($10^7$ cells/ml) and incubated in a water bath (37°C, 5 min). Fura-2/AM was added (final concentration 4 μM) and incubation carried on for 15 minutes. Then the cell suspension was diluted fivefold with medium containing 10% FCS and incubation continued another 15 minutes. Thereafter, the cells were washed once with assay buffer (10 mM HEPES, 1 mM $NaH_2PO_4$, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5.5 mM glucose), and resuspended in assay buffer at $10^6$ cells/ml. During the experiments cells were kept at room temperature. Fluorescence measurements were made using a Kontron SFM25 spectral fluorometer. Cells were placed in a cuvette holder equipped with a magnetic stirrer. The excitation wavelength was set to 340 nm, and emission was monitored at 510 nm. Maximum and minimum fluorescence of fura-2 were determined by lysing the cells with 0.1% Triton X-100/phosphate-buffered saline and by adding 1.4 mM Tris (pH 8.4), 24 mM EGTA. The $K_d$ of fura-2 for $Ca^{2+}$ was assumed to be 225 nM [29]. Figures of fluorescence tracings are representative of three or more experiments performed over several days.

### *Immuneprecipitation*

Serum-deprived cells were stimulated with 20 ng/ml PDGF-AB or -BB for the times indicated. Immuneprecipitation was performed essentially as described [30]. In brief, cells were lysed with 900 μl lysis buffer (0.05 M Tris/HCl, pH 7.2, 0.15 M NaCl, 1% triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonylfluoride, 0.2 mM sodium orthovanadate, 14 μg/ml aprotinin). Plates were washed with another 350 μl lysis buffer and both lysates combined. Anti-bovine $PLC_{\gamma 1}$ antibody (0.04 μg/400 μml) was allowed to bind overnight at 4°C. Protein G PLUS-Agarose was used to precipitate the complexes (2 hr, 4°C). After centrifugation and washing immunecomplexes were dissolved in 20 μl loading buffer, and 6 μl thereof were analyzed by PAGE (3.5% stacking gel, 6% resolving gel). Protein was transferred onto Hybond ECL-nitrocellulose by semi-dry blotting. As primary antibodies anti-bovine $PLC_{\gamma 1}$ (0.1 μg/ml) and anti-phosphotyrosine (1:4000) were used and detected with an anti-mouse-POD conjugate (1:10000). Protein bands were detected with ECL detection reagent. The size of $PLC_{\gamma 1}$ was determined to be about 150 kDa by comparison with known standard proteins.

### *Western blot analysis*

Western blot analysis was performed as described previously [19]. In brief, microsomal protein was obtained by differential centrifugation from cells disrupted by sonication. After SDS-polyacrylamide gel electrophoresis and blotting, immunoreactive protein was detected with the ECL Western blotting analysis system. The primary antibodies specific for PGHS-1 and PGHS-2 were kindly provided by D. DeWitt (Michigan State University, East Lansing, MI, USA). The secondary peroxidase-conjugated swine anti-rabbit antibody was purchased from Dako.

### *Determination of $PGE_2$*

$PGE_2$ concentration in the cell culture supernatants was determined by a specific ELISA similarly as described [30–32] with modifications of D. Schaefer (Institut für Immunologie, Universität Erlangen/Nürnberg, Germany). In brief, microtiter plates were coated with $PGE_2$ coupled to BSA. As standards, 10 different concentrations of $PGE_2$ (500 pg to 0.98 pg) were used in triplicates. The specific monoclonal mouse-anti-$PGE_2$ antibody was provided by K. Brune (Institut für Pharmakologie, Universität

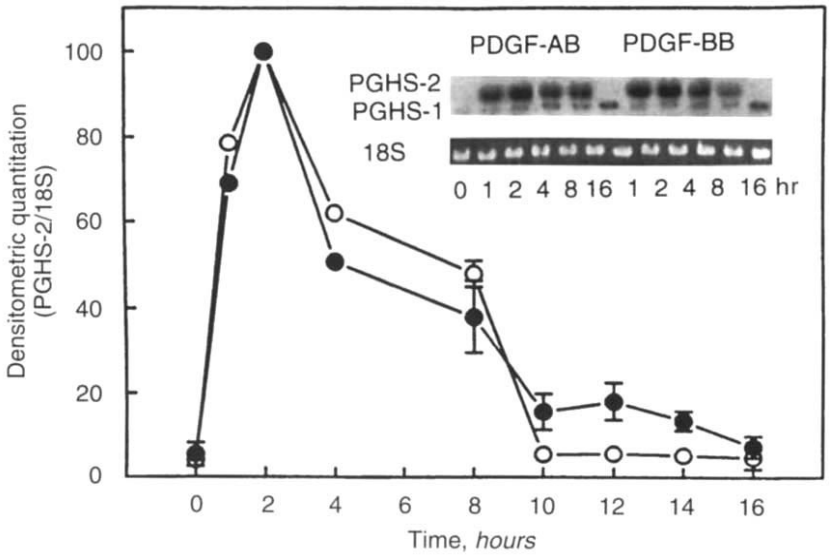


**Fig. 1.** *Induction of PGHS by PDGF-AB and PDGF-BB in mesangial cells.* Rat mesangial cells were incubated with 20 ng/ml PDGF-AB (●) or PDGF-BB (○) for the times indicated. PGHS mRNA was detected by Northern blot analysis as described in the **Methods** section. The insert shows one typical Northern blot. Densitometric data are means ± SEM of 2 to 3 experiments, with maximal expression (after 2 hr) set to 100%.

Erlangen/Nürnberg). Complexed antibody was detected by biotinylated goat-anti-mouse antibody and horseradish peroxidase complexed with streptavidin-biotin (Dako). Substrate was ABTS (2,2'-azino-di-[3-ethylbenzthiazoline-6-sulfonate]). Detection limit of the assay was 3 pg per well, corresponding to 0.03 ng/ml supernatant.

### *Determination of PGHS activity*

To determine PGHS activity, mesangial cells were incubated with 10 μM arachidonic acid for the last 15 minutes of the stimulation time. Thereafter, $PGE_2$ concentration was determined in the cell culture supernatants and corrected for $PGE_2$ released before the addition of exogenous arachidonic acid.

## Results

### *Induction of PGHS-2 mRNA expression by PDGF-AB and PDGF-BB*

In rat mesangial cells, PGHS-1 mRNA is expressed constitutively, and thus far no stimuli have been described that would significantly alter the expression of this isozyme in these cells. After prolonged stimulation with PDGF-AB or -BB (20 ng/ml each), however, PGHS-1 mRNA was up-regulated about twofold (2.4 ± 0.5-fold and 3.3 ± 0.7-fold with -AB and -BB, respectively, after 16 hr). PGHS-2 mRNA, in contrast, was barely visible in growth-arrested or cycling cells, but was induced by various stimuli. We could now show that two PDGF-isoforms, PDGF-AB and PDGF-BB, induced PGHS-2 mRNA (Fig. 1); PDGF-AA did not induce PGHS-2 mRNA (not shown). Induction of PGHS-2 mRNA by PDGF-AB and -BB was transient with maximal levels of mRNA reached after one to two hours, followed by a decline, which reached background levels after 10 hours. Similar kinetics were observed with growing or serum-deprived cells, although induction was more pronounced in serum-deprived cells. There was no significant difference between expression induced by either PDGF-AB or -BB. The slow decline of PDGF-induced PGHS-2 mRNA was at least partially due to mRNA stabilization. When mesangial cells were incubated with PDGF-AB for two hours and further transcriptional activity inhibited by actinomycin D (10 μg/ml), a very slow decay of PGHS-2 mRNA was observed, with an apparent half life of about four hours (Fig. 2).

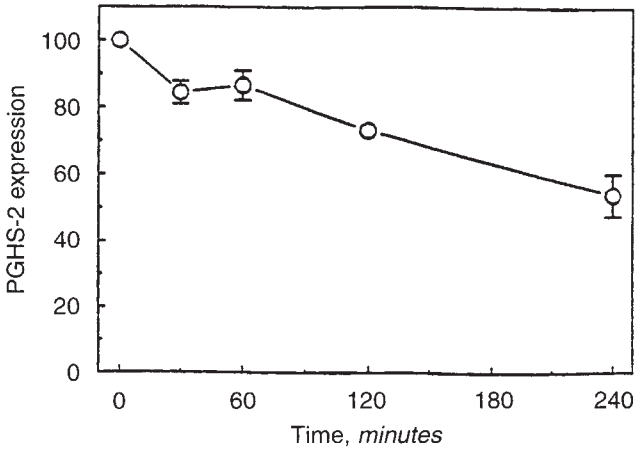


**Fig. 2.** *Apparent half life of PGHS-2 mRNA.* Mesangial cells were incubated with PDGF-AB for two hours. Thereafter, actinomycin D (10 μg/ml) was added to the cultures (t = 0, expression of PGHS-2 set to 100). Northern blot analyses were quantitated densitometrically. Data are means ± SEM of 3 independent experiments.

### *Effect of PDGF-isoforms on PGHS protein and activity*

Induction of PGHS-2 mRNA by PDGF was translated into increased PGHS-2 protein levels as demonstrated by Western blot analysis, whereas levels of PGHS-1 protein remained unchanged (Fig. 3; 4 hr stimulation). PGHS activity was determined by a 15 minutes incubation of mesangial cells with exogenous arachidonic acid and subsequent determination of $PGE_2$ in the supernatant. PDGF-AB and -BB enhanced PGHS activity after 4 and 24 hours two- to threefold (Table 1). Without exogenous addition of the precursor arachidonic acid, PDGF-AB or -BB did not stimulate production of $PGE_2$ (Table 1).

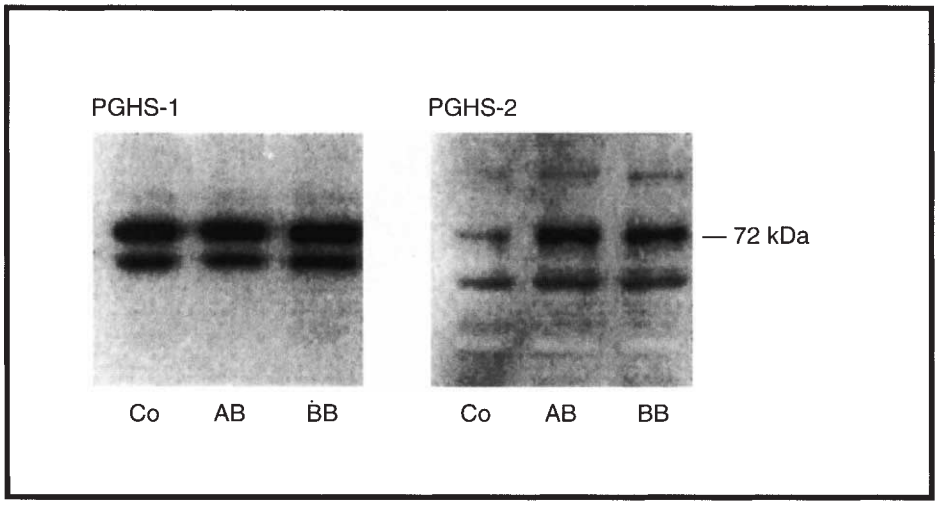


**Fig. 3.** *Expression of PGHS protein in mesangial cells.* Mesangial cells were stimulated with 20 ng/ml PDGF-AB or -BB for four hours. PGHS protein was detected by Western blot analysis as described in the **Methods** section. Blots shown are representative of 3 similar ones.

**Table 1.** $PGE_2$ release and PGHS activity in PDGF-stimulated mesangial cells

| | Co | PDGF-AB | PDGF-BB |
|---|---|---|---|
| $PGE_2$ release *pg/ml* | | | |
| 4 hours | 175 ± 50 | 133 ± 40 | 161 ± 40 |
| 24 hours | 150 ± 60 | 151 ± 30 | 127 ± 34 |
| PGHS activity *pg/ml/15 min* | | | |
| 4 hours | 1757 ± 222 | 5414 ± 957[a] | 3781 ± 756[a] |
| 24 hours | 1684 ± 330 | 4830 ± 1041[a] | 3041 ± 676[a] |

Serum-deprived mesangial cells were incubated with PDGF-AB or -BB (20 ng/ml each) for the times indicated. $PGE_2$ concentration in the culture supernatants was determined by ELISA as described in the **Methods** section. Conversion of exogenous arachidonic acid (10 μM) to $PGE_2$ within 15 minutes was taken as a measure of PGHS activity. Data are means ± SD of 2 independent experiments with duplicate samples and triplicate determination.

[a] $P < 0.01$ compared to corresponding controls (Student's *t*-test)

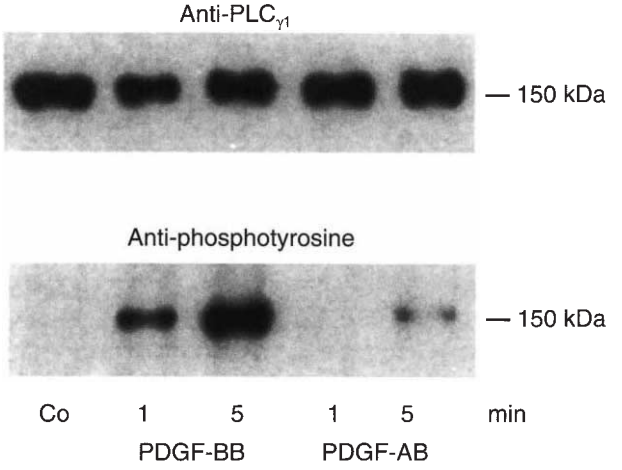


**Fig. 4.** *Differentiell phosphorylation of $PLC_{\gamma 1}$ by PDGF-AB and -BB.* Mesangial cells were incubated with 20 ng/ml PDGF-AB or -BB for the times indicated. $PLC_{\gamma 1}$ was precipitated from cell lysates. Parallel blots were detected with antibodies against $PLC_{\gamma 1}$ and phosphotyrosine. Blots shown are representative of 3 similar ones.

### *Differential effects of PDGF isoforms on $PLC_{\gamma 1}$ phosphorylation*

$PLC_{\gamma 1}$ is one of the enzymes which interacts with activated PDGF receptors. From mesangial cell lysates, $PLC_{\gamma 1}$ precipitated as a 150 kDa protein (Fig. 4). Phosphorylation of $PLC_{\gamma 1}$ on tyrosine residues was observed when mesangial cells were incubated with PDGF-BB for one to five minutes (Fig. 4). However, if cells were stimulated with PDGF-AB, phosphorylation was barely detectable.

### *Differential effect of PDGF isoforms on $Ca^{2+}$ mobilization*

Mesangial cells were loaded with the calcium indicator fura-2 and were then stimulated in suspension. When the cells were incubated with PDGF-AB, an influx of external $Ca^{2+}$ was induced, which was completely blocked by two minutes preincubation with EGTA (3 mM) in the medium (Fig. 5). Changes in fura-2 fluorescence were also abolished when $Ca^{2+}$ free medium was used (data not shown). PDGF-BB, in contrast, induced $Ca^{2+}$ release from internal stores as well as an influx of external $Ca^{2+}$ that resulted in a long lasting elevation of intracellular $Ca^{2+}$ levels. In the presence of extracellular EGTA or in nominally $Ca^{2+}$-free medium only the release from internal stores prevailed. $Ca^{2+}$ mobilization was concentration-dependent and observed at concentrations of PDGF-BB as low as 1 ng/ml (data not shown). The magnitude of the changes in $[Ca^{2+}]_i$ was comparable to the values obtained in rat and human mesangial cells grown on cover slips [33], confirming the reliability of the measurements in suspension. PDGF-AA was without effect on $[Ca^{2+}]_i$ in rat mesangial cells.

### *Role of intracellular $Ca^{2+}$ levels in PGHS-2 mRNA expression*

A role of elevated intracellular $Ca^{2+}$ concentrations for PGHS-2 mRNA expression in mesangial cells was demonstrated previously by the effect of calcium ionophore A23187 (0.1 μM; Fig. 6 and [17]). Furthermore, the effect of extracellular $Ca^{2+}$ on PDGF-mediated PGHS-2 mRNA expression was shown by incubating mesangial cells in medium containing EGTA (3 mM) during the stimulation period. This treatment almost completely

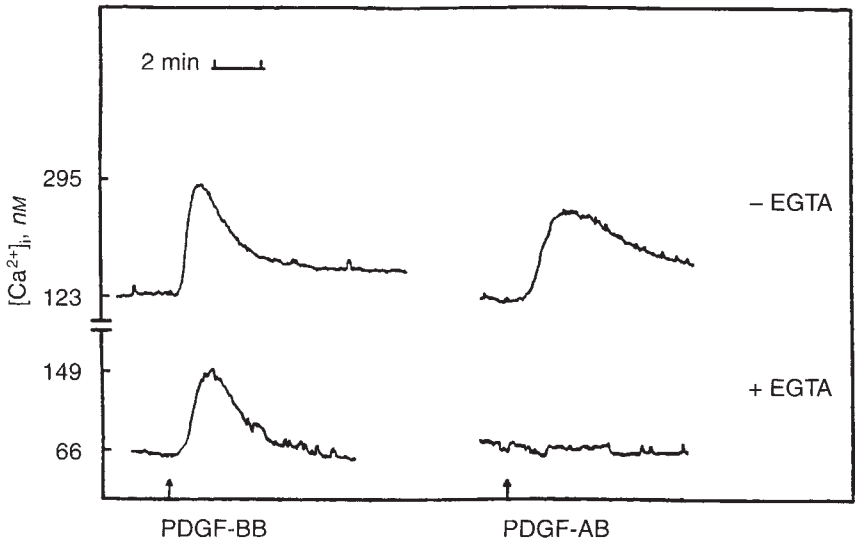


**Fig. 5.** *Calcium signaling of PDGF-AB and -BB in mesangial cells.* Mesangial cells were incubated with 20 ng/ml PDGF-AB or -BB in the presence or absence of 3 mM EGTA in the medium. Changes of intracellular $Ca^{2+}$ concentrations, calculated in nM, were detected with fura-2 as indicator. Fluorescence tracings are representative of 5 independent experiments.

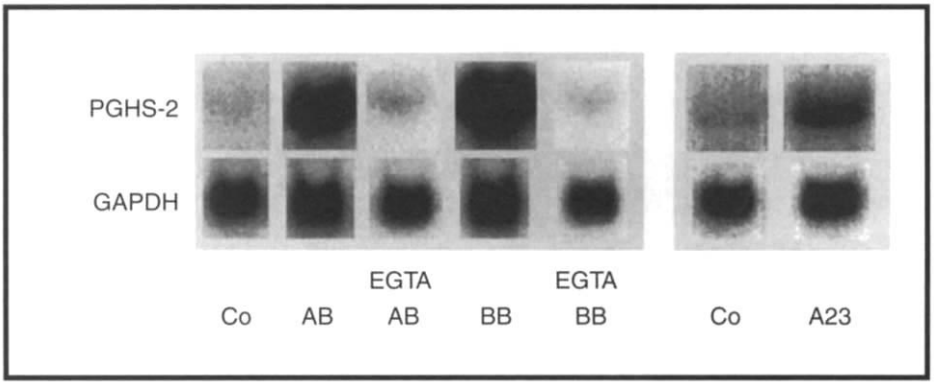


**Fig. 6.** *Sensitivity of PGHS-2 mRNA expression to calcium.* PGHS-2 mRNA expression was detected by Northern blot analysis. **Left panel.** Mesangial cells were incubated for two hours with 20 ng/ml PDGF-AB or -BB in the presence of 3 mM EGTA in the medium as indicated. **Right panel.** Mesangial cells were incubated with 0.1 μM calcium ionophore A23187 for two hours.

attenuated signal transduction after stimulation with PDGF-AB and -BB (Fig. 6). Similarly, complexation of intracellular calcium by the chelator BAPTA (100 μM) also inhibited PGHS-2 mRNA induction (data not shown).

### *Involvement of protein kinase C in PDGF-mediated PGHS-2 mRNA expression*

Protein kinase C (PKC) was down-regulated by treatment of mesangial cells with phorbol ester (TPA, 0.1 μM) overnight. TPA-induced PGHS-2 mRNA expression was completely prevented after down-regulation of PKC (Fig. 7) and PDGF-AB or BB-induced expression of PGHS-2 mRNA was impaired. Inhibition was 73 ± 5% and 72 ± 1% for PDGF-AB and -BB, respectively, in two independent experiments (means ± half range). Kinase inhibitors were used to confirm these results (Fig. 8). Induction of PGHS-2 mRNA was almost completely inhibited by genistein, a tyrosine kinase inhibitor. H7, an inhibitor of Ser/Thr kinases, reduced PGHS-2 mRNA induction by 48 ± 8% and 40 ± 10% for PDGF-AB and -BB, respectively (means ± half range of 2 independent experiments) comparable to the effect of PKC down-regulation. Since HA1004, which is more specific for protein kinase A and protein kinase G, had no effect on PGHS-2 mRNA induction, it was concluded that H7 exerted its effect by inhibition of PKC and thus diminished PGHS-2 mRNA expression.

## Discussion

PDGF is one of the growth factors critically involved in glomerular pathology. In mesangial cells, which by themselves produce PDGF, PDGF stimulates DNA synthesis and cell proliferation, chemotaxis, contraction and production of other cytokines and growth factors [4]. In the present study we investigated the effects of PDGF isoforms on key enzymes of prostaglandin synthesis, namely PGHS-1 and PGHS-2.

During short-term incubation, little or no effect of either PDGF isoform was observed with respect to PGHS-1 mRNA expression in line with previous results with other stimuli such as interleukin-1, serotonin, endothelin or FCS, which all failed to induce PGHS-1 in mesangial cells [17–19]. At later time points, after 8 to 12 hours, PGHS-1 mRNA was increased about twofold by PDGF-AB or -BB. The onset of enhanced PGHS-1 mRNA

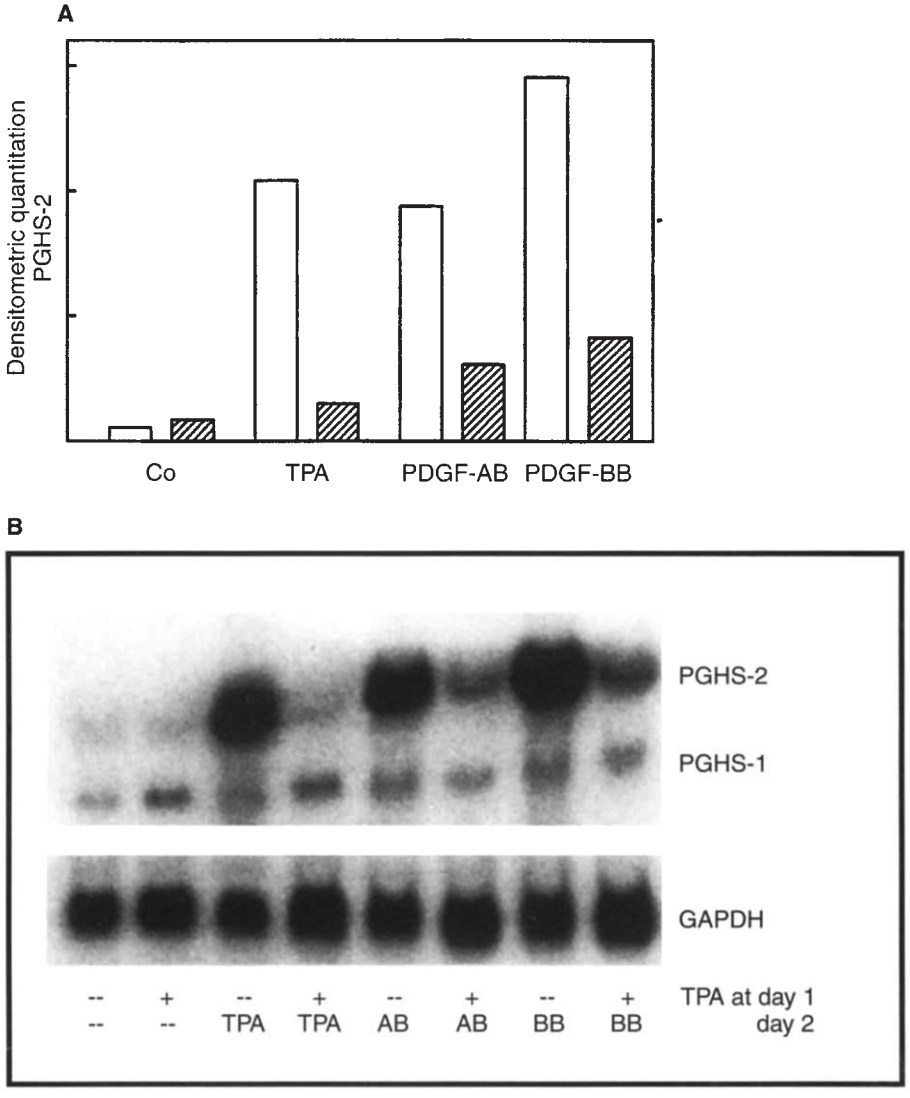


**Fig. 7.** *Effect of PKC down-regulation on PGHS mRNA expression.* **A.** Mesangial cells were preincubated overnight with or without TPA (0.1 μM) as indicated. Then the cells were stimulated with TPA (0.1 μM), PDGF-AB or -BB (20 ng/ml each). Symbols are: (□) induction of PGHS-2 mRNA without preincubation with TPA; (▨) induction of PGHS-2 mRNA after overnight incubation of the cells with TPA (0.1 μM). **B.** PGHS mRNA expression was detected by Northern blot analysis and quantitated by densitometry.

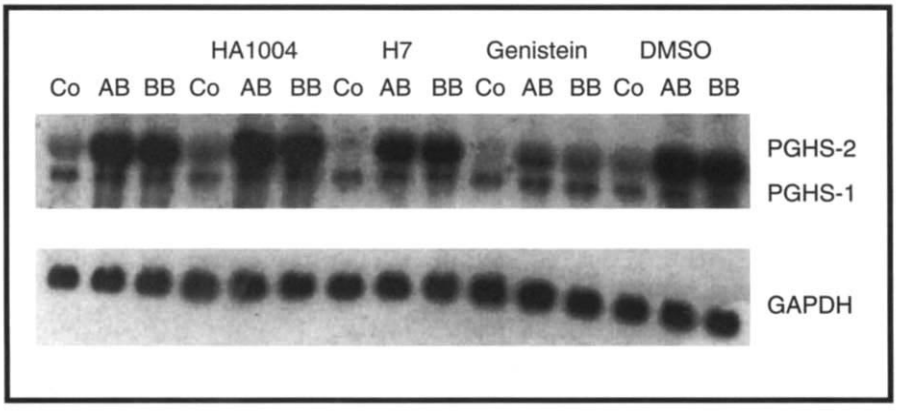


**Fig. 8.** *Effect of protein kinase inhibitors on PDGF-mediated PGHS-2 mRNA expression.* Mesangial cells were preincubated with HA1004 (10 μM), H7 (10 μM), genistein (50 μM) or the solvent DMSO (0.1%) for 30 minutes and then stimulated with PDGF-AB or -BB (20 ng/ml each) for two hours. PGHS mRNA expression was detected by Northern blot analysis.

expression seemed to be linked to the transition of the growth-arrested cells from G to S phase. These findings are consistent with results in other cell types, showing modest changes in PGHS-1 mRNA expression related to growth or differentiation (summarized in [16]). Furthermore, enhanced expression of PGHS-1 was recently observed in v-src transformed mesangial cells [34].

Of the three PDGF isoforms, only PDGF-AB and -BB induced

PGHS-2, while PDGF-AA was without effect. PDGF isoforms bind to different receptor dimers, PDGF-AA to the $\alpha\alpha$homologue [5]. Only a small number of this receptor type was found to be expressed in mesangial cells and may be occupied by intrinsically secreted PDGF-AA, which seems to be the major isoform secreted from mesangial cells in culture [7, 8, 33]. PDGF isoforms AB and BB induced PGHS-2 mRNA in mesangial cells with similar potency and kinetics. This was not expected regarding the binding characteristics of PDGF-AB, which is assumed to also require $\alpha$-receptors. It was recently suggested by Abboud et al [33] that binding of the B chain of AB isoform to $\alpha$-receptor may facilitate the binding of A chain to the receptor even in the presence of competing endogeneously produced AA isoform.

Induction of PGHS-2 was transient with a maximum after one to two hours, followed by a decline which persisted for several hours. These kinetics were different from those obtained with serotonin or TPA, which showed a more rapid disappearance of the mRNA [19]. The difference was also reflected in the apparent half lives determined in the presence of actinomycin D, where PDGF led to a marked increase in PGHS-2 mRNA stability with an apparent half life of about four hours, compared to 30 minutes observed after TPA stimulation [18].

Induction of PGHS-2 mRNA by PDGF was accompanied by enhanced protein levels and increased enzyme activity, the latter still being detectable after 24 hours. Despite the enhanced capacity of the cells to synthesize $PGE_2$, incubation of mesangial cells with PDGF did not induce the release of significant amounts of $PGE_2$ into the culture supernatant, indicating that availability of arachidonic acid was the rate-limiting step of prostanoid formation. This result is in line with investigations showing that phospholipase $A_2$ type II, the induction of which was related to stimulated $PGE_2$ formation in mesangial cells, was not induced by PDGF (unpublished observations and [35]). Synergism between IL-1 and PDGF with respect to $PGE_2$ synthesis was observed in human mesangial cells [7] and may relate to phospholipase $A_2$ activation by IL-1 [35, 36] and induction of PGHS-2 mRNA by both cytokines [17, 18].

PDGF receptors are coupled to different signaling pathways which include phospholipase $C_\gamma$ ($PLC_\gamma$), phosphatidylinositol-3 kinase, GTPase activating protein or members of the c-src kinase family [5]. In the present study, we investigated whether activation of $PLC_{\gamma 1}$, followed by the generation of diacylglycerol and inositol polyphosphates with subsequent release of $Ca^{2+}$ from internal stores and activation of protein kinase C (PKC), were part of the pathway leading to induction of PGHS-2. Activation of $PLC_{\gamma 1}$ by PDGF-AB and -BB was markedly different: PDGF-BB induced a rapid phosphorylation of the enzyme while PDGF-AB was without appreciable effect. Consistently only PDGF-BB induced a release of $Ca^{2+}$ from internal stores. These results add to the increasing evidence that signaling through $\alpha$- and $\beta$-receptors is qualitatively or at least quantitatively different [37–40].

Both PDGF isoforms induced $Ca^{2+}$ influx across the plasma membrane, which led to a long lasting elevation of $[Ca^{2+}]_i$. Influx of $[Ca^{2+}]_i$ was confirmed by measuring $Mn^{2+}$ influx into the cells (data not shown). $Mn^{2+}$ is able to pass through $Ca^{2+}$ channels [41] and quench fura-2 fluorescence [29]. Therefore, it can be used to distinguish between changes in fluorescence resulting from release or influx [42]. Influx seemed to be related to PGHS-2 mRNA expression because the omission of $Ca^{2+}$ from the external medium interfered with PDGF-AB or -BB-induced PGHS-2 mRNA expression. Furthermore, reduction of intracellular $Ca^{2+}$ levels by complexation inhibited the stimulated PGHS-2 mRNA expression, whereas the calcium ionophore A23187 was a potent inducer. So far, little information is available about the enzymes which take part in the signaling cascade leading to PGHS-2 mRNA induction. Induction was sensitive to cyclosporine A [17], suggesting an involvement of the calcium and calmodulin-dependent phosphatase calcineurin. Another calmodulin-dependent enzyme, $Ca^{2+}$/calmodulin-dependent kinase II, was shown to be involved in serotonin-mediated PGHS-2 mRNA expression (unpublished observation).

Earlier experiments with phorbol ester had shown that activation of PKC isoforms is one of the potential pathways to induce PGHS-2 mRNA in mesangial cells and is involved in serotonin but not endothelin signaling [19, 43]. The reduced induction of PGHS-2 mRNA after down-regulation of PKC by preincubation with phorbol ester indicated a role for PKC in PDGF-AB and -BB signaling. PGHS-2 mRNA induction was also reduced when PKC was inhibited by incubation of the cells with H7, whereas inhibition of protein kinase A or G by HA1004 was without effect. This was consistent with the inability of forskolin to induce PGHS-2 mRNA in mesangial cells [19]. The partial inhibition showed that additional PKC-independent pathways are part of the signaling cascade.

PKC was thus involved in PGHS-2 mRNA induction by both PDGF isoforms, whereas $PLC_{\gamma 1}$ was activated only by the BB isoform. Therefore, activation of $PLC_{\gamma 1}$ with its subsequent production of diacylglycerol is not the only pathway for PKC activation, which is essential for PGHS-2 mRNA expression. $PLC_\gamma$ is only one of the multiple enzymes associated with PDGF receptors, and it is more than likely that other signaling cascades are activated and participate in PGHS-2 gene activation. Activation of some PKC isoforms may be achieved by tyrosine phosphorylation as recently reported for PKC [44]: in fibroblasts, a positive correlation was determined between tyrosine phosphorylation, translocation and activation of PKC $\delta$ by PDGF $\beta$-receptor stimulation. Another potential source of diacylglycerol may be phosphatidylcholine, which is cleaved by phosphatidylcholine-specific PLC or phospholipase D.

This study demonstrates that binding of PDGF isoforms to PDGF $\alpha\beta$- and $\beta\beta$-receptors results in common as well as differential signaling in mesangial cells. As both PDGF isoforms potently induced PGHS-2 mRNA and protein, common or converging signal transduction pathways are essential for expression of this gene.

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